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Natural Compounds as Antimicrobial Agents

Edited by

Carlos M. Franco and Beatriz Vázquez Belda

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About the Special Issue Editors

Carlos M. Franco graduated in veterinary medicine and finished his Ph.D. at the University of Santiago de Compostela, Spain, in 1994, with a thesis regarding L. monocytogenes incidence in food as well as resistance to several antimicrobials. He completed his posdoctoral studies at the Veterinary Faculty of the Complutense University of Madrid, completing his formation in several food microbiology aspects as well as at the Laboratory of Chimie Analityque II of the University of Paris 11, where he studied several analytical methods. Subsequently, he was interested in antimicrobial resistance, the effect of the use of antibiotics in animal production, and antimicrobial resistance in food from ecological or conventional origins. He has researched the detection of antimicrobials and other drugs in food of animal origin as well as the effect of essential oils on the inhibition of bacteria. He has published more than 120 peer-reviewed papers. Currently, he is researching bacterial biofilms and their elimination or control as well as other food science topics.

Beatriz Vázquez Belda holds degrees in biology and in food technology and obtained her Ph.D. in 1997 at the Santiago de Compostela University (USC), Spain. She completed her posdoctoral studies at the Ecole Nationale d'Industrie Laitière et des Industries Agro-alimentaires (Surgères, France) and at the Faculté des Sciences Pharmaceutiques et Biologiques (Paris, France). She specializes in food micology, studying fungal contamination on Spanish cheeses as well as the development of fast detection techniques using microbiological and instrumental methods, taking advantage of the luminescence properties of aflatoxins as well as other micotoxins. After two years as lecturer at the Cardenal Herrera-CEU University at Valencia, she returned to the Veterinary Faculty (USC) with a Ramon y Cajal national grant. She continued her interest in researching the use of natural compounds such as essential oils to decrease mycotoxin production on dairy products. She is also the Technical Responsible for physico-chemical analysis at the Laboratory of Hygiene, Inspection and Food Control (LHICA-USC), which is accredited by the National Entity of Accreditation for testing agroalimentary products. Her research has mainly focused on the development of extraction and detection methods for antimicrobials, β-agonists, and corticosteroid drugs used for illicit purposes in animal production, methods that are used in the routine control analysis of feed and food samples. She has also co-authored more than 90 internationally publications in peer-reviewed journals apart from other publications.

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Editorial

Natural Compounds as Antimicrobial Agents

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Abstract: During the first two decades of this century, conventional antimicrobial compounds have been found out to have more bacterial resistance. What has also been worrying is the rediscovery of the so-called "natural compounds", which in turn have a good name among the average citizen because of the former's plant or animal origin. However, they do not form a well-classified group of substances. This Special Issue consists of five reviews focusing on clinical bacteria applications in food and their specific effects upon virulent bacterial factors. You will also find a research on much needed, new antimicrobials sourced in extreme environments, and secondary metabolites of *Burkholderia*. This issue includes 12 original research papers which will provide you with an in-depth coverage of the protein extract activity, as well as the activity of other plant extracts, on fighting bacteria, fungi or diarrhea. Their use in broilers or laying eggs for production purposes has also been focused on in order to improve gut microbiota. Last but not least, we should not forget about honey and its effect; Allium sativum-fermented extracts, as well as other "natural" compounds, have been studied in their fight against biofilms. Furthermore, we have also examined the use of essential oils, which are currently used in edibles such as fresh sausages. The present work also deals with other applications such as natural compound derivatives as well as compound mixtures.

This book details the manuscripts in the Special Issue of Antibiotics: "Natural Compounds as antimicrobial agents". Our in-depth study comprises 17 manuscripts, which focus on an important group of aspects related to biocontrol once this wide range of compounds has been used. Firstly, we start with two interesting reviews; one which deals with the use of traditional medicinal plants against clinical pathogens, documenting [1] a huge series of minimal inhibitory concentrations, using over 200 medicinal plants to fight pathogens off. The other is an analysis of the major applications of natural compounds for one of their main uses—food preservation so as to enhance food safety—in a review by prof. Quinto and colleagues [2]. Edible films as well as nanoparticles are also included. The food use of these compounds is perceived benevolently by consumers, so not only was the study of natural compounds as growth bacteria inhibitors examined in this present issue, but also the effect of these compounds on the virulent related factors of specific bacteria, i.e., T3SS (Type III Secretion System used by many Gram negative bacteria [3]). Further in the issue, you will also find two interesting reviews on the conventional meaning of "Natural Compounds" and conventional antibiotics, between which the borders are very thin. So thin, in fact, that many believe in the richness of extreme ecosystems for finding Actinobacteria-producing antibiotics [4], and in the use of secondary metabolites from *Burkholderia* as new sources in antibiotic development [5].

Additionally, there are, as has been stated above, 12 research papers studying, firstly [6], the effect of protein extracts from *Loranthus europaeus* berries against phytopathogenic fungi as well as foodborne bacteria. The already mentioned extracted experiments have shown an important activity against bacteria, though no effect against fungi was found. Bear in mind that you will also find the use of medicinal plants in this use against diarrhea [7]. This highlights an interesting aspect that needs further study as far as the metal content of these plants is concerned. Another research paper studies

the properties of witch-hazel plants and Green tea extracts upon the pathogenesis inhibition of staphylococci [8]. In another extract worth mentioning, Hibiscus activity against multidrug-resistant bacteria is thoroughly described [9], along with the elucidation of the extract compounds by means of magnetic resonance spectroscopy (as well as other techniques). Two more papers covering the research on the use of natural compounds in animal production are also included within this Special Issue. They comprise broiler and laying hens, using Chicory (*Chicorium intybus*) [10] resulting in gut microbiota improving. Fermented defatted Alperujo is also focused upon, including its role in enhancing the absorption capacity of intestinal mucosa [11]. Furthermore, not only have we studied the effect of natural compounds on gut microbiota, but we have also covered those natural compounds produced by gut microbiota—the short-chain fatty acids (studied by Lamas et al. [12]; the latter study regarding the effect on the biofilm formation, gen expression, and motility of *Salmonella*). Their effect against biofilm has also been observed using both honey [13] and *Allium sativum* fermented extract, and cannabinol oil extract [14]. The essential oil of *Zataria multiflora* and hops extracts have also been tested in fresh sausages [15], so as to avoid using other conventional preservatives. They have shown, however, that using natural compounds does not always imply an antimicrobial effect, which introduces the need to study which types of application are most advisable for these compounds. Finally, two extra chapters focusing on natural antimicrobial compounds are included. These natural antimicrobial compounds can be used directly, as well as either in a modified or mixed way, so as to increase their activity. You will find, for example, mixtures of chitosan oligomers with ε-polylysine acting as antifungals against three *Botryosphareiaceae* species [16]. Likewise, in the last paper [17], some berberine derivatives can be more potent than berberine itself in attenuating MexXY efflux-dependent aminoglycoside resistance in *Pseudomonas aeruginosa*, demonstrating that natural compounds are not only useful when used in direct applications, but also to obtain derivative compounds with enhanced antimicrobial properties.

The huge amount of natural antimicrobial compound applications and their direct inhibition of bacteria are detailed in terms of a range of applications—for instance, in the avoidance of biofilms. The food industry may well benefit from this in-depth study, and it is likewise useful as a basis from which to obtain more potent compounds. We do expect that this group of manuscripts will be of great help to every scientist or professional interested in the biocontrol of bacteria, fungi or even other biological agents, using a natural alternative instead of the classical chemical compounds.

Conflicts of Interest: The authors declare no conflict of interest.

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Review

Efficacy and Mechanism of Traditional Medicinal Plants and Bioactive Compounds against Clinically Important Pathogens

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Abstract: Traditional medicinal plants have been cultivated to treat various human illnesses and avert numerous infectious diseases. They display an extensive range of beneficial pharmacological and health effects for humans. These plants generally synthesize a diverse range of bioactive compounds which have been established to be potent antimicrobial agents against a wide range of pathogenic organisms. Various research studies have demonstrated the antimicrobial activity of traditional plants scientifically or experimentally measured with reports on pathogenic microorganisms resistant to antimicrobials. The antimicrobial activity of medicinal plants or their bioactive compounds arising from several functional activities may be capable of inhibiting virulence factors as well as targeting microbial cells. Some bioactive compounds derived from traditional plants manifest the ability to reverse antibiotic resistance and improve synergetic action with current antibiotic agents. Therefore, the advancement of bioactive-based pharmacological agents can be an auspicious method for treating antibiotic-resistant infections. This review considers the functional and molecular roles of medicinal plants and their bioactive compounds, focusing typically on their antimicrobial activities against clinically important pathogens.

Keywords: traditional medicinal plants; bioactive compounds; antimicrobial activities; mechanisms

1. Introduction

The incidence of microbial infectious diseases and their hitches consistently elevates, mostly due to microbial drug resistance to presently offered antimicrobial agents [1]. These multidrug-resistant microbes cause various infections globally and are connected with greater levels of morbidity and mortality [2]. These augmentations of antibiotic resistance and higher recurrence rates of such common infections have a great impact on our society [3–5]. Several investigations associated with antimicrobial resistance predict that the mortality toll owing to antimicrobial resistance may exceed 10 million by 2050, theoretically leading to greater mortality in the context of other infectious diseases and malignancies [6]. It is well known that infections are generally difficult to treat due to the development of biofilm in the host, which aids the proliferation of microbes as well as the aggressiveness of the infections [7]. Studies have also well-established that the physical structures of biofilm establishing organisms confer natural resistance to hostile environments, including antimicrobial agents [8]. Therefore, it is an urgent requirement to generate novel antimicrobial drugs which can inhibit the development of, or abolish the complete biofilms, and hence increase the vulnerability of microbes to antimicrobials. The requisite for new antimicrobials which could meritoriously fight against antimicrobial resistant clinical pathogens is extremely augmented.

Plant-derived antimicrobials have been established to be one of the most auspicious sources considered as safe due to their natural origin when compared with synthetic compounds [9,10]. There is an accumulating interest in the practice of either crude extract of medicinal plants, as well as the

screening plant-derived compounds as an alternative therapy for microbial infections [11]. Plants generally produce a diverse range of bioactive compounds which have been widely used in clinical practice [12]. Remarkably, a significant number of marketed drugs are obtained from nature or result in natural products through either chemical transformations or de novo synthesis [13]. Plant-derived compounds are a group of secondary metabolites that are used to treat chronic as well as infectious diseases. These traditional medicinal plants or active compounds remain included as part of the habitual treatment of various maladies [9]. These compounds could have other target sites than conventional antimicrobials as well as diverse mechanisms of action against pathogenic microbes. An electronic search was performed using PubMed, Science Direct, and Google Scholar using the keywords "medicinal plants" AND "bioactive compounds" AND "antimicrobial activities" AND "antibiotic resistance" in "Title/Abstract/Keywords" without date restriction in order to identify all published studies (in vitro, in vivo, clinical and case-control) that have investigated the connection between medicinal plants and their antimicrobial effects. Antimicrobial mechanisms were gathered and for review.

2. Traditional Medicinal Plants

The species of the plant kingdom are estimated to number about 500,000 and only a minor portion of them have been investigated for antimicrobial activity [9,14]. Traditional medicinal plants can be cultivated by humans over centuries without existing systematic standards and analysis due to their safety and efficacy. Hence, bioactive compounds derived from these medicinal plants apparently have more potential to succeed in toxicology screening when compared with the de novo synthesis of chemicals. The cumulative attention on traditional ethnomedicine may lead to the revealing of innovative therapeutic agents since traditional medicinal plant contains potential antimicrobial components that are beneficial for the development of pharmaceutical agents for the therapy of ailments. Nowadays, studies are progressively turning their consideration to traditional medicine and advancing better drugs to treat diabetes, cancer, and microbial infections [15,16]. A large number of studies have been piloted using medicinal plant extracts and their active principles on bacteria, fungi, algae, and viruses in different localities of the world [9,10]. Various families of traditional medicinal plants have been scientifically tested for their antimicrobial activities and are presented in Table 1. The extracts of plant organs, namely the root, stem, rhizome, bulb, leaf, bark, flower, fruit, and seed, may encompass distinctive phytochemicals with antimicrobial activities [17]. It is well-known that sole plant species of traditional medicine are habitually used to heal a great number of infections or diseases [18]. The plant extracts with an antiquity of folk use should be confirmed using contemporary methods for activities against human pathogens with the intention of identifying potential novel therapeutic drugs.

Table 1. Antimicrobial screening performed on various medicinal plants.

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Barleria prionitis L.	Acanthaceae	Leaves	Pet. Ether	3.33–33.3 mg/mL	*B. subtilis, M. luteus, B. cereus, S. mutans, S. aureus, L. sporogenes*	*S. typhi, V. Cholera, M. luteus, Citrobacter*	-	[19]
			Chloroform	5–50 mg/mL	*B. subtilis, L. sporogenes*	*S. typhi, V. cholerae, Citrobacter, Providencia*	-	
			Methanol	10–100 mg/mL	*B. subtilis, L. sporogenes*	*V. cholerae, S. typhi,*	-	
			Ethanol	50–600 µg/mL	-	*S. typhi*	-	
		Bark	Acetone	25, 50, 100 mg/mL				
			Ethanol	25, 50, 100 mg/mL	*Bacillus spp., S. mutans, S. aureus,*	*Pseudomonas* spp.,	*S. cerevisiae, C. albicans*	
			Methanol	25, 50, 100 mg/mL				
Adhatoda vasica L.	Acanthaceae	Leaves	Aqueous	4% v/v	*M. tuberculosis,*	*E. coli, S. typhi*	-	[20]
			Methanol	625 µg/mL	*S. aureus*	*E. coli, S. typhi*	-	
Pellaea calomelanos L.	Adiantaceae	Leaves, Rhizomes	Aqueous, Dichloromethane/ Methanol	250 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
				750–12,000 µg/mL				
Sambucus australis Cham. & Schltdl.	Adoxaceae	Leaves and Bark	Hexane Ethanol	50 µg/mL	*S. aureus, S. agalactiae*	*E. coli, S. typhimurium* and *K. pneumoniae*	*C. albicans*	[22]
Carpobrotus edulis L N.E.Br.	Aizoaceae	Leaves	Aqueous Dichloromethane/ Methanol	100 µg/mL	*S. mutans, S. sanguis, L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrataC. krusei*	[23]
				750–12,000 µg/mL				
Achyranthes aspera L.	Amaranthaceae	Root, Leaves, Stem	Ethanol	1 mg/mL	*S. aureus, B. subtilis,*	*E. coli, P. vulgaris, K. pneumoniae*	-	[16]
Alternanthera Sessile L.	Amaranthaceae	Leaves	Ethanol	75 µg/mL	*S. pyogenes*	*S. typhi*	-	[24,25]

Table 1. *Cont.*

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Amaranthus caudatus L.	Amaranthaceae	Leaves	Ethyl Acetate Chloroform Methanol	162.2–665 mg/mL 1.25 mg/mL 3–5 mg/mL	*S. aureus, Bacillus spp.*	*E. coli, S. typhi, P. mirabilis*	-	[26]
Amaranthus hybridus L.	Amaranthaceae	Leaves	Ethyl Acetate Chloroform Methanol	200–755 mg/mL 1.25 mg/mL 3–5 mg/mL	-	*E. coli, S. typhi, k. pneumoniae, P. aeruginosa*	-	[26]
Amaranthus spinosus L.	Amaranthaceae	Leaves	Ethyl Acetate Chloroform Methanol	129 mg/mL 1.25 mg/mL 3–5 mg/mL	-	*S. typhi*	-	[26]
Boophane disticha L.f.	Amaryllidaceae	Leaves	Aqueous, Dichloromethane/ Methanol	20–100 mg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Scadoxus puniceus (L.) Friis &Nordal.	Amaryllidaceae	Rhizomes, Roots	Aqueous Dichloromethane/ Methanol	50 µg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Harpephyllum caffrum Bernh. exKrauss	Anacardiaceae	Bark, Leaves	Aqueous Dichloromethane/ Methanol	125–500 µg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Lannea discolor Engl.	Anacardiaceae	Leaves	Aqueous Dichloromethane/ Methanol	50–200 µg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Polyalthia cerascides L.	Annonaceae	Stem Bark	Dichloromethane	100 µg/mL	*C. Dipthieriae*	-	-	[27]
Berula erecta Huds., Coville	Apiaceae	Rhizome, Leaves, Stem	Aqueous Dichloromethane/ Methanol	2–16 µg/mL 750–12,000 µg/mL	*S. mutans, S. sanguis, L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrata C. krusei*	[23]

Table 1. *Cont.*

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Acokanthera oppositifolia L. Codd.	Apocynaceae	Leaves, Stem	Aqueous Dichloromethane/ Methanol	25–200 µg/mL 750–12,000 µg/mL	*S. mutans*, *S. sanguis*, *L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrata C. krusei*	[23]
Plumeria ruba L.	Apocynaceae	Leaves	Aqueous Dichloromethane/ Methanol	50–200 µg/mL 100 µg/mL	*S. epidermidis*	*E. coli*	-	[16]
Acokanthera oppositifolia (Laim.) Codd.,	Apocynaceae	Leaves	Aqueous Dichloromethane/ Methanol	10–50 µg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Rauvolfia caffra Sond.	Apocynaceae	Leaves	Aqueous Dichloromethane/ Methanol	25, 50 µg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Calotropis gigantea L.	Apocynaceae	Latex	Ethanol	1–8 mg/mL	-	-	*C. albicans, T. mentagrophytes, T. rubrum*	[16]
Plumeria alba L.	Apocynaceae	Root	Methanol	10–40 µg/mL	-	*E. coli*		[16]
Ilex mitis Radlk.	Aquifoliaceae	Bark, Leaves	Aqueous Dichloromethane/ Methanol	1–8 mg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Anchomanes difformis Engl.	Araceae	Roots	Methanol	20–100 mg/mL	methicillin-resistant *S. aureus*	-	-	[28]
Zantedeschia aethiopica Spreng	Araceae	Leaves	Aqueous Dichloromethane/ Methanol	50 µg/mL 15–150 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Arum dioscoridis L.	Araceae	Leaves	Aqueous	125–500 µg/mL	*S. aureus*, *S. pneumoniae*	*E. coli*, *S. typhi*, *P. aeruginosa*	-	[29]
Aristolochia Indica L.	Aristolochiaceae	Leaves	Ethanol	1–8 mg/mL	-	-	*A. niger A. flavus A. fumigatus*	[3,4,30,31]

Table 1. *Cont.*

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Vernonia blumeoides Hook. f.	Asteraceae	Aerial Part	Ethanol	100 µg/mL	methicillin-resistant *S. aureus*	-	-	[28]
Artemisia afra Jacq. ex Willd.	Asteraceae	Leaves, Stem	Aqueous Dichloromethane/ Methanol	2–16 µg/mL 750–12,000 µg/mL	*S. mutans*, *S. sanguis*, *L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrata C. krusei*	[23]
Tarchonanthus camphoratus L.	Asteraceae	Leaves	Aqueous Dichloromethane/ Methanol	25–200 µg/mL 750–12,000 µg/mL	*S. mutans*, *S. sanguis*, *L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrata C. krusei*	[23]
Helichrysum paronychioides L.	Asteraceae	Whole Plant	Pet ether Methanol	50–200 µg/mL 50–200 µg/mL	*B. cereus*	*S. flexneri*	*C. glabrata*, *C. krusei*, *T. rubrum* and *T. tonsurans*	[2]
Senecio longiflorus L.	Asteraceae	Stem and Leaves	Pet ether Methanol	125–625 µg/mL 50–200 µg/mL	*B. cereus*	*S. flexneri*	*C. glabrata*, *C. krusei*, *T. rubrum* and *T. tonsurans*	[2]
Dahlia pinnata L.	Asteraceae	Leaves	Chloroform	2–16 µg/mL	–	*E. aerogenes*, *P. aeruginosa*	–	[16]
Athrixia phylicoides DC.	Asteraceae	Leaves	Aqueous Dichloromethane/ Methanol	25–200 µg/mL 750–12,000 µg/ml	*S. aureus*, *methicillin- resistant S. aureus*, *gentamycin– methicillin-resistant S. aureus*, *S. epidermidis*, *B. agri*, *P. acnes*	*P. aeruginosa*	*T. mentagrophytes*, *M. canis*	[21]
Dicoma anomala Sond.	Asteraceae	Tuber	Aqueous Dichloromethane/ Methanol	50–200 µg/mL 750–12,000 µg/mL	*S. aureus*, *methicillin- resistant S. aureus*, *gentamycin– methicillin-resistant S. aureus*, *S. epidermidis*, *B. agri*, *P. acnes*	*P. aeruginosa*	*T. mentagrophytes*, *M. canis*	[21]
Vernonia natalensis Sch. Bip. exWalp.	Asteraceae	Leaves, Roots	Aqueous Dichloromethane/ Methanol	10–50 µg/mL 750–12,000 µg/mL	*S. aureus*, *methicillin- resistant S. aureus*, *gentamycin– methicillin-resistant S. aureus*, *S. epidermidis*, *B. agri*, *P. acnes*	*P. aeruginosa*	*T. mentagrophytes*, *M. canis*	[21]
Achillea millefolium L.	Asteraceae	Leaves	Ethanol	100 µg/mL	*S. aureus*	*P. aeruginosa S. typhi*, *E. coli*	*C. albicans*	[29]
Blumea balsamifer (Linn.) D.C.	Asteraceae	Whole Plant	Ethanol	250 µg/mL	methicillin-resistant *S. aureus*	-	-	[32]
Impatiens balsamina L.	Balsaminaceae	Leaf	Ethanol	50–200 µg/ml	methicillin-resistant *S. aureus*	-	-	[28]
Berberis chitria L.	Berberidaceae	Roots	Ethanol, Methanol	5.5–6.5 mg/mL 2.5–3.5 mg/mL	*S. aureus*	*E. coli*	-	[33]

Table 1. *Cont.*

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Alnus nepalensis D. Don.	Betulaceae	TBL	Ethanol	50–200 µg/mL	Methicillin-resistant *S. aureus*	-	-	[32]
Tecoma capensis Lindl.	Bignoniaceae	Leaves, Stem	Aqueous, Dichloromethane/ Methanol	10–50 µg/mL 2.5 mg/mL	*S. mutans*, *S. sanguis*, *L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrata C. krusei*	[23]
Spathodea campanulata L.	Bignoniaceae	Leaves Flowers	Ethanol	221–254 µg/mL 156–173 µg/mL	*B. subtilis*, *S. aureus*,	*E. coli*, *K. pneumonia*, *P. vulgaris*, *S. typhi*, *Pseudomonas* spp., *V. cholerae*	-	[6,34,35]
Kigelia africana (Lam.) Benth.	Bignoniaceae	Fruit	Aqueous Dichloromethane/ Methanol	2–16 µg/mL 750–12,000 µg/mL	*S. aureus*, *methicillin- resistant S. aureus*, *gentamycin– methicillin-resistant S. aureus*, *S. epidermidis*, *B. agri*, *P. acnes*	*P. aeruginosa*	*T. mentagrophytes*, *M. canis*	[21]
Opuntia ficus-indica Mill.	Cactaceae	Leaves	Aqueous Dichloromethane Methanol	25–200 µg/mL 750–12,000 µg/mL	*S. aureus*, *methicillin- resistant S. aureus*, *gentamycin– methicillin-resistant S. aureus*, *S. epidermidis*, *B. agri*, *P. acnes*	*P. aeruginosa*	*T. mentagrophytes*, *M. canis*	[21]
Senna italic L.	Caesalpiniaceae	Leaves	Acetone	2.5 mg/mL	*B. cereus*, *B. pumilus*, *B. subtilis*, *S. aureus*, *E. faecalis*,	-	-	[36]
Cassia fistula L.	Caesalpiniaceae	Seeds	Aqueous Ethanol	780–6250 µg /mL 2–16 µg/mL	*S. aureus*	-	-	[6]
Warburgia salutaris (G. Bertol.) Chiov.	Canellaceae	Bark, Twigs	Aqueous	5.0–10 mg/mL	*S. mutans*, *S. sanguis*, *L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrata* *C. krusei*	[23]
			Dichloromethane, Methanol	50–200 µg/mL 750–12,000 µg/mL	*S. aureus*, *methicillin- resistant S. aureus*, *gentamycin– methicillin-resistant S. aureus*, *S. epidermidis*, *B. agri*, *P. acnes*	*P. aeruginosa*	*T. mentagrophytes*, *M. canis*	[21]
Cadaba fruticosa L.	Capparaceae	Leaves	Acetone Aqueous Benzene Butanol Chloroform Ethanol	100–200 µg/mL 4–16 µg/mL 4–16 µg/mL 4–16 µg/mL 4–16 µg/mL 4–16 µg/mL	*S. pyogens*, *S. aureus*, *B. subtilis*	*S. typhi*, *P. vulgaris*, *K. pneumoniae*, *P. aeruginosa*, *E. coli*	-	[37]

Table 1. *Cont.*

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Boscia senegalensis Del.	Capparidaceae	Roots	Methanol	10–20 µg/mL	methicillin-resistant *S. aureus*	-	-	[28]
Celastrus orbiculatus Thunb.	Celastraceae	Vane	Ethanol	1–2 mg/mL	methicillin-resistant *S. aureus*	-	-	[32]
Euonymus fortunei (Turcz.); Hand. Mazz.	Celastraceae	Leaves	Ethanol	10–40 µg/mL	methicillin-resistant *S. aureus*	-	-	[32]
Chenopodium ambrosioides Bert. ex Steud.	Chenopodiaceae	Leaves	Aqueous Dichloromethane/ Methanol	2–16 µg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Garcinia mangostana L.	Clusiaceae	Fruit Shell	Ethanol	25–200 µg/mL	methicillin-resistant *S. aureus*	-	-	[28]
Garcinia morella Desr.	Clusiaceae	Whole Plant	Ethanol	100–400 µg/mL	methicillin-resistant *S. aureus*	-	-	[32]
Terminalia paniculata L.	Combretaceae	Stem Bark	Ethyl Acetate Methanol	3.25, 3.5 mg/mL 5–20 µg/mL	*S. aureus, B. subtilis*	-	-	[38]
Terminalia sericea Burch. ex DC.	Combretaceae	Roots	Aqueous Dichloromethane/ Methanol	100–300 µg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Eupatorium odoratum L.	Compositae	Leaves	Benzene Aqueous Acetone	300–600 µg/mL 300–600 µg/mL 300–600 µg/mL	*B. cereus, S. aureus*	*E. coli, K. pneumoniae, V. cholerae*	*C. albicans*	[39]
Acmella paniculata L.	Compositae	Whole Plant	Chloroform Pet. ether Methanol	15 µg/mL 5–15 µg/mL 5–15 µg/mL	-	*E. aerogenes*	-	[40]

Table 1. *Cont.*

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Cotyledon orbiculata L.	Crassulaceae	Leaves	Aqueous Dichloromethane Methanol	5–30 µg/mL 750–12,000 µg/mL	*S. mutans, S. sanguis, L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrata* *C. krusei*	[23]
Cotyledono rbiculata Forssk.	Crassulaceae	Leaves	Aqueous Dichloromethane/ Methanol	25–200 µg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Mormodica basalmina L.	Cucurbitaceae	Whole Plant	Methanol	500 µg/mL	methicillin-resistant *S. aureus*	-	-	[28]
Coccinia grandis L.	Cucurbitaceae	Leaves	Aqueous Dichloromethane/ Methanol	500 µg/mL 2 mg/mL	*B. cereus, S. aureus*	-	-	[41]
Luffa acyntangula L.	Cucurbitaceae	Leaves	Aqueous Dichloromethane Methanol	5 mg/mL 2 mg/mL	*B. cereus, S. aureus*	-	-	[41]
Mukia maderspatana L.	Cucurbitaceae	Leaves	Aqueous Dichloromethane/ Methanol	5 mg/mL 1 mg/mL	*B. cereus, S. aureus*	-	-	[41]
Trichosanthes cucumerina L.	Cucurbitaceae	Leaves	Aqueous Dichloromethane/ Methanol	5 mg/mL 1 mg/mL	*B. cereus, S. aureus*	-	-	[41]
Momordica balsamina L.	Cucurbitaceae	Leaves, Roots	Acetone	500 µg/mL	*B. cereus, B. pumilus, B. subtilis, S. aureus, E. faecalis*	*E. coli, E. cloaceae, K. pneumoniae, P. aeruginosa, S. marcescens*	-	[42]
Carex prainii C.B. Clarke	Cyperaceae	Whole Plant	Ethanol	15–45 µg/mL	methicillin-resistant *S. aureus*	-	-	[32]

Table 1. *Cont.*

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Dioscorea dregeana T. Durand & Schinz.	Dioscoreaceae	Tuber	Aqueous Dichloromethane/ Methanol	5–30 µg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Sansevieria hyacinthoides L.	Dracaenaceae	Leaves, rhizome	Aqueous, Dichloromethane/ Methanol	1–4 mg/mL 750–12,000 µg/mL	*S. mutans, S. sanguis, L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrataC. krusei*	[23]
Diospyros mespiliformis Hochst. exA. DC.	Ebenaceae	Leaves	Aqueous Dichloromethane/ Methanol	15–45 µg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Phyllanthus amarus Schum. Thonn.	Euphorbiaceae	Whole Plant	Methanol	650–600 µg/mL	methicillin-resistant *S. aureus*	-	-	[28]
Croton gratissimus Burch.	Euphorbiaceae	Leaves, Stem,	Aqueous Dichloromethane/ Methanol	5 mg/mL 750–12,000 µg/mL	*S. mutans, S. sanguis, L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrataC. krusei*	[23]
Spirostachys africana Sond.	Euphorbiaceae	Leaves, Bark	Aqueous Dichloromethane/ Methanol	490 µg/mL 750–12,000 µg/mL	*S. mutans, S. sanguis, L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrataC. krusei*	[23]
Acalypha indica L.	Euphorbiaceae	Leaves	Aqueous	4% v/v	*M. tuberculosis*	-	-	[43]
Bridelia micrantha Baill.	Euphorbiaceae	Bark, Leaves	Aqueous Dichloromethane/ Methanol	5 mg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Emblica officinalis L.	Euphorbiaceae	Leaves	Benzene Aqueous Acetone	350–600 µg/mL 300–600 µg/mL 300–600 µg/mL	*B. cereus, S. aureus*	*E. coli, K. pneumoniae, V. cholerae*	*C. albicans*	[39]

Table 1. *Cont.*

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Hevea brasiliensis L.	Euphorbiaceae	Leaves	Benzene Aqueous Acetone	350–600 µg/mL 300–600 µg/mL 300–600 µg/mL	*B. cereus, S. aureus*	*E. coli, K. pneumoniae, V. cholerae*	*C. albicans*	[39]
Mallotus yunnanensis Pax et. Hoffm.	Euphorbiaceae	Tender Branches & Leaves	Ethanol	8–256 µg/mL	methicillin-resistant *S. aureus*	-	-	[32]
Acacia albida Del.	Fabaceae	Stem Bark	Methanol	50 µg/mL	methicillin-resistant *S. aureus*	-	-	[28]
Acacia catechu (L. f.) Willd	Fabaceae	Wood	Ethanol	100 µg/mL	methicillin-resistant *S. aureus*	-	-	[28]
Peltophorum ptercarpum (DC.)	Fabaceae	Bark	Ethanol	4% v/v	methicillin-resistant *S. aureus*	-	-	[28]
Acacia erioloba Edgew.	Fabaceae	Bark and Leaves	Aqueous Dichloromethane/ Methanol	1.56–3.12 mg/mL 750–12,000 µg/mL	*S. aureus, methicillin– resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Dichrostachys cinerea L.	Fabaceae	Stem	Aqueous Dichloromethane/ Methanol	129 mg/mL 750–12,000 µg/mL	*S. mutans, S. sanguis, L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrata C. krusei*	[23]
Albizia odoratissima (L.f.) Benth	Fabaceae	Leaves	Hexane Chloroform Ethyl Acetate Methanol	7.5–15 mg/mL 859–6875 µg/mL 136–546 µg/mL 136–546 µg/mL	*S. aureus*	*K. pneumoniae, E. coli, P. aeruginosa, P. vulgaris*	-	[44]
Prosopis juliflora L.	Fabaceae	Pod	Chloroform	250 µg/mL	*M. luteus, S. aureus, S. mutans*	-	-	[36]
Bauhinia macranthera Benth. Ex Hemsl.	Fabaceae	Leaves	Aqueous, Dichloromethane/ Methanol	1.56–3.12 mg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]

Table 1. *Cont.*

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Erythrina lysistemon Hutch.	Fabaceae	Leaves	Aqueous Dichloromethane/ Methanol	4 mg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes, S. mutans, S. sanguis, L. acidophilus L. casei*	*P. aeruginosa, P. gingivalis F. nucleatum*	*T. mentagrophytes, M. canis, C. albicans C. glabrata*	[21]
Elephantorrhiza elephantina (Burch.) Skeels	Fabaceae	Leaves, roots and rhizomes	Aqueous Dichloromethane/ Methanol	1–4 mg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes, B. cereus*	*P. aeruginosa, S. flexneri*	*C. krusei T. mentagrophytes, M. canis, C. glabrata, C. krusei, T. rubrum* and *T. tonsurans*	[21]
Albizia lebbeck L.	Fabaceae	Leaves	Benzene, Aqueous and Acetone	350–600 µg/mL	*B. cereus, S. aureus*	*E. coli, K. pneumoniae, V. cholera*	*C. albicans*	[39]
Adenanthera pavonina L.	Fabaceae	Leaves	Aqueous Dichloromethane/ Methanol	5 mg/mL 60 µg mg/mL	*B. cereus, S. aureus*	-	-	[41]
Alysicarpus vaginalis L.	Fabaceae	Leaves	Aqueous Dichloromethane/ Methanol	5 mg/mL 2 mg/mL	*B. cereus, S. aureus*	-	-	[41]
Bauhinia acuminate L.	Fabaceae	Leaves	Aqueous Dichloromethane/ Methanol	5 mg/mL 50 µg mg/mL	*B. cereus, S. aureus*	-	-	[41]
Bauhinia purpurea L.	Fabaceae	Leaves	Aqueous Dichloromethane/ Methanol	5 mg/mL 1 mg/mL	*B. cereus, S. aureus*	-	-	[41]
Bauhinia racemose L.	Fabaceae	Leaves, Stem Bark	Aqueous Dichloromethane/ Methanol	500 µg/mL 500 µg/mL	*B. cereus, S. aureus*	-	-	[41]
Cassia alata L.	Fabaceae	Leaves	Aqueous Dichloromethane/ Methanol	250 µg/mL 500 µg/mL	*B. cereus, S. aureus*	-	-	[41]
Cassia auriculata L.	Fabaceae	Leaves	Aqueous Dichloromethane/ Methanol	1 mg/mL 4 mg/mL	*B. cereus, S. aureus*	-	-	[41]
Cassia fistula L.	Fabaceae	Root Bark, Stem Bark	Aqueous Dichloromethane/ Methanol	1–5 mg/mL 500–1000 µg/mL	*B. cereus, S. aureus*	-	-	[41]

Table 1. *Cont.*

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Cassia tora L.	Fabaceae	Leaves, Root Bark, Stem Bark	Aqueous Dichloromethane/ Methanol	250–4000 µg/mL 1000–4000 µg/mL	*B. cereus*, *S. aureus*	-	-	[41]
Crotalaria retusa L.	Fabaceae	Leaves	Aqueous Dichloromethane/ Methanol	4 mg/mL 60 µg/mL	*B. cereus*, *S. aureus*	-	-	[41]
Crotalaria verrucosa L.	Fabaceae	Leaves	Aqueous Dichloromethane/ Methanol	1 mg/mL 1 mg/mL	*B. cereus*, *S. aureus*	-	-	[41]
Derris Scandens L.	Fabaceae	Leaves	Aqueous Dichloromethane/ Methanol	100 µg/mL 4 mg/mL	*B. cereus*, *S. aureus*	-	-	[41]
Desmodium triflorum (L.) DC. var. majus Wight & Arn.	Fabaceae	Stem Bark	Aqueous Dichloromethane/ Methanol	1 mg/mL 25 µg/mL	*B. cereus*, *S. aureus*	-	-	[41]
Erythuria variegate L.	Fabaceae	Leaves, Stem Bark	Aqueous Dichloromethane/ Methanol	1–5 mg/mL 250–1000 µg/mL	*B. cereus*, *S. aureus*	-	-	[41]
Indigofera tinctoria L.	Fabaceae	Leaves	Aqueous Dichloromethane/ Methanol	500 µg/mL 4 mg/mL	*B. cereus*, *S. aureus*	-	-	[41]
Mimosa pudica L.	Fabaceae	Stem Bark	Aqueous Dichloromethane/ Methanol	1–2 mg/mL 250–5000 µg/mL	*B. cereus*, *S. aureus*	-	-	[41]
Myroxylon balsamum L.	Fabaceae	Leaves	Aqueous Dichloromethane/ Methanol	1 mg/mL 500 µg/mL	*B. cereus*, *S. aureus*	-	-	[41]
Pterocarpus marsupium Roxb.	Fabaceae	Leaves	Aqueous Dichloromethane/ Methanol	4 mg/mL 250 µg/mL	*B. cereus*, *S. aureus*	-	-	[41]
Pterocarpus santalinus L.	Fabaceae	Leaves	Aqueous Dichloromethane/ Methanol	2 mg/mL 4 mg/mL	*B. cereus*, *S. aureus*	-	-	[41]

Table 1. *Cont.*

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Saraca asoca (Roxb.) Willd	Fabaceae	Leaves	Aqueous Dichloromethane/ Methanol	120 µg/mL 5 mg/mL	*B. cereus*, *S. aureus*	-	-	[41]
Sesbania grandiflora (L.) Poiret	Fabaceae	Stem Bark, Root Bark, Leaves	Aqueous, Dichloromethane/ Methanol	2 mg/mL 100 µg/mL	*B. cereus*, *S. aureus*	-	-	[41]
Tamarindus indica L.	Fabaceae	Leaves	Aqueous Dichloromethane/ Methanol	250–500 µg/mL 60–100 µg/mL	*B. cereus*, *S. aureus*	-	-	[41]
Tephrosia purpurea L. Pers.	Fabaceae	Leaves	Aqueous Dichloromethane/ Methanol	5 mg/mL 5 mg/mL	*B. cereus*, *S. aureus*	-	-	[41]
Butea monosperma L.	Fabaceae	Leaves	Aqueous Dichloromethane/ Methanol Ethanol	4 mg/mL 2 mg/mL 100–200 µg/mL	*B. cereus*, *S. aureus*, methicillin-resistant *S. aureus*	-	-	[41,45]
Senna alata	Fabaceae	Leaf	Ethanol	100 µg/mL	methicillin-resistant *S. aureus*	-	-	[46]
Quercus infectoria Olivier	Fagaceae	Nutgalls	Ethanol	100–200 µg/mL	methicillin-resistant *S. aureus*	-	-	[16]
Cyclobalanopsis austroglauca Y.T. Chang	Fagaceae	TBL	Ethanol	8–256 µg/mL	methicillin-resistant *S. aureus*	-	-	[32]
Scaevola spinescens L.	Goodeniaceae	Aerial parts	Ethyl Acetate, Methanol	500 µg/mL	*S. pyogenes*, *S. aureus*	-	-	[38]
Gunnera perpensa L.	Gunneraceae	Leaves, Rhizome	Aqueous, Dichloromethane/ Methanol	4 mg/mL 750–12,000 µg/mL	*S. aureus*, *methicillin- resistant S. aureus*, *gentamycin– methicillin-resistant S. aureus*, *S. epidermidis*, *B. agri*, *P. acnes*	*P. aeruginosa*	*T. mentagrophytes*, *M. canis*	[21]
Eucomis punctate L'Her.	Hyacinthaceae	Leaves	Aqueous, Dichloromethane/ Methanol	500 µg/mL 750–12,000 µg/mL	*S. mutans*, *S. sanguis*, *L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrata C. krusei*	[23]

Table 1. *Cont.*

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Drimia sanguinea L.	Hyacinthaceae	Bulb	Pet ether	18.75, 37.5, 300, 600, 1200 µg/mL	*B. cereus*	*S. flexneri*	*C. glabrata*, *C. krusei*, *T. rubrum* and *T. tonsurans*	[2]
Hypoxis hemerocallidea L.	Hypoxidaceae	Leaves	Pet ether	195–12,500 µg/mL	*B. cereus*	*S. flexneri*	*T. rubrum*, *T.urans*, *C. glabrata C. krusei*	[47]
			Methanol	390–3125 µg/mL				
Curculigo orchioides Gaertn.	Hypoxidaceae	Whole Plant	Ethanol	8–256 µg/mL	methicillin-resistant *S. aureus*	-		[32]
Illicium simonsii Maxim.	Illiciaceae	TBL	Ethanol	8–256 µg/mL	methicillin-resistant *S. aureus*	-	-	[32]
Aristea ecklonii Baker.	Iridaceae	Leaves and Roots	Aqueous	129 mg/mL	*S. aureus*, *methicillin- resistant S. aureus*, *gentamycin– methicillin-resistant S. aureus*, *S. epidermidis*, *B. agri*, *P. acnes*	*P. aeruginosa*	*T. mentagrophytes*, *M. canis*	[21]
			Dichloromethane/ Methanol	750–12,000 µg/mL				
Tetradenia riparia Hochst.	Lamiaceae	Leaves, Stem	Aqueous	200–755 mg/mL	*S. mutans*, *S. sanguis*, *L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrata C. krusei*	[23]
			Dichloromethane/ Methanol	750–12,000 µg/mL				
Thymus vulgaris L.	Lamiaceae	Leaves	Essential Oil	50 µg/mL	methicillin-resistant *S. aureus*	-	-	[48]
Mentha aquatica L.	Lamiaceae	Aerial Parts	Methanol	1.56–3.12 mg/mL	*S. aureus*	*E. coli*, *P. aeruginosa*, *S. heidelberg*, *K. pneumoniae*, *E. aerogenes*, *M. morganii*	-	[49]
			Chloroform	128 µg/mL				
			Acetone	32–128 µg/mL				
Stachys guyoniana Noë ex. Batt.	Lamiaceae	Leaves	*n*-Butanol	4 mg/mL	*S. aureus*	*E. coli*, *P. aeruginosa*, *S. heidelberg*, *K. pneumoniae*, *E. aerogenes*, *M. morganii*	-	[49]
			Ethyl Acetate	128 µg/mL				
			Chloroform	32–128 µg/mL				
Ocimum basilicum L.	Lamiaceae	Stem, leaves	Ethanol	1–4 mg/mL	*S. aureus*	-	-	[38]
Ocimum gratissimum L.	Lamiaceae	Leaves	Methanol	780–6250 µg/mL	*S. aureus*	*S. typhi*, *E. coli*, *S. paratyphi*	-	[38]

Table 1. *Cont.*

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Ocimum sanctum L.	Lamiaceae	Whole Plant	Methanol	360 µg/mL	*S. aureus, S. saprophyticus*	*S. typhi, E. coli, S. paratyphi*	-	[6]
Mentha longifolia Huds.	Lamiaceae	Leaves	Aqueous Dichloromethane/ Methanol	150, 300, 600 µg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Melissa officinalis L.	Lamiaceae	Leaves	Ethanol	49 µg/mL	-	*K. pneumoniae*	-	[42]
Ocimum americanum L.	Lamiaceae	Leaves	Acetone	2.5 mg/mL	*B. cereus, B. pumilus, B. subtilis, S. aureus, E. faecalis*	-	-	[16]
Machilus salicina Hance.	Lauraceae	Tender Branches & Leaves	Ethanol	500 µg/mL	methicillin-resistant *S. aureus*	-	-	[32]
Meliosma squamulata Hance.	Lauraceae	TBL	Ethanol	1–4 mg/mL	methicillin-resistant *S. aureus*	-	-	[32]
Sophora alopecuroides	Leguminosae	Aerial Parts, Seeds	Ethanol	129 mg/mL	*B. subtilis, S. aureus, B. subtilis*	*P. aeruginosa*	-	[50]
Acacia karroo Hayne.	Leguminosae	Leaves, Stem	Aqueous, Dichloromethane/ Methanol	200–755 mg/mL 750–12,000 µg/mL	*S. mutans, S. sanguis, L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrata C. krusei*	[23]
Acacia polyacantha Willd.	Leguminosae	Leaves, Stem	Aqueous	50 µg/mL	*S. mutans, S. sanguis, L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrata C. krusei*	[23]
			Dichloromethane/ Methanol	750–12,000 µg/mL				
Dalbergia obovate E. Mey.	Leguminosae	Leaves, stem	Aqueous Dichloromethane/ Methanol	1.56–3.12 mg/mL 750–12,000 µg/mL	*S. mutans, S. sanguis, L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrata C. krusei*	[23]

Table 1. *Cont.*

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Sophora jaubertii	Leguminosae	Aerial Parts, Seeds	Ethanol	4 mg/mL	*B. subtilis, P. aeruginosa, S. aureus*	-	-	[38]
Glycyrrhiza glabra L.	Leguminosae	Leaves	Methanol	1–4 mg/mL	*K. kristinae, M. luteus, S. auricularis, B. megaterium*	*A. bohemicus, E. coli*	-	[51]
Allium cepa L.	Liliaceae	Bulb	Aqueous	780–6250 µg/mL	*M. tuberculosis*	-	-	[43]
Allium sativum L.	Liliaceae	Bulb	Aqueous	4% v/v	*M. tuberculosis*	-	-	[43]
Allium vera L.	Liliaceae	Gel	Aqueous	4% v/v	*M. tuberculosis*	-	-	[43]
Lobelia nicotianaefolia L.	Lobeliaceae	Root	Chloroform Acetone Ethanol	129 mg/mL 6 mg/mL 6 mg/mL	*S. aureus*	*P. aeruginosa*	-	[39]
Woodfordia fruticose L.	Lythraceae	Flower	Aqueous Dichloromethane/ Methanol	200–755 mg/mL 100 mg/mL	*S. aureus, B. cereus*	*S. typhi, E. coli, S. dysenteriae. V. cholerae*	-	[37]
Manglietia hongheensis Y.m Shui et. W.H. Chen.	Magnoliaceae	TBL	Ethanol	50 µg/mL	methicillin-resistant *S. aureus*	-	-	[32]
Malva parviflora L.	Malvaceae	Leaves	Aqueous Dichloromethane/ Methanol	500 µg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Sida rhombifolia L.	Malvaceae	Stem	Chloroform	162.2–665 mg/mL	*S. lutea, B. subtilis,*	*E. coli, Shigella shiga*	-	[38]
Walsura robusta L.	Meliaceae	Wood	Ethanol	250 µg/mL	methicillin-resistant *S. aureus*	-	-	[28]
Swietenia mahagoni	Meliaceae	Seed	Ethanol	500 µg/mL	methicillin-resistant *S. aureus*	-	-	[52]
Azadirachta indica	Meliaceae	LeavesStem	MethanolAqueous	1.56–3.12 mg/mL	*M. luteusS. aureus, S. pyogenes*	*P. vulgarisE. coli, P. aeruginosa*	–	[53]

Table 1. *Cont.*

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Ekebergia capensis Sparrm.	Meliaceae	Bark, Leaves	Aqueous Dichloromethane/ Methanol	1.59–25 mg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Trichilia emetica Vahl	Meliaceae	Leaves	Aqueous Dichloromethane/ Methanol	50–600 µg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Melia azedarach L.	Meliaceae	Leaves	Methanol Ethanol Pet.ether Aqueous	3.33–33.3 mg/mL 500 µg/mL 1.56–3.12 mg/mL 10–30 mg/mL	*B. cereus, S. aureus*	*E. coli, P. aeruginosa*	*A. niger, A. flavus, F. oxysporum, R. stolonifer*	[16]
Melianthus comosus Vahl.	Melianthaceae	Leaves	Aqueous Dichloromethane/ Methanol	50 mg/mL 4–64 mg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes,* methicillin-resistant *S. aureus*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[28]
Melianthus major L.	Melianthaceae	Leaves	Ethanol	10–100 mg/mL	methicillin-resistant *S. aureus*	-	-	[28]
Melianthus major L.	Melianthaceae	Leaves	Aqueous Dichloromethane/ Methanol	5–50 mg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Cissampelos torulosa E. Mey. Ex Harv.	Menispermaceae	Leaves, Stem	Aqueous Dichloromethane/ Methanol	25, 50, 100 mg/mL 750–12,000 µg/mL	*S. mutans, S. sanguis, L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrata C. krusei*	[23]
Tinospora crispa L.	Menispermaceae	Stem	Ethanol	10 mg/mL	methicillin-resistant *S. aureus*	-	-	[21]
Cissampelos capensis Thunb.	Menispermaceae	Leaves	Aqueous Dichloromethane/ Methanol	3.33–33.3 mg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]

Table 1. *Cont.*

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Ficus natalensis Hochst.	Moraceae	Leaves	Aqueous Dichloromethane/ Methanol	250 mg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Ficus sur Forssk.	Moraceae	Bark, Leaves	Aqueous, Dichloromethane/ Methanol	10–100 mg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Moringa oleifera Lam.	Moringacceae	Leaf	Ethanol	5–50 mg/mL	methicillin-resistant *S. aureus*	-	-	[28]
Myrothamnus flabellifolia Welw.,	Myrothamnaceae	Leaves	Aqueous Dichloromethane/ Methanol	156–625 µg/mL 750–12,000 µg/mL	*S. mutans, S. sanguis, L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrata C. krusei*	[23]
Embelia ruminate (E. Mey.exA.Dc.) Mez	Myrsinaceae	leaves	Aqueous Dichloromethane/ Methanol	350–600 µg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Embelia burm f.	Myrsinaceae	Leaves	Ethanol	500 µg/mL	methicillin-resistant *S. aureus*	-	-	[32]
Callistemon rigidus R.Br.	Myrtaceae	Leaf	Methanol	800 mg/disc	methicillin-resistant *S. aureus*	-	-	[28]
Psidium guajava L.	Myrtaceae	Leaf	Ethanol	600, 1200 µg/mL	methicillin-resistant *S. aureus*	-	-	[28]
Heteropyxis natalenesis Harv.	Myrtaceae	Leaves, Stem	Aqueous, Dichloromethane/ Methanol	5 mg/mL 750–12,000 µg/mL	*S. mutans, S. sanguis, L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrata C. krusei*	[23]
Eucalyptus camaldulensis Dehnh.	Myrtaceae	Bark	Aqueous Dichloromethane/ Methanol	9.375, 18.75, 37.5, 75, 150, 300, 600 µg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]

Table 1. *Cont.*

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Eucalyptus deglupta	Myrtaceae	Leaves	Benzene Aqueous Acetone	37.5, 75, 150, 300, 600 µg/mL 4–8 mg/mL 6 mg/mL	*B. cereus, S. aureus*	*E. coli, K. pneumoniae, V. cholerae*	*C. albicans*	[39]
Myrtus communis L.	Myrtaceae	Leaves	Ethanol	12.5–50 mg/mL	*B. cereus, L. monocytogenes*	*E. coli*	*C. albicans*	[42]
Nelumbo nucifera L.	Nelumbonaceae	Flower	Ethanol	8–32 mg/mL	*B. subtilis, S. aureus,*	*E. coli, K. pneumonia, P. aeruginosa*	-	[54]
Nymphaea lotus L.	Nymphaeaceae	Leaf	Ethanol	500 µg/mL	methicillin-resistant *S. aureus*	-	-	[21]
Oxalis corniculata L.	Oxalidaceae	Leaves	Aqueous Benzene Acetone	5 mg/mL 37.5, 75, 150, 300, 600 µg/mL 6 mg/mL	*B. cereus, S. aureus*	*E. coli, K. pneumoniae, V. cholera*	*C. albicans*	[39]
Paeonia lactiflora Pall.	Paeoniaceae	Leaves	Ethanol	22.4–52.3 µg/mL	*K. kristinae, M. luteus, S. auricularis, B. megaterium*	*A. bohemicus, E. coli*	-	[51]
Argemone mexicana	Papaveraceae	Stem	Chloroform	32.4–55.8 µg/mL	*S. aureus*	*E. coli, P. aeruginosa, k. pneumoniae*	-	[55]
Passiflora Mexicana L.	Passifloraceae	Aerial Parts	Ethanol	33.7–58.3 µg/mL	*S. aureus*	-	-	[21]
Cleistanthus collinus	Phyllanthaceae	Leaves	Benzene Aqueous Acetone	100 mg/mL 4–8 mg/mL 5 mg/mL	*B. cereus, S. aureus*	*E. coli, K. pneumoniae, V. cholerae*	*C. albicans*	[39]
Piper nigrum L.	Piperaceae	Bark, Seeds	Ethanol Acetone Dichloromethane/ Methanol	500 µg/mL 6 mg/mL 12.5–50 µg/mL	*S. aureus, B. cereus, S. fecalis*	*P. aeruginosa, E. coli, S. typhi*	-	[38]

Table 1. *Cont.*

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Pittosporum viridiflorum Sims.	Pittosporaceae	Leaves	Aqueous Dichloromethane/ Methanol	600 µg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Spinifex littoreus	Poaceae	Grass	Acetone	2.5 mg/mL	-	-	Dermatophytes	[27]
Polygonum molle D. Don.	Polygonaceae	Whole Plant	Ethanol	25–50 µg/mL	Methicillin-resistant *S. aureus*	-	-	[32]
Eichhornia crassipes L.	Pontederiaceae	Leaves, Shoot	Ethanol Chloroform Aqueous	500–4000 µg/mL 32.4–55.8 µg/mL 2.5–15 µg/mL	*M. luteus*	*R. rubrum*	*M. ruber, A. fumigates*	[56]
Punica granatum L.	Punicaceae	Fruit Shell	Ethanol	70 mg/mL	Methicillin-resistant *S. aureus*	-	-	[28]
Clematis brachiate Thunb.	Ranunculaceae	Flower, Leaves, Stem, Root	Aqueous, Dichloromethane/ Methanol	1 mg/mL 750–12,000 µg/mL	*S. mutans, S. sanguis, L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrata C. krusei*	[23]
Ziziphus mucronata Willd.	Rhamnaceae	Bark, Leabes	Aqueous Dichloromethane/ Methanol	2.5 mg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes, S. mutans, S. sanguis, L. acidophilus L. casei*	*P. aeruginosa, P. gingivalis F. nucleatum*	*T. mentagrophytes, M. canis, C. albicans C. glabrata C. krusei*	[21]
Eriobotrya japonica (Thunb.) Lindl.	Rosaceae	Leaves	Ethanol	2–16 µg/mL	*K. kristinae, M. luteus, S. auricularis, B. megaterium*	*A. bohemicus, E. coli*		[51]
Pavetta crassipes K. Schum.	Rubiaceae	Leaf	Methanol	12.5–50 mg/mL	methicillin-resistant *S. aureus*	-	-	[28]
Uncaria gambir (Hunter) Roxb.	Rubiaceae	Leaf, Stem	Ethanol	8–32 mg/mL	methicillin-resistant *S. aureus*	-	-	[28]
Vangueria spinose L.	Rubiaceae	Leaves	Ethyl Acetate	500 µg/mL	*S. aureus*	*E. coli, K. pneumoniae, P. aeruginosa*	-	[57]

Table 1. *Cont.*

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Pentanisia prunelloides Walp.	Rubiaceae	Root Bark	Aqueous Dichloromethane/ Methanol	5 mg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Rothmannia capensis Thunb.	Rubiaceae	Leaves	Aqueous Dichloromethane/ Methanol	22.4–52.3 µg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Geophila repens L.	Rubiaceae	Leaves, Stem Bark	Aqueous Dichloromethane/ methanol	1 mg/mL 250 µg/mL	*B. cereus, S. aureus*	-	-	[41]
Guettarda speciose L.	Rubiaceae	Leaves	Aqueous Dichloromethane/ Methanol	2 mg/mL 2 mg/mL	*B. cereus, S. aureus*	-	-	[41]
Haldina cordifolia L.	Rubiaceae	Leaves	Aqueous Dichloromethane/ Methanol	1 mg/mL 500 µg/mL	*B. cereus, S. aureus*	-	-	[41]
Hedyotis auricularia L.	Rubiaceae	Leaves	Aqueous Dichloromethane/ Methanol	300 µg/mL 250 µg/mL	*B. cereus, S. aureus*	-	-	[41]
Knoxia zeylanica L.	Rubiaceae	Leaves, Stem	Aqueous Dichloromethane/ Methanol	250 µg/mL 1 mg/mL	*B. cereus, S. aureus*	-	-	[41]
Mitragyna parvifolia L.	Rubiaceae	Leaves	Aqueous Dichloromethane/ Methanol	300 µg/mL 1 mg/mL	*B. cereus, S. aureus*	-	-	[41]
Morinda umbellate L.	Rubiaceae	Leaves, Stem Bark	Aqueous Dichloromethane/ Methanol	100 µg/mL 250 µg/mL	*B. cereus, S. aureus*	-	-	[41]
Nauclea orientalis L.	Rubiaceae	Leaves	Aqueous Dichloromethane/ Methanol	500 µg/mL 500 µg/mL	*B. cereus, S. aureus*	-	-	[41]
Oldenlandia biflora L.	Rubiaceae	Leaves	Aqueous Dichloromethane/ Methanol	2 mg/mL 5 mg/mL	*B. cereus, S. aureus*	-	-	[41]
Oldenlandia herbacea L.	Rubiaceae	Stem, Root	Aqueous Dichloromethane/ Methanol	5mg/mL 60 µg/mL	*B. cereus, S. aureus*	-	-	[41]

Table 1. *Cont.*

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Ophiorrhiza mungos L.	Rubiaceae	Leaves	Aqueous Dichloromethane/ Methanol	2 mg/mL 500 µg/mL	*B. cereus, S. aureus*	-	-	[41]
Paederia foetida L.	Rubiaceae	Leaves, Stem	Aqueous Dichloromethane/ Methanol	300 µg/mL 60 µg/mL	*B. cereus, S. aureus*	-	-	[41]
Pavetta lanceolate Eckl.	Rubiaceae	Leaves	Aqueous Dichloromethane/ Methanol	1 mg/mL 250 µg/mL	*B. cereus, S. aureus*	-	-	[41]
Spermacoce hispida L.	Rubiaceae	Leaves, Stem	Aqueous Dichloromethane/ Methanol	300 µg/mL 120 µg/mL	*B. cereus, S. aureus*	-	-	[41]
Wendlandia bicuspidate Wight & Arn.	Rubiaceae	Leaves	Aqueous Dichloromethane/ Methanol	60 µg/mL 5 mg/mL	*B. cereus, S. aureus*	-	-	[41]
Chassalia kolly	Rubiaceae	Whole Plant	Mthanol	5 mg/mL	*S. aureus*	*E. coli, P. aeruginosa, S. typhi, P. aeruginosa*	-	[16]
Randia dumetorum L.	Rubiaceae	Fruits	Methanol	9.375, 18.75, 37.5, 75, 150, 300, 600 µg/mL	*S. aureus, S. epidermidis, B. subtilis*	*E. coli, S. typhi*	-	[23]
Mitragyna speciosa L.	Rubiaceae	Leaves	Methanol	37.5, 75, 150, 300, 600 µg/mL	*S. typhi*			[42]
Clausena anisate (Willd) Hook. f. ex.	Rutaceae	Leaves, Stem, Twigs	Aqueous Dichloromethane/ Methanol	12.5–50 mg/mL 750–12,000 µg/mL	*S. mutans, S. sanguis, L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrata C. krusei*	[23]
Zanthoxylum capense Harv.	Rutaceae	Stem	Aqueous Dichloromethane/ Methanol	8–32 mg/mL 750–12,000 µg/mL	*S. mutans, S. sanguis, L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrata C. krusei*	[23]
Aegle marmelos L.	Rutaceae	Leaves and Fruits	Methanol	500 µg/ml	*S.aureus, B. cereus*	*E. coli, S. typhi, P. aeruginosa, S. boydii, K. aerogenes, P.vulgaris,*		[20]

Table 1. *Cont.*

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Evodia daneillii (Benn) Hemsl.	Rutaceae	Tender Branches & Leaves	Ethanol	3.33–33.3 mg/mL	Methicillin-resistant *S. aureus*	-	-	[32]
Skimmia arborescens Anders.	Rutaceae	TBL	Ethanol	250 mg/mL	Methicillin-resistant *S. aureus*	-	-	[32]
Salvadora australis	Salvadoraceae	Leaves	Acetone	10–100 mg/mL	*B. cereus, B. pumilus, B. subtilis, S. aureus, E. faecalis*	-	-	[18]
Viscum capense L.f.	Santalaceae	Leaves	Aqueous Dichloromethane/ Methanol	5–50 mg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Dodonaea angustifolia (L.f.) Benth	Sapindaceae	Leaves	Ethanol	156–625 µg/mL	methicillin-resistant *S. aureus*	-	-	[28]
Dodonaea viscosa Jacq.	Sapindaceae	Leaves, Stem	Aqueous Dichloromethane/ Methanol	350–600 µg/mL 750–12,000 µg/mL	*S. mutans, S. sanguis, L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrata C. krusei*	[23]
Cardiospermum halicacabum L.	Sapindaceae	Leaves	*n*-Butanol Ethyl acetate Chloroform	500 µg/mL 60 µg/mL 40 µg/mL	*S. aureus, S. agalactiae*	*E. coli, S. typhimurium* and *K. pneumoniae*	*T. rubrum, C. albicans*	[58]
Dodonaea angustifolia L. f.	Sapindaceae	Leaves	Aqueous Dichloromethane/ Methanol	800 mg/disc 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Englerophytum magalismontanum Sonder.	Sapotaceae	Leaves, Stem	Aqueous Dichloromethane/ Methanol	600, 1200 µg/mL 750–12,000 µg/mL	*S. mutans, S. sanguis, L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrataC. krusei*	[23]
Schisandra viridis A.c. Smith.	Schisandraceae	Vane	Ethanol	5 mg/mL	Methicillin-resistant *S. aureus*	-	-	[32]

Table 1. *Cont.*

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Halleria lucida L.	Scrophulariaceae	Leaves Stem	Aqueous Dichloromethane/ Methanol	1–8 mg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Brandisia hancei Hook.f.	Scrophulariaceae	Whole Plant	Ethanol	3.33–33.3 mg/mL	Methicillin-resistant *S. aureus*	-	-	[32]
Selaginella tamariscina (Seauv.) Spring.	Selaginellaceae	Whole Plant	Ethanol	250 mg/mL	Methicillin-resistant *S. aureus*	-	-	[32]
Datura stramonium L.	Solanaceae	Leaves, Stem, Fruit	Aqueous Dichloromethane/ Methanol	10–100 mg/mL 750–12,000 µg/mL	*S. mutans, S. sanguis, L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrata C. krusei*	[23]
Solanum incanum L	Solanaceae	Leaves	Aqueous Dichloromethane/ Methanol	5–50 mg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Solanum trilobatum L.	Solanaceae	Leaves	Acetone Aqueous Benzene Butanol Chloroform Ethanol	156–625 µg/mL 250 mg/mL 10–100 mg/mL 5–50 mg/mL 60 µg/mL 5 mg/mL	*S. pyogens, S. aureus, B. subtilis*	*S. typhi, P. vulgaris, K. pneumoniae, P. aeruginosa, E. coli*	-	[37]
Datura metel L.	Solanaceae	Leaves	Aqueous Dichloromethane/ Methanol	350–600 µg/mL 1 mg/mL	*B. cereus, S. aureus*	-	-	[41]
Solanum macrocarpon L.	Solanaceae	Leaves, Stem	Aqueous Dichloromethane/ Methanol	500 µg/mL 60 µg/mL	*B. cereus, S. aureus*	-	-	[41]

Table 1. *Cont.*

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Solanum melongena L.	Solanaceae	Leaves, Root Stem	Aqueous Dichloromethane/ Methanol	800 mg/disc 100 µg/mL	*B. cereus*, *S. aureus*	-	-	[41]
Solanum nigrum L.	Solanaceae	Leaves, Stem	Aqueous Dichloromethane/ Methanol	600, 1200 µg/mL 1 mg/mL	*B. cereus*, *S. aureus*	-	-	[41]
Solanum torvum Sw.	Solanaceae	Leaves	Aqueous Dichloromethane/ Methanol	3.33–33.3 mg/mL 60 µg/mL	*B. cereus*, *S. aureus*	-	-	[41]
Solanum virginianum L.	Solanaceae	Leaves, Stem, Root	Aqueous Dichloromethane/ Methanol	250 mg/mL 4 mg/mL	*B. cereus*, *S. aureus*	-	-	[41]
Withania somnifera (L.) Dunal	Solanaceae	Roots & Leaves	Aqueous Dichloromethane/ Methanol	10–100 mg/mL 1 mg/mL	*B. cereus*, *S. aureus*, methicillin-resistant *S. aureus*	-	-	[41,59]
Cola acuminate L.	Sterculiaceae	Stem	Acetone Methanol	5–50 mg/mL 100 µg/mL	*S. aureus*	-	*C. albicans*	[16]
Schima sinensis (Hemsl. et. Wils) Airy-shaw.	Theaceae	Tbl	Ethanol	156–625 µg/mL	methicillin-resistant *S. aureus*	-	-	[32]
Coriandrum sativum	Umbelliferae	Seeds	Aqueous	350–600 µg/mL	*S. aureus*	*K. pneumoniae*, *P. aeruginosa*,	*A. niger*, *P. lilacinum*	[27]
Clerodendrum inerme L	Verbenaceae	Leaves	Methanol	500 µg/mL	*S. aureus*	-	*A. niger*	[60]
Lantana rugosa Thunb.	Verbenaceae	Leaves	Aqueous Dichloromethane/ Methanol	800 mg/disc 750–12,000 µg/mL	*S. aureus*, *methicillin- resistant S. aureus*, *gentamycin– methicillin-resistant S. aureus*, *S. epidermidis*, *B. agri*, *P. acnes*	*P. aeruginosa*	*T. mentagrophytes*, *M. canis*	[21]

Table 1. *Cont.*

Botanical Name	Family	Plant Used	Extracts	MIC *	Gram Positive	Gram Negative	Fungi	References
Lantana camara L.	Verbenaceae	Leaves, Flower	Chloroform Acetone Methanol Aqueous	600, 1200 µg/mL 5 mg/mL 1–8 mg/mL 1–2 mg/mL	*S. aureus, B. cereus*	*E. coli, S. typhi, P. aeruginosa, K. aerogenes, P. vulgaris, S. Boydii, K. pneumoniae, V. cholerae*	*A. fumigatus, A. flavus, A. niger, C. albicans*	[39]
Lantana indica L.	Verbenaceae	Leaves	Methanol Aqueous	3.33–33.3 mg/mL 4 mg/mL	*B. subtilis, S. aureus, S. pyogenes,*	*E. coli, P. vulgaris, K. pneumoniae*	*C.albicans,*	[61]
Cyphostemma lanigerum Harv.	Vitaceae	Leaves, Stem	Aqueous Dichloromethane/ Methanol	250 mg/mL 750–12,000 µg/mL	*S. mutans, S. sanguis, L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrata C. krusei*	[23]
Cyphostemma setosum Roxb.	Vitaceae	Leaves, Stem, Fruit	Aqueous Dichloromethane/ Methanol	10–100 mg/mL 750–12,000 µg/mL	*S. mutans, S. sanguis, L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrata C. krusei*	[23]
Aloe arborescens Mill.	Xanthorrhoeaceae	Leaves	Aqueous Dichloromethane/ Methanol	5–50 mg/mL 750–12,000 µg/mL	*S. aureus, methicillin- resistant S. aureus, gentamycin– methicillin-resistant S. aureus, S. epidermidis, B. agri, P. acnes*	*P. aeruginosa*	*T. mentagrophytes, M. canis*	[21]
Siphonochilus aethiopicus Schweinf.,	Zingiberaceae	Leaves, Stem, Root	Aqueous Dichloromethane/ Methanol	156–625 µg/mL 750–12,000 µg/mL	*S. mutans, S. sanguis, L. acidophilus L. casei*	*P. gingivalis F. nucleatum*	*C. albicans C. glabrata C. krusei*	[23]
Curcuma xanthorrhiza	Zingiberaceae	Rhizome	Ethanol	350–600 µg/mL	methicillin-resistant *S. aureus*	-	-	[46]
Kaempferia pandurata Roxb.	Zingiberaceae	Rhizome	Ethanol	500 µg/mL	methicillin-resistant *S. aureus*	-	-	[46]
Peganum harmala L.	Zygophyllaceae	Seeds	Ethanol	800 mg/disc	*S. aureus*	*E. coli*	-	[21]

* **MIC** (minimum inhibitory concentration) is the lowest drug concentration at which a given antimicrobial extract inhibits the visible growth of a tested organism. **MIC absolute value**: the given absolute value of drug concentration inhibits the growth of all tested organisms/ **MIC ranges**: the given range of drug concentrations (minimum to maximum) inhibit the growth of the individual to all tested organisms.

Phytocomponent Fractions and Antimicrobial Methods

Fresh or dried plant extracts were prepared using aqueous and different organic solvents in traditional extraction techniques (maceration, percolation, Soxhlet extraction). During the extraction method, the solvents penetrate into the plant material and dissolve active compounds with a related polarity [62]. At the completion of the technique, solvents have been vaporized, resulting in the formation of a concentrated mixture that yields the active compounds [63]. A successful extraction is mainly reliant on the nature of the solvent utilized during the extraction. The most regularly established extracts are aqueous extract followed by organic solvents, which include using methanol, ethanol, hexane, isopropanol, ethyl acetate, benzene, acetone, chloroform, and dichloromethane [64].

Two popular types of antibacterial susceptibility test, namely diffusion and dilution methods, are generally performed to determine the antibacterial efficacy of the plant materials. The method of diffusion is a screening test to classify bacteria that aid susceptibility or resistance to the tested plant material based on the size or diameter of the inhibition zone [62]. On the other hand, the activity of plant materials is determined as minimum inhibitory concentration (MIC) in the dilution method. In the MIC method, the lowest concentration is capable of inhibiting bacterial growth. Redox indicators and turbidity are most often measured for the analysis of results in broth dilution methods. The turbidity can be calculated colorimetrically while changing the indicator color represents the inhibition of bacterial growth [62]. The screening of traditional plant extracts has been of great attention to researchers investigating novel bioactive compounds effective in the treatment of microbial infections. Plant extracts exhibit: (a) direct antimicrobial activity presenting effects on metabolism and development of microbes and (b) indirect activity as antibiotic resistance adapting substances which, joint with antibiotics, upsurge their efficiency. Numerous studies have considered the antimicrobial screening of traditional plant extracts. The studies of medicinal plants from diverse topographical areas include: Armenia [65], Iran [66], Mexico [67], Saudi Arabia [68], Libya [26], Ethiopia [64], India [63], Poland [69], Cameroon [70], Nigeria [71], and other Middle Eastern countries [72]. Based on the available information, the traditional plant extracts showed antimicrobial activity against a huge number of pathogenic bacteria, fungi, viruses, algae, protozoan, and Trypanosoma [26,63,64,66].

3. Bioactive Compounds (Bioactive Phytocomponents)

Traditional medicinal plants possess various chemical substances that support certain physiological and biochemical activities in the human body and they are known as phytochemicals or phytocomponents. These chemicals are non-nutritive substances used to heal various infectious diseases, as well as provide disease preventive properties [9,10]. With advances in phytochemical practices, numerous active principles have been isolated from medicinal plants and presented as a valuable drug in contemporary systems of medicine. Mostly, the pharmacological activity of medicinal plants resides in their secondary metabolites, which are relatively smaller in quantity in contrast to the primary molecules such as carbohydrates, proteins, and lipids. Plant secondary metabolites are commonly accountable for their antimicrobial properties [62]. These metabolites offer clues to manufacture new structural types of antimicrobial and antifungal chemicals that are comparatively safe to humans [62]. The classes of secondary metabolites that have greater antimicrobial properties are flavonoids (flavones, flavonols, flavanols, isoflavones, anthocyanidins), phenolic acids (hydroxybenzoic, hydroxycinnamic acids), stilbenes, lignans, quinones, tannins, coumarins (simple coumarins, furanocoumarins, pyranocoumarins), terpenoids (sesquiterpene lactones, diterpenes, triterpenes, polyterpenes), alkaloids, glycosides, saponins, lectins, steroids, and polypeptides [6,16,56,62,73–83]. These compounds have copious mechanisms that underlie antimicrobial activity, e.g., disturbing microbial membranes, weakening cellular metabolism, control biofilm formation, inhibiting bacterial capsule production, attenuating bacterial virulence by controlling quorum-sensing, and reducing microbial toxin production [3–6,73–85]. Various bioactive compounds have been scientifically tested for their antimicrobial activities and are presented in Table 2.

Table 2. Antimicrobial activities of bioactive compounds.

Botanical Name	Family	Extracts	Bioactive Compounds	MIC *	Organism Inhibited	References
Allium sativum L.	Alliaceae	Methanol	Cyanidin-3-(6′-malonyl)-glucoside, vanillic acid caffeic acid, p-coumaric acid, ferulic acid, sinapic acid, L-alliin, alliin isomer and methiin	-	*B. cereus, L. monocytogenes S. aureus, P. aeruginosa, E. coli*	[11]
Searsia chirindensis (Baker f.) Moffett	Anacardiaceae	Ethanol	Methyl gallate myricetin-3-O-arabinopyranoside myricetrin-3-O-rhamnoside kaempferol-3-O-rhamnoside quercetin-3-O-arabinofuranoside	30–130 µg/mL 60–250 µg/mL 60–250 µg/mL 130–250 µg/mL 250 µg/mL	*C. jejuni, E. coli, S. flexneri, S. aureus*	[86]
		Dichloromethane/ Methanol		250–6250 µg/mL		
		n-butanol		130–3125 µg/mL		
		Ethyl Acetate		60–780 µg/mL		
		Crude		60–780 µg/mL		
Xylopia aethiopica (Dunal) A. Rich.	Annonaceae	Aqueous	1R-a-Pinene, β-Pinene, 2-Carene, Cyclohexene,5-methyl-3-(1-methylethenyl)-trans-(-)-Bicyclo [3.1.0] hexane,6-isopropylidene-1-methyl-, Eucalyptol, Ethyl 2-(5-methyl-5-vinyltetrahydrofuran-2-yl) propan-2-yl carbonate, Isogeraniol, α-Campholenal, L-trans-Pinocarveol, Pinocarvone, Myrtenal, (-)-Spathulenol	1–256 µg/mL	*S. aureus, B. licheniformis, E. coli, K. pneumoniae*	[87]

Table 2. *Cont.*

Botanical Name	Family	Extracts	Bioactive Compounds	MIC *	Organism Inhibited	References
Polyalthia cerasoides	Annonaceae	Hexane Dichloromethane	N-(4-hydroxy-β-phenethyl-4-hydroxy cinnamide	64–128 µg/mL 32–256 µg/mL	*C. diphtheria, B. subtilis, B. cereus, M. lutens*	[88]
Unonopsis lindmanii R. E. Fries	Anonaceae	Hexane	Gallic acid, kaempferol, ellagic acid, epicatechin, vitexin, corilagin	25–250 µg/mL	*C.albicans*	[89]
Allagoptera leucocalyx (Drude) Kuntze,	Arecaceae	Hexane	Gallic acid, kaempferol, ellagic acid, epicatechin, vitexin, corilagin	162.2–665 mg/mL	*C.albicans*	[89]
Bactris glaucescens Drude	Arecaceae	Hexane	Gallic acid, kaempferol, ellagic acid, epicatechin, vitexin, corilagin	200–755 mg/mL	*C.albicans*	[89]
Scheelea phalerata Mart	Arecaceae	Hexane	Gallic acid, kaempferol, ellagic acid, epicatechin, vitexin, corilagin	129 mg/mL	*C.albicans*	[89]
Artemisia herba-alba Asso	Asteraceae	Aqueous	1,8-cineole, β-thujone, α-thujone, camphor	640–2500 µg/mL	*T. rubrum and E. floccosum*	[90]
Vernonia adoensis Sch. Bip. ex Walp.	Asteraceae	Acetone	Chondrillasterol	50 µg/mL	*S. aureus, K. pneumonia, P. aeruginosa*	[1]

Table 2. *Cont.*

Botanical Name	Family	Extracts	Bioactive Compounds	MIC *	Organism Inhibited	References
Matricaria chamomilla	Asteraceae	Ethanol	Phenolic acid	1.56–3.12 mg/mL	*S. typhimurium*	[19]
Solidago graminifolia L. Salisb.	Asteraceae	Ethanol Methanol Aqueous	di-C-glycosylflavones (schaftoside, isoschaftoside), caftaric acid, gentisic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid, hyperoside, rutin, quercitrin, quercetin, Luteolin, kaempferol, gallic acid, protocatechuic acid, vanillic acid, syringic acid, rosmarinic acid	40–3120 µg/mL 90–3120 µg/mL 190–6250 µg/mL	*S. aureus, C. albicans, C. parapsilosis.*	[12]
Baccharis trimera	Asteraceae	Crude	Polyphenols, flavonoids, alkaloids, and terpenes	7.8–500 µg/mL	*E. coli, S. aureus, P. aeruginosa, C. albicans, C. tropicalis, C. parapsilosis, Epicoccum sp., C. sphaerospermum, C. neoformans, P. brasiliensis, C. gatti, Pestalotiopsis sp., C. lunatus, Nigrospora sp.*	[88]
Tecoma stans	Bignoniaceae	Aqueous	Phenolic compounds	50–600 µg/mL	*S. aureus*	[91]
Bixa orellana L.	Bixaceae	Aqueous	Bixin, catechin, chlorogenic acid, chrysin, butein, hypolaetin, licochalcone A, and xanthohumol.	16–32 µg/mL	*B. cereus, S. aureus*	[9]
Trichodesma indicum	Boraginaceae	Ethanol	Lanast-5-en-3β-D- glucopyranosyl-21(24)-oilde	2.4–19.2 µg/mL	*S. aureus*	[92]

Table 2. *Cont.*

Botanical Name	Family	Extracts	Bioactive Compounds	MIC *	Organism Inhibited	References
Boswellia dalzielii Hutch.	Burseraceae	Crude	Oleic acid, squalene and n-hexadecanoic acid	-	*S. pyogenes*, *S. aureus*, *E. coli*, *E. faecalis*, *K. pneumonia*, *P. aeruginosa*, *P. mirabilis*, *S. typhi*, and *C. albicans*	[93]
Caesalpinia coriaria (Jacq) Willd	Caesalpiniaceae	Aqueous Ethanol	Methyl gallate and gallic acid	1.56–25 mg/mL 390–6250 µg/mL	S. typhi, E. coli, P. aeruginosa, L. monocytogenes, S. aureus.	[94]
Senna aculeate (Bth.) Irw et Barn	Ceasalpinioideae	Hexane	Gallic acid, kaempferol, ellagic acid, epicatechin, vitexin, corilagin	25, 50, 100 mg/mL	*C.albicans*	[89]
Kochia scoparia	Chenopodiaceae	Crude	Polyphenols, flavonoids, alkaloids, and terpenes	3.125 mg/mL	*C. graminicola*, *T. deformans*, *A. flavus*, *H. carbonum*, *C. zeaemaydis*, *C. macrocarpum*, *P. innundatus*, *S. japonicas*, *E. ficariae*, *P. herbarum*, *M. verticillata*, *Rhisoclosmatium* sp., *S. pseudodichotomus*, *S. kneipii*, *R. solani*, *P. sojae*.	[8]
Buchenavia tomentosa (Mart) Eichler	Combretaceae	Hexane	Gallic acid, Kaempferol, Ellagic acid, epicatechin, Vitexin, Corilagin	10 mg/mL	*C.albicans*	[89]

Table 2. *Cont.*

Botanical Name	Family	Extracts	Bioactive Compounds	MIC *	Organism Inhibited	References
Terminalia phanerophlebia Engl. & Diels	Combretaceae	Crude Dichloromethane Hexane Ethyl Acetate n-butanol	Methyl gallate (methyl-3,4,5-trihydroxybenzoate) and a phenylpropanoid glucoside, 1,6-di-O-coumaroyl glucopyranoside	125 µg/mL 16–250 µg/mL 31–250 µg/mL 8–125 µg/mL 31–250 µg/mL	*M. aurum*, *M. tuberculosis*, *S. aureus*, *K. pneumoniae*	[95]
Buchenavia tomentosa L.	Combretaceae	Crude	Gallic acid, quinic acid, kaempferol, (-) epicatechin, ellagic acid, buchenavianine, eschweilenol b, eschweilenol c, vitexin, corilagin, 1α,23β-dihydroxy-12-oleanen-29-oicacid-23β-o-α-l-4-acetylramnopiranoside and punicalin	200–12500 µg/mL	*Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Candida glabrata*, *Candida krusei and Candida dubliniensis.*	[96]
Diadema setosum f. depressa Dollfus & Roman.	Diadematidae	Acetone	Polyunsaturated fatty acids (PUFAs) and β-carotene	500–4000 µg/mL	*S. typhi*, *S. typhimurium*, *S. flexneri*, *P. aeruginosa*, *A. hydrophila*, *Acinetobacter sp*, *C. freundii and K. pneumonia*, *B. subtilis*, *S. epidermidis S. aureus*	[1]
Monotes kerstingii Gilg	Dipterocarpaceae	Crude	Stilbene-coumarin derivative, coumarin-carbinol and fatty glycoside	1–8 mg/mL	*B. subtilis*, *Septoria tritici* Desm	[7]
Croton doctoris S Moore	Euphorbiaceae	Hexane	Gallic acid, kaempferol, ellagic acid, epicatechin, vitexin, corilagin	500 µg/mL	*C.albicans*	[89]

Table 2. *Cont.*

Botanical Name	Family	Extracts	Bioactive Compounds	MIC *	Organism Inhibited	References
Jatropha weddelliana Baillon	Euphorbiaceae	Hexane	Gallic acid, kaempferol, ellagic acid, epicatechin, vitexin, corilagin	4–32 µg/mL	*C.albicans*	[89]
Cassia alata	Fabaceae	Ethanol	4-butylamine, cannabinoid, dronabinol, methyl-6-hydroxy	1.25, 1.5 mg/mL	*S. aureus, E. coli, P. aeruginosa, C. albicans*	[28]
Dalbergia scandens Roxb., Corom.	Fabaceae	Ethanol	Dalpanitin, vicenin-2 and 3, rutin	780–6250 mg/mL	*B. cereus, S. aureus, E. coli, P. aeruginosa, C. albicans*	[41]
Acacia nilotica	Fabaceae	Crude	Alkaloids	600–1200 µg/mL	*S. aureus*	[27]
Salvia sessei Benth	Lamiaceae	Hexane Dichloromethane Methanol	Sessein, isosessein	12.5–100 µg/mL 100 µg/mL 12.5–100 µg/mL	*S. haemolyticus, S. hominis, E. faecalis, S. epidermis, S. pyogenes, S.aureus*	[14]
Mentha piperita	Lamiaceae	Methanol	1,1-diphenyl-2-picrylhydazyl-hydrate	1–4 mg/mL	*S. aureus, E. coli, C. albicans*	[97]

Table 2. *Cont.*

Botanical Name	Family	Extracts	Bioactive Compounds	MIC *	Organism Inhibited	References
Ocimum basilicum L.	Lamiaceae	Ethanol	Gallic acid, 3,4-dihydroxy benzoic acid, 4-hydroxy benzoic acid, 2,5 dihydroxybenzoic acid, chlorogenic acid, vanillic acid, Epicatechin, caffeic acid, p-coumaric acid, ferulic acid, rutin, ellagic acid, naringin, quercetin, cinnamic acid, α-pinene, camphene, sabinene, β-pinene, myrcene, 3-octanol, α-terpinene, *p*-cymene, limonene, 1,8-cineole, *(Z)*-β-ocimene, *(E)*-β-ocimene, γ-terpinene, *cis*-sabinene hydrate, terpinolene, linalool, nonanal, pentylisovalerate, 1-octen-3-yl acetate, *cis*-*p*-menth-2-en-1-ol, 3-octyl acetate, α-campholenal, camphor, *trans*-verbenol, δ-terpineol, 4-terpineol, α-terpineol, *cis*-dihydrocarvone, *trans*-carveol, *(Z)*-3-hexenyl isovalerate, pulegone, neral, carvone, linalyl acetate, bornyl acetate, dihydroedulan IA, isodihydrocarvyl acetate, α-terpinyl acetate, *cis*-carvyl acetate, neryl acetate, geranyl acetate, β-elemene, *(Z)*-jasmone, β-caryophyllene, β-copaene, aromadendrene, α-humulene, *(E)*-β-farnesene, *cis*-muurola-4(14), 5-diene germacrene D, bicyclogermacrene, germacrene A, δ-cadinene, *(E)*-α-bisabolene, *(E)*-nerolidol, Spathulenol, caryophyllene oxide, viridiflorol, 1, 10-di-*epi*-cubenol, T-cadinol, T-muurolol, monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, apocarotenes non-terpene derivatives	16–256 µg/mL	*S. epidermidis S. aureus, B. subtilis, E. coli, P. aeruginosa, K. pneumoniae, C. glabrata, C. albicans*	[98]

Table 2. *Cont.*

Botanical Name	Family	Extracts	Bioactive Compounds	MIC *	Organism Inhibited	References
Thymus algeriensis Boiss. & Reut	Lamiaceae	Ethanol	Gallic acid, 3,4-dihydroxy benzoic acid, 4-hydroxy benzoic acid, 2,5 dihydroxybenzoic acid, chlorogenic acid, vanillic acid, epicatechin, caffeic acid, p-coumaric acid, ferulic acid, rutin, ellagic acid, naringin, quercetin, cinnamic acid, α-pinene, camphene, sabinene, β-pinene, myrcene, 3-octanol, α-terpinene, *p*-cymene, limonene, 1,8-cineole, *(Z)*-β-ocimene, *(E)*-β-ocimene, γ-terpinene, *cis*-sabinene hydrate, terpinolene, linalool, nonanal, pentylisovalerate, 1-octen-3-yl acetate, *cis*-*p*-menth-2-en-1-ol, 3-octyl acetate, α-campholenal, camphor, *trans*-verbenol, δ-terpineol, 4-terpineol, α-terpineol, *cis*-dihydrocarvone, *trans*-carveol, *(Z)*-3-hexenyl isovalerate, pulegone, neral, carvone, linalyl acetate, bornyl acetate, dihydroedulan IA, isodihydrocarvyl acetate, α-terpinyl acetate, *cis*-carvyl acetate, neryl acetate, geranyl acetate, β-elemene, *(Z)*-jasmone, β-caryophyllene, β-copaene, aromadendrene, α-humulene, *(E)*-β-farnesene, *cis*-muurola-4(14), 5-diene germacrene D, bicyclogermacrene, germacrene A, δ-cadinene, *(E)*-α-bisabolene, *(E)*-nerolidol, spathulenol, caryophyllene oxide, viridiflorol, 1, 10-di-*epi*-cubenol, T-cadinol, T-muurolol, monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, apocarotenes non-terpene derivatives	32–512 µg/mL	*S. epidermidis S. aureus, B. subtilis, E. coli, P. aeruginosa, K. pneumoniae, C. glabrata, C. albicans*	[98]

Table 2. *Cont.*

Botanical Name	Family	Extracts	Bioactive Compounds	MIC *	Organism Inhibited	References
Cinnamomun inerme	Lauraceae	Ethyl Acetate Hexane Acetone n-butanol	5-(1,5-dimethyl-2-4-hexenyl)- methyl phenol)	100–800 µg/mL 8000 µg/mL 8000 µg/mL 100–800 µg/mL	*S. aureus, E. coli*	[99]
Allium sativam	Liliaceae	Crude	Allicin	49 µg/mL	*C. albicans*	[100]
Strychnos nigritana Baker	Loganiaceae	Crude	Nigritanine, Speciociliatine, Mytragine Paynantheine Rhyncophylline	128–256 µg/mL	S. aureus	[10]
Mascagnia benthamiana (Gries) WR Anderson	Malpighiaceae	Hexane	Gallic acid, kaempferol, ellagic acid, epicatechin, vitexin, corilagin	17.84 mg/mL	*C.albicans*	[89]
Mouriri elliptica Mart	Memecylaceae	Hexane	Gallic acid, kaempferol, ellagic acid, epicatechin, vitexin, corilagin	100 µg/mL	*C.albicans*	[89]
Artocarpus communis	Moraceae	Crude	Atonin E, 2-(3,5-dihydroxy)-(Z)-4-(3 methyl but-1-etnyl	4–512 µg/mL	*P. aeruginosa, S.typhi, S.aureus, K.pneumoniae*	[101]
Myrtus nivellei Batt. & Trab.	Myrtaceae	Crude	1,8-cineole, limonene, isoamylcyclopentane, di-nor-sesquiterpenoids	5 mg/mL	*C. neoformans*	[102]

Table 2. *Cont.*

Botanical Name	Family	Extracts	Bioactive Compounds	MIC *	Organism Inhibited	References
Myrtus communis L	Myrtaceae	Crude	α-pinene, 1,8-cineole, linalool, and linalyl acetate	156–625 µg/mL	*E. floccosum*, *M. canis*, *T. rubrum*	[102]
Piper nigrum	Piperaceae	Aqueous	Piperine	500–1000 µg/mL	*E. coli*, *M. luteus*	[91]
Citrus aurantium L.	Rutaceae	Ethanol	Polyphenols, flavonoids, alkaloids, and terpenes	1562–6250 µg/mL	Amoxycillin resistant *B. cereus*	[13]
Salix babylonica L.	Salicaceae	Hydroalcoholic	Luteolin, luteolin 7-*O*-glucoside	1.56–100 mg/mL	*E. coli*, *S. aureus* and *L. monocytogenes*	[103]
Verbascum glabratum subsp. bosnense (K. Malý) Murb	Scrophulariaceae	Ethanol	quercitrin and rosmarinic acid, 4-hydroxybenzoic acid, salicylic acid, morin, and apigenin	600, 1200 µg/mL	*E. coli*, *S. aureus*, *Candida albicans*	[17]
Simaba ferruginea A. St.-Hil	Simaroubaceae	Methanol	Canthin-6-one, indole β-carboxylic	12.5–200 µg/mL	S. flexneri, S. aureus and S. aureus	[91]
Camellia sinensis	Theaceae	Aqueous	Catechin	7.81–31.25 µg/mL	*S. mutans*	[104]
Talaromyces sp.	Trichocomaceae	Aqueous	Talaropeptide A and B	5 mg/mL	*B. subtilis*	[18]
Hybanthus enneasperm us	Violaceae	Crude	Flavonoids, Tannins	37.5, 75, 150, 300, 600 µg/mL	*P. vulgaris*, *V. cholera*	[100]

4. Mechanism of Actions of Antibacterial Bioactive Compounds

As proven by in vitro experiments, medicinal plants produce a boundless quantity of secondary metabolites that have great antimicrobial activity [9,10,18]. These plant-produced low molecular weight antibiotics are classified according to two types, namely phytoanticipins, which are involved in microbial inhibitory actions, and phytoalexins, which are generally anti-oxidative and synthesized de novo by plants in response to microbial infection [16,74]. Plant antimicrobial secondary metabolites are generally categorized into three broad classes, namely phenolic compounds, terpenes, and alkaloids. Numerous studies have shown that the antimicrobial activity of the plant extracts and their active compounds have the following potential: to promote cell wall disruption and lysis, induce reactive oxygen species production, inhibit biofilm formation, inhibit cell wall construction, inhibit microbial DNA replication, inhibit energy synthesis, and inhibit bacterial toxins to the host [75,85,105–109]. In addition, these compounds may prevent antibacterial resistance as well as synergetics to antibiotics, which can ultimately kill pathogenic organisms (Figure 1).

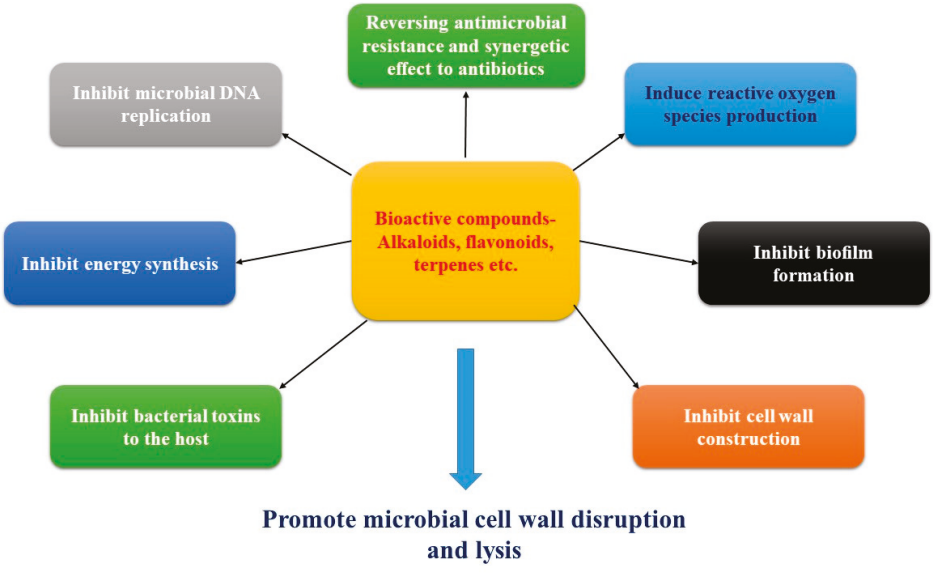
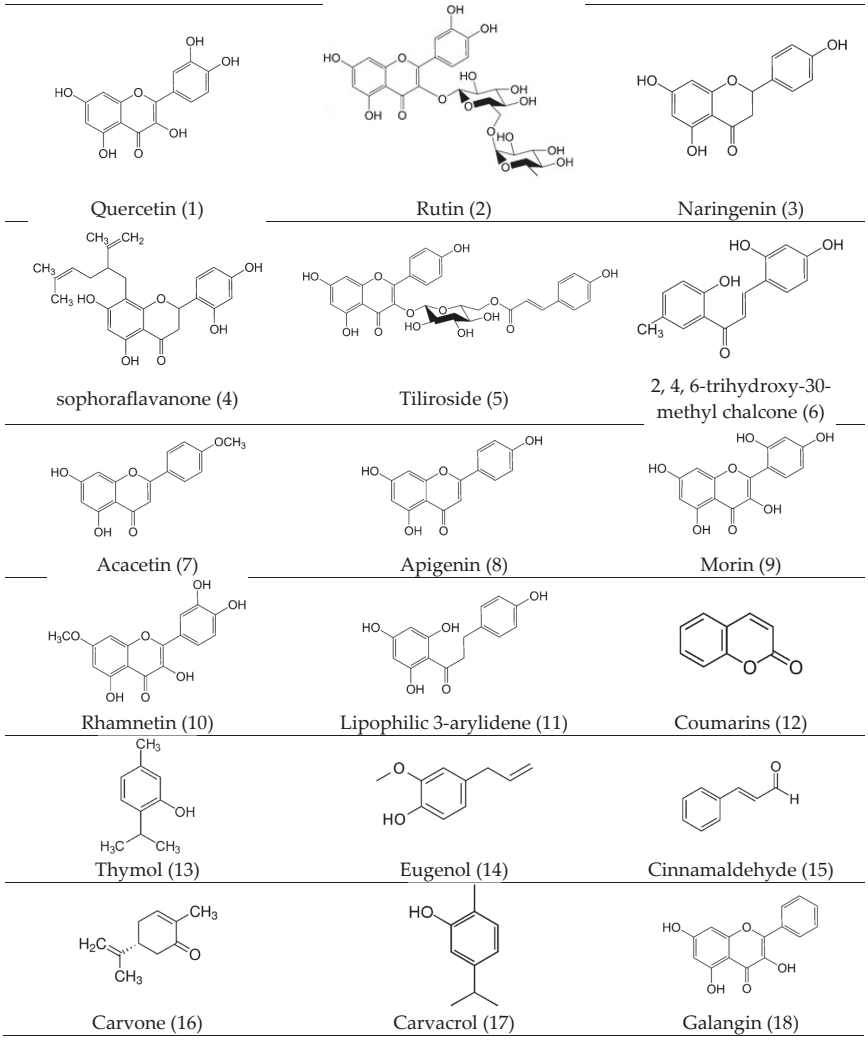


Figure 1. Mechanisms of antimicrobial activity of bioactive compounds.

4.1. Promote Cell Wall Disruption and Lysis

Phenolic compounds are a family of aromatic rings consisting of a hydroxyl functional group (-OH) which is alleged to absolute toxicity to microorganisms, although increased reactions of hydroxylation result in microbial cell lysis [110]. Quinones also have aromatic rings with two ketone molecules, which enables the production of an irreversible complex with nucleophilic amino acids, resulting in greater antimicrobial properties. These potential aromatic compounds are usually targeted to microbial cell surface adhesins, membrane-bound polypeptides, enzymes, and eventually lysis of the microbes [111]. Flavonoids are hydroxylated phenolic substances which are also able to complex with bacterial cell walls and disrupt microbial membranes [75,105]. Highly active flavonoids, quercetin (1), rutin (2), naringenin (3), sophoraflavanone (4), tiliroside (5) and 2, 4, 6-trihydroxy-30-methyl chalcone (6) (Figure 2) decreased lipid bilayer thickness and fluidity levels and increased membrane permeability, supporting the leaking of intracellular protein and ions in *S. aureus* and *S. mutans* [112,113]. These compounds contribute to the synergistic effect with ampicillin and tetracycline [114]. The other active flavonoids, acacetin (7), apigenin (8), morin (9), and rhamnetin (10) (Figure 2) cause weakening of the

bacterial cell wall by disarrangement and disorientation of the lipid bilayer and ultimately persuade vesicle leakage [115–117]. The synthetic flavonoid lipophilic 3-arylidene (11) was found to be very active against *S. aureus*, *S. epidermidis*, and *E. faecalis* due to a bacterial cell clump that influences the integrity of the cell wall as a result of biofilm disruption [118]. Tannins are classes of another polymeric phenolic substance, characterized as astringency, which is capable to deactivate microbial adhesins, enzymes, and membrane transporter systems [105,119]. Coumarins (12) are benzo-α-pyrones known to stimulate macrophages, which could have an adverse effect on infections [7,120]. Terpenes are organic compounds containing isoprene subunits, which involve microbial membrane disruption [121,122]. Thymol (13), eugenol (14), Cinnamaldehyde (15), carvone (16), and carvacrol (17) (Figure 2) disintegrate the external membrane of various Gram-negative bacteria, releasing LPS and increasing the permeability [123–125].



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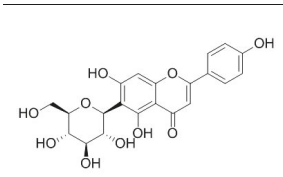
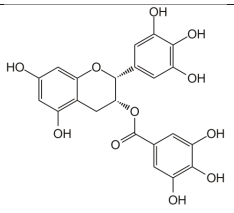
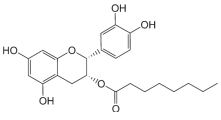


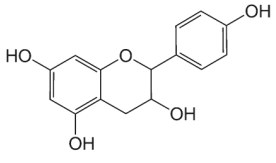
Isovitexin (19)



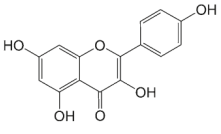
EGCG (20)



3-O-octanoyl-epicatechin (21)



5, 7, 40-trihydroxyflavanol (22)



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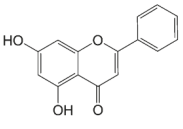

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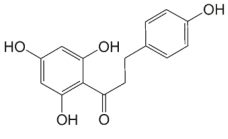

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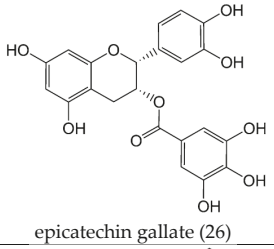
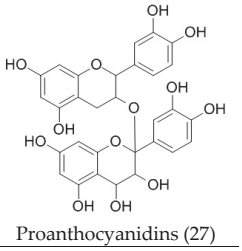
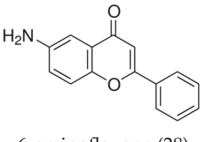


epicatechin gallate (26)



Proanthocyanidins (27)



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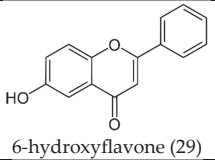

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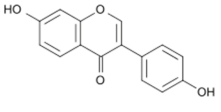

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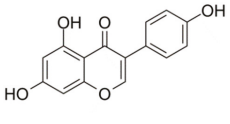

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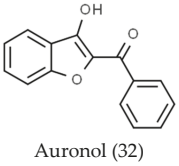

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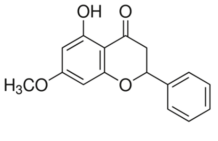

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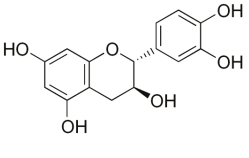

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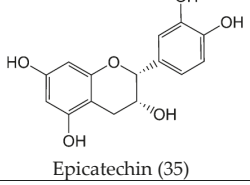

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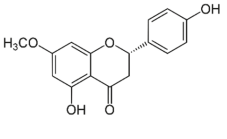

Sakuranetin (36)

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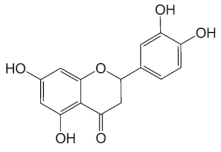	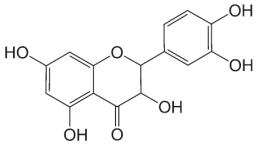	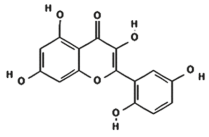
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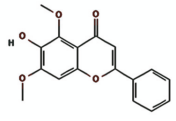	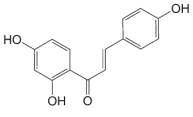	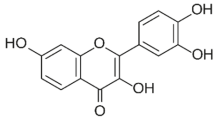
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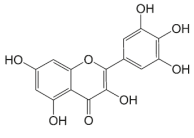	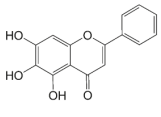	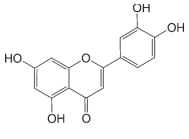
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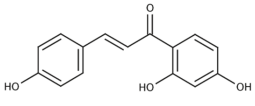
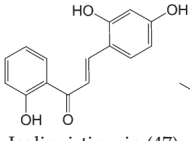
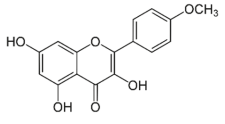
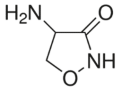
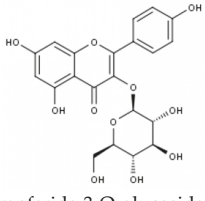
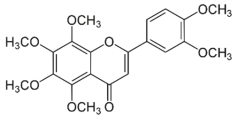
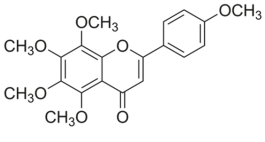
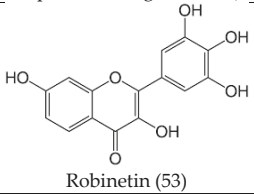
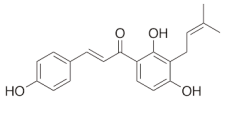
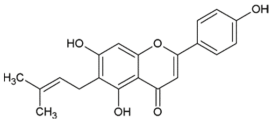
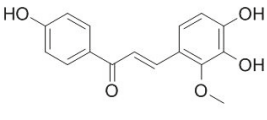
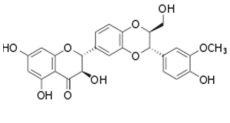
		
Butein (46)	Isoliquirtigenin (47)	Kaempferide (48)
		
DL-cycloserine (49)	kaempferide-3-O-glucoside (50)	Nobiletin (51)
		
Tangeritin (52)	Robinetin (53)	Iso bavachalcone (54)
		
6-prenylapigenin (55)	Licochalcones (56)	Silibinin (57)

Figure 2. *Cont.*

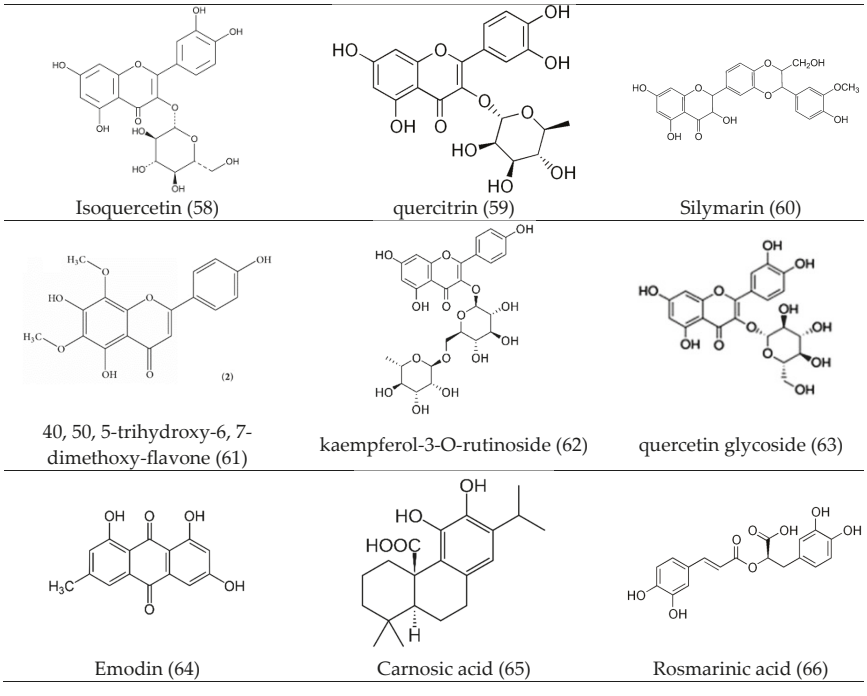


Figure 2. Chemical structures of antibacterial bioactive compounds.

4.2. Inhibition of Biofilm Formation

The key features of bacteria developing biofilms are generally 100–1000 times more resistant to antimicrobial drugs while related to their usual planktonic forms [64]. Interestingly, numerous researchers have described how flavonoids cause the aggregation of multicellular composites of bacteria and inhibit bacterial growth after aggregation, which indicates that flavonoids are potent antibiofilm compounds. The bioactive flavonoids such as galangin (18), isovitexin (19), EGCG (20) and 3-O-octanoyl-epicatechin (21), as well as 5, 7, and 40-trihydroxyflavanol (22) induce pseudo multicellular aggregation of *S. aureus* and *S. mutans* [106–109]. Quorum sensing involves cell signaling molecules called autoinducers present in *E. coli*, *Vibrio cholerae*, and *S. typhi*, which is a notable regulatory factor for biofilm formation [126]. Interestingly, apigenin (8), kaempferol (23), quercetin (1), and naringenin (3) are effective antagonists of cell–cell signaling [126,127] that have been revealed to inhibit enteroaggregative biofilm formation in *E. coli* and *P. aeruginosa* in a concentration-dependent manner [128,129]. Moreover, chrysin (24), phloretin (25), naringenin (3), kaempferol (23), epicatechin gallate (26), proanthocyanidins (27), and EGCG (20) (Figure 2) inhibited N-acyl homoserine lactones-mediated QS [130–132]. Hydrophilic flavonoids such as 6-aminoflavone (28), 6-hydroxyflavone (29), apigenin (8), chrysin (24), daidzein (30), genistein (31), auronol (32), and phloretin (25) (Figure 2) have inhibitory effects on *E. coli* biofilm formation [133,134]. In addition, Phloretin (25) inhibited fimbriae formation in *E. coli* by reducing the expression of the curli genes (csgA, csgB) and toxin genes (hemolysin E, Shiga toxin 2) [6], eventually inhibiting the formation of biofilm. Hence, phloretin (25) is well known as an antibiotic resistant compound. Pinostrobin (33), EGCG (20) and prenylated flavonoids enhanced membrane permeability in *E. faecalis*, *S. aureus*, *E. coli*, and *P. aeruginosa*, *Porphyromonas gingivalis*, which is consistent with its effect on efflux-pump inhibitors and anti-biofilm formation [34,135,136].

4.3. Inhibition of Cell Wall Construction

The bacterial cell wall is accountable for osmoregulation, respiration, the transport mechanism, and biosynthesis of lipids. For the execution of these functions, membrane integrity is very important, and its disruption can directly or indirectly cause metabolic dysfunction eventually leads to bacterial death. Catechins (34) attract lipid bilayers of the membrane which involves the following mechanisms [137]. Catechins form hydrogen bonds, which attract polar head groups of lipids at the membrane edge. Epicatechin (35) and epigallocatechin gallate (26) alter phospholipids, which can alter structural changes in the cell membrane. Moreover, these catechins promote the inactivation or inhibition of intracellular and extracellular enzyme synthesis [137]. Generally, the inhibition of enzymes in fatty acid biosynthesis is an excellent target for antimicrobial agents for blocking bacterial growth, especially the key enzyme fatty acid synthase II (FAS-II) inhibitor is significant as an antimicrobial drug. Quercetin (1), apigenin (8), and sakuranetin (36) have been demonstrated to inhibit 3-hydroxyacyl-ACP dehydrase from *Helicobacter pylori* [138] and eriodictyol (37). Further, naringenin (3) and taxifolin (38) (Figure 2) inhibit 3-ketoacyl- ACP synthase from *E. faecalis* [139]. Flavonoids such as Epigallocatechin gallate (EGCG) (20), 5, 6, 7, 40, 50- pentahydroxyflavone (39), and 5-hydroxy-40, 7-dimethoxyflavone (40) inhibit the malonyl CoA-acyl carrier protein transacylase that regulates bacterial FAS-II [140,141]. EGCG (20) inhibits 3-ketoacyl-ACP reductase and enoyl-ACP reductase and prevents fatty acid biosynthesis [142]. Quercetin (1), kaempferol (23), 4, 20, 40-trihydroxychalcone (41), fisetin (42), morin (9), myricetin (43), baicalein (44), luteolin (45), EGCG (20), butein (46), and isoliquirtigenin (47) (Figure 2) inhibit various enzymes involved in fatty acid synthesis, including, FAS-II, enoyl-ACP-reductase, β-ketoacyl-ACP reductase, and β-hydroxy acyl-ACP dehydratases in *Mycobacterium sp*. [143]. Baicalein (44), EGCG (20), galangin (18), kaempferide (48), DL-cycloserine (49), quercetin (1), apigenin (8), and kaempferide-3-O-glucoside (50) (Figure 2) inhibit the synthesis of peptidoglycan, which is an essential component of the bacterial cell wall, resulting in cell wall damage [144–146].

4.4. Inhibition of Prokaryotic DNA Replication

Alkaloids are nitrogenous compounds characterized by their alkaline nature, which aids the inhibition of cell respiration, intercalates with DNA, and inhibits various enzymes involved in replication, transcription, and translation [147]. Plant-based bioactive compounds such as quercetin (1), nobiletin (51), myricetin (43), tangeritin (52,) genistein (31), apigenin (8), chrysin (24), kaempferol (23), and 3, 6, 7, 30, 40-pentahydroxyflavone (39) have been recognized as noteworthy DNA gyrase inhibitors, which are essential for DNA replication in prokaryotes including *V. harveyi*, *B. subtilis*, *M. smegmatis*, *M. tuberculosis*, and *E. coli* [146,148–151]. These bioactive compounds binding to the β subunit of gyrase and the corresponding blockage of the ATP binding pocket eventually contribute to the antimicrobial activity. Bioactive compounds have mediated the dysfunction of DNA gyrase functions in a dose-dependent manner that leads to the impairment of cell division and/or completion of chromosome replication, resulting in the inhibition of bacterial growth [149]. Luteolin (45), morin (9), and myricetin (43) have been demonstrated to inhibit the helicases of *E. coli* [152]. Helicases consititute another significant replicative enzyme responsible for separating and/or rearranging DNA double-strands [153]. Furthermore, myricetin (43) and baicalein (44) have been proposed as potent inhibitors of numerous DNA and RNA polymerases, as well as viral reverse transcriptase, resulting in the inhibition of bacterial growth [154]. EGCG (20), myricetin (43), and robinetin (53) have been demonstrated as inhibitors of dihydrofolate reductase in *Streptomonas maltophilia, P.vulgaris, S. aureus, M. tuberculosis*, and *E. coli* [43,155,156]. Dihydrofolate reductase is key enzyme for the synthesis of the purine and pyrimidine rings of nucleic acid, resulting in reduced DNA, RNA, and protein synthesis [156].

4.5. Inhibition of Energy Production

Energy production or ATP synthesis is the supreme vital requirement for the existence and development of bacteria as these chemicals are the main source of living systems. The treatment of flavonoids such as isobavachalcone (54) and 6-prenylapigenin (55) with *S. aureus* cause membrane depolarization, resulting in bacterial cell wall lysis [101]. Similarly, licochalcones (56) inhibited oxygen consumption in *M. luteus*, interruping the electron transport system eventually killing the bacteria [6]. It has been described that flavonoids such as baicalein (44), morin (9), silibinin (57), quercetin (1), isoquercetin (58), quercitrin (59), and silymarin (60) can constrain the F1FO ATPase system of *E. coli* and result in the obstruction of ATP synthesis [157–159]. Additionally, EGCG (20), 40, 50, 5-trihydroxy-6, 7-dimethoxy-flavone (61), and proanthocyanidins (27) have also inhibited *S. mutans, P. aeruginosa* and *S. aureus* through the enzymatic activity of F1FO ATPase respectively [100,104,141].

4.6. Inhibition of Bacterial Toxins

It is noteworthy that catechins and other flavonoids can cause bacterial cell wall destruction, resulting in an inability to discharge toxins [160,161]. Catechins (34), pinocembrin, kaempferol, EGCG (20), gallocatechin gallate (26), kaempferol-3-O-rutinoside (62), genistein (31), quercetin glycoside (63), and proanthocyanidins (27) (Figure 2) are suggested to neutralize bacterial toxic factors initiating from *V. cholerae, E. coli, S. aureus, V. vulnificus, B. anthracis, N. gonorrhoeae*, and *C. botulinum* [162–165]. Bacterial hyaluronidases are enzymes formed by both Gram-positive and Gram-negative bacteria and directly interact with host tissues, causing the permeability of connective tissues and reducing the viscosity of body fluids due to hyaluronidase-mediated degradation [166]. Flavonoids such as myricetin (43) and quercetin (1) have been identified as hyaluronic acid lyase inhibitors in *Streptococcus equisimilis* and *Streptococcus agalactiae* [167].

4.7. Mechanism of Resistance to Antibacterial Agents

Pathogenic bacteria generally receive the resistance to various antibiotics through diverse mechanisms. Such mechanisms include: (a) bacteria can share the resistance genes through transformation, transduction, and conjugation; (b) bacteria produce various enzymes to deactivate the antibiotics through the process of phosphorylation, adenylation, or acetylation; (c) damage or alteration of the drug compound; (c) prevent the interaction of the drug with the target; (d) efflux of the antibiotic from the cell [168–170]. Emodin (1, 2, 8-trihydroxy-6-methylanthraquinone) (64) is an anthraquinone derivative which prevents the transformation of resistance genes in *S. aureus* [171]. Baicalein is a potent inhibitor of the expression of the SOS genes, *RecA, LexA*, and *SACOL1400* that prevent rifampin-resistant mutation in *S. aureus* [172]. Phenolic compounds such as Carnosic (65) and rosmarinic acids (66) inactivate cmeB, cmeF, and cmeR genes in *Campylobacter jejuni* [173].

4.8. Antimicrobial Action with Generation of Reactive Oxygen Species

Reactive oxygen species (ROS) can be formed by the partial reduction of molecular oxygen that targets the exertion of antimicrobial activity, which aids host defense against various disease-causing pathogens. The suggested method of antimicrobial activity of catechins (34) involves augmentation of the production of oxidative stress (ROS and RNS), which can alter membrane permeability and cause as cell wall damage [174]. In addition, catechins damage liposomes as they contain a high amount of negatively charged lipids and are susceptible to damage [175]. An earlier study indicated that catechins support the leaking of potassium and disturbs the membrane transport system in a methicillin-resistant *S. aureus* strain [85]. This team has further demonstrated that acylated 3-O-octanoyl-epicatechin (21) is a lipophilic compound that produces more outcomes in antibacterial activity.

5. Conclusions

Since time immemorial, traditional medicinal plants have been cultivated by diverse populations to treat a great number of infectious diseases. Various investigations on the pharmacognostics and kinetics of medicinal plants have shown that crude extracts and plant-derived bioactive compounds may enhance the effects of traditional antimicrobials, which may be cost-effective, have fewer side effects, and improve the quality of treatment. Numerous studies have shown that the antimicrobial activity of plant extracts and their active compounds have the following potential: promote cell wall disruption and lysis, induce reactive oxygen species production, inhibit biofilm formation, inhibit cell wall construction, inhibit microbial DNA replication, inhibit energy synthesis, and inhibit bacterial toxins to the host. In addition, these compounds may prevent antibacterial resistance as well as synergetics to antibiotics, which can ultimately kill pathogenic organisms. Based on these comprehensive antimicrobial mechanisms, the cultivation of traditional plant extracts and bioactive compounds offers a promising treatment for disease-causing infectious microbial pathogens. Hence, this mechanism constitutes an encouraging ally in the development of pharmacological agents required to combat the growing number of microbial strains that have become resistant to extant antibiotics in clinical practice.

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Abbreviations

A. bohemicus	*Acinetobacter bohemicus*
A. flavus	*Aspergillus flavus*
A. fumigatus	*Aspergillus fumigatus*
A. niger	*Aspergillus niger*
A. solani	*Alternaria solani*
B. agri	*Brevibacillus agri*
B. brevis	*Brevibacillus brevis*
B. cereus	*Bacillus cereus*
B. megaterium	*Bacillus megaterium*
B. pumilus	*Bacillus pumilus*
B. subtilis	*Bacillus subtilis*
C. albicans	*Candida albicans*
C. Dipthieriae	*Corynebacterium Dipthieriae*
C. dubliniensis	*Candida dubliniensis*
C. glabrata	*Candida glabrata*
C. graminicola	*Colletotrichum graminicola*
C. jejuni	*Campylobacter jejuni*
C. krusei	*Candida krusei*
C. lunat	*Candida lunat*
C. lunatus	*Cochliobolus lunatus*
C. macrocarpum	*Cladosporium macrocarpum*
C. neoformans	*Cryptococcus neoformans*
C. parapsilosis	*Candida parapsilosis*
C. sphaerospermum	*Cladosporium sphaerospermum*
C. tropicalis	*Candida tropicalis*
C. maydis	*Cercospora zeae-maydis*
D. turcica	*Drechslera turcica*

E. aerogenes	*Enterobacter aerogenes*
E. cloacae	*Enterobacter cloacae*
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
E. ficariae	*Entyloma ficariae*
E. floccosum	*Epidermophyton floccosum*
F. nucleatum	*Fusobacterium nucleatum*
F. oxysporum	*Fusarium oxysporum*
F. verticillioides	*Fusarium verticillioides*
H. carbonum	*Helminthosporium carbonum*
H. pylori	*Helicobacter pylori*
K. aerogenes	*Klebsiella aerogenes*
K. kristinae	*Kocuria kristinae*
K. pneumonia	*Klebsiella pneumonia*
L. acidophilus	*Lactobacillus acidophilus*
L. casei	*Lactobacillus casei*
L. innocua	*Listeria innocua*
L. monocytogenes	*Listeria monocytogenes*
L. sporogenes	*Lactobacillus sporogenes*
M. canis	*Microsporum canis*
M. luteus	*Micrococcus luteus*
M. morganii	*Morganella morganii*
M. ruber	*Monascus ruber*
M. smegmatis	*Mycobacterium smegmatis*
M. tuberculosis	*Mycobacterium tuberculosis*
M. verticillata	*Mortierella verticillata*
P. acnes	*Propionibacterium acnes*
P. aeruginosa	*Pseudomonas aeruginosa*
P. brasiliensis	*Paracoccidioides brasiliensis*
P. fluorescens	*Pseudomonas fluorescens*
P. gingivalis	*Porphrymonas gingivalis*
P. herbarum	*Pleospora herbarum*
P. innundatus	*Protomyces innundatus*
P. intermedia	*Prevotella intermedia*
P. lilacinum	*Purpureocillium lilacinum*
P. mirabilis	*Proteus mirabilis*
P. sojae	*Phytophthora sojae*
P. vulgaris	*Proteus vulgaris*
R. rubrum	*Rhodospirillum rubrum*
R. solanacearum	*Ralstonia solanacearum*
R. solani	*Rhizoctonia solani*
R. stolonifera	*Rhizopus stolonifera*
S. agalactiae	*Streptococcus agalactiae*
S. anginosus	*Streptococcus anginosus*
S. aureus	*Staphylococcus aureus*
S. auricularis	*Staphylococcus auricularis*
S. boydii	*Shigella boydii*
S. dysenteriae	*shigella dysenteriae*
S. epidermidis	*Staphylococcus epidermidis*
S. fecalis	*Streptococcus fecalis*
S. flexneri	*Shigella flexneri*
S. gordonii	*Streptococcus gordonii*
S. haemolyticus	*Staphylococcus haemolyticus*

S. heidelberg	*Salmonella heidelberg*
S. hominis	*Staphylococcus hominis*
S. japonicas	*Schizosaccharomyces japonicas*
S. kneipii	*Spizellomyces kneipii*
S. lutea	*Sarcina lutea*
S. marcescens	*Serratia marcescens*
S. mutans	*Streptococcus mutans*
S. para typhi	*Salmonella para typhi*
S. pneumoniae	*Streptococcus pneumoniae*
S. pseudodichotomus	*Spizellomyces pseudodichotomus*
S. pyogenes	*Streptococcus pyogenes*
S. sanguis	*Streptococcus sanguis*
S. saprophyticus	*Staphylococcus saprophyticus*
S. shiga	*Shigella shiga*
S. typhi	*Salmonella typhi*
T. deformans	*Taphrina deformans*
T. mentagraphytes	*Trichophyton mentagraphytes*
T. rubrum	*Trichophyton rubrum*
T. tonsurans	*Trichophyton tonsurans*
T. urans	*Trichophytontonsurans*
V. cholerae	*Vibrio cholerae*
V. fischeri	*Vibrio fischeri*
X. axonopodis Pv. malvacearum	*Xanthomonas axonopodis pv. Malvacearum*
X. vesicatoria	*Xanthomonas vesicatoria*
Y. enterocolitica	*Yersinia enterocolitica*

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antibiotics

MDPI

Review

Food Safety through Natural Antimicrobials

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Abstract: Microbial pathogens are the cause of many foodborne diseases after the ingestion of contaminated food. Several preservation methods have been developed to assure microbial food safety, as well as nutritional values and sensory characteristics of food. However, the demand for natural antimicrobial agents is increasing due to consumers' concern on health issues. Moreover, the use of antibiotics is leading to multidrug resistant microorganisms reinforcing the focus of researchers and the food industry on natural antimicrobials. Natural antimicrobial compounds from plants, animals, bacteria, viruses, algae and mushrooms are covered. Finally, new perspectives from researchers in the field and the interest of the food industry in innovations are reviewed. These new approaches should be useful for controlling foodborne bacterial pathogens; furthermore, the shelf-life of food would be extended.

Keywords: natural antimicrobials; preservation; plants; spices; bacteria; viruses; algae; mushrooms; bacteriocins; bacteriophages

1. Introduction

Microbial pathogens are the cause of many foodborne diseases after the ingestion of contaminated food. Several preservation methods have been developed to assure microbial food safety, as well as nutritional values and sensory characteristics of food. Those methods sometimes have undesired effects on the nutritional and/or organoleptic aspects of food; synthetic preservatives are well known for causing health problems such as allergic reactions: nitrates, benzoates, sulfites, sorbates, formaldehyde, and phenolic antioxidants are good examples [1,2]. The use of natural antimicrobial food preservatives—biopreservation—could ensure the safety and quality of food being an alternative to other systems of preservation such as chemical or thermal ones. An excellent overview of natural antimicrobials applications can be seen in [1]. Biopreservation uses natural preservatives against a high number of pathogenic microorganisms related to food; those preservatives are obtained from animals, plants, bacteria, as well as mushrooms, algae, and viruses [2]. Figure 1 shows a general view of natural antimicrobials and their different roles in food safety.

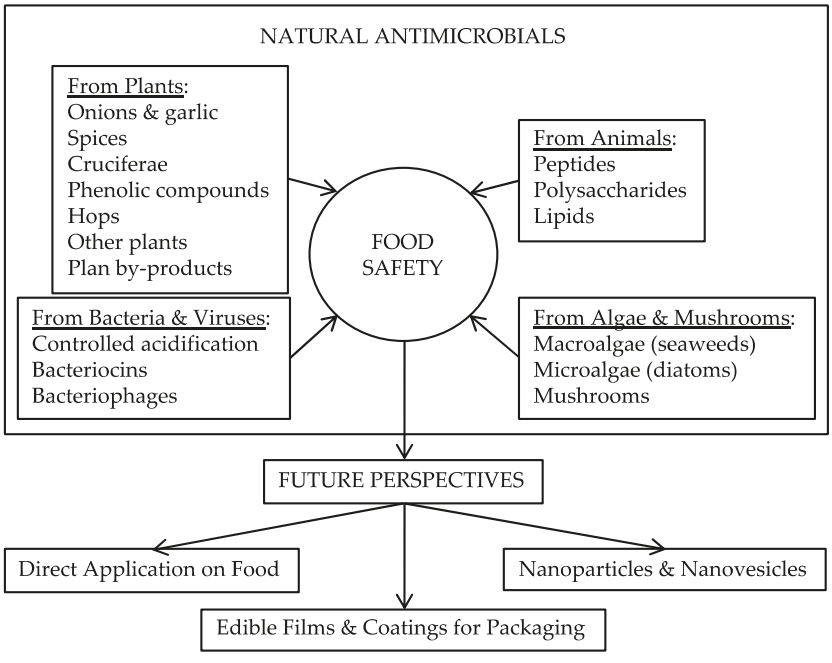

Figure 1. Overview of natural antimicrobials and their role in food safety.

The demand for natural antimicrobial agents is expected to increase steadily for replacing synthetic compounds [3]. A novel trend is arising from health-conscious consumers expecting that natural antimicrobials act only against foodborne pathogens leaving the consumers' microbiome out of their scope [1]. The negative effect of some synthetic preservatives on consumers' health is leading to more research to evaluate that natural antimicrobials fulfil food safety regulations [4]; the inadequate use of antibiotics leading to multidrug-resistant microorganisms also justify and reinforce the focus on natural antimicrobials [2]. Natural antimicrobials ensure food safety from a new perspective increasing its shelf-life; furthermore, their direct incorporation to different foods from different origins such as meat or vegetables as well as to their packaging give, as a result, the extension of their shelf-life [1,5]. They also constitute a viable alternative to microbial resistance caused by antibiotics.

Recent studies comparing natural derivatives from plants with synthetic antimicrobials have shown that natural substances could be safer [3,6,7]. The mechanisms of action of natural antimicrobials include the rupture of the cell membrane, affect the nucleic acids mechanisms, the decay of the proton motive force, and depletion of adenosine triphosphate (ATP). Antimicrobials from plants (polyphenols, essential oils), animals (lysozyme, lactoperoxidase, lactoferrin), metabolites from microorganisms, or extracts from algae use those mechanisms of action against foodborne bacteria [1,8].

2. Natural Antimicrobials from Plants

Herbs and spices have most of the antimicrobials derived from plants [9–11]. These compounds have different structural configurations, having different antimicrobial actions against foodborne pathogens [12]. A fine review showing the different structural variations of plant-derived components and their effect on their antimicrobial capacities was published by Gyawali and Ibrahim [2]. The structural configuration of these compounds has big impact on their antimicrobial action, i.e. the hydroxyl (−OH) groups are thought to be the cause; the reason behind that fact is the interaction of the

hydroxyl groups with the bacterial cell membrane disrupting its structures and causing leakage of its components.

Growing interest in using antimicrobial plant-derived extracts is caused by the need to reduce the use of synthetic additives in food [13]. Antioxidant capacity usually joins the antimicrobial characteristics of these natural products; both properties together in one molecule makes the compound even more effective [1]. Plants and herbs (oregano, garlic, parsley, sage, coriander, rosemary, and lemongrass), spices (cinnamon, clove), oils (citral) or organic compounds (vanillin) have been used alone for their antimicrobial and antioxidant properties or in combination with other techniques for food preservation [14–16]. These authors also reported lower activity from products such as ginger, pepper, cumin, chilli, and curry. Gutierrez et al. [14] assessed combinations of essential oils from thyme, sage, rosemary, oregano, lemon, and basil against different microorganisms: *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes* and *Pseudomonas aeruginosa*. Oregano showed efficacy against *B. cereus*; furthermore, the oregano combinations with basil or thyme were active against *B. cereus*, *E. coli* and *P. aeruginosa*. These authors also studied the effect of the pH and different ingredients from foods on the activity of thyme and oregano against *L. monocytogenes*; the kinetic parameters of the microorganism were more affected in foods with acidic pH and a high content in proteins. Proestos et al. [15] studied extracts from five plants—meadowsweet, hawthorn, polygonum, silverweed, and little robin—showing their antioxidant capacity, with total phenolic contents between 7.2–28.2 gallic acid equivalents/mg or mL; their activity against the microorganisms showed that Gram-negative bacteria were less sensitive than Gram-positive. Numerous studies have been carried out using natural compounds extracted from plants against several microbial genera and/or species. Nanasombat and Lohasupthawee [17] studied the antimicrobial activity of extracts and essential oils from 14 spices tested against 20 serotypes of *Salmonella* and other members of the *Enterobacteriaceae* family, founding the following trend from greater to lesser degree of antimicrobial activity: Clove, cardamom, coriander, nutmeg, ginger, garlic, and basil among others. *E. coli* was the non-salmonellae strain more susceptible to most of the spice oils.

2.1. Onions and Garlic

The growth of many microorganisms is inhibited by onion and garlic. Several authors reported on the antimicrobial capacities of onions and garlic a long time ago [18–24]. Juices and vapours of these plants inhibit the growth of several microorganisms including bacteria (*Bacillus cereus*, *Clostridium botulinum*, *Escherichia coli*, *Lactobacillus*, *Salmonella*, *Staphylococcus aureus*, etc.) and fungi (*Aspergillus* spp., *Candida*, *Saccharomyces*, etc.) [23]. Conner et al. [18] reported that essential oils of onion (500 μg/mL) reduced the ethanol production by *Sacchraromyces cerevisiae*, suppressed the production of ethanol by *Hansenula anomala*, and delayed sporulation of *Lodderomyces elongisporus*. González-Fandos et al. [19] studied the inhibition of *S. aureus* growth and enterotoxin and thermonuclease production by garlic in brain heart infusion (BHI) broth. These authors found that *S. aureus* was inhibited at levels of 1.5% and over; enterotoxins A, B, and C1 were found with less than 1% of garlic, but at a 2% concentration the enterotoxin D was synthesized. Garlic inhibited thermonuclease production completely at levels greater or equal to 1.5%. Barone et al. [22] reported fungicidal activity of garlic extracts (68 μg/mL) against 39 of 41 clinical strains of *Candida albicans* in standing culture; the extract was fungistatic (50–300 μg/ mL) and fungicidal (>400 μg/mL) in shake culture. A very interesting fact found by Barone et al. [22] was a loss of antimicrobial activity against *C. albicans* when the garlic extract was heat treated at 37 °C, having food safety implications in culinary processes; moreover, the activity against the microorganism was stable under acidic conditions, but unstable under base conditions. Kim et al. [24] studied the activity of garlic and onion essential oils and their sulfides against several bacteria and yeasts: *S. aureus*, *E. coli*, *Enterobacter aerogenes*, *Leuconostoc mesenteroides*, *Pediococcus pentosaceus*, *Lactobacillus plantarum*, *Pichia membranefaciens*, *Saccharomyces cerevisiae*, *Candida utilis*, *Candida albicans*, *Zygosaccharomyces bisporus*, and *Zygosaccharomyces rouxii*. The minimum inhibitory concentrations (MIC) of garlic and onion oils, diallyl-trisulfide and -tetrasulfide, and dimethyl-trisulfide were 2–45 ppm for the yeasts

studied; however, these compounds had weak activity against most of the bacteria (MIC > 300 ppm). The activity against the tested yeasts was not influenced by the storage or the pH.

2.2. Spices

As stated by Taylor and Davidson [23], spices are different parts (roots, seeds, leaves, fruits, etc.) of aromatic plants added as flavouring components to foods; among them, oregano, cinnamon, clove, and rosemary showed the greatest activity against microorganisms. Eugenol and cinnamic aldehyde are the major constituents of clove and cinnamon, respectively [23]. Cinnamon and cinnamic aldehyde have shown activity against bacteria (*Aeromonas hydrophila*, *Bacillus* spp., *Campylobacter jejuni*, verotoxin-producing *E. coli*, *Lactobacillus*, *Listeria monocytogenes*, *Salmonella*, *Shigella*, *S. aureus*, and *Streptococcus*) and fungi (*Aspergillus*, *Candida*, *Penicillium*, and *Saccharomyces*) [23,25–31]. Clove and eugenol are inhibitory to similar bacteria and fungi [28–39]. The antimicrobial effects of cinnamon alone or combined with potassium sorbate or sodium benzoate were tested against *Escherichia coli* O157:H7 at different temperatures in apple juice by Ceylan et al. [25]; the microorganism counts were reduced by approximately 2.0 log colony forming units (CFU)/mL at 8 or 25 °C by 0.3% cinnamon. Between cinnamon and the studied preservatives a synergistic activity was found: 0.3% of cinnamon combined with 0.1% of sodium benzoate or potassium sorbate killed 5 log CFU/mL in 11 or 14 d at 8 °C, respectively; the inhibitory effect was similar in 3 d by the same combinations at 25 °C. Thyme, oregano, dictamus, marjoram, lavender, rosemary, and sage were tested against *Penicillium digitatum* by Daferera et al. [26]; the growth and germination were inhibited by the essential oils of dictamus, marjoram, oregano, and thyme at 250–400 μg/mL, while lavender, rosemary, and sage were less effective. Friedman et al. [28] studied the activity of 119 essential oils against bacteria isolated from foods and clinical sources (*Campylobacter jejuni*, *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella enterica*) founding that 39 oils were active against all four species of bacteria. Nielsen et al. [30] investigated the effect of spices and herbs oils and oleoresins against bread spoilage fungi (*Penicillium commune*, *P. roqueforti*, *Aspergillus flavus*, and *Endomyces fibuliger*) as an alternative to modified atmosphere packaging. Cinnamon, clove, garlic, and mustard had high activity, while oregano had weak activity against the growth of fungi; the more resistant microorganisms was *A. flavus*, and *P. roqueforti* the most sensitive.

The activities against the microorganisms of oregano and thyme have been assigned to carvacrol and thymol, respectively [23], showing activity against the bacteria *Aeromonas* spp., *B. cereus*, *Brochothrix thermosphacta*, *Campylobacter jejuni*, *Escherichia coli*, *Enterobacter faecalis*, *Lactobacillus plantarum*, *Listeria monocytogenes*, *Pediococcus cerevisiae*, *Pseudomonas*, *Proteus*, *Salmonella*, *Shigella*, *Staphylococcus aureus*, *Vibrio parahaemolyticus*, and *Yersinia enterocolitica* [7,34,40–51], and the moulds and yeasts *Aspergillus*, *Candida*, *Geotrichum*, *Penicillium*, *Pichia*, *Rhodotorula*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe* [52–59]. Burt and Reinders [7] quantified the antibacterial effect against *E. coli* O157:H7 of several essential oils with or without a stabilizer (such as agar) and an emulsifier (lecithin) at different temperatures. Oregano and thyme essential oils had the strongest properties; 0.05% of agar reinforced the activity of the essential oils at 10 °C, whereas the addition of 0.25% of lecithin reduced their activity. These authors reported that the combination of oregano or thyme with agar reduces the number of *E. coli* O157:H7 preventing its growth. Of 17 spices and herbs tested at 0.5–1%, only clove, basil, marjoram, oregano, rosemary, and thyme showed activity against *Shigella* spp. [34]. These authors combined temperature (12, 22, and 37 °C), pH (5.0, 5.5, and 6.0), NaCl (1–4%), and thyme or basil (0 or 1%), establishing that both can contribute as an inhibitory factor: *S. flexneri* did not grow for 7 d with basil and/or thyme, while growth was noted without them. The practical side of the study was the use of these spices in spaghetti sauce founding that, at 12 °C, the population of *S. sonnei* decreased after 16 d; the population did not reduce its counts at 4 °C. Seaberg et al. [41] addressed the fact that different batches of the same plant species have a genetic heterogeneity that represents a problem for their use against microbial growth and for achieving the "clean label" for the food industry. To overcome the situation, a clonal line of oregano was isolated, and its ethanol extracts together with

its main constituents—thymol and carvacrol—were used in both broth and meat systems to study its activity against *Listeria monocytogenes*; all thymol and carvacrol (150–200 ppm) and the clonal line (1200 ppm) inhibited the *L. monocytogenes* growth in both systems. Singh et al. [42] also evaluated the activity of essential oils from different plants against *Listeria monocytogenes* in peptone water and hotdogs, finding that thyme and clove (1 mL/L) were highly effective inhibiting the population of *L. monocytogenes* below detection limits. Carvacrol was also investigated by Ultee et al. [43] for its effect on *Bacillus cereus* and the production of diarrheal toxin; its counts were reduced with concentrations of about 0–0.06 mg/mL in BHI broth—an 80% decline in toxin production was detected with 0.06 mg/mL. Carvacrol, thymol, cymene, and terpinene were studied against *E. coli* O157:H7 by Burt et al. [45] and Kiskó and Roller [47]; carvacrol and thymol were additive in combination showing bacteriostatic and bactericidal activities (1.2 mmol/L), and cymene and terpinene did not show antibacterial activity up to 50 mmol/L. The inhibitory activity of several natural compounds (thymol, carvacrol, eugenol, cinnamic acid, and diacetyl) alone or in combination with nisin against *E. coli* and *Salmonella enterica* serovar Typhimurium [48], or *Bacillus subtilis* and *Listeria innocua* [49] was studied. Nisin alone showed no antibacterial activity. Thymol was the most effective with concentrations of 1.0–1.2 mmol against *S. enterica* and *E. coli*; the combination of nisin showed no improvement of the antimicrobial activity. All the organic compounds exhibited activity against the Gram-positive microorganisms with concentrations between 0.8 and 15.0 mM; the interaction between the organic compounds and nisin showed different patterns, varying from synergistic (carvacrol, eugenol, or thymol; nisin plus cinnamic acid only against *L. innocua*) to antagonistic (nisin plus diacetyl). The anticandidal activity of the major phenolic compounds of oregano (carvacrol at 0.1%) and clove (eugenol at 0.2%) essential oils was studied by Chami et al. [53]. Both compounds were fungicidal in exponentially growing *Candida albicans*. Also using *Candida albicans*, the activity of origanum, carvacrol, nystatin, and amphotericin B were tested by Manohar et al. [57]. *C. albicans* growth was completely inhibited by origanum oil at 0.25 mg/mL; origanum oil and carvacrol inhibited both germination and mycelial growth in a dose-dependent manner.

Sage (containing thujone) and rosemary (with borneol, pinene, camphene, and camphor) also have antimicrobial activity [23,44]. Oregano, thyme and savoury [7], and sage and rosemary [60,61] essential oils showed pronounced bactericidal properties against *E. coli* O157:H7 and other foodborne pathogens. Pirbalouti et al. [62] reported antibacterial activity against *L. monocytogenes* by several plant extracts including essential oils from *Thymus* spp. In contrast, other authors [42,54,59,63] have found that essential oil of spices had little antimicrobial activity against bacteria and yeasts may be due to the assays utilized [23].

Several other spice essential oils have shown potential for antibacterial and antifungal activity. Sweet basil demonstrated activity against fungi such as *Mucor* and *Penicillium* although little activity against bacteria [23,64]; the main agents are linalool and methyl chavicol [23]. Essential oils from different varieties of sweet basil were tested for their activity against Gram-positive and Gram-negative foodborne bacteria, yeasts, and moulds by Lachowicz et al. [64]; all basil's essential oils showed activity against the microorganisms tested with the exception of *Flavimonas oryzihabitans* and *Pseudomonas* spp. Vanilla beans have vanillin as their major constituent, being most active against moulds and Gram-positive bacteria [23,65]. Delaquis et al. [65] studied the activity of vanillin and vanillic acid against *Listeria monocytogenes*, *L. innocua*, *L. grayi*, and *L. seeligeri*. All strains were inhibited by concentrations of about 23–33 mM; concentrations of about 100 mM vanillic acid at pH > 6.0 was not effective against the microorganisms, but with 10 mM at pH 5.0 the inhibition was complete. A declining pH increased the lethal activity of vanillic acid, and vanillin plus vanillic acid gave additive inhibitory effects.

Other essential oils from spices have potential antimicrobial activity as well as antifungal, such as cilantro-also known as coriander, fingerroot, lemongrass, savory, and tea tree oil [23,28,44,58,61,66–70].

2.3. Cruciferae

Cabbage, cauliflower, broccoli, Brussels sprouts, horseradish, kale, kohlrabi, mustard, turnips, and rutabaga are members of this family. Isothiocyanates are reported as antimicrobial agents [23] against bacteria (*E. coli* O157:H7, *L. monocytogenes*, *Salmonella*, *S. aureus*, *Serratia*, *Lactobacillus sake*, *Pseudomonas*, and *Enterobacteriaceae*) [71–73] and fungi and yeast (*Penicillium expansum*, *Aspergillus flavus*, and *Botrytis cinereal*) [74].

Delaquis et al. [71] and Ward et al. [72] tested the behaviour of bacteria in pre-cooked roast beef with vaporized horseradish essential oil at 4 °C for 28 d. *Pseudomonas* spp. and some members of the family *Enterobacteriaceae* were inhibited; lactic acid bacteria were more resistant. Interestingly, the colour of the cooked meat was preserved in samples stored with horseradish essential oil. The growth of *S. aureus*, *E. coli* O157:H7, *S. typhimurium*, *L. monocytogenes*, and *Serratia grimesii* was inhibited at 12 °C for 7 d of storage under aerobic conditions.

2.4. Phenolic Compounds

Monophenols, diphenols, and triphenols are simple phenolic compounds. The use of wood smoke for food preservation implies the use of simple phenols (cresol, hydroquinone, gallic acid); additionally, their use gives a desirable flavour [23]. Liquid smoke is a method widely used in cheese surface inhibiting the growth of fungi such as *Aspergillus oryzae*, *Penicillium camemberti*, and *Penicillium roqueforti* [75]; these authors found that only isoeugenol inhibited all these molds. Cresol (forms m- and p-) slightly inhibited the growth of *P. camemberti*, and guaiacol, 4-methylguaiacol, and m- and p-cresol inhibited the growth of *A. oryzae*.

The phenolic acids are present in plants and can inhibit bacteria such as *Aeromonas hydrophila*, *E. coli*, *E. faecalis*, *Salmonella* serovar Enteritidis, *L. monocytogenes*, and *S. aureus*, [23,76]. The most effective compound was the phenolic antioxidant tertiary butylhydroquinone with MIC of 64 μg/mL [76].

Hydroxycinnamic acids (such as caffeic, coumaric, ferulic, and sinapic acids) have different inhibition effects against *B. cereus* and *S. aureus*; *P. fluorescens* and *E. coli* are more resistant to them [23,77]. It has been reported the antifungal properties of hydroxycinnamic acids, i.e., inhibiting the production of aflatoxins from *A. flavus* and *A. parasiticus* [23,78]. Herald and Davidson [77] reported the antibacterial activity of caffeic, ferulic, and p-coumaric acids against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus*; p-coumaric acid was the most effective against *E. coli* with concentrations of about 1000 μg/mL at pH 5.0 for 48 h, and *S. aureus* and *B. cereus* with concentrations of about 500 μg/mL at pH 5.0 for 48 h or at pH 7.0 for 9 h, respectively. Inhibition increased as pH decreased with *E. coli* and *S. aureus* but not *B. cereus*. Chipley and Uraih [78] studied the antimicrobial activity of o-nitrobenzoate, p-aminobenzoate, ethyl aminobenzoate, ethyl- and methyl-benzoate, salicylic acid, trans-cinnamic acid, trans-cinnamaldehyde, ferulic acid, o-acetoxy benzoic acid, and anthranilic acid on *Aspergillus flavus* and *A. parasiticus* growth and aflatoxin production at 27 °C. Both methyl- and ethyl-benzoate were the most effective at concentrations of about 2.5–5.0 mg/25 mL of medium reducing the mycelial growth and the aflatoxin production.

Furocoumarins are present in carrots, celery, citrus fruits, parsley, and parsnips. Several authors reported their antimicrobial activity against *E. coli* O157:H7, *Erwinia carotovora*, *L. monocytogenes*, and *Micrococcus luteus* [23,79]. The antimicrobial activity of furanocoumarins against *L. monocytogenes*, *E. coli* O157:H7, and *Micrococcus luteus* was investigated in a model food system (25% commercial vegetable baby food in peptone water) by Ulate-Rodríguez et al. [79]. The growth of *L. monocytogenes* was inhibited with lime peel extract and cold-pressed lime oil, but not the growth of *E. coli* O157:H7; *M. luteus* counts were inhibited only by the cold-pressed lime oil. The minimum inhibitory and the minimum bactericidal concentrations of *L. monocytogenes* were 32 or 43 μg/g, respectively.

Flavonoids, such as catechins, flavons, flavonols, and their glycosides, are present in apples, barley, grapes, plums, sorghum, and strawberries [23,80]. Cushnie and Lamb [80] reported antifungal, antiviral, and antibacterial activity. Quercetin activity was attributed to the inhibition of the enzyme DNA gyrase; the inhibition of the cell membrane functions by the activity of sophoraflavone G and

(-)-epigallocatechin gallate was reported and, moreover, the inhibition of the energy metabolism by the licochalcones A and C [80]. Other studied flavonoids are 2,4,2′-trihydroxy-5′-methyl chalcone, apigenin, galangin, lonchocarpol A, myricetin, robinetin, and rutin.

2.5. Hops

The hop (*Humulus lupulus* L.) flower's resin is used in the brewing industry for the bitter flavour it gives to beer [23]. Hop contains compounds (prenylated acylphloroglucinols and xanthohumol) that have inhibited bacteria growth, mostly Gram-positive [81–87]. The use of bitter acids as antimicrobials was approved by the Food Safety and Inspection Service (FSIS), USA [88]. Kramer et al. [81] studied the effect of hop extracts against some pathogens related with food using in vitro and meat model applications at 2 and 8 °C. The MIC of hop extracts with bitter acids (α- and β-acids) or xanthohumol were tested against *E. coli*, *S. aureus*, *S. enterica*, and *L. monocytogenes*. The xanthohumol and the β-acid inhibited the growth of the Gram-positive bacteria (MICs of 12.5 and 6.3 ppm, respectively), and the α-acid was less active (MIC of 200 ppm); in contrast, the Gram-negative bacteria were highly resistant. These authors concluded that "hop extracts could be used as natural preservatives in food applications to extend the shelf life and to increase the safety of fresh products." Bogdanova et al. [82] investigated the antibiofilm properties of hop compounds (humulone, lupulone, and xanthohumol) against *Staphylococcus* spp., including strains that were methicillin-susceptible and resistant. All compounds showed antimicrobial activity against all strains; lupulone, followed by xanthohumol had the strongest effect. Lupulone and xanthohumol penetrated the biofilm reducing the number of cells or reducing completely their number at the higher concentrations (lupulone: 125 μg/mL; xanthohumol: 60 μg/mL). Hop extracts showed different grades of inhibition against *L. monocytogenes* in food [86]: In coleslaw, 1 mg/g of hop extract increased the inactivation; in milk, 0.1–1 mg/mL was inhibitory; and in cottage cheese, hop extract was bactericidal at 0.1–3 g/kg. These authors concluded that, overall, the activity against *L. monocytogenes* in food was enhanced with acidity and lower fat content.

Some fungi are inhibited by hop acids [89,90] as well as protozoa [91]. Mizobuchi et al. [89] isolated a new flavonone (6-isopentenylnaringenin) from hard resins of hops; it was tested together with xanthohumol and isoxanthohumol showing antifungal activities against *Candida albicans*, *Fusarium oxysporum*, *Trichophyton mentagrophytes* and *T. rubrum*, and *Mucor rouxianus*. Srinivasan et al. [91] studied the antimicrobial spectrum of hop acid components for antiprotozoal activity, founding that ciliated protozoa were more sensitive than amoebae; plasmodia were sensitive but at a lower level than to the anti-malarial drugs. Xanthohumol was particularly potent, and the effect was enhanced by carbon dioxide.

2.6. Other Plants

Ahn et al. [92] focused their studies on the extracts from grape seed and pine bark; these authors found that their extracts can be used against *E. coli* O157:H7, *S. enterica* serovar Typhimurium, and *L. monocytogenes* in vitro and ground beef. The populations of *E. coli* O157:H7, *S. enterica* serovar Typhimurium, and *L. monocytogenes* decreased below 10 CFU/mL after 16 h. Markin et al. [93] studied olive leaves extracts founding deadly effects on bacteria, dermatophytes, and yeast. Olive leaf 0.6% extract killed within 3 h almost all cells from cultures of *E. coli*, *B. subtilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *S. aureus*; 1.25% after 3 d inhibited the growth of dermatophytes such as *Microsporum canis*, *Trichophyton mentagrophytes* and *T. rubrum*, whereas 15% of plant extract killed after 24 h of incubation all the cells from the yeast *Candida albicans*. Dogasaki et al. [94] and Ibrahim et al. [95] mentioned the antibacterial properties of coffee and its compounds such as caffeic acid, chlorogenic acid, and protocatechuic acid; these compounds inhibited the growth of *Legionella pneumophila* and *E. coli* O157:H7, respectively. Furthermore, tea (*Camellia sinensis*) was also demonstrated to feature antimicrobial properties [96–98] through its predominant catechin, epigallocatechin gallate, against methicillin-resistant *S. aureus*. Shan et al. [99] reported the activity of cinnamon stick extracts (*Cinnamomum burmanii* Blume) against *B. cereus*, *E. coli*, *L. monocytogenes*, *S. aureus*, and *Salmonella*

anatum. Major compounds in the cinnamon stick were identified: E-cinnamaldehyde and polyphenols; both components significantly contributed to the antimicrobial properties.

2.7. Plant By-Products

Large amounts of by-products are generated during the food processing of plants, such as fruit pomace, husks, kernels, peels, pulps, seeds, and unused flesh [2]. Usually considered as a waste, these by-products possess bioactive compounds with antimicrobial activity being promising sources for their commercial exploitation; Gyawali and Ibrahim [2] list some plant by-products as antimicrobials.

Extracts of grape pomane [100] and olive pomace [101,102] have shown to be able to inhibit the growth of *E. coli*, *Enterobacter* spp., *S. aureus*, *Salmonella* spp., and *L. monocytogenes*, and other spoilage and pathogenic bacteria. Sagdic et al. [100] incorporated grape pomace extracts into beef patties at different concentrations: 1–10% for 12, 24 and 48 h. All the microorganisms tested (*Enterobacteriaceae* and spoilage microorganisms) were inhibited at a concentration of 10% in all the storage periods. Friedman et al. [101] evaluated the bactericidal activity of 10 food-based powders against *E. coli* O157:H7, *S. enterica*, *S. aureus*, and *L. monocytogenes*. Olive pomace, juice powder, and leaves were active against all bacteria. All powders had strong activity against *S. aureus*.

Fruit peels are also important. Pomegranate fruit peels extracts showed their antimicrobial activity enhancing the shelf-life of chicken products [103], and their ability to inhibit the growth of *E. coli*, *B. cereus*, *L. monocytogenes*, *S. aureus*, and *Y. enterocolitica* [104–106]. Pomegranate peel showed good activity against *S. aureus* and *B. cereus* (MIC of 0.01%); concentrations of 0.1% inhibited *Pseudomonas* but *E. coli* and *S. typhimurium*. The shelf life of chicken products was enhanced by 2–3 weeks with the addition of pomegranate peel during chilled storage [103]. Li et al. [105] investigated the activity of the tannin-rich fraction from pomegranate rind against *L. monocytogenes*; punicalagin and ellagic acid were detected, and the MICs against *L. monocytogenes* strains were 1.25–5.0 mg/mL. The same research group [106] evaluated the effects of the same fraction on both the virulence gene expression and the *L. monocytogenes* interaction with the epithelial cells. The adhesion to and the invasion of Caco-2 cells were reduced at 2.5 mg/mL. Guava, jackfruit, mango, papaya, plum, tamarind, and their seeds were effective against *B. subtilis*, *E. coli*, *S. aureus*, and *P. aeruginosa* [107]. A major fruit by-product is tomato seeds from the tomato processing industry; tomato seeds extracts have shown inhibition of Gram-positive bacteria and fungi [108]. These authors studied the antimicrobial potential of tomato seed extracts against Gram-positive (*Bacillus cereus*, *Enterococcus faecalis*, *Micrococcus luteus*, *Staphylococcus aureus* and *S. epidermidis*) and Gram-negative (*E. coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *S. typhimurium*) bacteria and fungi (*Aspergillus fumigatus*, *Candida albicans*, and *Trichophyton rubrum*). *E. faecalis* was the most susceptible Gram-positive bacteria (MIC of 2.5–10 mg/mL). *C. albicans* was the most susceptible fungal species (MIC of 5–10 mg/mL).

Coffee husks, peel, and pulp are some of the main by-products obtained from coffee processing industry [2,109,110]; extracts from these by-products contain large amounts of phenolic compounds (tannins, flavonols, flavandiols, flavonoids, and phenol acids) and are potential natural preservatives for food [2]. Quoting Taveira et al. [108] and Gyawali and Ibrahim [2], "the waste produced by the food-processing industry could be incorporated into antimicrobial packaging or utilized as edible antimicrobial films".

3. Natural Antimicrobials from Animals

Some of the animal defence mechanisms have antimicrobial properties [1,23] destroying the cell membranes [1,60] and killing both Gram-negative and -positive bacteria [1].

3.1. Peptides

Antimicrobial peptides from animal origin have a broad range of antibacterial activities as well as antiviral [111].

Pleurocidin is a peptide with antimicrobial activity found in the skin secretions of the winter flounder (*Pleuronectes americanus*) [112], and it is active against Gram-positive and -negative bacteria such as *E. coli* O157:H7, *L. monocytogenes*, *Saccharomyces cerevisiae*, *Penicillium expansum*, and *Vibrio parahemolyticus* [113,114]. Burrowes et al. [113] evaluated pleurocidin in food applications using 18 microbial species. Pleurocidin was effective against *E. coli* O157:H7, *L. monocytogenes*, *P. expansum*, *S. cerevisiae*, and *V. parahemolyticus* with MIC of 5.3, 23.0, 20.6, 5.5, and 69 μM, respectively; no haemolytic or cytotoxic effect on intestinal cells were found. Patrzykat et al. [114] identified peptide effects studying a flounder pleurocidin and frog dermaseptin hybrid. At 2 μg/mL, dermaseptin inhibited the growth of *E. coli* but did not kill the cells within 30 min; concentrations equal to or higher than 20 μg/mL reduced the viable counts by 2 log within 5 min. Pleurocidin showed variations of this antimicrobial pattern.

Other antimicrobial peptides are defensins, protamine, magainin, and casocidin [1]. Defensins are produced by vertebrates-phagocytes of mammals and epithelial cells, with antimicrobial properties against bacteria and fungi, as well as viruses [60]. Protamine and magainin are active against bacteria and fungi [60,115]: protamine is a protein obtained from sperm cells of vertebrates [116], and magainin from the skin of the frog *Xenopus laevis* [117,118]. Protamine is a cationic peptide; its activity against microorganisms is probably due to its electrostatic affinity to negatively charged bacteria. Potter et al. [115] tested this hypothesis in model broth (tryptic soy broth) and food systems (milk and ground beef). The analysis of 21 bacteria revealed that the most negatively charged were also the most susceptible. Kim et al. [116] investigated the suppressive effects of protamine on the growth of oral pathogens; 12 strains of streptococci, *Actinomyces naeslundii* and *A. odontolyticus*, *Aggregatibacter actinomycetemcomitans*, *Candida albicans*, *Enterococcus faecalis*, *Fusobacterium nucleatum*, *Lactobacillus acidophilus*, and *Porphyromonas gingivalis* were inhibited (MIC of 0.009–20 mg/mL). Zasloff [117] tested magainin for its antibacterial activity founding that, at low concentrations, inhibited the growth of numerous species of bacteria (*E. coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Citrobacter freundii*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Serratia marcescens*, *Proteus mirabilis*, and *Streptococcus fecalis*) and fungi (*Saccharomyces cerevisiae*, *Cryptococcus neoformans*, and *Candida albicans*) and induce osmotic lysis of protozoa (*Amoeba proteus*, *Euglena gracilis*, and *Paramecium caudatum*). Casocidin is another peptide obtained from bovine milk with antibacterial activity against *E. coli* and *Staphylococcus carnosus* [119]. The primary structure of casocidin is a fragment of 39 amino acids of bovine αs2-casein. The casein-αs2 is not present in human milk, so Zucht et al. [119] hypothesized that "these findings could explain the different influence of human and bovine milk on the gastrointestinal flora of the suckling."

Lactoferrin is a peptide with capacity against Gram-positive and -negative bacteria, fungi, and parasites [2,120,121]. Murdock et al. [120] determined whether nisin and lactoferrin would act synergistically against *L. monocytogenes* and *E. coli* O157:H7. *L. monocytogenes* was inhibited with 1000 μg/mL of lactoferrin, although *E. coli* O157:H7 counts initially decreased and then recovered to cell counts similar to the control. Lactoferrin (500 μg/mL) plus nisin (250 IU/mL) effectively inhibited the *E. coli* O157:H7 growth, whereas 250 μg/mL plus 10 IU/mL, respectively, had an inhibitory effect suggesting that lactoferrin and nisin act synergistically to inhibit both microorganisms. López-Expósito et al. [121] studied whether the antimicrobial activity of nisin could be enhanced by lactoferrin f and αs2-casein f against *Escherichia coli*, *Listeria monocytogenes*, *Salmonella choleraesuis*, and *Staphylococcus epidermidis*. Results showed a synergistic effect against *E. coli* and *S. epidermidis*; moreover, another synergistic effect was found between αs2-casein f and nisin against *L. monocytogenes* because of its capacity to develop resistance to nisin. Lactoferrin binds iron [1,2] and is used as antimicrobial in meat products [122]. Murdock et al. [120] and Al-Nabulsi and Holley [123] reported the antimicrobial activity of lactoferrin against foodborne bacteria such as *E. coli*, *Carnobacterium* spp., *Klebsiella*, and *L. monocytogenes*. Lactoferrin in a concentration of about 8 mg/mL killed 4 log CFU/mL of *Carnobacterium viridans* at 4, 10 and 30 °C and neutral pH in a broth system [123].

Lactoperoxidase is a protein (glycoprotein enzyme) present in raw milk, colostrum, saliva, and other secretions [23,124]. Lactoperoxidase reacts with thiocyanate and hydrogen peroxide forming

the termed lactoperoxidase system (LPS) with antimicrobial capacities. Thiocyanate is found in milk and other animal secretions by the metabolism of amino acids and glucosides from the diet or by detoxification of thiosulfates. Hydrogen peroxide is not present in milk, so it needs to be added, or obtained from lactic acid bacteria activity or enzymatic action [23,124,125]. Potential mechanisms used by the LPS were reviewed by Bafort et al. [125]. The LPS inhibits both Gram-positive and -negative pathogens including *E. coli* O157:H7, *Y. enterocolitica*, *Salmonella*, *S. aureus*, *L. monocytogenes*, *C. jejuni* and *P. aeruginosa* in different foods [23,124,126–130]. Elliot et al. [127] assessed the growth of *E. coli* O157:H7, *L. monocytogenes*, *S. aureus*, *S. enterica* subsp. enterica serovar Typhimurium, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, and beef microbiota on meat surfaces treated with the LPS at 37 °C for 24 h, 12 °C for 7 d, 12 to −1 °C for 1 week, and −1 °C for 4 weeks. LPS was more effective at refrigeration temperatures, inhibiting the growth of *Pseudomonas* from beef microbiota but of lactic acid bacteria. McLay et al. [128] evaluated an LPS-monolaurin system (5–200 mg/kg of lactoperoxidase and 50–1000 ppm of monolaurin) for the inhibition of *E. coli* O157:H7 and *Staphylococcus aureus* in growth, milk and ground beef. In broth, the growth of both microorganisms was inhibited more strongly than in milk, and in milk more than in ground beef. The potential use of the LPS in broth at 37 °C and ground meat preparations at 0, 6, and 12 °C was examined by Kennedy et al. [129]. *L. monocytogenes* was the most sensitive, followed by *Staphylococcus aureus* and *E. coli* O157:H7. The inhibition was highly dependent of the temperature: it was maximal at a temperature adequate but not optimal for the bacterial growth. The LPS was tested against *Salmonella enteritidis* in tomato and carrot juices, milk, liquid whole egg, and chicken skin extract under different conditions [130]; at low pH and 30 °C, LPS was more effective in vegetables than in animal products.

Avidin is present in egg albumen and stable to heat and pH [23]. Avidin is a glycoprotein that binds biotin, creating one of the strongest non-covalent interactions in nature [131]. Biotin probably plays a role in the immune system because it is an enzyme cofactor in the tricarboxylic acid cycle and in the biosynthesis of fatty acids [132–134]. Avidin has been extensively used for biochemical assays, diagnosis, and drug delivery [135]. Recently, there has been growing interest in studying this avidin-biotin interaction in nanoscale drug delivery systems [131,136]. Korpela et al. [137] studied the binding of avidin to Gram-negative and -positive bacteria (*Enterobacter cloacae*, *E. coli*, *B. cereus*, *P. aeruginosa*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Streptococcus pyogenes*, and *Staphylococcus aureus* and *S. epidermidis*). Avidin showed binding capacity to all the tested Gram-negative bacteria and to some Gram-positive. The *E. coli* K-12 avidin receptor was the outer membrane's porin protein (OmpFOmpC); these authors hypothesised that avidin traps biotin preventing its entry into the cell. Moreover, the avidin binding to the cell membrane may also imply a role in the mechanism of infection.

Ovotransferrin or conalbumin is another glycoprotein that also occurs in egg albumen and with inhibitory activity against both Gram-positive and -negative bacteria, and some yeasts [23,138–140]. Bacterial sensitivity to ovotransferrin varied among species, being *E. coli*, *Pseudomonas*, and *Streptococcus mutans* the most sensitive, and *Staphylococcus aureus, Proteus*, and *Klebsiella* the more resistant [138]. Although ovotransferrin's antimicrobial property is thought to be the result of its capacity to bind the iron used by the microorganisms for growth, recent studies suggest that its role is also iron independent [138,139]. Ovotransferrin properties imply applications such as ingredients for infant formula, food additive, or antimicrobial agent for animal health [139,141]. Furthermore, ovotransferrin has similarities to the homologous mammalian lactoferrin in terms of its protective roles, suggesting a "direct relationship between egg consumption and human health" [141].

Lysozyme is an enzyme found in egg white and milk, and even in blood [1,142–145]. Masschalck and Michiels [146] reviewed the properties against the microorganisms as well as the mode of action of lysozyme against Gram-positive and -negative bacteria, also providing insight into the causes of bacterial resistance. The antimicrobial capacities of lysozyme against bacteria are mainly due to its enzymic activity; lysozyme acts through peptidoglycan hydrolysis and cell lysis [1,143]. However, a new nonlytic activity has been shown by new findings [146,147]. As Gram-negative bacteria are resistant to lysozyme because of their outer membrane, different strategies were developed to extend

its spectrum, including denaturation, modification (by attachment of polysaccharides, fatty acids, etc.), genetic modification, membrane permeabilizing agents (ethylenediaminetetraacetic acid, EDTA), and/or permeabilizing treatments such as high hydrostatic pressure treatments [146,148–155]. In order to study the role of the lysozyme enzymatic activity on its capacity against Gram-positive bacteria (*S. aureus* and *Bacillus subtilis*), Ibrahim et al. [148] constructed an inactive mutant of lysozyme. These authors revealed that the lysozyme activity against Gram-positive bacteria is independent of its muramidase activity; thus, the antibacterial action is due to structural factors.

3.2. Polysaccharides

Chitosan is produced commercially from chitin, a by-product obtained from exoskeletons of crustaceans and arthropods [1,23], with capacity to inhibit the growth of moulds and yeasts (*Aspergillus flavus*, *Botrytis cinerea*, *Byssochlamys* spp., *Mucor racemosus*, *Rhizopus stolonifer*, *Saccharomyces cerevisiae*, and *Zygosaccharomyces bailii*) and bacteria (*E. coli*, *Lactobacillus fructivorans*, *L. monocytogenes*, *Salmonella*, *S. aureus*, and *Y. enterocolitica*) from food [150,156–164]. Oh et al. [156] tested the antimicrobial activities of chitosan against food spoilage microorganisms in mayonnaise (*Lactobacillus plantarum* and *L. fructivorans*, *Serratia liquefaciens*, and *Zygosaccharomyces bailii*) founding an important decrease of the counts of *L. fructivorans* and *Z. bailii* at 25 °C. Roller and Covill [158] studied the use of chitosan (3 g/L) in mayonnaise with 0.16% of acetic acid or 1.2–2.6% of lemon juice and a population of ca. 5–6 log CFU/g of *S. enteritidis*, *Z. bailii*, or *L. fructivorans* at 5 or 25 °C for 8 d; the results of these authors showed that chitosan with acetic acid could be used as a natural preservative under such conditions. Chitosan at a concentration of 0.005% combined with sodium benzoate (0.025%) were synergic against yeasts (*Saccharomyces exiguus*, *Saccharomycodes ludwigii* and *Torulaspora delbrueckii*) in saline solutions [159]. Recently, chitosan's films with antimicrobials attached (garlic oil, sorbic acid, and nisin) to the polymer were used for packaging applications or in combination with ethanolic extract and polypropylene in order to contact the surface of the food; these films inhibited the growth of *E. coli*, *Cronobacter sakazakii*, *Salmonella*, *Staphylococcus*, *L. monocytogenes*, and *B. cereus* [150,158,165–171]. Sagoo et al. [159] and Devlieghere et al. [167] theorized that chitosan may interact with the cytoplasmic membrane's anionic polysaccharides and/or interfere the cell protein synthesis, both resulting in cell inhibition by altered permeability and/or compromised protein transport. The molecular weight of chitosan, its role as water binding agent and enzyme inhibitor, the improvement of membrane permeability, its activity as a bio-absorbent competing against bacteria nutrients, and its capacity to bind to DNA and inhibit the synthesis of mRNA and proteins have been reported [163,172–179].

3.3. Lipids

Food lipids may inhibit the proliferation of microorganisms [1], e.g., those present in milk can inhibit Gram-positive and -negative bacteria, and fungi [180–185]. The activity of triglycerides and lipids from bovine milk was investigated against *E. coli* O157:H7, *S. enteritidis*, *Campylobacter jejuni*, *L. monocytogenes*, and *Clostridium perfringens* by Sprong et al. [180]. C10:0 and C12:0 fatty acids and sphingolipids showed bactericidal activity, whereas phosphoglycerides were moderately bactericidal. These authors [181] also studied the activity of sphingolipids combined with C10:0 and C12:0 fatty acids, and unsaturated C18 fatty acids; *L. monocytogenes* and *Campylobacter jejuni* were very sensitive, whereas *E. coli* O157:H7 and *S. enteritidis* were less vulnerable. Eicosapentaenoic acid and docosahexaenoic acid, two fatty acids from animal origin, can inhibit the growth of bacteria [186–188]. The knowledge about their mode of action is limited; their first action is the electron transport chain's disruption and the cell membrane's oxidative phosphorylation [188]. Furthermore, the mechanism of action also implies the inhibition of the enzymatic activity by the cell, the damage of the nutrient uptake, or peroxidation. The combination of fatty acids and monoglycerides was studied showing an increase in the antimicrobial activity [1]. Their broad spectrum of activity makes them very useful as agents against microorganisms for various applications in food safety increasing food quality through its preservation [188].

4. Natural Antimicrobials from Bacteria and Viruses: Biopreservation

Biopreservation is defined as "the use of microorganisms (including bacteriophages), their metabolic products, or both to preserve foods that are not generally considered fermented" [189].

4.1. Controlled Acidification

The production of acid by lactic acid bacteria (LAB) under controlled acidification conditions is a preservation form very important in food production; the pH of the food, the characteristics of the targeted microorganism, the fermented carbohydrate that is going to be used by the biopreservatives, the LAB growth kinetics, the temperature (refrigeration or abuse), and the targeted pathogens are factors to be taken into account [189]. The use of LAB for biopreservation comes back to the 1950s for preventing the production of botulinum toxin [190–192]; *Clostridium botulinum* is unable to grow at pH < 4.6, so LAB is added to the food for acid production after the use of a fermentable carbohydrate also added to it and, consequently, the pH decreases [190,193]. Hutton and Chehak [193] described the "Wisconsin process" (lactic acid starter culture combined with sucrose) as a similar method to be applied in bacon with a reduced content of nitrite. These authors also found a similar effect with the combination of *Pediococcus acidilactici* plus dextrose in chicken salad; it was interesting to report that conditions of abuse temperature were the most effective for the production of acid by *P. acidilactici* after the use of dextrose.

4.2. Bacteriocins

Some lactic acid bacteria produce ribosomally synthesized peptides, called bacteriocins, with antimicrobial capacity that are not lethal to the host; these proteins inhibit both pathogenic and spoilage bacteria without changing the physicochemical characteristics of the food, that is, the inhibition is not exerted by acidification, protein denaturation, or other processes [189,194–204]. The interest in LAB-produced bacteriocins has grown dramatically because of their antimicrobial capacities and the use of LAB as starter cultures [198]. The use of bacteriocins or bacteriocinogenic LAB or both is important to the food industry because of the demand for natural products by consumers that are also increasing concern about foodborne pathogens [189].

Gram-positive bacteria are inhibited by most bacteriocins. Gram-negative bacteria can be increasingly sensitive to bacteriocins after the use of chelating agents or hydrostatic pressure [205,206]; these techniques can also be synergic enhancing the action of bacteriocins against Gram-positive bacteria [189,207], or even with the use of nanoparticles [208,209] or nanovesicles [210].

Bacteriocins can be used in different ways in foods [189]: (i) They can be directly added to foods inhibiting the growth of both pathogenic and spoilage bacteria; nisin is the only bacteriocin commercially available. Nisin is added to milk, cheese, canned foods, mayonnaise, and other foods. It is considered as a Generally Recognized as Safe (GRAS) food preservative, and once it is absorbed onto surfaces it inhibits the growth of *Listeria* spp. and prevents the formation of biofilms [211–216]. Pediocins also inhibit the growth of *L. monocytogenes* and they are used in salads and salad dressings, cream, cheese and meats [216–225]. Reuterin is secreted by *Lactobacillus reuteri* and possess activity against Gram-positive and -negative pathogenic bacteria [226,227]. (ii) To add bacteriocin-producing bacteria to non-fermented foods or use them as starter cultures for the improvement of food safety. For a fine description see Montville and Chikindas [189]. Natamycin is produced by fermentation using *Streptomyces* spp. acting against foodborne moulds and yeasts; however, it is inactive against bacteria and viruses [1,228]. (iii) A third way is to use bacteriocin-producing bacteria as starter cultures to direct the fermentation. The benefits of defined starter cultures depend on their capacity to predominate over the indigenous microbiota [229–233].

An increasing problem is resistance to bacteriocins. The emergence of pathogens resistant to bacteriocins can undermine their use as antimicrobial agents. For example, nisin-resistant isolates have been generated from *C. botulinum*, *L. monocytogenes*, *S. aureus*, and *Bacillus licheniformis*, *B. subtilis*, and

B. cereus [234–238]. Ming and Daeschel [234] evaluated Gram-positive pathogenic and food spoilage bacteria for their resistance to nisin obtaining a *L. monocytogenes* mutant (resistant to nisin at 2000 U/mL). The resistant mutant had straight-chain fatty acids at a higher percentage, and less percentage of branched-chain fatty acids; thus, both the cell membrane structure and function suffered changes as a resistance response to nisin. Turovskiy et al. [235] investigated the quorum sensing mediated by the autoinducer AI-2 as a mechanism for triggering the stress response in *L. monocytogenes*; thus, they examined the acquisition of resistance to nisin and lactic acid by the microorganism. After pre-exposing the cells to the autoinducer and being challenged with specific stresses, the resistance to nisin and lactic acid was not mediated through quorum sensing. The frequencies of colony formation on agar media with different concentrations of nisin by different strains of *Clostridium botulinum* were determined by Mazzotta et al. [236]. Increasing concentrations of nisin generated resistant isolates, and the cells' nisin resistance was maintained by their spores. Naghmouchi et al. [237] developed variants of *Listeria monocytogenes* resistant to pediocin PA-1, divergicin M35, and nisins A and Z. These authors reported that the resistance decreased antibiotic sensitivity to ampicillin, chloramphenicol, erythromycin, and tetracycline. Laursen et al. [238] studied if the exposure to a *Lactobacillus plantarum* pediocin could lead to resistance in *L. monocytogenes*. These authors observed changes in the expression of genes regulated by the LisRK system and the SigB and SigL sigma factors; thus, a single exposure to a sublethal concentration of the bacteriocin initiates a response leading to resistance. Some authors have suggested the use of bacteriocin mixtures to overcome the problem of resistance [239,240] although this method is effective only when different mechanisms of resistance are implicated [189]. Cross-resistance among bacteriocins have been observed [237,241–244] complicating the situation. The stability of *Listeria monocytogenes* mutant's resistance to LAB bacteriocins (mesenterocin, curvaticin, and plantaricin) was estimated by Rekhif et al. [241] who found that it was maintained for several generations even when the bacteriocins were not present. Furthermore, the mutants resistant to one of the bacteriocins showed a cross-resistance to the two other bacteriocins, but not to nisin. Nisin-resistant variants of *Listeria monocytogenes* as well as resistant to pediocin produced by *Pediococcus pentosaceus* 34, and enterocin produced by *Enterococcus faecium* FH99 were developed by Kaur et al. [242,243]. Cross-resistance between pediocin 34 and enterocin FH 99 was found, but not with nisin. The understanding of bacteriocin resistance is incomplete and further investigations are needed.

4.3. Bacteriophages

Bacteriophages are viruses whose only hosts are bacteria [245–247]. The addition of virulence factors to the host has been reported, although reductions in virulence have been also described [248] with potential consequences in phage therapy.

Bacteriophages are consumed with the diet because they are natural components of food microbiota [189,249]. The use of bacteriophages to control foodborne bacteria is characterized by the low numbers of non-growing pathogens together with large populations of indigenous bacteria [250–255]. Moreover, the repeated use of bacteriophages in food could create resistance [189,256]. The complexity of this issue caused Montville and Chikindas [189] to conclude that "like bacteriocins, bacteriophages are not silver bullets but need to be used from a perspective that considers the microbial ecology of the food".

5. Natural Antimicrobials from Algae and Mushrooms

Macroalgae (seaweeds) and microalgae (diatoms) produce substances with antimicrobial activity. Pharmaceutical and food industries search for promising marine algae derivatives [257–259]. There is limited research to evaluate the antimicrobial activity of algae in food biopreservation.

Several authors have studied the antimicrobial characteristics of algae. Herrero et al. [260] carried out the screening for this type of compounds in a macro- and a micro-algae (*Himanthalia elongata* and *Synechocystis* spp., respectively); extracts from both had antioxidant capacities and antimicrobial action against *S. aureus* and *E. coli*. Similar results were obtained by Devi et al. [261] when they studied

extracts from *Haligra* spp. active against *S. aureus*. *Hymanthalia elongata*, *Saccharina latissima*, *Laminaria digitate*, *Padina*, and *Dictyota* were reported to have antimicrobial activity against *L. monocytogenes*, *Salmonella*, *Enterococcus faecalis*, *P. aeruginosa*, *B. cereus*, and *E. coli* [262,263].

Algae-derived compounds such as carrageenan and alginates are useful for food coatings and films; these compounds together with other natural antimicrobials will enhance their applications [264]. Carrageenan and alginates have been used in a variety of ways in food industry: forming nanocomposite films enriched with essential oils enhancing its effectiveness against *L. monocytogenes* [265], combined with chitosan and isothiocyanate in a film used for food packaging and active against *C. jejuni* [266], or combined with EDTA in a film increasing the potential to reduce *Salmonella* populations [267]. Alboofetileh et al. [265] developed nanocomposite films with antimicrobial characteristics to control the growth of foodborne pathogens. Firstly, they tested the antibacterial effects of the essential oils of caraway, cinnamon, clove, coriander, cumin, and marjoram against *E. coli*, *S. aureus*, and *L. monocytogenes*. Then, the essential oils of marjoram, clove, and cinnamon—the most potent against the microorganisms—were incorporated into nanocomposite films made with alginate or clay and tested for 12 d. Marjoram (1.5%) showed the highest activity against microorganisms in all matrices decreasing the populations of the three microorganisms up to 6.3, 4.5, and 5.8 log, respectively. Four *Campylobacter jejuni* strains were tested with allyl isothiocyanate contained into an edible coating (with 0.2% κ-carrageenan and 2% chitosan) on vacuum-packaged chicken breasts at 4 °C [266]; the coatings with 50 or 100 μL/g of allyl isothiocyanate reduced the number of cells of the microorganism to levels below the detection limit after 5 d. Olaimat and Holley [267] tested the same carrageenan/chitosan coating previously described for its ability to inhibit the population of *Salmonella* on fresh chicken breasts; the edible coating contained allyl isothiocyanate, mustard, EDTA or their combinations. Coatings containing 50 μL/g of allyl isothiocyanate or 250 mg/g of mustard reduced Salmonella's counts about 2.3 log CFU/g at 4 °C after 21 d; LAB was also reduced.

Among fungi, mushrooms have antimicrobial and antioxidant capacities [2]. Wild *Laetiporus sulphureus* (Bull.) Murrill fruiting bodies extracts have shown antimicrobial activity in vitro against bacteria such as *Candida albicans*, *Candida parapsilopsis*, *E. coli*, *S. aureus*, *Enterococcus faecalis*, and *S. epidermidis*. Antimicrobial activity was also detected by edible mushrooms extracts of *Aphyllophorales* [268], *Agaricus* [269], and *Armillaria mellea*, *Meripilus giganteus*, *Morchella costata* and *M. elata*, *M. esculenta* var. vulgaris, *M. hortensis*, *M. rotunda*, *Paxillus involutus*, and *Pleurotus eryngii* and *P. ostreatus* [270]. Methanolic extracts of 6 wild edible mushrooms (*Cantharellus cibarius*, *Clavaria vermiculris*, *Lycoperdon perlatum*, *Marasmius oreades*, *Pleurotus pulmonarius*, and *Ramaria formosa*) were used by Ramesh and Pattar [268]. All the isolates showed high content of phenols and flavonoids with antimicrobial activity against several of pathogenic bacteria (*E. coli*, *B. subtilis*, *P. aeruginosa*, and *S. aureus*) and fungi (*Candida albicans*) indicating that the concentrations of the components directly influence the capability of the isolated mushrooms against the microorganisms. Öztürk et al. [269] investigated the fatty acids from *Agaricus essettei*, *A. bitorquis* and *A. bisporus* extracts founding that linoleic and palmitic acids were dominant and active against Gram-positive bacteria (*Micrococcus luteus*, *Micrococcus flavus*, *Bacillus subtilis*, and *Bacillus cereus*). Kalyoncu et al. [270] determined the antimicrobial activities of ethanol extracts from the mushrooms cited above against 11 microorganisms (*Bacillus cereus* and *Bacillus subtilis*, *E. coli*, *Enterobacter aerogenes* and *E. cloacae*, *Enterococcus faecalis*, *Proteus vulgaris*, *S. typhimurium*, *Sarcina lutea*, *S. aureus*, and the yeast *Candida albicans*). *P. ostreatus* and *M. giganteus* were the species with the greater activity against both bacteria and yeast.

Mushrooms' antimicrobials have not been sufficiently investigated to date for their food application. A fine review of them can be found in Gyawali and Ibrahim [2]. Further information can be found in References [271–274].

6. Future Perspectives

The food industry is receiving increasing pressure from consumers for the use of natural components in its products. The major objective is to use them as natural antimicrobials, and three methods are the most promising in food systems.

6.1. Direct Application on Food

The use of natural antimicrobials in food as biopreservatives is often limited due to the smell and taste given to the foods and the difficulties for achieving a good solubility in them [2,275]. Antimicrobial activity against *B. cereus* in rice has been demonstrated after the inclusion of basil, thyme, or oregano essential oils [43,276,277]; 1% of fresh garlic was active against *E. coli* O157 and *S. enterica* serovar Enteritidis in mayonnaise [37]; inhibitory activity against *L. monocytogenes* was found with ground cinnamon in pasteurized apple juice [278] and with essential oils of cinnamon, bark, and clove in semi-skimmed milk [279]; the essential oils of clove, cinnamon, thyme, and bay were active against *L. monocytogenes* in cheese [280,281]; thyme, oregano and lemongrass essential oils combined with modified atmosphere packaging were used to evaluate the inhibition of the total mesophilic population in cabbage and radish sprouts resulting in almost total inhibition of the microorganisms [282].

The shelf-life of meat and meat products has been improved using extracts or essential oils of natural antimicrobials compounds. Thyme and oregano essential oils at 0.1–0.3% were active against meat-based products dipped in them and combined with modified atmosphere packaging [283]. Thyme essential oil combined with nisin significantly decreased the population of *L. monocytogenes* [284] and *E. coli* O157:H7 [285] in minced beef meat under refrigeration conditions. *C. jejuni* populations were reduced in chicken meat after the application of rosemary extracts combined with a pre-freezing period [286] or after the use of *Inula graveolens*, *Laurus nobilis*, *Satureja montana*, and *Pistacia lentiscus* essential oils combined with packaging under microaerophilic conditions [287].

6.2. Edible Films and Coatings for Packaging

Food-packaging related industries are showing interest in the use of natural antimicrobials in edible films and coatings to improve food quality. At the same time, consumers' concerns created by plastic packaging are reduced. Lysozyme-chitosan composite films (a 2% chitosan film with an incorporated solution of 10% lysozyme at 0, 20, 60, or 100%) were developed by Park et al. [150] for improving the antibacterial capacities of chitosan films. The efficacy of chitosan films was enhanced with 60% lysozyme against both *Streptococcus faecalis* (reduction of 3.8 log cycles) and *Escherichia coli* (reductions of 2.7 log cycles). Bayarri et al. [153] determined the properties of lysozyme with methoxyl pectin for developing an edible film with antimicrobial activity. The formation of these complexes considerably decrease the lysozyme antimicrobial activity; however, after their use to manufacture an edible antimicrobial film, the lysozyme release was controlled and the enhancement of the lysozyme release was reported, allowing the use of the edible film to protect foods against microorganisms sensible to lysozyme activity. In their study, Güçbilmez et al. [154] incorporated lysozyme into zein films together with chickpea albumin, bovine serum albumin, and EDTA; that combination gave zein films effective activity against *E. coli* and *Bacillus subtilis*. Chitosan was combined with sodium caseinate to create films by Moreira et al. [162]; these authors evaluated their effectiveness against microbiota of cheese, salami, and carrots. Assays with the film-forming solutions applied on foods showed a significant antimicrobial action on the mesophilic, psychrotrophic, and yeasts and moulds populations with reductions of about 2.0–4.5 log CFU/g.

Edible films containing different extracts or essentials oils have shown their efficacy against foodborne pathogens such as *E. coli* O157:H7 [288–290], *L. monocytogenes*, and *Salmonella* [289,290]. Jang et al. [288] manufactured an edible film for strawberries containing 10% of grapefruit seed extract. The film inhibited the growth of *E. coli* O157:H7 and *L. monocytogenes*, and the populations of aerobic bacteria, yeast and moulds decreased after 14 d of storage. Ready-to-eat minimally processed salads

were packaged under modified atmosphere conditions with films of polypropylene plus ethylene-vinyl alcohol copolymer (with a 29% ethylene molar content) containing oregano and citral [289,290]. The results showed that antimicrobial activity reduced spoilage microbiota on the salad as well as inhibit the growth of *E. coli*, *S. enterica*, and *L. monocytogenes*; the inhibition was greater against Gram-negative bacteria. Chitosan-based films have been very effective in increasing the shelf-life of different products such as fruits and vegetables [291–296], and different meats and products [297–300]. Cé et al. [291] reported the increase in activity against several bacteria in minimally processed pear after the addition of nisin and peptide P34 to chitosan films: *E. coli*, *B. cereus*, *Clostridium perfringens*, *Lactobacillus acidophilus*, *L. monocytogenes*, *S. aureus*, *S. enteritidis*, *Aspergillus phoenicis*, and *Penicillium stoloniferum*. Films containing natamycin showed similar inhibition than those with chitosan alone. Vodnar et al. [297] developed chitosan-based films with bioactive compounds from green and black teas for the control of *L. monocytogenes* on vacuum-packaged ham steak at 20 °C for 10 d and 4 °C for 8 weeks. *L. monocytogenes* growth was inhibited in a dose-dependent manner: 4% of green tea extract was the most effective at both temperatures; 2% of green tea or 2% and 4% of black tea showed less antibacterial activity. Chitosan lactate was included into low-density polyethylene [298]; these films were applied on the surfaces of red meat and tested against *E. coli*, *L. monocytogenes*, and *S. enteritidis*. The microorganisms on the meat surface were not inhibited; however, a significant extension of the red colour shelf-life was observed. Soy protein edible films with EDTA or nisin have been studied for their physical and antimicrobial properties [301]; the films incorporated with 1% of grape seed extract, 10,000 IU/g of nisin, and 0.16% of EDTA showed the greatest activity against *L. monocytogenes* reducing its population by approximately 3 log CFU/mL. *E. coli* O157:H7 and *S. typhimurium* counts were reduced by approximately 2 and 1 log CFU/mL, respectively.

6.3. Nanoparticles and Nanovesicles

Nanotechnology is increasing its role in the food industry and some studies have been carried out over the last years. Applications of nanotechnology to deliver natural antimicrobial compounds in foods are very limited because of the complexity of the technology needed and the food matrix. Eby et al. [149] reported that hen egg-white lysozyme catalyzed the formation of silver nanoparticles that were able to maintain the hydrolase function of the enzyme; they were effective against *E. coli*, *Bacillus anthracis*, *S. aureus*, and *Candida albicans*. These nanoparticles had strong activity against silver-resistant strains of *Proteus mirabilis* as well as against an antibiotic- and silver-resistant *E. coli* strain. Human epidermal keratinocytes studies showed that these nanoparticles were non-toxic at the concentrations used to inhibit microbial growth. Nisin nanoparticles have been tested against *L. monocytogenes* and *S. aureus* with good results [302–304], as well as bacteriocin nanovesicles [210] or nanoparticles [208,209] against different pathogens. Zou et al. [302] evaluated the prolonged antimicrobial stability of liposome nanoparticles loaded with nisin against *L. monocytogenes* and *S. aureus*. The MIC of the nanoparticles against both microorganisms was 320 UI/mL, reducing their populations by more than 6 log CFU/mL after 48 and 72 h of incubation, respectively. Field et al. [303] identified a nisin A variant with a serine to glycine change at position 29 and with enhanced efficacy against *S. aureus*. Three more derivatives were developed and tested against *E. coli*, *Cronobacter sakazakii*, and *S. enterica* serovar Typhimurium showing enhanced antimicrobial activity. Encapsulation provides stability to bacteriocins; thus, de Mello et al. [210] encapsulated the peptide pediocin in nanovesicles of soybean phosphatidylcholine. The nanovesicles maintained 50% of the pediocin antimicrobial activity for 13 d at 4 °C against *Listeria monocytogenes*, *L. innocua*, and *L. ivanovii*. Gold nanoparticles with *Lactobacillus acidophilus* CH1 bacteriocin were used by Mossallam et al. [208] against intestinal microsporidiosis in immunosuppressed mice. The anti-microsporidia activity of the bacteriocin was potentiated, showing a sustained reduction in faecal spore shedding and intestinal spore load.

7. Conclusions

Since consumers increasingly demand food free of synthetic preservatives, it is necessary to identify and study new alternatives. These new approaches should be useful for controlling foodborne pathogens and for extending the foods' shelf-life. From the economic point of view, the search for natural antimicrobials must be cost-effective, and one alternative approach would be the mixture of several natural antimicrobials combined with food preservation techniques.

Due to the complexity of food matrices, natural antimicrobial compounds could bind with some food components limiting their action. Nanoparticles and/or nanovesicles have enormous potential in food safety as an effective antimicrobial delivery system, although this technology has raised concerns over consumers' safety. Therefore, further research is needed to determine the best antimicrobial delivery technology and the best concentrations of such natural antimicrobial compounds.

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antibiotics

MDPI

Review

Natural Product Type III Secretion System Inhibitors

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Abstract: Many known inhibitors of the bacterial type III secretion system (T3SS), a virulence factor used by pathogenic bacteria to infect host cells, are natural products. These compounds, produced by bacteria, fungi, and plants, may have developed as prophylactic treatments for potential attack by bacterial pathogens or as an attempt by symbiotic organisms to protect their hosts. Regardless, better understanding of the structures and mechanisms of action of these compounds may open opportunities for drug development against diseases caused by pathogens utilizing the T3SS. This review will cover selected known natural products of the T3SS and detail what is known of their origin and mechanism of action. These inhibitors highlight nature's ability to modulate interactions between organisms at a cellular level.

Keywords: natural products; pathogenesis; type III secretion system; probiotics; prophylaxis

1. Introduction

The type III secretion system (T3SS) is a virulence factor utilized by many Gram-negative pathogens to enable and perpetuate infection of a host [1–3]. Pathogens known to encode a T3SS include enteropathogenic and enterohemorrhagic *Escherichia coli* (EPEC and EHEC, respectively) [4–6], *Salmonella enterica* serovar Typhimurium [1], *Chlamydia* species [7], *Yersinia pestis* [2,8,9], *Vibrio* spp., *Shigella* spp., and *Pseudomonas* spp. [2]. The T3SS is indispensable in the ability of a pathogen to cause infection, with knockouts of the T3SS being avirulent [10]. Chemical inhibition of the T3SS has emerged as a strategy to combat these pathogens [11]. Inhibition of the T3SS results in an inability of a pathogen to cause infection in a host; in vivo studies in mice have shown that T3SS inhibitors allowed the host immune system to clear the infection better than a placebo [12–14]. In addition, a functioning T3SS is not necessary for bacterial cell viability, and inhibition of the T3SS is not toxic to the pathogen [15]. This removes the selective pressure for the formation of resistance to treatment [16].

The T3SS functions like a molecular syringe by injecting or secreting effector proteins directly from the cytosol of the bacterial pathogen through the host cell membrane, earning it the nickname "the type III injectosome" [2,4,17–20]. The structure of the injectosome can be broken down into three major regions, the sorting platform, the basal body and the needle (Figure 1). The sorting platform, which is within the cytoplasmic region of the bacterial cell, contains an ATPase (purple) to power secretion of linear unfolded proteins, as folded proteins are too wide to go through the ~2.5 nm needle [21,22]. The ATPase also functions as the recognition domain for effectors and unfolds the effectors for secretion. The basal body (yellow, blue, red) is made of a dual ring system that spans the inner and outer bacterial membranes and anchors the needle to the bacterial cell surface. The needle is composed of helical monomers (orange) that form a tube-like structure after polymerization [20]. The needle differs in length from species to strain depending on the host that the pathogen has evolved to infect [23]. The tip of the needle (green) also varies in length from one species to another. In *E. coli*, for instance, the needle tip is longer than the needle itself, which is not the case for other T3SSs [20]. The final structural

component to the injectosome is the pore (dark blue) that is formed in the host cell membrane, made up of two pore-forming proteins that allow effectors to pass into the host (for *E. coli*, EspB and D) [24–27].

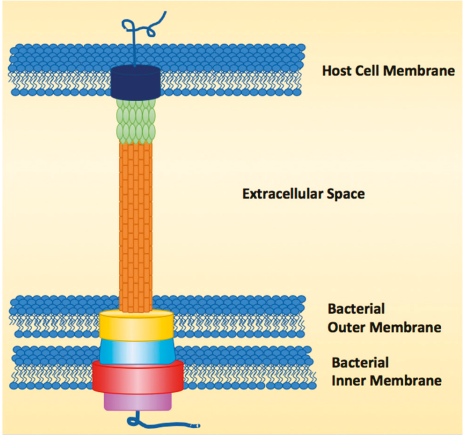


Figure 1. Example of type III secretion system (T3SS) structure.

Once the effectors pass into the host cell, they elicit specific responses from the host. In the case of *E. coli*, one such effector is translocated intimin receptor (Tir) [3,10,28–30]. Tir is secreted in an unfolded form into the host cell. Once there, it folds and presents itself on the surface of the host cell. Intimin, which is presented on the outer membrane of *E. coli*, recognizes the translocated Tir and binds. This forms an intimate attachment between the bacteria and the host cell. This attachment is necessary for the propagation of infection [26]. Other activities elicited by effector proteins include mimicking host signaling proteins and enzymes to hijack host cell machinery, inducing host cell death directly, or evading immune response in the host cell. Up to 30 different effector proteins can be secreted by a single pathogen [31–33].

The T3SS and its components are typically encoded in pathogenicity islands (e.g., *Salmonella* pathogenicity islands, SPIs [34–36]); however, in the case of *Yersinia* species, the genes for the T3SS can also be found on a ~70 kbp virulence plasmid [8,37]. The ~35 kbp pathogenicity island that encodes the T3SS in *E. coli* is referred to as the locus of enterocyte effacement (LEE) [5,38–40], and encodes 41 different genes under the control of 5 promoter regions (LEE1–LEE4 and Tir, Figure 2). The T3SS is not constitutively active, and expression is tightly controlled by environmental signaling factors [8,34,36,37,39].

Figure 2. Gene map for the locus of enterocyte effacement (LEE) in *E. coli*, indicating the five major promoter regions [40].

This review will cover a selection of natural product inhibitors of the T3SS, which were chosen to highlight the structural diversity of T3SS inhibitors as well as the diversity of bacteria, plants and fungi that make them. The discovery of natural products as inhibitors of the T3SS introduces new chemical scaffolds for exploration and development of drug-like compounds for clinical investigation, and understanding their mechanisms of action will create opportunity for rational drug design for more potent compounds.

2. Natural Products

2.1. Caminosides

The first inhibitor of the T3SS discovered was caminoside A (Figure 3) [15]. The caminosides are glycolipids isolated from the marine sponge *Caminus sphaeroconia* found in the upper walls of the Toucari Caves on the island of Dominica. Marine invertebrates were collected and transported to the Anderson lab facilities in Canada, where they were extracted repetitively with methanol before screening for T3SS inhibitory activity in EPEC. The isolation of caminoside A was a result of a bioassay guided fractionation approach using a protocol designed to screen for T3SS inhibitors using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and analyzing secretion of effector proteins (Esps). Samples having cytotoxic effects against EPEC were dropped from the study.

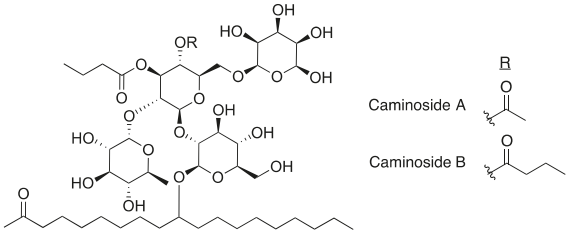


Figure 3. Structures of caminosides, inhibitors isolated from marine sponge *C. sphaeroconia*.

Caminoside A decreased the secretion of EspB, but not EspC. Since EspC is also secreted via the type IV secretion system this result indicates that caminoside A is specifically targeting the T3SS [41]. The structure of caminoside A was elucidated and potency was characterized (IC_{50} = 20 μm), although details on the mechanism of action are still not well understood [15]. Interestingly, although caminoside A has no cytotoxic effect against EPEC, it does have cytotoxic activity against Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (MIC = 12 μg/mL for each). Since the discovery of the caminosides, full syntheses of caminoside A and B have been published [42,43], detailing a synthetic process of 33 and 25 steps, respectively. Despite the promising activity of the caminosides against EPEC, very little has been done since their discovery to further develop these natural products for use as T3SS inhibitors due to difficulties in production or synthesis of the compounds.

2.2. Aurodox

Aurodox, a polyketide produced by *Streptomyces goldiniensis*, was originally isolated and characterized as having antibiotic activity in 1973 (Figure 4) [44]. Aurodox was originally named antibiotic X-5108 by the Grunberg lab when they elucidated its structure and determined aurodox's antibiotic activity against Gram-positive bacteria. The Chinali lab investigated the biological activity of aurodox by performing structure activity relationship (SAR) studies against the biological target elongation factor Tu (EF-Tu) [45].

Aurodox was identified as a T3SS inhibitor by the Abe group in 2011 after the development of a method to screen for inhibitors of the T3SS in EPEC known as EPEC-mediated hemolysis [14,46]. The molecular components of the translocon, EspB and EspD, typically form the end of the T3SS needle complex and allow for passage of effectors into the target host cell [24–26]; however, they also form pores on the surface of red blood cells (RBCs) [46,47]. Formation of these pores results in leakage of hemoglobin into the extracellular space. The supernatant may then be separated from cellular components and the concentration of hemoglobin may be measured indirectly by absorbance measurements. Thus, hemoglobin concentrations can be tied to T3SS expression.

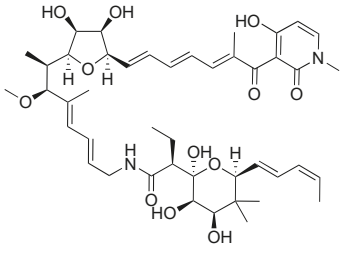


Figure 4. Structure of aurodox, a polyketide isolated from *Streptomyces* sp. K06-0806.

A screen was performed on 13,300 biological extracts from actinomycetes, fungi, plants, and invertebrates [14]. After extracts from *Streptomyces* sp. K06-0806 showed potent inhibition of EPEC-mediated hemolysis of RBCs without significantly affecting bacterial growth, a large culture of *Streptomyces* sp. K06-0806 was fermented and aurodox was purified. Further testing with purified compound confirmed inhibitory potency of aurodox in the RBC assay (IC_{50} = 1.5 μg/mL). According to analysis by SDS-PAGE followed by Western blotting, aurodox reduces the amount of secreted proteins EspA, EspB, EspD, EspF and Map (an effector that targets and damages host cell mitochondria [48]) without significantly affecting overall protein levels. It was shown that T3SS inhibition (IC_{50} = 1.5 μg/mL) occurs at a concentration much lower than the concentration at which aurodox shows signs of toxicity against Gram-negative bacteria (~10 μg/mL) [14].

The Abe lab collected further data using an *in vivo* mouse model using *Citrobacter rodentium* [40] to analyze the effectiveness of aurodox on mitigating infection. *C. rodentium* is a commonly used model of EPEC infection in mice, due to a high identity of sequence between the EPEC LEE and the LEE in *C. rodentium* [14,40]. Mice were initially infected with *C. rodentium*, then treated either with 10% dimethyl sulfoxide as a control, a single dose of tetracycline (200 mg/kg), or aurodox (25 mg/kg) every 24 h for four days. All of the mice that were treated with aurodox survived while none of those that were treated with tetracycline survived past day 13. These results show the power of T3SS inhibitors to protect against an otherwise lethal dose of pathogen.

A recent study has been published investigating the mechanism of action of aurodox [49]. The Roe lab showed that aurodox decreased the secretion of effector proteins via Western blotting. Aurodox was also shown to decrease infectability of epithelial cells by EHEC. Transcriptomal analysis on gene expression revealed that aurodox downregulates more than 100 genes cell-wide and downregulates 25 of 41 genes related to the T3SS. This suggests that the inhibitory activity of aurodox is a result of a change in gene expression, and not a result of physical manipulation of the T3SS needle complex. One of the genes downregulated by aurodox is *ler*, a major activator of the LEE [28]. In addition, aurodox downregulated the expression of EspG and NleB, which are non-LEE encoded effectors. Importantly, treatment with aurodox does not induce Shiga toxin production in EHEC, suggesting promise for the use of aurodox to treat EHEC infection. If the binding target of aurodox were identified, efforts could be made to strengthen that binding and increase the potency of aurodox further.

2.3. Piericidin A

Piericidin A was originally discovered for its insecticidal properties in 1963 (Figure 5) [50]. Soil samples were collected from Chiba Prefecture, a region encompassing the outskirts of Tokyo, and their microbial makeup was analyzed for their toxicity against a variety of larval species. The microorganism that exemplified the highest toxicity was *Streptomyces* sp. 16–22. Piericidin A was purified via bioactivity-guided fractionation approaches and its structure and chemical properties were characterized. Notably, this same study found that piericidin A presented limited cytotoxicity against Gram-negative bacteria such as *E. coli* and *Xanthymonas oryzae* [50]. In 1966, piericidin A was investigated for antibiotic properties against Gram-positive bacteria [51]. It was discovered that piericidin A targets nicotinamide adenine dinucleotide (NADH) dehydrogenase within complex I

(100% inhibition at 0.03 nmol piericidin per mg protein), a complex important in mitochondrial electron transport. In 2010, the Svatos lab described a symbiotic relationship between beewolf digger wasps and certain strains of piericidin-producing *Streptomyces* [52]. The wasps cultivate *Streptomyces* on their antennae, and incorporate the cells into their larval cocoons as prophylaxis against pathogenic bacteria. This is just one example of nature's purposeful use of natural products as a defensive mechanism against pathogens.

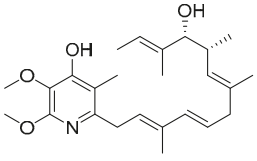


Figure 5. Piericidin A, a natural product T3SS inhibitor isolated from *Streptomyces* sp. 16–22 [50].

In 2014, the Auerbach lab performed a high-throughput screen to discover new inhibitors of the *Yersinia pseudotuberculosis* T3SS and uncovered piericidin A's inhibitory activity [53]. *Y. pseudotuberculosis* possesses a unique ability to trigger Nf-κB signaling in HEK293T cells, a process that is dependent on YopB and YopD transport of effectors into the host cytosol [54]. T3SS function was measured using an Nf-κB luciferase reporter plasmid and changes in activity were monitored to identify potent inhibitors [53]. After eliminating compounds that produced cytotoxicity against either the mammalian or bacterial cells, the group identified piericidin A as one of their hit compounds. SDS-PAGE analysis indicated that secretion of YopE was decreased by 65% at 71 μm piericidin A. Piericidin A was also shown to potently inhibit translocation of YopM (75% decrease at 71 μm) into Chinese hamster ovary (CHO) cells.

A clue into the mechanism of action of piericidin A as a T3SS inhibitor has recently been discovered. Inhibition of the T3SS by piericidin A resulted in decreased formation of Ysc-type needle units on the surface of *Y. pseudotuberculosis* without interfering with gene expression, indicating the mechanism of action is related directly to needle assembly [54]. Although piericidin has a known antibacterial target (Complex I), an alternative Complex I inhibitor, rotenone, has no T3SS inhibitory activity, indicating the T3SS inhibitory activity of piericidin A may be independent of complex I inhibition [55]. Further work to find the binding partner to elicit T3SS inhibition by piericidin A would aid in the ability to rationally design more potent analogs that selectively inhibit the T3SS without producing antibiotic effects related to Complex I binding.

2.4. Cytosporone B

Cytosporone B (Csn-B) was originally identified as a naturally occurring substrate to nuclear orphan receptor Nur77 in 2008 [56]. This octaketide natural product (Figure 6), isolated from the endophytic fungus *Dothiorella* sp. HTF3, has since been extensively studied as a potential anti-cancer agent due to its ability to stimulate Nur77-mediated apoptosis in multiple cancer cell models [56–58]. Csn-B was later identified by the Shen lab as an inhibitor of the T3SS in *Salmonella enterica* serovar Typhimurium during a screen of Csn-B and analogues [59].

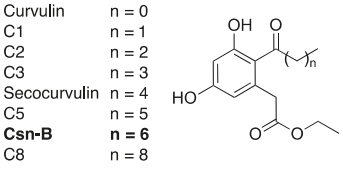
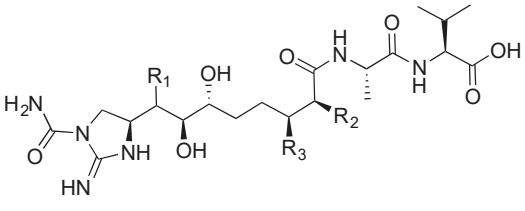


Figure 6. Analogs of cytosporone B (Csn-B) synthesized and analyzed for T3SS inhibitory activity by the Shen group.

Western blots indicated that secocurvulin, C5, Csn-B, and C8 (Figure 6) were all capable of inhibiting secretion of SPI-1 effectors in *S. enterica* [60]. In addition, secocurvulin, C5, and Csn-B inhibited *S. enterica* invasion of HeLa cells, a process dependent on SPI-1, with Csn-B proving to be the most potent. General SAR analysis suggests that the inhibitory potency is maximized at $n = 6$ (Csn-B), and that the potency decreases with increased or decreased chain length. Csn-B also showed dose-dependent inhibition of SPI-1 effector secretion (IC_{50} = 6.25 μm) with no toxicity to bacterial cells. Although the molecular target for T3SS inhibition is unknown, Csn-B inhibition could be overcome by overexpression of HilA, a positive regulator of the *S. enterica* T3SS. This result suggests that Csn-B interferes with the HilA expression pathway. A route for Csn-B total synthesis was published in 2010 [61], but since then, very little has been done to further this compound as a T3SS inhibitor.

2.5. Guadinomines

The guadinomines were discovered by Ōmura and colleagues using EPEC-mediated hemolysis to screen natural product extracts. [62,63]. *Streptomyces* sp. K01-0509 was found to produce potent inhibitors of RBC hemolysis [45,46]. Cultures were scaled up, and guadinomine A, B, C1, C2, and D were isolated, purified and analyzed (Figure 7) [62]. Guadinomines A and B are the most potent natural product TTSS inhibitors with IC_{50} = 0.007~0.01 μg/mL. The mechanism of action of the guadinomines is not well understood and yields of guadinomines from culture are low, making further research difficult. The lengthy total syntheses of guadinomine B and C2 have been published, with 33 steps in the longest linear synthetic sequence [64]. In 2012, a study on the biosynthetic pathway of guadinomine A was published by Khosla and coworkers [65]. Notably, guadinomine D, having an acylated amine at R_2 (Figure 7), is 1000-fold less potent than guadinomine B. This shows the importance of the vicinal diamine to biological activity. The acyl group on guadinomine D may be installed by enzymes apart from the guadinomine synthetase, since no obvious acylation enzyme is part of the gene cluster. While guadinomines do not appear to produce any antimicrobial activity, Ōmura found that guadinomine B is cytotoxic to Jukat cells at a concentration 100 times higher than the IC_{50} for TTSS inhibition [62].



	R_1	R_2	R_3	IC_{50}
Guadinomine A	OH	NH_2	NH_2	38 nM
Guadinomine B	H	NH_2	NH_2	14 nM
Guadinomine C1 and C2 (diasteromers)	H	HN, H_3C, NH, O		inactive
Guadinomine D	H	HN, O	NH_2	16 μM

Figure 7. Guadinomines isolated from *Streptomyces* sp. K01-0509, and their activity against enteropathogenic *Escherichia coli* (EPEC).

2.6. Butyric Acid

Initial studies relating to the biological effects of butyric acid predate knowledge of the T3SS (Figure 8). In the 1960s, William R. Martin and coworkers investigated how the infectious dose (ID_{50}) of *Salmonella enteritidis* changed when mice were pretreated with antibiotics [66]. The authors showed that the ED_{50} went from 10^6 to <10 cells when a single dose of streptomycin was administered 24 h before infection. The authors noted that treatment with antibiotic increased the gut pH and identified that butyric acid and acetic acid were being produced by the gut bacteria. The authors attributed the ability of the mice to tolerate *Salmonella* to a low gut pH and not a specific inhibitory effect caused by butyric acid. Since these experiments were performed before the discovery of the T3SS, the authors were unaware that they were completing initial studies on the effect that T3SS inhibitors have on severity of infection.

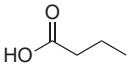


Figure 8. Butyric acid, a product of Bacterioidetes and other intestinal microbes [67].

Butyric acid is classified as a short-chain fatty acid (SCFA), and is produced as a fermentation product by commensal Bacterioidetes species in mammals. In human intestines butyric acid is typically present at 10–20 mm [67–69]. Butyric acid is a major energy source for colonocytes, and the ability of colonic cells to absorb and utilize sodium butyrate is seen as a sign of good health [69]. Administration of butyric acid to the intestines of mice infected with *C. rodentium* results in decreased inflammation and increased mucus production from colonic cells.

Butyric acid interacts with the epigenetic modifier Lrp, a major regulator of gene expression in bacteria [5,66–69]. Lrp does not control the expression of genes in the same pattern from one organism to another. As a result, butyrate acts as a T3SS inhibitor for some organisms and as a T3SS activator in others [68,70–72]. A notable example involves LEE-encoding bacteria EPEC and *C. rodentium*. EPEC and *C. rodentium* have 90% sequence identity in their LEE pathogenicity islands [40]. Their T3SSs are so similar that *C. rodentium* is often used as a mouse model for EPEC infection [14]. Lrp is a non-LEE encoded T3SS regulator, and activation of Lrp has opposite responses in these two organisms. Activation of Lrp upregulates expression of the LEE in EPEC, while activation of Lrp downregulates expression of the LEE in *C. rodentium* [68,70,72].

Research into SCFAs as T3SS regulators has focused primarily on the effects of probiotics on infection [67,70,71]. By increasing the concentration of SCFA-producing bacteria in the gut, concentrations of a variety of SCFAs are altered. Depending on the pathogen attempting to infect the gut, differing ratios of SCFAs could have dramatically different results, from improving to worsening infection. Given the prevalence and widespread use of probiotics, this area requires further investigation.

2.7. Fusaric Acid

Fusaric acid is a toxin produced by fungal species *Fusarium oxysporum*, a common inhabitant of soil (Figure 9) [73,74]. Fusaric acid causes a variety of negative outcomes in plants, and it is thought to be a virulence factor in Fusarium wilt in banana, tomato, and cotton crops [73–75] and heavy decline disease in grapevine [76]. Fusaric acid was first studied as a potential inhibitor of the T3SS in *S. enterica* in 2014 as part of a screen of a small library [77]. In SDS-PAGE and Western blot analysis, fusaric acid potently inhibited secretion of SPI-1 effector proteins when cells were treated with 100 µm fusaric acid, without disrupting cell growth. Inhibition was dose-dependent with a calculated IC_{50} = 53.5 µm. In a gentamycin-protection assay, fusaric acid markedly inhibited *Salmonella* invasion into HeLa cells, with no toxicity toward the HeLa cells observed [77].

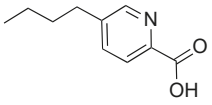


Figure 9. Fusaric acid, a fungal toxin isolated from *Fusarium oxysporum.*

Some studies attempting to elucidate the mechanism of action of fusaric acid gave conclusive results that the inhibitory effect of fusaric acid cannot be overcome by overexpression of T3SS activator HilA, unlike the case of Csn-B [77]. Also, fusaric acid does not change the level of PgrH, an assembly protein for the needle complex, and does not interfere with the SicA/InfV transcriptional pathway for T3SS initiation. Further studies on the mechanism of action are needed in order to determine the pathway through which fusaric acid elicits an inhibitory response. The cytotoxicity of fusaric acid against plants and other organisms will need to be considered when moving forward with this compound as a potential T3SS inhibitor.

2.8. (-)-Hopeaphenol

Many plants produce inhibitors to protect against infection caused by Gram-negative pathogens that utilize a T3SS. (-)-Hopeaphenol (Figure 10) was isolated as part of a bioassay-guided fractionation study to find natural product T3SS inhibitors from two rainforest plants from Papua New Guinea, *Anisoptera thurifera* and *A. polyandra* [78]. (-)-Hopeaphenol is a tetramer of resveratrol, a common building block used in nature to synthesize natural products. (-)-Hopeaphenol has also been explored for its anti-oxidant properties [79]. In their work, Eloffson and coworkers analyzed (-)-hopeaphenol for inhibition of T3SS in *Y. pseudotuberculosis*, *P. aeruginosa*, and *C. trachomatis* [78]. (-)-Hopeaphenol exhibited inhibition of YopE expression in a reporter gene assay and found the compound had inhibitory activity (IC_{50} = 6.6 μm). Western blot analysis showed dose-dependent inhibition of secretion and expression of YopD. When cells grown in the presence of (-)-hopeaphenol were moved to T3SS-inducing environments, they were incapable of expressing the T3SS, regardless of whether (-)-hopeaphenol was still present. This suggests an irreversible mechanism of inhibition.

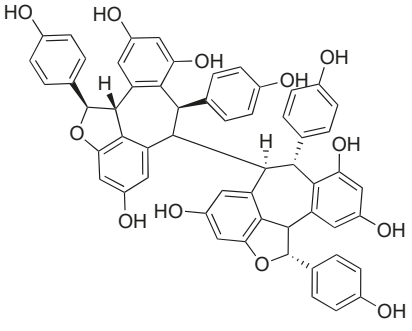


Figure 10. (-)-Hopeaphenol, a tetramer of resveratrol isolated from rainforest plants.

(-)-Hopeaphenol was also found to inhibit expression and secretion of ExoS, an effector from the *P. aeruginosa* T3SS [79]. In infection model assays, (-)-hopeaphenol completely inhibited infection of HeLa cells by *P. aeruginosa* at a concentration of 100 μm. In addition, (-)-hopeaphenol was observed to inhibit intracellular growth of *C. trachomatis* in HeLa cells in a dose-dependent manner. When tested for cytotoxicity against a panel of Gram-positive and Gram-negative organisms, (-)-hopeaphenol did not affect cell growth or viability. Despite the promise of this structural class, there are large barriers for chemical synthesis. In addition, *Anisoptera* spp. that produce (-)-hopeaphenol are in danger of extinction; 6 of 10 within the genus are either endangered or critically endangered according to the International Union for Conservation of Nature (IUCN) Red List, with the remainder being

vulnerable [80]. Without the ability to easily access samples of (-)-hopeaphenol and analogs to test for T3SS inhibitory activity, further development of this structural class as inhibitors will be difficult.

2.9. Sanguinarine Chloride

Sanguinarine chloride is a natural product isolated from the extracts of the bloodroot plant *Sanguinaria canadensis* (Figure 11) [81]. In the 1970s and 1980s, sanguinarine chloride was studied as a potential treatment for gingivitis due to its anti-inflammatory properties. It has since been studied as a chemotherapeutic agent [82]. Sanguinarine chloride was found to be a T3SS inhibitor against *Salmonella enterica* serovar Typhimurium [83]. It inhibited SipA-lactamase fusion translocation into HeLa cells at a concentration of 4 μm. In a gentamycin-protection assay, sanguinarine chloride was effective against pathogenic invasion of HeLa cells. Expression of SipA and SipB was also inhibited at 5 μm sanguinarine chloride. Overexpression of HilA overcomes the inhibitory effects of sanguinarine chloride, indicating a possible mechanism of action. While sanguinarine chloride shows promise as a T3SS inhibitor, efforts to reduce the cytotoxic affects that the compound has toward human cells must be made for this drug to move forward, as well as further characterizing its biological target.

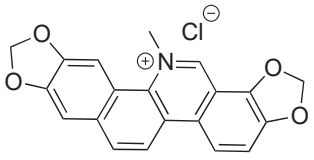


Figure 11. Sanguinarine chloride, a natural product from bloodroot *Sanguinaria canadensis*.

2.10. Thymol

The Deng lab recently identified thymol during a study aimed at identifying T3SS inhibitors from traditional Chinese medicine (Figure 12) [84]. Thymol is a component of an essential oil derived from plants belonging to the *Thymus* genus [85]. The translocation of a SipA-lactamase fusion from *Salmonella* into HeLa cells in the presence or absence of thymol was monitored. At a concentration of 0.2 mm thymol, translocation was almost completely inhibited, while cytotoxicity was not observed until thymol concentrations reached 0.6 mm. In a gentamycin protection assay, preincubation with 0.2 mm thymol resulted in a 90% reduction of T3SS-dependent internalization of *Salmonella* by HeLa cells. Doses of 50 mg/kg thymol resulted in a 100% survival rate of mice administered lethal doses of *Salmonella* after a 10-day infection period. This dose also alleviated pathophysiology related to *Salmonella* infections to colonocytes. These promising results show that further investigations are needed into traditional medicines.

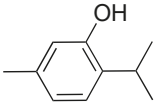


Figure 12. Thymol, a major component of essential oils from *Thymus* plants.

2.11. Cinnamic Acid and Derivatives

In 2008, the Yang lab investigated the influence of plant-derived compounds on the expression of the T3SS in *Dickeya dadantii* 3937 [86]. They chose to monitor change in expression of the T3SS via the two major regulatory pathways in *D. dadantii* 3937, the HrpX/HrpY-HrpS-HrpL and GacS/GacA-rsmB-RsmA pathways. The group found that *trans*-cinnamic acid (TCA, Figure 13) acts as an activator of the *D. dadantii* T3SS, increasing the expression of the gene *hrpN*, an indicator for the HrpX/HrpY-HrpS-HrpL pathway. Specifically, a 3-fold increase in expression of *hrpN* was observed at 5 μm TCA.

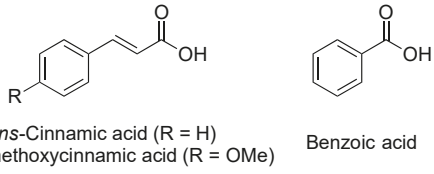


Figure 13. Structures of *trans*-cinnamic acid (TCA), *trans*-4-methoxycinnamic acid (TMCA), and benzoic acid (BA), regulators of the T3SS.

In later studies, the Yang lab investigated derivatives of TCA, including *trans*-4-methoxycinnamic acid (TMCA) and benzoic acid (BA, Figure 13) on T3SS expression in *Erwinia amylovora*, the fire blight pathogen [87]. Their results indicated that TMCA and BA act as inhibitors of T3SS expression in a fused-green fluorescence protein (GFP)-*hrp* reporter assay. TCA, TMCA and BA (100 μm) all decreased fluorescence to approximately 30%, 20%, and 3% of the control, respectively. Based on this assay, the IC_{50} concentrations for TCA (0.5 μm) and BA (1 μm) were approximated. Northern blot analysis indicated that TMCA inhibits the T3SS by reducing expression of $rsmB_{Ea}$ and *hrpN*, while BA only inhibits the expression of *hrpN*, suggesting potential differences in their mechanisms of action. None of the compounds altered the expression of the regulatory genes *hrpX/hrpY*. Further analyses of this structural class, their mechanisms of action, and the differences in inhibitory/inducing behavior between pathogenic species will need to be conducted for this group of compounds to move forward as T3SS inhibitors.

3. Conclusions

Many natural products have been shown to possess T3SS inhibitory properties over the last decade. These compounds are made by a variety of different biological sources and cover a diverse range of chemical scaffolds. Notwithstanding these successes, most inhibitors of the T3SS have unknown biological targets. This complicates the rational design of new and more potent analogs. With a better understanding of their binding partners and mechanism of action, modern methods of analog design (e.g., computational modeling) can be employed effectively. More natural product T3SS inhibitors are still being discovered, indicating that there remains a lot to learn about how nature employs this strategy.

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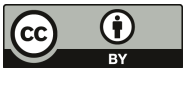

antibiotics

MDPI

Review

Actinobacteria Derived from Algerian Ecosystems as a Prominent Source of Antimicrobial Molecules

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Abstract: Actinobacteria, in particular "rare actinobacteria" isolated from extreme ecosystems, remain the most inexhaustible source of novel antimicrobials, offering a chance to discover new bioactive metabolites. This is the first overview on actinobacteria isolated in Algeria since 2002 to date with the aim to present their potential in producing bioactive secondary metabolites. Twenty-nine new species and one novel genus have been isolated, mainly from the Saharan soil and palm groves, where 37.93% of the most abundant genera belong to *Saccharothrix* and *Actinopolyspora*. Several of these strains were found to produce antibiotics and antifungal metabolites, including 17 new molecules among the 50 structures reported, and some of these antibacterial metabolites have shown interesting antitumor activities. A series of approaches used to enhance the production of bioactive compounds is also presented as the manipulation of culture media by both classical methods and modeling designs through statistical strategies and the associations with diverse organisms and strains. Focusing on the Algerian natural sources of antimicrobial metabolites, this work is a representative example of the potential of a closely combined study on biology and chemistry of natural products.

Keywords: actinobacteria; antimicrobial; antibiotic; antifungal; secondary metabolites; chemodiversity; rare actinobacteria; Saharan ecosystem

1. Introduction

By receiving the Nobel Prize for the discovery of penicillin in 1945, Alexander Fleming informed the scientific community that the misuse of antibiotics would lead, in the near future, to the emergence of microbial pathogens resistant to these substances. Fleming's prediction was true as we are confronted in recent decades with the emergence of multidrug-resistant bacteria that threaten global health. [1]. The inappropriate use of antibiotics has created a selective pressure that drives the emergence and spread of multidrug-resistant pathogens, like those of the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.), to all antibiotics currently used in therapy. Bacteria have developed diverse resistance mechanisms to avoid antimicrobial agents action, classified as follows: (i) antimicrobial molecule modification, (ii) decrease in permeability or extrusion of the antimicrobial compound through the overexpression of efflux pumps, (iii) changes and/or bypass of target sites by mutations in their encoding genes (either by protecting or modifying the target site), and (iv) resistance due to global cell adaptions. Moreover, new resistance mechanisms are constantly being described, for example, for colistin, which is the last-resort antibiotic for multidrug-resistant bacteria infections [2]. Despite the alarming situation, the number of new antibiotics placed on the market has been in decline in recent years. At the present, the limited choice of antibiotic classes for treating multidrug-resistant bacteria encourages scientists to search for unknown molecules displaying new mechanisms of action.

Natural microbial sources are considered the most important source of bioactive metabolites that are promising for new therapeutic drugs [3–5]. The *Actinobacteria* phylum represents one of the largest taxonomic groups in the *Bacteria* domain. It includes mycelium-forming or not, Gram-positive bacteria, with a high G + C content reaching 70% for some species of the *Streptomyces* genus. As might be expected from a large phylum, representatives of *Actinobacteria* are found in a wide range of ecological niches, including aquatic ones such as marine and oceanic sediments, seawater, freshwater ecosystems, and marine invertebrates. In terrestrial environments, different lifestyles are found in actinobacteria like plant commensals, nitrogen-fixing symbionts, as well as animal and plant pathogens. Thus, they constitute a significant proportion of the telluric microflora [6].

Streptomyces genus is known for its complex development cycle which has been extensively studied, indicating that secondary metabolites are synthesized as a defence against antagonistic microorganisms and also ensuring a major role in the cycling of organic matter in the soil and sediments ecosystem. Details on this topic have been efficiently reviewed [7,8]. The filamentous actinobacteria belonging to the *Actinobacteriaceae* family have the potential to produce chemically diverse and relevant metabolites counting known antibiotic, antifungal, antitumor, and anti-inflammatory agents, along with plant-growth-promoting substances and regulators. These substances find different applications including medicine, biotechnology, and agriculture [9] in addition to the industrially relevant enzymes (e.g., cellulases, chitinases, and xylanases) responsible for the production of biofuels and biochemicals [10,11]. The actinobacteria genome is rich in biosynthetic gene clusters (a group of two or more genes that together encode a biosynthetic pathway) coding for known and/or novel metabolites with potential to discover new therapeutic agents [12,13]. In particular, actinobacteria synthesize a large number of bioactive metabolites of which antibiotics cover the major proportion. For almost a century, actinobacteria have contributed significantly to the development of the antibiotic arsenal required for human health, so they are responsible for the production of more than 70% of relevant anti-infective natural products. Antimicrobial agents have been the first isolated natural compounds, starting from actinomycin from *Streptomyces antibioticus* in 1940, followed by a significant number of antibiotics discovered in the so-called "golden age" corresponding to the period 1940s–1960s, when the production of about half of all known antibiotics is due to *Streptomyces* [14].

Besides antibacterials, the interest was on the search for new and more effective antifungal agents, particularly against opportunistic molds and fungal infections caused by *Aspergillus* and *Candida albicans*, the latter responsible of nosocomial infections. Furthermore, while the frequency of fungal infections is increasing alarmingly, current antifungal therapy has a limited number of drugs due to their side effects and toxicity. In addition to antibiotics and antifungals, actinobacteria are known to produce a wide range of secondary metabolites with a broad range of bioactivities including antitumor, antioxidant, and herbicide- and plant-promoting agents.

Although actinobacteria are known for their rich metabolism, it has become always more difficult to find novel bioactive substances due to the frequent rediscovery of already known compounds. One of the main strategies in the search for new sources of bioactive compounds is the isolation of rare actinobacteria (non-Streptomycete actinobacteria) from underexplored and uncommon habitats [15]. The potential of this approach has been extensively reported [16,17] since the first isolation in 1964 of a thermophilic actinobacterial strain from an Italian soil sample, which led to discovery of the antibiotic thermorubin [18]. Moreover, conditions such as temperature, light radiation, and salt concentration of arid and semiarid ecosystems proved capable in affecting the metabolite profile of the extremophilic actinobacteria [19].

It must be remembered the highly complex structures of many bioactive metabolites from actinobacteria and the rich presence of stereogenic centres with defined configurations due to the enantioselective synthesis occurring in nature. Therefore, the employment of suitable bacteria strains still remains the method of choice for their production. It is a more advangeous method than the organic synthesis to obtain enantiomerically pure forms of bioactive molecules and in large-scale access for therapeutic applications.

To this purpose, a wide range of methods have been used for the selective isolation of actinobacteria and have applied combined physical and chemical approaches, such as thermic treatments [20], or the addition of chemicals (calcium carbonate and chitin, calcium chloride, phenol [21–24], and sodium dodecyl sulfate). The incorporation of antibacterial (e.g., nalidixic acid and kanamycin) and antifungal agents (e.g., nystatin and amphotericin B) to the culture media is also an effective approach for the selective isolation of these bacteria.

Another aspect is related to the generally low production of secondary metabolites. To overcome this restriction, a particular attention was focused on the employment of both conventional and new methodologies able to enhance their production. It is widely accepted that culture parameters significantly affect the performance of microbial metabolism [25]; therefore, an optimization of culture media and physical chemical conditions of the fermentation process usually comes before any production on a large scale. Different upscaling techniques are also used by industries for yield maximization during both the fermentation process and the extraction steps [26].

In detail, one factor at time (OFAT) approach has been widely applied in the past for any upscaling production due to its simplicity [27], although it was reduced mainly during the initial steps of medium formulation due to its drawbacks (time-consuming process, expensive, needs many experiments, and parameter interactions not taken into account). It has been replaced by statistical methodologies such as design of experiments procedure (DOE), which is more effective, quick, and accurate, requiring less experiments; and estimating the effects of several culture parameters simultaneously [28,29]. Through statistical strategies of culture conditions, the Plackett–Burman design (PBD) and the Tagushi design (TD) have been successfully applied as modeling methodologies to evaluate the culture parameters affecting the metabolite production. The most widely used tools for the optimization step are central composite (CCD) and Box and Behnken designs (BB) in response surface methodology (RSM) [30].

Mixed cultivation of actinobacteria/actinobacteria, actinobacteria/bacteria, or actinobacteria/fungi allows to activate cryptic biosynthetic pathways. Co-cultivation at a laboratory scale can reproduce the original and natural conditions of microorganisms, creating an antagonism and competitive environment able to stimulate and enhance the production of new secondary metabolites. The effectiveness of this strategy is well represented by the production of new bioactive molecules derived from the dual culture of desert-derived isolated *Streptomyces leeuwenhoekii* strains with a marine-derived fungi *Aspergillus fumigatus* [31].

An additional attractive source of novel bioactive compounds is given by the endophytic actinobacteria acting as biocontrol agents of plant disease, responsible for plant-promoting growth via the production of diverse substances [32], in particular, screening of native plants from the Algerian Sahara to isolate endophytic actinobcateria for the biocontrol of *Rhizoctonia solani* damping-off and the improvement of tomato growth [33,34], which is in line with the microbial symbiotic association with insects and which led to the discovery of a number of new and diverse chemical structures [35].

Moreover, genome analysis of actinobacteria turned out to be a promising approach for the study of the metabolic potential and identification of novel biosynthetic gene clusters. Indeed, genomic sequencing has revealed the presence of silent genes and cryptic biosynthetic pathways encoding for secondary metabolites not expressed under conventional culture conditions [36]. The significant success of the genomic tools led to the exponential increase of the number of available actinobacteria genome sequences reported in suitable databases. Furthermore, ribosome engineering has been successfully applied to increase the production of bioactive secondary metabolites, in particular, to obtain antibiotics from *Streptomyces* [37].

In the present review, we highlight the diversity of actinobacteria coming from ecosystems in Algeria and the chemical structures of the corresponding secondary metabolites associated to their biological activities. Moreover, the attention is focused on the novel species and genera studied and on new bioactive compounds reported since 2002. Additionally, we discuss the biosynthesis improvement of these metabolites through the application of classical and advanced methodologies. Data reported in this paper were obtained from PubMed and SciFinder (Chemical Abstract online) databases. To the

best of our knowlege, this is the first overview on the biology and chemistry of actinobacteria from Algerian ecosystems reported so far.

2. Algerian Sampling Sites Providing Culturable Actinobacteria

With an area of more than 2 million square kilometers, Algeria has an impressive climatic diversity ranging from snow-capped mountains in the northern regions overlooking the Mediterranean Sea to the world's hottest Saharan desert. This affects a great biodiversity, rich and diversified in actinobacteria, to which corresponds a wide chemodiversity of metabolites. A number of actinobacteria has been isolated from different ecosystems including Saharan plants [34,38,39], caves [40], waste water [41,42], river sediments [43], hypersaline areas [44–51], Saharan desert soil [22,52], and derived algae [53]. The most studied sampling sites for the isolation of actinobacteria are listed in Figure 1.

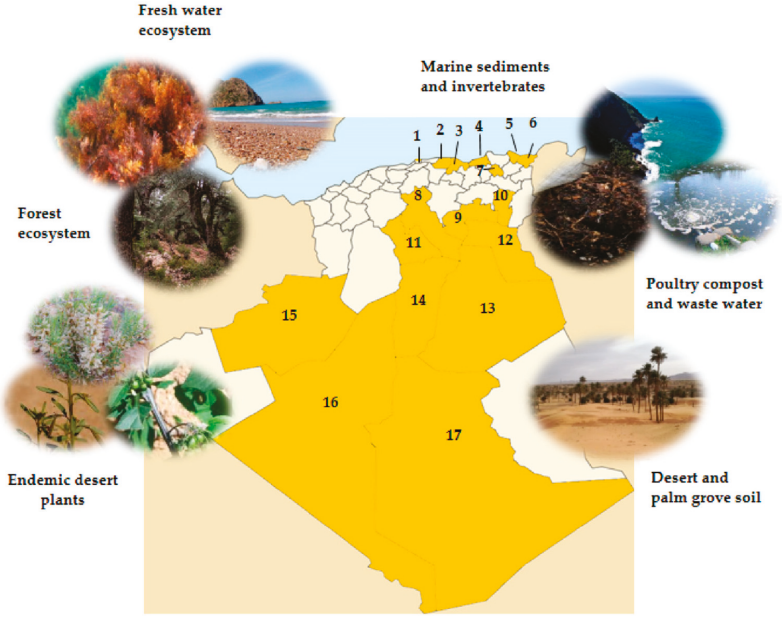

Figure 1. Distribution of the most explored *Actinobacteria* sampling sites in Algeria. **1**: Algiers, **2**: Tizi Ouzou, **3**: Bejaia, **4**: Jijel, **5**: Annaba, **6**: El Taref, **7**: Constantine, **8**: Djelfa, **9**: Biskra, **10**: Khenchela, **11**: Laghouat, **12**: El Oued, **13**: Ouargla, **14**: Ghardaia, **15**: Bechar, **16**: Adrar, and **17**: Tamarasset. Details are in Table 1.

Under the research topic of *Actinobacteria* as a resource of novel potential therapeutic agents, a total of 134 articles were published starting from 2002 (Figure 2). They cover different aspects, including the isolation and diversity of bacteria along with the investigation on secondary metabolite production targeting a broad spectrum of bioalogical activities (mainly antimicrobial but also cytotoxic and plant promoting agents) and with a look at potential biotechnological applications [54].

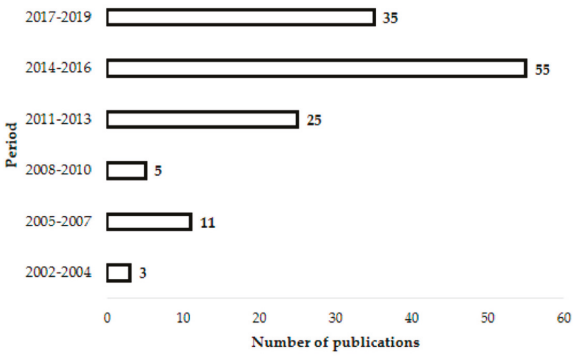

Figure 2. Variation in the number of publications dealing with the isolation and metabolic profile investigation of actinobacteria in Algeria based on data since 2002 to the present.

3. Biodiversity of Rare and Novel Genera and Species of Actinobateria

The interest in actinobacteria biodiversity present in Algerian ecosystems started more than two decades ago [55–58]. From a historical point of view, the research activities carried out in 1974 by the group of Bounaga, director of the arid zone research center (CRZA) in Algeria, focused on biological control methods including the use of actinobacteria against the appearance of Bayoud's disease caused by *Fusarium*, a serious date palm disease that threatens all date-producing countries. The Sabaou team started within CRZA, and it was only in 2000s that the activity in this field improved due to the official creation of university research laboratories. The studies by Sabaou et al. have shown the great potential of Algerian habitats for novel actinobacteria species, indicating the richness of their metabolism for the production of bioactive compounds [22]. Thereafter, a large number of new species have been discovered, mainly from the Saharan ecosystem, which represents 85% of the total area of the country (Figure 3). The number of novel species and rare actinobacteria isolates reported from the Algerian ecosystems [24,44–50,59–80] has increased in the last years (Table 1), with the discovery of six new species between 2012 and 2013, to reach a peak of 20 new species and one genus between 2014 and 2017 and a total of 10 species within 2 years in the periods of 2014–2015 and 2016–2017 (Figure 3).

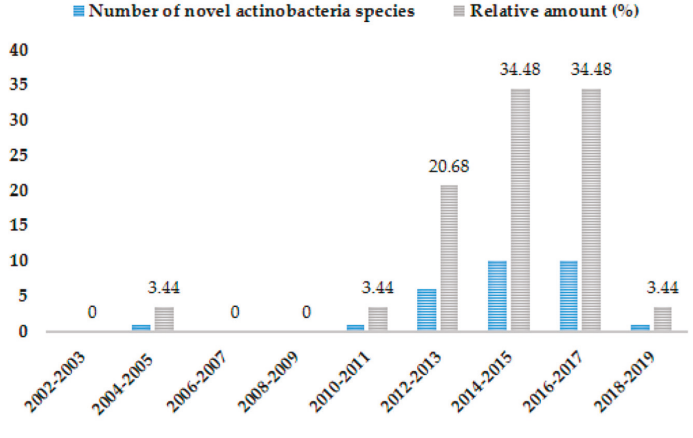

Figure 3. Variation in number of culturable novel actinobacteria species collected from Algerian sources since 2002.

Table 1. List of novelly discovered rare actinobacteria species isolated from different ecosystems in Algeria since 2002.

Microorganism	Ecological Niches and Climate	References
Saccharothrix algeriensis sp. nov.	Saharan Palm grove, Adrar; Saharan climate	[24]
Mycobacterium algericum sp. nov.	Goat-lung lesion, Souk El Tenine slaughterhouse, Bejaia; Mediterranean climate	[59]
Actinopolyspora algeriensis sp. nov.	Saharan salin soil, Ouargla; Saharan climate	[44]
Actinopolyspora saharensis sp. nov.	Saharan soil, El Oued; Saharan climate	[45]
Actinopolyspora righensis sp. nov.	Saharan soil, El Oued; Saharan climate	[46]
Actinopolyspora mzabensis sp. nov.	Saharan soil, Ghardaia; Saharan climate	[47]
Saccharothrix saharensis sp. nov.	Saharan palm grove, Adrar; Saharan climate	[60]
Saccharothrix hoggarensis sp. nov.	Saharan soil, Hoggar–Tamenrasset; Saharan climate	[61]
Saccharopolyspora ghardaiensis sp. nov.	Saharan soil, Ghardaia; Saharan climate	[48]
Halopolyspora algeriensis comb. nov.	Saharan soil, Mzab region, Ghardaia; Saharan climate	[62,63]
Streptomonospora algeriensis sp. nov.	Soil sample, Djelfa; semiarid climate	[49]
Actinokineospora mzabensis sp. nov.	Saharan soil, Beni izguen region, Ghardaia; Saharan climate	[64]
Bounagaea algeriensis gen. nov., sp. nov.	Saharan soil, El-Goléa, Ghardaia; Saharan climate	[50]
Actinopolyspora biskrensis sp. nov.	Saharan soil, Biskra; Saharan climate	[65]
Prauserella isguenensis sp. nov.	Saharan soil, Beni izguen region, Ghardaia; Saharan climate	[66]
Nocardiopsis algeriensis sp. nov.	Saharan soil, Adrar; Saharan climate	[67]
Actinoalloteichus hoggarensis sp. nov.	Saharan soil, Hoggar region, Tamanrasset; Saharan climate	[68]
Saccharothrix tamanrassetensis sp. nov.	Saharan soil, Hoggar region, Tamanrasset; Saharan climate	[69]
Streptosporangium algeriense sp. nov.	Saharan soil, palm grove in Adrar; Saharan climate	[70]
Actinomadura algeriensis sp. nov.	Saharan soil, Hoggar region, Tamanrasset; Saharan climate	[71]
Mycobacterium icosiumassiliensis sp. nov.	Water lake surface, Algiers; Mediterranean climate	[72]
Actinomadura adrarensis sp. nov.	Saharan soil, Adrar; Saharan climate	[73]
Saccharothrix isguenensis sp. nov.	Saharan soil, Mzab region, Ghardaia; Saharan climate	[74]
Actinophytocola algeriensis sp. nov.	Saharan soil, Mzab region, Ghardaia; Saharan climate	[75]
Streptosporangium becharense sp. nov.	Saharan soil, Beni Abbes region, Bechar; Saharan climate	[76]
Streptosporangium saharense sp. nov.	Saharan soil, Mzab region, Ghardaia; Saharan climate	[77]
Saccharothrix ghardaiensis sp. nov.	Saharan soil, Mzab region, Ghardaia; Saharan climate	[78]
Planomonospora algeriensis sp. nov.	Saharan soil, Beni Abbas, Bechar; Saharan climate	[79]
Streptomyces massilialgeriensis sp. nov.	Saline soil, dry lake, Oum el Bouaghi; semiarid climate	[80]

Figure 4a illustrates the proportion of novel species, genera, and rare actinobacteria described since 2002 from different Algerian ecosystems. More than 29 novel species and the new genus *Bounagaea* have been discovered, as was the new family *Mzabimycetaceae* proposed by Saker et al. [62]. The same authors published *Mzabimyces algeriensis* as a novel strain isolated from a palm grove soil sample of the Mzab region-Ghardaia (south of Algeria). It must be specified that the strain was recently reclassified regarding the genus *Halopolyspora* and identified as *Halopolyspora algeriensis* comb. nov. by comparison of phenotypic, chemotaxonomic, and phylogenetic data and DNA–DNA hybridization [63]. Among 29 new species and 16 genera belonging to the order *Actinomycetales*, *Saccharothrix* is the most recovered and abundant genus, accounting for 20.68% of the total novel actinobacteria species from Southern Algeria regions, followed by the *Actinopolyspora* genus with a proportion of 17.24%. In summary, both amount and diversity of rare actinobacteria genera deriving from the Saharan desert evidence the wealth of this particular ecosystem.

The sampling sites regarded for the isolation and investigation of rare actinobacteria have been essentially focused on the Saharan soil and palm groves. The Adrar, Ghardaia, and Tamanrasset regions are together associated with the discovery of 18 new species: *Saccharothrix algeriensis* sp. nov., *Saccharothrix saharensis* sp. nov., *Nocardiopsis algeriensis* sp. nov., *Actinomadura adrarensis* sp. nov., *Streptosporangium algeriense* sp. nov., *Saccharopolyspora ghardaiensis* sp. nov., *Actinopolyspora mzabensis* sp. nov., *Halopolyspora algeriensis* comb. nov., *Actinokineospora mzabensis* sp. nov., *Saccharothrix isguenensis* sp. nov., *Actinophytocola algeriensis* sp. nov., *Streptosporangium saharense* sp. nov., *Saccharothrix ghardaiensis* sp. nov., *Saccharothrix hoggarensis* sp. nov., *Saccharothrix tamanrassetensis* sp. nov., *Actinomadura algeriensis* sp. nov., and *Actinoalloteichus hoggarensis* sp. nov., belonging to 11 different genera (Figure 4b). A Venn diagram assembling shared and unique genera obtained from each of the three studied regions (Adrar, Ghardaia, and Tamanrasset) is presented in Figure 5. Just one genus, *Saccharothrix*, shared across the Adrar, Ghardaia, and Tamanrasset sites, represents the most abundant recovered genus from the arid

soil. The Adrar and Tamanrasset locations shared only the *Actinomadura* genus, and the Adrar and Ghardaia sites showed *Streptosporangium* as the common genus. Thus, so far, the Saharan desert is associated with 28 new species of actinobacteria that are related to soil and palm groves.

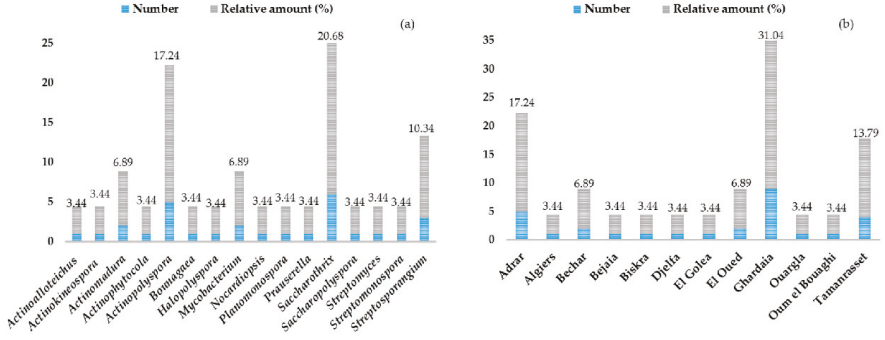

Figure 4. Proportion of novel species, genera, and rare actinobacteria strains from different Algerian ecosystems reported since 2002 (**a**), according to the places of collection (**b**). Details are in Figure 1.

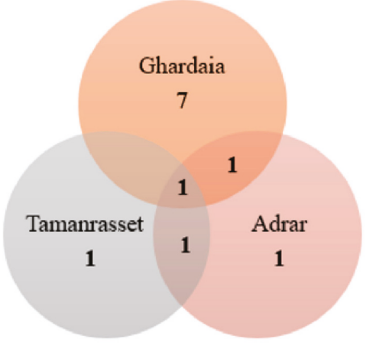

Figure 5. Venn diagram showing the numbers of shared and unique rare actinobacterial genera among the three most dominant Saharan soil samples sites.

4. Secondary Metabolites Derived from Algerian Actinobacteria

Bioactive natural products are reported to be classified according to their biological activities, focusing on isolation and elucidation of their chemical structures (**1**–**50** in Figures 6 and 7) and on their production by both classical changes of culture conditions and new statistical approaches, when present in the original works. Table 2 lists the metabolites, summarizing their data on sources, producer strains, and bioactivies.

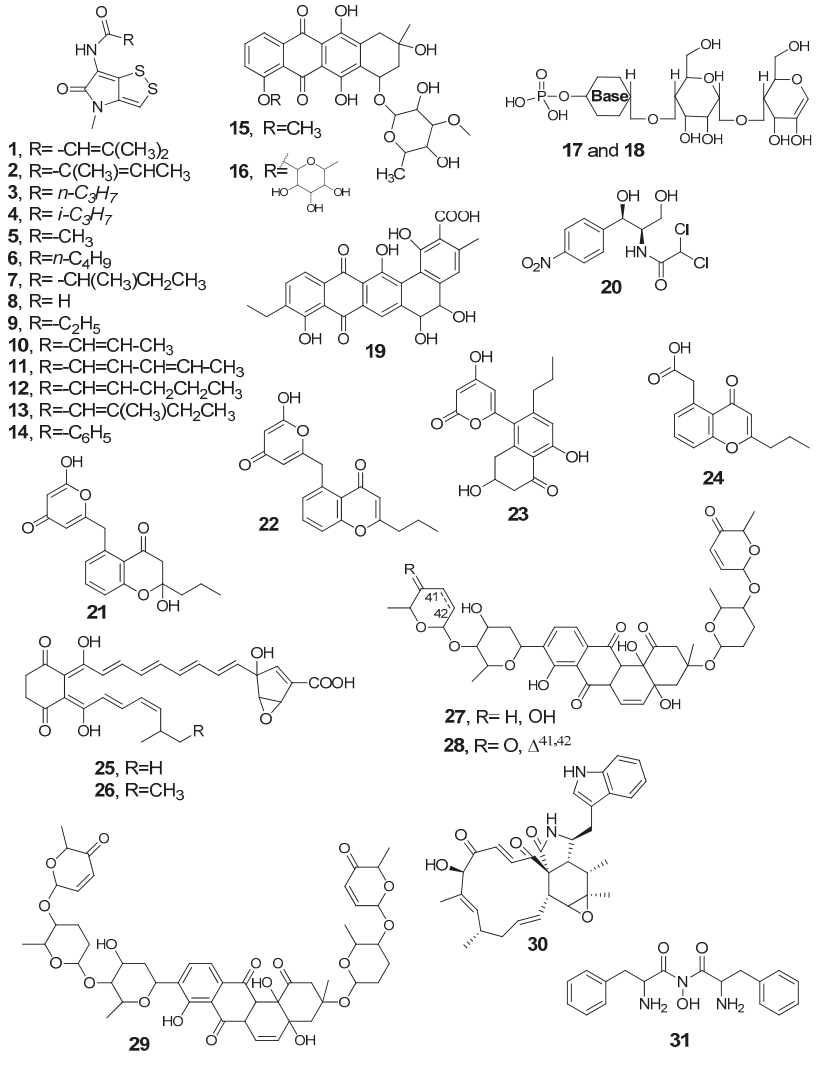

Figure 6. Molecular structures of the bioactive metabolites **1**–**31** isolated from Algerian actinobacteria: The stereochemical details are according the cited references.

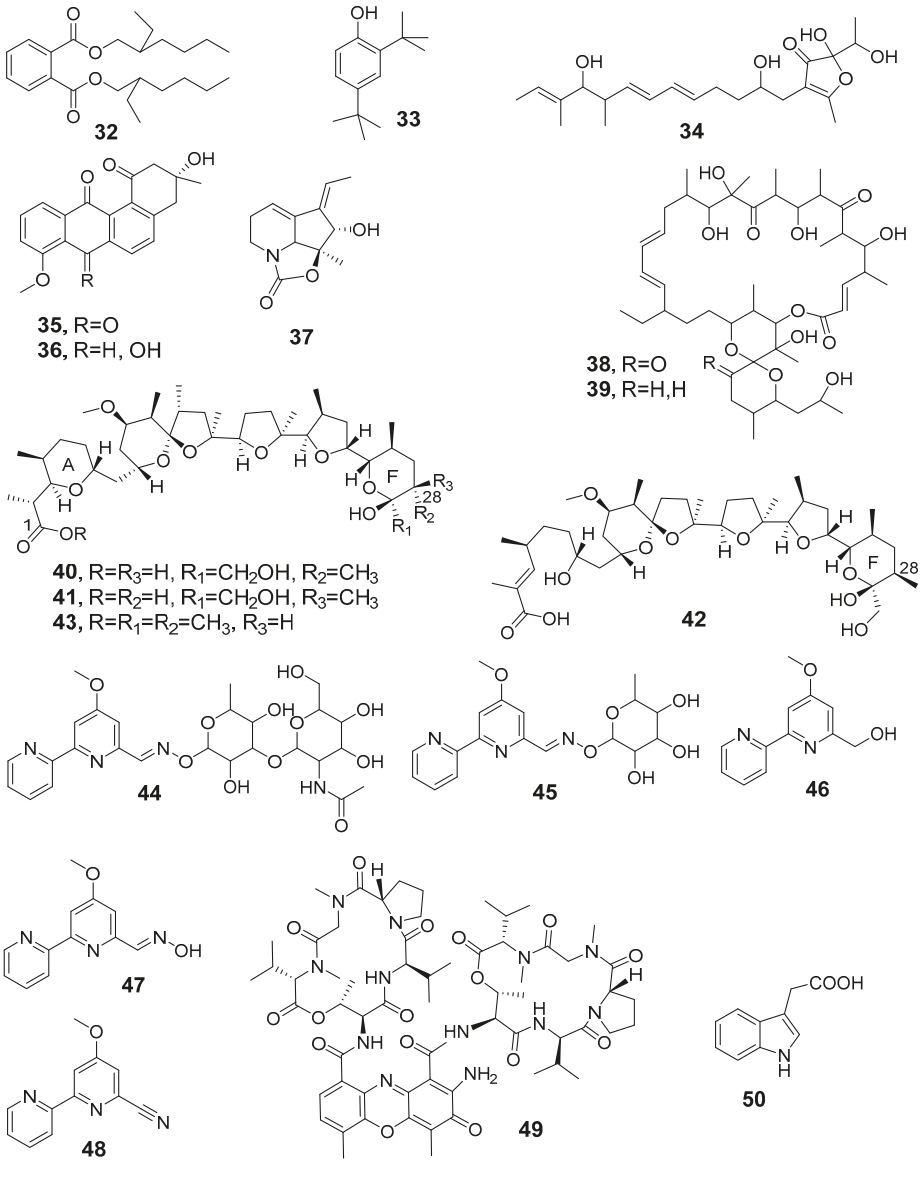

Figure 7. Molecular structures of the bioactive metabolites **32**–**50** isolated from Algerian actinobacteria. The stereochemical details are according the cited references.

4.1. Antimicrobials

Lamari et al. [23] isolated the new dithiopyrrolone metabolites, namely 3-methyl-2-butenoylpyrr othine (**1**), tigloylpyrrothine (**2**), and *n*-butyropyrrothine (**3**), along with the known iso-butyropyrrothine (**4**) and thiolutine (**5**) from a Saharan palm grove soil strain *Saccharothrix* sp. SA233 (Figure 6). These products were isolated after a workup including centrifugation of the fermentation broth, extraction with dichlormethane and purification by preparative thin layer chromatography (TLC) on silica gel, followed by reversed phase HPLC. The new metabolites (**1**)–(**3**) displayed high antibacterial

activity against *Bacillus coagulans*, *Bacillus subtilis*, and *Micrococcus luteus*, specifically with minimum inhibitory concentration (MIC) values of <0.2, 0.5, and 1 μg/mL (corresponding to <0.7, 1.8, and 3.9 μM), respectively, against *Bacillus coagulans*. The three compounds exhibited also a higher activity against *Saccharomyces cerevisiae* and *Mucor ramanniamus* compared to the phytopathogenic fungi *Fusarium oxysporum* f. sp. *albedinis*, *F. o.* f. sp. *Lini*, and *F. culmorum*. The structural characterization of the metabolites was reported by the same authors [81]. Later, from the fermentation broth of *Saccharothrix algeriensis* NRRL B-24137 (fully sequenced strain) isolated from a Saharan soil sample collected in 1992 at a palm grove in Adrar (southwest of Algeria), Merrouche et al. [82,83] isolated the new dithiolopyrrolone derivatives valerylpyrrothine (**6**), isovalerylpyrrothine (**7**), and formylpyrrothine (**8**) by addition of valeric acid to the culture medium and the known aureothricin (**9**), exhibiting a moderate bioactivity against some filamentous fungi and yeasts such as *Mucor ramanniamus*, *Penicillium expansum*, and *Aspergillus carbonarius*. Moreover, the new crotonyl-pyrrothine (**10**), sorbyl-pyrrothine (**11**), 2-hexonyl-pyrrothine (**12**), and 2-methyl-3-pentenyl-pyrrothine (**13**) were obtained by addition of sorbic acid to the culture medium. Compound **11** resulted in the most active in the series against Gram-positive bacteria. All metabolites showed a moderate inhibition of the tested fungi and yeast, except compound **13**, which gave a higher activity against *Aspergillus carbonarius* and *Candida albicans* with MICs evaluated at 2 μg/mL (MIC = 7 μM) for both germs [83]. Very recently, the same authors have reported on the new benzoyl-pyrrothine dithiolopyrrolone (**14**), obtained after the supplementation of cinnamic acid into the culture medium of *S. algeriensis* NRRL B-24137. In the antibacterial evaluation, *Listeria monocytogenes* resulted in the most sensitive to the treatment with this pure metabolite (MIC = 4 μg/mL corresponding to 13.8 μM) [84].

Zitouni et al. [85] purified the new anthracycline antibiotic mutactimycin PR (**15**) and the already reported mutactimycin C (**16**) from the *Saccharothrix* sp. SA 103 strain collected in the Tamanrasset, an arid region of Southern Algeria. The culture broth was extracted using *n*-butanol and the residue subjected to reversed phase HPLC under isocratic conditions to give the pure metabolites. The compounds displayed moderate activity against some Gram-positive bacteria and fungi, especially *Bacillus subtilis*, *Saccharomyces cerevisiae*, and *Mucor ramannianus*. Some of the same authors isolated also 54 *Nocardiopsis* and 32 *Saccharothrix* strains from Algerian Saharan soils, identified by morphological and chemotaxonomic features. These strains showed antibacterial (against *B. subtillis* and *M. lutesus*) and antifungal activity against *M. ramannianus* and *S. cerevisiae* and also against mycotoxinogenic fungi *Fusarium graminearum*, *F. solani*, *F. culmorum*, *Aspergillus carbonarius*, *A. ochraceus*, and *penicillium citrinum*. A medium containing ammonium sulfate, starch, and yeast extract produced nucleotidic and nucleosidic molecules named ZA01 (**17**) and ZA02 (**18**), which were HPLC purified and of which their structures were partially characterized by electrospray–mass spectrometry (ESI-MS) analysis including tandem fragmentation experiments [86].

The phylogenetic analysis on a strain collected from the Saharan soil in a southwest location of Algeria identified the *Streptomsporangium* Sg10 strain as a potential new genomic species. It produced compounds active against Gram-positive bacteria and fungi. Only a partial structural characterization was described, able to identify a generic glycosylated aromatic nature of these metabolites [87]. The same authors reported also on the actinomycete strain *Streptomsporangium* sp. Sg3 collected from Adrar (southwest of Algeria), which produced three pigments, called R1, R2, and R3, that display no activity against fungi and Gram-negative bacteria, with the highest inhibition against Gram-positive bacteria shown by R2. UV-visible, IR, and NMR spectrtoscopic analyses allowed to give a partial elucidation, supporting a quinone-anthracycline aromatic structure for these pigments [88]. Later, the authors established the structure of R2 by extensive NMR analysis and high-resolution mass spectrometry as the new angucyclinone (**19**), related to tetracyclines and anthracyclines. In detail, the optically active molecule was defined in its planar structure and no streochemical assignments have been reported. MIC values evaluated by the conventional agar dilution method showed potent activities against *Micrococcus luteus* ATCC 9314 and *Bacillus subtilis* ATCC 6633 (MICs = 0.5 and 1 μg/mL, corresponding to 1 and 2 μM, respectively) [89].

The novel isolate recovered from a desert soil sample collected in Beni-Abbes (southwest Algeria) and named *Nonomuraea* sp. NM94 was studied under liquid fermentation condition. It produced five bioactive compounds, which were HPLC purified and partially characterized by IR, ^{1}HNMR, and ESI-MS investigation. It was only possible to define the same chemical class for all compounds, containing an aromatic unit substituted by aliphatic chains. In detail, one of the metabolites showed a molecular mass of 340 Da, as established by ESI-MS spectra recorded in negative ion mode. The crude dichloromethane extract of the strain was evaluated by a paper disc method, resulting in active against some Gram-positive bacteria, yeast, and fungi [90].

Saccharothrix sp. PAL54A strain isolated from a Saharan soil in Ghardaïa produced the known chloramphenicol (**20**); therefore, it is the first production of this antibiotic by a *Saccharothrix* species. [91].

Actinobacteria of marine origin and, in particular, marine endophytic actinobacteria are also promising sources of new classes of antimicrobial compounds. Mutualistic or parasitic interactions of actinobacteria with marine macroorganisms and invertebrates have been proven to affect the production of novel metabolites. One of the most representative examples is the production of the new polyketide **21** along with phaeochromicins B (**22**), C (**23**), and E (**24**). The metabolites were isolated from a solid-state fermentation of *Streptomyces* sp. WR1L1S8 obtained from a marine brown algae *Fucus* sp. The structure of **21** was established regarding its 2-hydroxy-γ-pyrone tautomeric form by both NMR study on the products from deuterium incorporation using CD_3OD solvent and the comparison of experiments with density functional theory (DFT)-calculated IR spectra. The Cotton effect observed by circular dichroic analysis is in favor of the enantiomeric purity of the natural product, denying the idea to be a product by water addition during the workup. However, the absolute configuration of the molecule remains undetermined. The new metabolite represents the lacking member in the series of phaeochromycins A–E, which are the first polyketides bearing the *n*-propyl chain. Compound **21** exhibited a selective bacteriostatic activity against methicillin-resistant *Staphylococcus aureus* (MRSA) (MIC = 6 μM) [53]. Culture conditions on antibacterial activity and mycelial growth were later evaluated, changing the parameters able to affect the production of metabolites **21**–**24**. The optimal conditions to increase the yield of the new anti-MRSA compound **21** were established using the OFAT approach on the culture of the strain *Streptomyces sundarbansensis* WR1L1S8 (on the starch casein agar medium in freshwater or 50% seawater at pH 7 or 9 at 28 °C using agar-state fermentation). In this study, the analysis carried out by HPLC equipped with a diode array detector evaporative light scattering detection (DAD-ELSD) or online coupled to an ESI-MS apparatus emerges as an efficient method to evaluate the chemical profile of the metabolites present in the crude extracts derived by different culture comditions. Compound **21** resulted in being also the most abundant by culturing the strains on starch casein agar medium in freshwater or 50% seawater at pH 7 or 9 using agar-state fermentation method [92].

The novel *Saccharothrix* SA198 strain from a Saharan soil sample (collected at Tamanrasset in southern Algeria at a 1370-m altitude) provided the new antibiotics A4 (**25**) and A5 (**26**). Their production was evaluated by changing culture media and pH values, and they were HPLC purified starting from the crude dichloromethane extract. The planar structures of **25** and **26** were established by MS data and 2D-NMR analysis. Pure metabolites displayed moderate activities against Gram-positive and -negative bacteria and potent effects against phytopathogenic and toxinogenic fungi: *Mucor ramannianus* (MICs: 5 μg/mL for **25** and 1 μg/mL for **26**), *Aspergillus carbonarius* (MICs: 10 μg/mL for **25** and 2 μg/mL for **26**), and *Penicillium expansum* (MICs: 2 μg/mL for each **25** and **26**) [93].

The anthracycline saquayamycin A (**27**) and C (**28**), known for their antibacterial and anticancer activities, were obtained from a culture broth of a novel *Streptomyces* spp. PAL114 strain collected in Ghardaïa. MIC values of pure compounds were evaluated using conventional agar dilution method on a series of microorganisms, observing moderate activities, with the highest effects against *Bacillus subtilis* ATCC 6633 and *Candida albicans* M3 [94].

Another metabolite belonging to the family of anthracyclines, the aquayamycin-like vineomycin A1 (**29**), was purified from the same strain: *Streptomyces* sp. PAL114. The strain produced also the

cytochalasin derivative chaetoglobosin A (**30**). It is remarkable that chaetoglobosin A is known to be produced only by fungi and that this is the first report in prokaryotes. Both metabolites exhibited moderate effects against *B. subtilis* and *Candida albicans* and on filametous fungi [95].

The novel hydroxamic acid (**31**) was purified from the culture broth of *Streptomyces* WAB9, a strain isolated from the Saharan soil collected in Bechar region. The pure compound was obtained by HPLC purification of the *n*-butanol extract from the culture filtrate, and its planar structure was established by ESI-MS spectra recorded in negative ion mode and extensive NMR investigation. It exhibited antibacterial activitiy towards a range of multidrug-resistant microorganisms, in particular, *Pseudomonas aeruginosa* IPA1 (10 μg/mL = 30 μM) and *E. coli* E52 (20 μg/mL = 60 μM) [96].

Driche et al. [97] reported the isolation of di-(2-ethylhexyl) phthalate (**32**) from the novel strain *Streptomyces* sp. G60 obtained from a Ghardaia soil sample by a workup including the use of a series of organic solvents (*n*-hexane, dichloromethane, and n-butanol and ethyl acetate). There is doubt that the compound is an actual metabolite, although the molecules have been also reported isolated from other natural sources, as cited by authors themselves. In fact, it is known that di-(2-ethylhexyl) phthalate (DEHP) is the most common member of the phthalates class used as a plasticizer. Moreover, the solvent power able to extract this plasticizer from polymeric bags indicated *n*-hexane, methanol, chloroform, and ethyl acetate, in increasing order [98]. Compound **32** was tested for its activity against different strains of *Staphylococcus aureus* and MRSA, obtaining strong effects [97].

Belghit et al. [99] isolated 2,4-di-tert-butylphenol (**33**) from a culture of *Streptomyces mutabilis* strain from a Saharan soil collected in Metlili (Ghardaïa region). The known compound was active against pathogenic fungi exhibiting a MIC value of 5 μg/mL against *C. albicans* M3.

From the broth culture of the novel strain *Streptomyces* sp. AT37 obtained from Adrar Saharan soil (southwest Algeria) the furanone derivative **34** was detected by bioautography of the crude extract, purified by reversed phase HPLC, and identified as the known as antibiotic E-975. The compound exhibited a moderate activity against multidrug-resistant *S. aureus* and inhibited the biofilm formation, which were reduced by 50% at a concentration of 10–15 μg/mL [100].

Nocardiopsis species are known to be present in Saharan soils, characterized by saline and hypersaline properties. The new halotolerant *Nocardiopsis* sp. HR-4 strain, collected from the salt-lake soil named Sebkha of Ain, provided two angucyclinone aromatic polyketides. In particular, the stereochemistry of the known (−)-7-deoxy-8-*O*-methyltetrangomycin (**35**) was assigned by comparison with the polarimetric value obtained for the same molecule by stereoselective total synthesis [101]. Compound **36**, corresponding to the reduced form of one carbonyl group in the quinone unit, had been already isolated from an Indonesian *Streptomyces* spp. and reported without the stereochemical assignment at this centre [102]. These metabolites exhibited good antibacterial activities only against Gram-positive bacteria [103].

Recently, Djinni et al. [43] purified (+)-streptazolin (**37**), produced as a major compound in an appreciable amount from *Streptomyces thermoviolaceus* SRC3, a fresh-water sediment-derived strain. The structural characterization of streptazolin, including its absolute configuration previously defined by X-ray crystallographic analysis on a derivative, was established by comparison of NMR, MS, and optical activity. Pure streptazolin was evaluated for its antimicrobial effects against ATCC pathogenic germs obtaining, as known, a moderate activity. However, recent studies have focused on the role of this compound as an antibiotic adjuvant. A sequential modelisation using PBD and CCD statistical methods allowed to maximize the antimicrobial activity under the following conditions: KCl (0.01%), K_2HPO_4 (0.1%), and $MgSO_4{\cdot}7H_2O$ (0.02%) with 9 days of incubation for inhibiting *Salmonella Typhi* ATCC 14028; KCl (0.051%) and $MgSO_4{\cdot}7H_2O$ (0.05%) with 5 days of incubation for improving effects against *Candida albicans* ATCC 10231.

Oligomycins A (**38**) and E (**39**) were produced as major metabolites by the *Streptomyces* sp. HG29 strain isolated from a Saharan soil collected in Hoggar (Tamanrasset, Southern Algeria) [104]. Their structure assignment is based on MS and NMR spectra, but no indication on the several stereocentres is given, neither are polarimetric data reported to allow a comparison with known

oligomycins. Both metabolites have been already described to have a broad spectrum of bioactivies, mainly antifungal. Khebizi et al. [104] reported significant antifungal activity observed for **38** and **39** (with MIC values estimated between 2 and 75 μg/mL against representatives of the *Aspergillus*, *Fusarium* and *Penicillium* genera as well as *C. albicans*), but their known high toxicity to eukaryotic cells prevents any clinical applications.

A series of polyether antibiotics including nigericin (**40**), epinigericin (**41**), abierixin (**42**), and the new grisorixin methyl ester (**43**) were isolated from the *Streptomyces youssoufiensis* SF10 strain collected from Chélia Mountain, in Khenchela (North-eastern Algeria) [105]. The online coupled HPLC-ESIMS analysis provided the full polyether profile, and the preparative HPLC technique in the reversed phase condition gave pure compounds, which were identified by extensive NMR and ESI-MS spectra in comparison with reported data. Nigericin, the main member of the series, is known for its strong antibacterial antagonism and for its behavior as potassium ionophore, whereas the related metabolites grisorixin and abierixin exhibit weak activity against Gram-positive bacteria. A computational analysis on the structural epimerization at C-28 positon in the F ring of these metabolites (Figure 7) carried out by density functional theory (DFT) calculations allowed to compare their relative stability, providing structural considerations applicable to the other several members of the polyether class. The strain was cultured under different conditions (solid state or submerged fermentation, using several carbon sources, presence or absence of iron (II) sulfate, changing pH values, in co-culture with other *Streptomyces* species), and the production of nigericin present in the corresponding crude extracts was evaluated using a calibration curve by HPLC apparatus equipped with an evaporative light scattering detector (ELSD), a sensitive detector for the analysis of molecules lacking of chromophore units as nigericin. The best culture conditions provided a concentration of nigericin of 0.490 mg/mL in the extract. By the co-culture with *Streptomyces* sp., the formation of phenylacetic acid was observed, a metabolite previously reported from *S. humidus* cultures showing inhibition on some plant-pathogenic fungi. Otherwise, co-culturing SF10 strain with *S. coeruleorubidus* neither nigericin nor phenylacetic acid were observed [106].

Very recently, a novel *Saccharothrix xinjiangensis* ABH26 strain, isolated from a soil sample collected in the Adrar region (Southern Algeria), was studied by Lahoum et al. [107]. The new metabolites, named cyanogriside I (**44**) and J (**45**), were purified, and the suggested structures were indicated as a methoxy-bipyridine linked to sugar units through an O-oxime moiety. The long-range heterocorrelations by 2D-NMR experiments allowed to define the connectivities but not the configuration of the sugar unit. In the structures of cyanogrisides A–D, already reported from the marine-derived actinomycete *Actinoalloteichus cyanogriseus* WH1-2216-6, the sugar was fully assigned by X-ray diffractometric analysis [108] from nuclear Overhouser effect (NOE) experiments for cyanogrisides E–H [109]. Moreover, these last compounds showed different connectivities (the unit sugar replacing the methoxy group of **44**/**45**) so that cyanogriside names look like it is not strictly appropriate for metabolites **44** and **45**, and furthermore, their putative biosynthesis was not discussed in correlation with cyanogrisides A–H. Isolated from the same Algerian strain, Lahoum et al. reported also the identification of the known methoxy-bipyridines caerulomycin A (**46**), caerulomycin F (**47**), and caerulomycinonitrile (**48**). The five metabolites exhibited moderate inhibition on Gram-positive pathogenic bacteria and low effects on filamentous fungi and pathogenic yeast (with MICs > 100 μg/mL). Compound **47** emerged as the most active in the series, mainly on Gram-positive bacteria and fungi (MICs in the range 1–50 μg/mL corresponding to 4.6–230 μM).

Table 2. Bioactivities of Actinobacteria metabolites derived from Algerian ecosystems.

Compound	Bioactivity	Producer Strain	Source	Reference
3-Methyl-2-butenoylpyrrothine (**1**), Tigloylpyrrothine (**2**), *n*-Butyropyrrothine (**3**), iso-Butyropyrrothine (**4**), Thiolutin (**5**)	antibacterial, antifungal	*Saccharothrix* sp. SA 233	Saharian palm grove soil (Adrar)	[24,81]
Valerylpyrrothine (**6**), Isovalerylpyrrothine (**7**), Formylpyrrothine (**8**), Aureothricin (**9**)	antibacterial and antifungal	*Saccharothrix algeriensis* NRRL B-24137, fully sequenced strain	Saharan soil	[82]
Crotonyl-pyrrothine (**10**), Sorbyl-pyrrothine (**11**), 2-Hexenyl-pyrrothine (**12**), 2-Methyl-3-pentenyl-pyrrothine (**13**)	antibacterial and antifungal	*Saccharothrix algeriensis* NRRL B-24137	Saharan soil	[83]
Benzoyl-pyrrothine dithiolopyrrolone (**14**)	antibacterial, antifungal	*Saccharothrix algeriensis* NRRL B-24137	palm grove soil (Southern Algeria)	[84]
Mutactimycin PR (**15**), Mutactimycin C (**16**)	moderate anti-Gram-positive bacteria	*Saccharothrix* sp. SA 103	Saharan soil sample (Tamanrasset, South Algeria)	[85]
ZA01 (**17**), ZA02 (**18**)	antibacterial antifungal	*Nocardiopsis* SA 103	non-rhizospheric soil samples, Saharan regions	[86]
Angucyclinone R2 (**19**)	antibacterial, antifungal, antitumor, antiviral, enzyme inhibitor, platelet aggregation inhibitor	*Streptosporangium* sp. Sg3	Saharan soil, Adrar region	[87–89]
D(-)-threo chloramphenicol (**20**)	antibacterial	*Saccharothrix* sp. PAL54	Saharan soil of Ghardaïa	[91]
2-Hydroxy-5-((6-hydroxy-4-oxo-4H-pyran-2-yl)methyl) -2-propylchroman-4-one (**21**), Phaeochromycins B (**22**), C (**23**), E(**24**)	antibacterial, anti-inflammatory	*Streptomyces sundarbansensis* WR1L1S8	Endophitic strain, inner tissue of marine algeae *Fucus* sp.	[53]
Compound A4 (**25**), Compound A5 (**26**)	anti-Gram-positive and -negative bacteria, anti-phytopathogenic and toxinogenic fungi	*Saccharothrix* SA198	Saharan soil, Tamanrasset (Southern Algeria)	[93]
Saquayamycin A (**27**), Saquayamycin C (**28**)	antifungal and antibacterial	*Streptomyces* spp. PAL114	Saharan soil, Béni-isguen-Ghardaïa (South of Algeria).	[94]
Vineomycin A1 (**29**), chaetoglobosin A (**30**)	antibacterial and antifungal	*Streptomyces* sp. PAL114.	Saharan soil	[95]
Novel hydroxamic acid (**31**)	antibacterial	*Streptomyces* WAB9	Saharan soil, Bechar	[96]
Di-(2-ethylhexyl) phthalate (**32**)	antibacterial	*Streptomyces* sp. G60	Saharan soil, Ghardaïa	[97]
2,4-Di-tert-butylphenol (**33**)	against *Candida albicans* and other pathogenic fungi	*Streptomyces mutabilis* G61	Soil sample Metlili, Ghardaïa	[99]

Table 2. *Cont.*

Compound	Bioactivity	Producer Strain	Source	Reference
AT37-1 (**34**)	against multidrug-resistant *S. aureus*	*Streptomyces* sp. AT37	Saharan soil sample (Adrar)	[100]
(−)-7-Deoxy-8-*O*-methyltetrangomycin (**35**), (−)-8-Methyltetrangomycin (**36**)	anti-Gram-positive bacteria, antifungal	*Nocardiopsis* sp. HR-4	Salt-lake soil, Sebkha of Ain Salah (Saharan desert)	[103]
Streptazolin (**37**)	antimicrobial adjuvant antibiotic	*Streptomyces thermoviolaceus* SRC3	Fresh water river sediments	[43]
Oligomycin A (NK1) (**38**), Oligomycin E (NK2) (**39**)	anti-Gram-positive bacteria, antifungal	*Streptomyces* sp. HG29	Saharan soil sample (Hoggar, Tamanrasset)	[104]
Nigericin (**40**), Epinigericin (**41**), Abierixin (**42**), Grisorixin methyl ester (**43**)	glioblastoma stem-cell inhibitor	*Streptomyces youssoufiensis* SF10	soil derived	[105]
Cyanogriside I (**44**), Cyanogriside J (**45**), Caerulomycin A (**46**), Caerulomycin F (**47**), Caerulomycinonitrile (**48**)	anti-Gram-positive bacteria, antifungal	*Saccharothrix xinjiangensis* ABH26	Saharan soil (Adrar)	[107]
Actinomycin D (**49**)	antimicrobial, antitumor	*Streptomyces* sp. IA1. *Streptomyces* sp. GSBNT10	Saharan soil (Ain amenas) Saharan soil (Beni Abbes-Bechar)	[110,111]
Indole-3-acetic acid (**50**)	plant-growth-promoting activity	*Streptomyces* sp. PT2	Spontaneous herbaceous plants (*Cleome arabica*, *Solanum nigrum*, *Astragallus armatus*, *Aristida pungens*, and *Panicum turgidum*) (Sahara, Hassi R'mel region)	[38]

Toumatia et al. [110] and, recently, Djinni et al. [111] isolated novel strains producing actinomycin D (**49**) and showed that genus *Streptomyces* isolated from Saharan soil of Ain amenas (*Streptomyces* sp. IA1) and Beni Abbes-Bechar (*Streptomyces* sp. GSBNT10) had potent antibacterial and antifungal activity against a wide range of plant pathogenic fungi. The production of the metabolite from *Streptomyces* sp. GSBNT10 was successfully enhanced using PBD and CCD methods, as detected by LC-MS analysis of crude extracts. Under the optimized culture conditions, a 58.56% increase of actinomicyn D formation was obtained, arriving at the value of 656.46 mg/L. These results suggested interest in scaling-up the process for access to this molecule, which is currently employed to treat some highly aggressive tumors, alone or in combined chemotherapies [111].

Messis et al. [112] used the Box–Behnken design approach to improve the antifungal activity of the *Streptomyces* sp. TKJ2 strain collected from a forest soil origin, but this study did not include the isolation and structural elucidation of the bioactive metabolites.

The same modeling approach, including optimization of pH and temperature values, was applied to select the factors affecting antifungal activity of the *Streptomyces* sp. SY-BS5 strain isolated from an arid soil sample collected in Bou-Saada [51]. Similarly, the *Streptomyces albidoflavus* S19 strain, derived from wastewater collected in Bejaia region, was studied as antifungal producer. In detail, the best conditions for the production of anti-*Candida albicans* compounds were selected, evaluating a rise from 13 to 34 mm of the diameter inhibition zone. The data have highlighted the requirements of next studies to characterize the metabolites responsible for this activity [113].

In summary, the biological evaluation on the metabolites isolated so far from Algerian actinobacteria is mainly focused on the first bacterial and fungal inhibition, with the aim to find solutions to the urgent problems of increased bacterial resistance and of the incidence of fungal infection even potentially lethal in immuno-compromised people. Regarding antibacterial metabolites, studies are currently underway on their contribution in improving the efficacy of therapeutic antibiotics when used in combination with them. A few other reports are on their potential role as antitumor agents, also based on present studies of known antibiotics as promising agents able to inhibit in vitro and in vivo the development of human tumors.

4.2. Other Activities

4.2.1. Tumor Cells Growth Inhibitors

Actinobacteria are responsible for more than half of cytotoxic compounds of microbial origin approved in cancer therapy [113,114]. Few studies have focused on finding cytotoxic compounds derived from Algerian microorganisms and actinobacteria, except for cases of some cytotoxic antibiotics.

The polypeptide lactone actinomycin D (**49**), also known as dactinomycin, was the first antibiotic presenting anticancer activity and is now commonly used as a drug in mono and combined therapy in the treatment of a variety of highly aggressive malignancies, including Wilm's tumor and Ewing's sarcoma [115]. It is known for its inhibitory effect of cellular transcription by intercalating between adjacent base pairs in DNA. It was first identified from *Actinomyces antibioticus* in 1940s, later produced by various *Streptomyces* and *Micromonospora* species in the world, and also isolated from the Algerian desert soil (Ain Amenas and Beni Abbes)-derived *Streptomyces* strains *Streptomyces* sp. IA1 [110,111], demonstrating the effectiveness of the compound for biocontrol against chocolate spots of field bean and *Fusarium* wilt of flax diseases.

Recently, the polyether antibiotics nigericin (**40**) and the new grisorixin methyl ester (**43**) isolated from *Streptomyces youssoufiensis* SF10 have provided significant cytotoxic activities against glioblastoma stem cells, with a higher activity for grisorixin methyl ester (GI_{50} values of 3.85 and 3.05 μM for two human glioblastoma stem cell lines), corresponding to a higher growth-inhibion cell-proliferation than the drug temozolomide [105]. The data are remarkable due to both the nature of glioblastoma multiforme as the most malignant primary brain tumors and the effect against stem cells which are

resistant to the conventional therapies. Nigericin had also shown activity in suppressing colorectal cancer metastasis [116].

4.2.2. Plant-Growth-Promoting Agents

Among actinobacteria-derived metabolites, the plant-growth-stimulating agents play important roles in agriculture, both in improving plant growth and in controlling or inhibiting phytopathogens infecting host plants. A number of reports on the isolation of plant-associated endophytic actinobacteria, mainly of *Streptomyces* genus [38] from various plants families and even soil [117], have been reported. It was described their metabolic potential as biological control agents and plant-growth promoters [118], which can replace chemicals and pesticides. In detail, according to Rugthaworn et al. [119], the biocontrol effect of actinobacteria can be either by lysis of fungal cell walls or by antibiosis through their capability of growth inhibition, competition, or hyperparasitism on several plant pathogenic fungi.

A plant-growth-promotion effect on seed germination and root elongation was observed by Goudjal et al. [38] through the production of indole-3-acetic acid (**50**), a phytohormone which is widespread among bacteria. This metabolite acts as a common natural auxin produced by the L-tryptophane metabolism pathway for eighteen strains of *Streptomyces* isolated from five spontaneous desert plants well adapted to the arid climatic conditions of the Algerian Sahara. The highest produced amount was estimated at 127 µg/mL by cultivating *Streptomyces* sp. PT2 strain in yeast extract-tryptone broth supplemented with 5 mg of L-tryptophane/mL. Moreover, Goudjal et al. [33] isolated two potent strains (CA-2 and AA-2 related to *Streptomyces mutabilis* NBRC 12800^{T} and *S. cyaneofuscatus* JCM 4364^{T}, respectively) from native Algerian Saharan plants roots, which exhibited both in vivo biocontrol potential on *Rhizoctonia solani* damping-off, a largely common fungal pathogen affecting a wide range of crops seedlings, and the promotion of tomato plant growth. Similarly, Zamoum et al. [34] reported the production of indole-3-acetic acid and siderophores by the endophytic strain, *Streptomyces caeruleatus* ZL2. They observed the enhancement of tomato plant resistance to *Fusarium oxysporum* f. sp. *radicis lycopersici* root rot as well as the ability to promote seedlings growth, proposing therefore the possible application of the isolate ZL2 in crop protection. Furthermore, the study carried out by Toumatia et al. [120] on plant-growth-stimulating properties of the *Streptomyces mutabilis* IA1 strain derived from Saharan soil, demonstrated a potent and promising protective effect on wheat seedlings against *F. culmorum*, which is the causal agent of seedling blight, showing its growth-promoting ability by the production of indole-3-acetic acid and gibberellic acid (GA3). The study by Merrouche et al. [118] allowed to highlight the potent antifungal effect of *Saccharothrix algeriensis* NRRL B-24137 due to the production of dithiolopyrrolones compounds acting against *Fusarium oxysporum*, which induces wilt disease affecting flax, lentil, chickpea, and tomato.

A study by Goudjal et al. [33] on endophytic actinobacteria pertaining to *Streptomyces* genus, collected from spontaneous Saharan plants, allowed the isolation of indole-3-acetic acid (**50**) and showed a growth-promoting activity of tomato plants.

5. Conclusions

In the last years, a growing interest in the exploration of less studied environments (such as marine, forest, sebkha, and arid ecosystems) and of the symbiotic associations has been observed in Algeria for isolation of new actinobacteria species and the isolated bioactive metabolites. This report provides the first comparative overview of the full diversity of actinobacteria phyla reported from the Algerian ecosystems. Compared to all geographical niches which provided 29 novel species, it is evident a high abundance of new actinobacteria species is associated with Algerian Saharan soil, yielding 27 novel species belonging to 15 genera. Fifty secondary metabolites have been isolated and identified, including 17 new molecular structures (**1**–**3**, **6**–**8**, **10**–**15**, **18**, **21**, **31**, and **43**–**45**), and then evaluated for their biological activities, mainly focusing on antibacterial and antifungal but also including cytotoxicity and promotion of plant growth. The following points are proposed and emphasized for future research in this topic: (i) the exploration of understudied ecological niches (telluric and marine) including

associations of diverse nature as well as the reinforcement of the Algerian desert investigations; (ii) the investigation of the actinobacteria diversity in these ecosystems; (iii) the developement of more suitable cultivation techniques for the isolation of new and rare actinobacteria species from these niches; (iv) in-depth metabolic and genomic studies of new isolated species; and (v) the development of new biotechnologically exploitable species.

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antibiotics

MDPI

Review

Do Global Regulators Hold the Key to Production of Bacterial Secondary Metabolites?

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Abstract: The emergence of multiple antibiotic resistant bacteria has pushed the available pool of antibiotics to the brink. Bacterial secondary metabolites have long been a valuable resource in the development of antibiotics, and the genus *Burkholderia* has recently emerged as a source of novel compounds with antibacterial, antifungal, and anti-cancer activities. Genome mining has contributed to the identification of biosynthetic gene clusters, which encode enzymes that are responsible for synthesis of such secondary metabolites. Unfortunately, these large gene clusters generally remain silent or cryptic under normal laboratory settings, which creates a hurdle in identification and isolation of these compounds. Various strategies, such as changes in growth conditions and antibiotic stress, have been applied to elicit the expression of these cryptic gene clusters. Although a number of compounds have been isolated from different *Burkholderia* species, the mechanisms by which the corresponding gene clusters are regulated remain poorly understood. This review summarizes the activity of well characterized secondary metabolites from *Burkholderia* species and the role of local regulators in their synthesis, and it highlights recent evidence for the role of global regulators in controlling production of secondary metabolites. We suggest that targeting global regulators holds great promise for the awakening of cryptic gene clusters and for developing better strategies for discovery of novel antibiotics.

Keywords: antibiotics; biosynthetic gene clusters; *Burkholderia*; gene regulation; global transcriptional regulator; MftR; ScmR; secondary metabolites

1. Introduction

A large number of antibiotics are in clinical use, and they target a variety of pathways that range from cell wall, protein, and DNA synthesis to folate and nucleotide metabolism [1]. Over the last decade, occurrences of multiple drug resistant bacteria have been on the rise, and the development of new effective methods or drugs to combat such strains has become critical [2]. Studies focused on the identification and isolation of novel natural products from microbes that are effective against pathogenic bacteria or possess other clinical significance, e.g., anti-fungal, anti-cancer, and immunosuppressant activities, have become a priority [3,4]. A considerable number of current pharmaceutical drugs have been directly derived from, or inspired by, bacterial natural products or secondary metabolites. These secondary metabolites, which are not essential for growth under normal conditions, are produced by bacteria in response to environmental stress or host interaction and provide them with a competitive advantage. Many bacterial species produce bioactive secondary metabolites, of which members of the genus *Streptomyces* and lately the genus *Burkholderia* are prominent sources. *Streptomyces* is the source of about 80% of the antibiotics that are produced today, which includes neomycin, kanamycin, vancomycin, streptomycin, tetracycline, and chloramphenicol [5,6]. Proteins that are essential for the production of these bioactive compounds are generally encoded by large cryptic gene clusters, which remain silent under normal laboratory conditions, a circumstance that creates a hurdle in discovery

of novel compounds [7,8]. This review article focuses on such cryptic gene clusters in the genus *Burkholderia* and on potential mechanisms for eliciting their expression.

When originally coined in 1992, the genus *Burkholderia* comprised seven species [9]. At present, nearly a hundred validly named *Burkholderia* species exist [10]. These *Burkholderia* species occupy diverse ecological niches (free living, saprophytic, obligate endosymbionts, phytopathogens, opportunistic pathogens, or obligate parasites) and they include species that can serve as biocontrol and bioremediation agents as well as pathogens. Some *Burkholderia* species, mainly *B. pseudomallei*, *B. mallei,* and members of the *B. cepacia* complex (Bcc), have caught attention because of their pathogenicity [11]. Genome sequencing of *Burkholderia* spp., driven largely by a desire to understand virulence mechanisms, has led to the discovery of a large number of cryptic natural product biosynthetic gene clusters [12]. The regulatory mechanism of some of these cryptic gene clusters has been studied in detail, primarily in *B. thailandensis*. *B. thailandensis*, which is a relatively non-pathogenic strain, shares a large number of genes with other members of the *B. pseudomallei* complex (Bpc), some of which are involved in synthesis of bioactive compounds.

The *B. thailandensis* genome features ~22 natural product biosynthetic gene clusters [12,13]. Understanding how these gene clusters are regulated by local or global regulators (repressors or activators) and how different stress conditions (such as oxidative stress, osmolarity stress, phosphate starvation, and amino acid starvation) or inducing ligands can elicit gene expression is key to unlocking the potential of these cryptic gene clusters. Here, we focus on how the gene clusters that are responsible for encoding proteins involved in the synthesis of well-characterized bioactive compounds are regulated by local transcription factors dedicated to a specific gene cluster and how global regulators exert control over multiple gene clusters. Based on recent reports that a multitude of compounds are concurrently produced under conditions such as antibiotic stress and that the corresponding gene clusters are induced by inactivating a single transcription factor, we propose that such global regulators hold the key to the discovery of novel compounds.

2. Bioactive Secondary Metabolites

2.1. Malleilactone

Bacterially produced cytotoxic products are an important contributor to pathogenesis during host infection. To conserve energy, such compounds are only produced when needed and in response to inducing signals; they are, therefore, generally not synthesized under normal laboratory conditions, and this renders their identification and characterization particularly challenging. Malleilactone (also identified as Burkholderic acid [14]), is a polyketide synthase (PKS)-derived cytotoxic product, produced by species in the *B. pseudomallei* complex (Bpc), and it has received much attention, as it has been shown to be essential for *B. pseudomallei* and *B. thailandensis* to cause infection in *Caenorhabditis elegans* (Figure 1). Malleilactone can also inhibit growth of Gram-positive bacteria and it is cytotoxic to cultured mammalian cells [15–17]. Proteins that are encoded by the *mal* gene cluster produce it (Table 1).

In *B. thailandensis*, the *mal* cluster is an ~35 kb cryptic gene cluster with 13 open reading frames (BTH_II2088 to BTH_II2099). The *mal* cluster is highly conserved in *B. pseudomallei* and *B. mallei* with about 80–90% amino acid identity across these three species [14,15] (Figure 2A). The *mal* cluster has the same gene content in *B. pseudomallei, B. mallei,* and *B. thailandensis,* except that two annotated hypothetical genes that are upstream of *malA* and upstream of *malC* are absent in *B. thailandensis*. The *mal* cluster is divergent from the gene encoding the transcription factor MalR.

MalR has been shown to be essential for the expression of the *mal* cluster, and in both *B. thailandensis* and *B. pseudomallei*, MalR was shown to be required for the bacteria to infect *C. elegans* [18–21]. MalR is an orphan LuxR, which means that no cognate LuxI acyl homoserine lactone (AHL) synthase has been identified. The intergenic region between *malR* and *malA* contains a lux box-like region, to which MalR likely binds, and an intact lux box is required for the expression of *malA,* which indicates that

MalR functions as an activator [13,15,18,19]. Generally, LuxRs respond to either endogenously and/or exogenously produced AHLs, but MalR does not respond to AHLs. However, the deletion of all three *luxI* genes in *B. pseudomallei* Bp82 led to ~10-fold greater production of Malleilactone, and expression was restored to wild type levels upon the addition of all three AHLs exogenously to the mutant culture. This led to the inference that AHL levels indirectly regulate the production of Malleilactone [19]. As outlined below, the AHL-mediated repression is likely achieved *via* the global regulator ScmR (Secondary Metabolite Regulator).

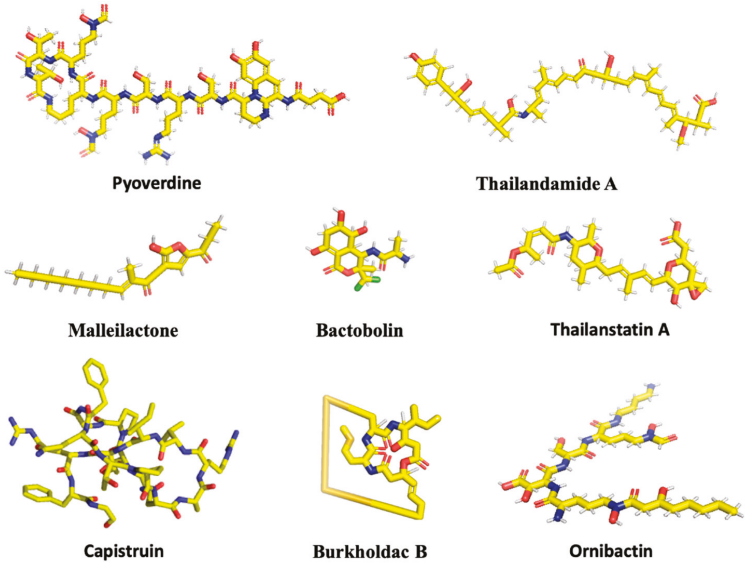

Figure 1. Chemical structures of selected secondary metabolites. Two-dimensional structures were obtained from PubChem (Malleilactone under the name Burkholderic Acid), except for Burkholdac B, which was obtained from ChEMBL (under the name Thailandepsin A) and Capistruin, for which the three-dimensional structure represents its conformation in complex with RNA polymerase (PDB ID 6N61). The complete amino acid sequence of Capistruin is **GTPGFQTPD**ARVISRFGFN, where bold letters denote residues linked by a backbone-sidechain lactam linkage to form the cyclic structure through which the C-terminal residues are threaded. Images were rendered with PyMol. C, yellow; O, red; N, blue; H, grey; S, orange; Cl, green.

Table 1. Local regulators of biosynthetic gene clusters.

Bioactive Compound	Gene Cluster	Local Regulator	Cellular Target/Function
Malleilactone	*mal*	MalR (Orphan LuxR)	Unknown
Bactobolin	*bta*	BtaR2 (LuxR)	50S Ribosomal Subunit
Capistruin	*cap*	Unknown	RNA Polymerase
Thailandamide	*tha*	ThaA (Orphan LuxR)	Acetyl-CoA Carboxylase
Burkholdacs	*bhc*	BhcM (AraC)	Histone Deacetylase
Pyoverdine	*pvc*	BTH_II2035 (LTTR)? Unknown	Siderophore
Ornibactin	*orb*	OrbS (ECF)	Siderophore
Thailanstatin	*tst*	TstA (Orphan LuxR)	Spliceosome

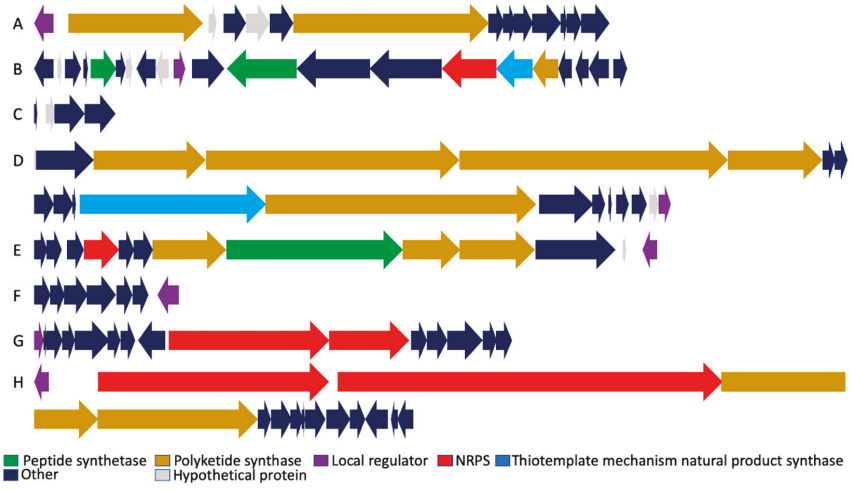

Figure 2. Organization of biosynthetic gene clusters. (**A**) Malleilactone. (**B**) Bactobolin. (**C**) Capistruin. (**D**) Thailandamide. (**E**) Burkholdacs. (**F**) Pyoverdine. (**G**) Ornibactin. (**H**) Thailanstatin. Genomic loci and individual gene annotations correspond to *B. thailandensis* E264 and are obtained from the Burkholderia Genome Database (https://www.burkholderia.com), except for the Ornibactin biosynthetic gene cluster (*B. cenocepacia* J2315; Burkholderia Genome Database [22]) and the Thailanstatin gene cluster (*B. thailandensis* MSMB43; NCBI GenBank JX307851.1).

2.2. Bactobolin

Bactobolin, which is a polyketide-peptide (a C_6-polyketide fused to a chlorinated hydroxy-valine residue), is produced by *B. thailandensis* (Figure 1). It was initially identified and characterized in *Pseudomonas* BMG13-A7 (1979 AD), where the compound was shown to have antibacterial as well as anti-tumor effects [23]. A number of studies have been conducted in *Burkholderia* spp. to understand the effect of the compound, its mechanism of action, and the genes that are involved in its synthesis. Approximately eight structurally different Bactobolin compounds have been identified in *B. thailandensis* (Bactobolin A-H) [24]. Bactobolins A-D have been tested for cytotoxicity to mammalian cells and antibacterial activity, with compounds A and C exhibiting more antibacterial activity than B and D. Further, there is a direct correlation between cytotoxicity and antibacterial activity [25]. The compounds seem to be particularly effective against Gram-positive bacteria. A mutational study in *B. subtilis* confirmed that Bactobolin targets the ribosome, but a different site than other known ribosome inhibitors [25]. Specifically, Bactobolin A's biological activity derives from inhibiting protein synthesis by binding to a site in the 50S ribosomal subunit, which displaces tRNA from the P site of the ribosome [26].

Genes that are involved in synthesis of Bactobolin have been identified and characterized for *B. thailandensis* [25,27]. It is one of the few gene clusters that include both polyketide synthase (PKS)-and nonribosomal peptide synthetase (NRPS)-encoding genes (Figure 2B). An ~37 kb gene cluster, *btaA* to *btaU* (BTH_II1222-BTH_II1242), which includes the LuxI-LuxR system *btaI2* (BTH_II1227) and *btaR2* (BTH_II1231), is involved in the synthesis and regulation of Bactobolin (Table 1) [25,27]. The gene cluster is relatively conserved in *B. pseudomallei*, but not in *B. mallei*. The *btaI2-btaR2* quorum sensing pair is absent in *B. mallei* (most likely lost due to deletion of genes not required for virulence) [25,28]. The presence of the *btaI2-btaR2* system in the cluster suggests that the production of Bactobolin would be regulated in a quorum sensing-dependent manner, an inference that was confirmed by the generation of a quorum sensing-defective mutant [29]. That BtaI2 and BtaR2 control the production of Bactobolin has been established while using the *btaR2* mutant, which showed no antibiotic activity

against Gram-positive bacteria. It is interesting to note that *btaR2* could also be regulated by the product of BtaI3 [27]. As discussed below, the expression of genes encoding BtaR2 and BtaI2, and therefore the *bta* gene cluster, is under the control of the global regulators ScmR and MftR (Major Facilitator Transport Regulator). Moreover, the production of Bactobolin has been suggested to be influenced by temperature, as its production was higher when *B. thailandensis* was grown at 30 °C as compared to 37 °C [25].

2.3. Capistruin

Capistruin belongs to the family of lasso peptides. Lasso peptides are bioactive peptides, which are ribosomally synthesized and post-translationally modified. They are characterized by an N-terminal macrolactam ring through which a C-terminal peptide tail is threaded [30–32]. The first lasso peptide, Anantin, was discovered in 1991 from a strain of *Streptomyces coerulescens* [33]. Capistruin from *B. thailandensis* was the first lasso peptide to be identified based on a genome-mining approach. This approach has since led to the more efficient identification of lasso peptides, and about 50 lasso peptides have been discovered so far. They have been classified into class I (which contains two disulfide bonds), class II (the most abundant type, which has no disulfide bonds, but the topology is held by steric hindrance), and classes III and IV (which both contain one disulfide bond that connects the ring to the tail or is within the tail region, respectively) [34]. Capistruin belongs to class II and it is similar to *E. coli* MccJ25 (Microcin J25; Figure 1).

The mature Capistruin is a 19 amino acid peptide (excised from a 47 amino acid precursor), in which the N-terminal 9 residues form the macrolactam ring through which the 10-residues long C-terminal tail is threaded [30,35,36]. Unlike other antibacterial compounds, Capistruin from *B. thailandensis* is effective against closely related *Burkholderia* and *Pseudomonas* strains [36]. This leads to the suggestion that Capistruin-producing *Burkholderia* species either encode an immunity protein or feature some modification of the target. Capistruin and Microcin J25 biological activity is due to their ability to inhibit RNA polymerase (RNAP), although for Microcin J25, the over-production of reactive oxygen species (ROS) through a possible secondary target has also been suggested [37–39]. Microcin J25 and Capistruin share the RNAP secondary channel as their binding site. Although Capistruin binds to *E. coli* RNAP as effectively as Microcin J25 in vitro, a nearly 10-fold higher concentration of Capistruin is required for inhibiting *E. coli* growth. Similarly, Microcin J25 can bind *Pseudomonas* RNAP as effectively as Capistruin, which inhibits *Pseudomonas* and *Burkholderia* growth, but no such inhibitory effect was seen with Microcin J25 [35,36]. A recently discovered lasso peptide, named Citrocin, which was isolated from *Citrobacter pasteurii* and *Citrobacter braakii,* showed similar effect; the lasso peptide was nearly 100-fold more potent as an RNAP inhibitor compared to Microcin J25, but a higher concentration of Citrocin was required to inhibit *E. coli* growth when compared to Microcin J25 [40]. Despite having the same functional target, the effect of the compounds seems to be species-specific. The most likely reason for this species specificity is a variation in cellular uptake/export [35]. Another plausible cause could be a variation in how the compounds inhibit RNAP. The crystal structure of Microcin J25 and Capistruin in complex with RNAP has revealed that Microcin J25 binds within the active site of RNAP, which limits or prevents access to NTPs, and its inhibition of RNAP is partially competitive with respect to NTP binding. This is not the case for Capistruin, which binds further away from the active site, its inhibition is partially non-competitive with NTP binding, and it lowers the rate of phosphodiester bond formation by eight-fold. This suggests that the Capistruin-mediated inhibition of RNAP catalysis is primarily due to interference with the proper folding of the trigger loop, a mobile element within the RNAP catalytic subunit [39].

By comparison to the *E. coli* MccJ25 gene cluster (*mcjABCD*), a similar cluster was found in *B. thailandensis* and was determined to be the Capistruin biosynthetic gene cluster (*capABCD*; Figure 2C). *capABCD* encodes (i) the capistruin precursor protein CapA (BTH_I2437a), (ii) two modifying enzymes for converting the precursor to mature lasso peptide, a putative protease CapB (BTH_I2438) and asparagine synthase CapC (BTH_I2439), and (iii) an ABC transporter CapD involved in export/immunity

(BTH_I2440). CapD is most likely involved in the export of Capistruin from the cell, thereby mediating both transport and detoxification. Overproduction of the CapD homolog McjD in *E. coli* was sufficient to establish resistance against Capistruin [41]. The 4.5 kb lasso peptide gene cluster is conserved in *B. pseudomallei*.

The mechanism of expression of lasso peptide biosynthetic genes is poorly understood (Table 1). The *E. coli mcjABCD* was shown to be up-regulated under iron deficient conditions [42]. Similarly, Capistruin production in *Burkholderia* was upregulated when cells were grown in minimal media under heat stress (42 °C). Both cases can be related in terms of nutrient deficient conditions, but if nutrient deficiency were the main trigger for expression, one would expect Capistruin production to be higher in the stationary phase as compared to the exponential phase. However, unlike many other secondary metabolites with antibacterial activity, Capistruin can be detected in exponential phase, and its synthesis is arrested while transiting from late exponential to early stationary phase [36], which suggests a different mode of regulation as compared to other biosynthetic gene clusters. While no local regulator has been reported, Capistruin biosynthesis genes are under the control of ScmR and MftR, as discussed below.

2.4. Thailandamide

Thailandamide, which is a linear, long-chain, unstable polyene natural product, is a fatty acid synthesis blocker (Figure 1). It is a broad-spectrum antibiotic that has effect against both Gram-positive and Gram-negative bacteria. The structure of three distinctive forms of Thailandamide have been elucidated (Thailandamide A, Thailandamide B, and Thailandamide lactone). Thailandamide A is effective against Gram-positives, but it is less effective against Gram-negative bacteria (except *Neisseria gonorrheae*). It is interesting to note that a change in the *E. coli* cell wall structure, which increased uptake, led to an increased efficiency of Thailandamide A. This prompted the suggestion that the otherwise broad-spectrum activity of Thailandamide is limited by poor uptake in Gram-negative bacteria [43]. A study involving insertional mutation for characterization of new antibiotics revealed Thailandamide B to be the major product formed by *B. thailandensis* [44]; this is contrary to other analyses, where Thailandamide A was shown to be the major product [43,45–47]. Thailandamide B was revealed to have bactericidal activity and it was shown to be toxic to human cells as well. Since Thailandamide is unstable, these differences might derive from variations in the extraction techniques; alternatively, mutations in genes encoding regulatory factors could lead to variations in the types of Thailandamide produced. For example, Thailandamide lactone, which was only detected in cells in which the *tha* gene cluster was highly active, displayed moderate anti-proliferative activity against tumor cell lines [46].

B. subtilis is sensitive to Thailandamide, and it was shown that a mutation in the *accA* gene, which encodes acetyl-CoA carboxylase, conferred resistance against Thailandamide A. Similarly, a single base mutation in *accA* was found in Thailandamide B-resistant *Salmonella* mutants. Further, in *B. subtilis*, over-expression of mutant AccA was sufficient to relieve the Thailandamide-induced inhibition. Thus, the cellular target for Thailandamide was suggested to be AccA protein or the AccA/AccD complex, which is involved in the first committed step of fatty-acid biosynthesis [43,44].

The *B. thailandensis* gene locus BTH_II1662-1681 encodes proteins that are involved in the synthesis of Thailandamide (BTH_II1662-1676), resistance (BTH_II1679), and regulation of its production (BTH_II1681/ThaA; Figure 2D). *thaC* or *accA*-2 is responsible for resistance against Thailandamide (*B. thailandensis* and closely related species are resistant to Thailandamide). The presence of a second copy of *accA* (*thaC*) likely affords the resistance to Thailandamide, which further strengthens the inference that AccA is the target for Thailandamide [43,44], as cells that express *thaC* are less susceptible to Thailandamide activity. It is interesting to note that several *Burkholderia* spp., which lack the *tha* gene cluster still encode a *thaC* homolog [43]. This could explain the Thailandamide resistance characteristic of these *Burkholderia* spp.

The mode of regulation of the *tha* biosynthetic gene cluster has not been elucidated or agreed upon completely (Table 1). Thailandamide production seems to be regulated by more than one gene product, and environmental cues appear to be responsible for its regulation. Under normal growth conditions, only vanishingly small amounts of Thailandamide are produced and exclusively in the early growth phase. The disruption of *thaA* (*luxR5*) abolished production of Thailandamide A, whereas the disruption of the *thaA* promoter resulted in increased production, likely on account of increased *thaA* expression. ThaA (LuxR5) has an AHL motif and is similar to LuxR regulators, but it has no known cognate LuxI [46]. In a study that was designed to elucidate a quorum sensing-controlled regulon in *B. thailandensis*, it was shown that ThaA is autoregulatory and represses *thaA*. Interestingly, *thaA* was activated upon addition of exogenous AHLs [48]. Whether ThaA directly responds to AHL levels or if *thaA* is activated by another transcription factor remains to be determined. Most likely, ThaA activates the expression of genes that are involved in Thailandamide synthesis. As cells progress from early growth phase, levels of AHLs increase, which should lead to an increased production of ThaA; therefore, the observed decrease in Thailandamide production speaks to the repression of the *tha* cluster by a different mechanism. As noted below, increased AHL-dependent production of ScmR might explain such repression. Recently, it has been shown that transposon-mediated disruption of *momS* (*BTH_I0633*) led to increased production of Thailandamide B. MomS has 66% sequence identity to AtsR (Adhesion and Type Six Secretion System Regulator), which has been shown to be a global regulator in *B. cenocepacia* [44].

2.5. Burkholdacs

Burkholdacs belong to the class of histone deacetylase (HDAC) inhibitors, which includes drugs, such as vorinostat, romidepsin, belinostat, and panobinostat (Figure 1). HDACs are a relatively new class of anti-cancer agents that induce death, apoptosis, and cell-cycle arrest. In eukaryotic cells, expression of genes is regulated through chromatin remodeling. One of the mechanisms by which such remodeling can be brought about is through either acetylation or deacetylation of lysine residues in core histones. While HATs (histone acetyl transferases), as the name suggests, acetylate the core histones, leading to uncoiled or less compact DNA, providing access to the transcription machinery, HDACs remove acetyl groups leading to the condensation of DNA around histone and repression of transcription. Thus, HDAC inhibitors prevent deacetylation, leading to an accumulation of hyperacetylated nucleosomes and differential gene expression [49].

Burkholdacs A and B from *B. thailandensis* were first isolated based on the over-expression of transcription factors linked to genes encoding secondary metabolite biosynthetic enzymes. In *B. thailandensis*, the *bhc* gene cluster includes two adjacent operons BTH_I2357-2358 and BTH-2359-2367, a hybrid-NRPS/PKS biosynthetic gene cluster (Figure 2E). The gene cluster has been shown to be under control of BTH_I2369 (encoded by *bhcM*), an AraC family transcription factor (Table 1) [50]. Members of the AraC/XylS family have three common regulatory functions: carbon metabolism, stress response, and pathogenesis [51]. Most members function as transcriptional activators, but some act as repressors or both, depending upon promoter architecture or the presence or absence of effectors [52,53].

The above-mentioned study involving an overexpression of transcription factors within or adjacent to NRPS/PKS gene clusters identified BhcM as an activator of the *bhc* gene cluster, but it did not reveal how *bhcM* expression is controlled. [50]. Members of AraC/Xyls can be classified into two groups: Either the signal receptor and regulatory function resides in same polypeptide or transcription of the regulatory protein is controlled by another regulator, be it repressor or activator [51]. BhcM seems to fall in the latter group, as discussed below.

2.6. Pyoverdines

Pyoverdines, fluorescent yellowish-green pigments, are the primary siderophores in *P. aeruginosa* (Figure 1). Siderophores are small, metal-chelating molecules with high affinity for Fe (III), which are

produced by almost all bacteria and generally under iron limiting conditions. The role of siderophores becomes even more critical for pathogens that face a challenging low iron environment inside a host [54,55]. Besides acting as iron chelators, siderophores (catecholate types) can serve an anti-oxidant role during host-pathogen interactions [56]. Siderophores can also bind other essential metals, such as Mn, Mo, Co, and Ni, and deliver them to the microbe [57]. Various strains of *Pseudomonas* secrete different Pyoverdines, but commonalities in their structure include: (i) a fluorescent chromophore that is quenched upon binding of Fe^{3+}, (ii) a strain-specific peptide that interacts with Fe^{3+} by chelating it, and (iii) an acyl side-chain bound to the chromophore whose functionality has not been completely understood [58]. Pyoverdines have been shown to be involved in both acquisition of iron and as signaling molecules for production of virulence factors [59–62]. No systematic analyses of Pyoverdine function have been reported in *B. thailandensis*. However, a study was carried out to determine the interactions between different cystic fibrosis pathogens discovered that Pyoverdine produced by *P. aeruginosa* inhibited the growth of *B. cenocepacia* J2315 [63].

The *B. thailandensis* chromosome harbors a predicted Pyoverdine gene cluster (BTH_II0229-0234) that is similar to *P. aeruginosa pvcABCD* (PyoVerdine Chromophore) cluster, which encodes proteins needed to synthesize Pyoverdine [17,64]. In *B. thailandensis*, a convergently oriented LysR-type transcriptional regulator (LTTR; BTH_II0235) is encoded immediately downstream of the cluster (Figure 2F). Similarly, *P. aeruginosa pvcABCD* has a convergently oriented *ptxR* that encodes an activator PtxR, also an LTTR. *ptxR* has two promoter sites, of which one is regulated in an iron dependent manner, while the other promoter is iron-independent [65].

pvcABCD expression has been shown to be repressed by presence of iron and to be positively regulated by the alternate sigma factor PvdS and by the activator PtxR [65,66]. A *ptxR* mutant did not produce detectable Pyoverdine, even under iron deficient conditions, which suggests that PtxR is an essential activator for expression of *pvc*ABCD in *P. aeruginosa* [66]. PvdS synthesis is repressed by another transcription factor, called Fur (Ferric uptake regulator), which utilizes Fe^{2+} as a corepressor [67]. A low level of iron causes the dissociation of ferrous ion from Fur, leading to the derepression of *pvdS*; in turn, PvdS can possibly facilitate the expression of *ptxR*. *B. thailandensis* encodes PtxR and several extracytoplasmic function (ECF) sigma factors, whose roles have not been determined. A Fur homolog, BTH_I1206, is also present in *B. thailandensis*. Thus, the Pyoverdine gene cluster may be similarly regulated in *B. thailandensis* as in *P. aeruginosa* (Table 1).

2.7. Ornibactin

Ornibactin, which is a tetrapeptide siderophore with an L-ornithine-D-hydroxyaspartate-L-serine-L-ornithine backbone (Figure 1), was first identified in *B. cepacia* [68]. Various strains of *Burkholderia* produce Ornibactin, but it has been primarily characterized in members of the Bcc. Ornibactin is produced under iron deficient conditions and its expression is completely inhibited by the presence of more than 15 μM of ferric iron in the media [69]. The function of Ornibactin, besides being an iron-acquiring molecule, has been established by studies of its function in different strains of *Burkholderia*. In *B. cepacia*, Ornibactin was shown to be critical for establishing infection in a murine chronic respiratory infection model. Moreover, it was noted that Ornibactin was critical in adherence and colonization [55,70]. Evolution of the role of Ornibactin was highlighted by a study that was conducted in *B. contaminans* MS14, where the production of Ornibactin was shown to be critical for the production of an antibacterial compound, which is effective against a wide range of plant-pathogenic bacteria [71].

The Ornibactin gene cluster, *orbA* through *orbS*, has been described in *B. cenocepacia* J2315 (BCAL 1688-1702). The gene cluster includes a gene encoding an ECF sigma factor, *orbS* (*BCAL 1688*), whose product OrbS has a high degree of similarity to PvdS (Figure 2G). The Ornibactin gene cluster contains a promoter region, to which OrbS could possibly bind and thus activate the expression of the gene cluster. Further, no production of Ornibactin was detected in an *orbS* mutant, suggesting that OrbS is an essential activator (Table 1). The *orbS* promoter has a region to which Fur protein may bind and

repress its expression under iron sufficiency [69]. Thus, regulation of Ornibactin synthesis by OrbS and Fur could work in similar fashion as described for Pyoverdine.

An interesting thing to note in the case of the Ornibactin gene cluster, which is present in a large number of *Burkholderia* species, is that it has conserved NRPS genes (*orbI* and *orbJ*), but contains diversity within the genes that are involved in initiation, transport, regulation, and modification, suggesting the possibility for differential roles and regulation across species, as exemplified by the antibacterial activity of the *B. contaminans* MS14-derived Ornibactin [71]. While Ornibactin has been characterized from members of the Bcc, members of the Bpc produce the related siderophore malleobactin, with the biosynthetic genes being organized and regulated in a similar fashion [72].

2.8. Thailanstatin

Thailanstatin belongs to the spliceostatin class of natural products, which inhibit the spliceosome. Four forms of the compound (Thailanstatin A-D; Figure 1) have been isolated and characterized from *B. thailandensis* MSMB43 [73–75]. Alternative splicing, which is carried out by spliceosomes, generates an abundance of protein variants, however, cancer cells exhibit increased splicing levels, mutations in the splicing machinery, and aberrant alternative splicing. Thus, compounds that belong to the spliceostatin class can serve as potent anti-cancer agents [73,76].

FR901464, the first natural product of the spliceostatin family, was identified in 1996 from *Pseudomonas* sp. No. 2663 (subsequently, 16S rRNA sequence analysis showed the correct phylogenetic classification to be *Burkholderia sp.* FERM BP-342117 [77,78]), and it was shown to have marked anti-tumor activity [79]. As the compound is chemically unstable, a more stable methylated derivative, Spliceostatin A, was produced. In vitro assays revealed that Thailanstatin A, which was shown to be the most potent, could inhibit pre-mRNA splicing as efficiently as FR901464; moreover, it possesses anti-proliferative activity and it is more chemically stable than FR901464 [73,74]. Overall, Thailanstatin A has been shown to be less toxic to normal human cells and effective against human cancer cells lines. Spliceostatin A and FR901464 target the splicing factor 3b (SF3b) subcomplex of the U2 small nuclear ribonucleoprotein particle of the spliceosome, leading to the inhibition of pre-mRNA splicing and causing pre-mRNA leakage to the cytoplasm [80].

A gene cluster with homology to the *fr9* gene cluster, which encodes proteins that are required for production of FR901464, was discovered in *B. thailandensis* MSMB43 and it was named the *tst* gene cluster (Figure 2H); this gene cluster is not conserved in closely related species such as *B. thailandensis* E264. The *tst* gene cluster is a 78.1 kb DNA region comprising 15 ORFs (*tstA* through *tstR*). *tstA*, which is divergently oriented to the rest of the gene cluster, encodes a LuxR type transcriptional factor with no cognate LuxI within the gene cluster (Table 1). TstA has been suggested to be involved in the regulation of the gene cluster [73]. This genomic locus and its arrangement is very similar to the *mal* gene cluster arrangement in *B. thailandensis* E264 suggesting that TstA could possibly serve as an activator of the gene cluster similar to MalR. Whether TstA responds to AHL levels or any other ligand has not been reported.

3. Global Regulators of Biosynthetic Gene Clusters

3.1. ScmR (Secondary Metabolite Regulator)

As discussed above, dedicated pathway-specific regulators control the expression of many biosynthetic gene clusters. However, efforts to elucidate mechanisms by which cryptic biosynthetic gene clusters might be activated have recently led to the discovery of global regulators with a role in controlling an array of biosynthetic gene clusters. One of these regulators is ScmR, an LTTR, which has been shown to be involved in the production of various secondary metabolites in *B. thailandensis* E264. ScmR is conserved in *B. thailandensis* (BTH_I1403) with orthologs in *B. pseudomallei* and *B. mallei*. The *scmR* promoter contains a lux box, and gene expression was shown to be about two-fold greater when the cells were grown to higher cell density [16,48,81]. Moreover, no activation of *scmR* was seen

in a mutant strain that was deleted for all three *btaI* genes, and therefore deficient in AHL synthesis. Similarly, a *B. pseudomallei* Bp82 triple *btaI* mutant exhibited ~3-fold lower *scmR* transcript levels when compared to wild type. Interestingly, the *B. thailandensis* triple *btaI* mutant featured an increased production of cryptic secondary metabolites, as also seen in the Δ*scmR* strain, which suggests that ScmR—and therefore production of some secondary metabolites—is regulated by quorum sensing. Further, production of AHLs is drastically reduced in the Δ*scmR* strain, indicating reciprocal regulation of AHL synthesis by ScmR. In the *B. thailandensis* Δ*scmR* strain, 13 of the >20 predicted biosynthetic gene clusters in *B. thailandensis* were differentially regulated [16]. We will focus on the role of ScmR in regulation of some of the compounds that are discussed above.

The inactivation of *scmR* leads to a 7- to 13-fold upregulation of genes in the *bta* cluster, indicating that ScmR represses the production of Bactobolin [16]. The *bta* gene cluster is locally activated by BtaR2, which is induced by AHLs. AHLs are produced at a higher cell density and they serve to activate both BtaR2 and ScmR. The repression of genes in the *bta* cluster by ScmR could therefore be achieved by ScmR binding directly to the *btaI2* promoter and repressing transcription, consistent with the ~5-fold increased expression of *btaI2* in Δ*scmR*, forming a negative feedback loop that is designed to limit bactobolin production by attenuating activation by the AHL-dependent BtaR2 (Figure 3). Recently, a proteomic profiling of *B. thailandensis* during host infection revealed that ScmR is overexpressed in host-associated bacteria; while nine of 11 proteins encoded by genes that are repressed by ScmR were detected at lower levels, as expected, a Bactobolin biosynthetic enzyme was an exception. BtaC, encoded by *BTH_II1224*, was overproduced during infection. This suggests that, at least under infection conditions, Bactobolin synthesis is not solely determined through ScmR-mediated transcriptional repression [82].

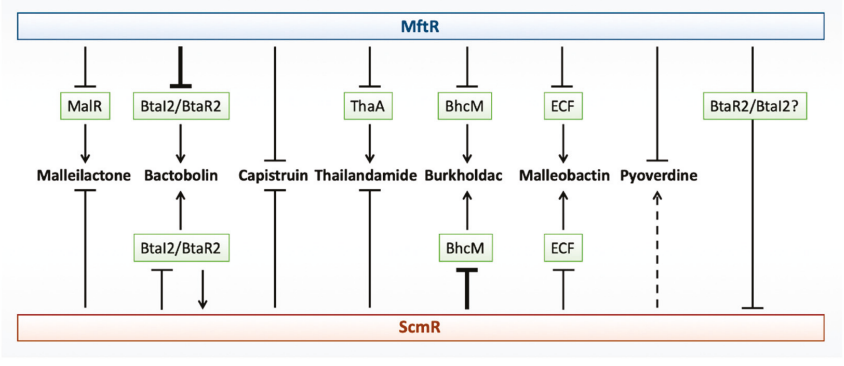

Figure 3. Regulation of biosynthetic gene clusters by Major Facilitator Transport Regulator (MftR) and Secondary Metabolite Regulator (ScmR). Cluster-specific regulators, where known, are identified in green. Repression is shown as lines, and activation as arrows. Deletion of *mftR* or *scmR* results in the greatest upregulation of Bactobolin and Burkholdac, respectively, as indicated by heavier lines. ScmR-mediated activation of Pyoverdine biosynthetic genes is marginal (dotted line). For ScmR-mediated regulation of the Bactobolin biosynthetic gene cluster, direct repression of *btaI2* was inferred, with BtaR2 reciprocally activating *scmR*.

The deletion of *B. thailandensis scmR* also revealed upregulation of genes involved in Capistruin biosynthesis, implicating ScmR as a repressor. During the stationary phase, AHL levels increase and *scmR* expression has been shown to be higher in presence of AHLs. Thus, the decrease in Capistruin production that was observed as cells enter stationary phase might be due to more efficient repression of Capistruin biosynthetic genes by ScmR [16]. Whether ScmR directly controls the Capistruin gene cluster or whether it controls the expression of a cluster-specific regulator remains to be determined.

The metabolomics analysis showed 61-fold increased production of Burkholdac A in Δ*scmR* cells, and the corresponding transcriptome analysis revealed 50- to 135-fold upregulation of the various genes in the *bhc* cluster [16]. Further, the complementation of the Δ*scmR* strain resulted in reduced production of Burkholdac, as seen in wild type cells, which indicated that ScmR represses the *bhc* gene cluster. Among the genes that were upregulated in Δ*scmR* cells is *BTH_I2369* (*bhcM*), encoding the AraC-family activator of the *bhc* gene cluster, BhcM. The repression of the *bhc* cluster by ScmR can therefore be explained by its repression of *bhcM*, possibly by direct binding to its promoter, resulting in failure to produce an essential activator. As AHLs are produced at higher cell density even under normal laboratory settings, which leads to increased production of ScmR and repression of *bhcM*, this would result in little to no production of Burkholdacs under these conditions.

The regulation of Malleilactone production by ScmR seems to be more complex. The metabolomics analysis of Δ*scmR* cells showed 210-fold overproduction of Malleilactone A, previously shown to be essential for the bacteria to cause infection in *C. elegans*. This overproduction was supported by the 8- to 18-fold upregulation of the different genes in the *mal* gene cluster and by the ~90% worm killing in a span of just 20 minutes when only ~5% of the worms were killed by wild type *B. thailandensis* [16]. Complementing the Δ*scmR* strain with plasmid-encoded *scmR* resulted in diminished production of Malleilactone, which verifies that ScmR acts as a repressor of Malleilactone synthesis. MalR has been established as an essential activator of the *mal* gene cluster, yet no change in expression of *malR* was noticed in the Δ*scmR* cells. This led the authors to the suggestion that ScmR possibly competes with MalR for binding to the *mal* promoter or that the accumulation of an unknown molecule only occurs in Δ*scmR* cells and functions as a MalR coinducer to activate the *mal* gene cluster further; we favor the first interpretation as no coinducers for MalR have been suggested in other studies. As discussed below, the global regulator MftR represses the *mal* gene cluster by also repressing the *malR* gene, which suggests an even more complex regulatory network. In a *B. pseudomallei* Bp82 Δ*scmR* strain, only ~4-fold increased production of Malleilactone was reported as compared to wild type cells, and the production was reduced to wild type levels upon complementing the mutant. In *B. pseudomallei* Bp82, *malR* expression was reported to modestly increase in a mutant deficient in AHL synthesis and *malR* transcript levels were two-fold higher in a Δ*scmR* strain, but only when cells were grown to late stationary phase and not in exponential or early stationary phase [16,19]. While the change in *malR* expression in the AHL mutant would be consistent with repression of *malR* by the quorum-sensing activated ScmR, the growth phase-dependent *malR* expression in Δ*scmR* cells points to regulation by a different mechanism.

3.2. Major Facilitator Transport Regulator (MftR)

B. thailandensis encodes 12 annotated MarR (Multiple Antibiotic Resistance Regulator) homologs, all of which are conserved in *B. mallei* and *B. pseudomallei* [83]. Members of the MarR family are transcription factors that are ubiquitous in the domains bacteria and archaea, and they have been shown to regulate various biological functions, such as response to environmental stress (for instance, antibiotic and oxidative stress or a change in pH), regulation of genes that are involved in virulence, and catabolism of aromatic compounds [84–86]. The genomic locus in which *mftR*, *BTH_I2391* in *B. thailandensis*, is divergently oriented to the *mftP-fenI* operon (*BTH_I2392* and *BTH_2393*) is conserved across members of the Bpc. MftR is a MarR homolog, while MftP (Major Facilitator Transport Protein) encodes an efflux pump and FenI is a predicted glycosyl hydrolase. MftR binds to the intergenic region between these divergently oriented genes, thereby repressing the expression of both *mftR* as well as the *mftP-fenI* operon [87].

MftR has been previously classified as a member of the MarR subfamily UrtR (Urate Responsive Transcriptional Regulator) [87,88]. Urate and xanthine, which are products of purine metabolism [8,89], bind MftR and attenuate its binding to DNA. Urate was predicted to bind MftR in a pocket that spans the DNA-binding and dimerization regions of the protein, a prediction that was based on the structure of the homologous urate-binding MarR protein HucR [87,90]. By binding in this pocket, the ligand is

predicted to reconfigure the disposition of DNA recognition helices to create a conformation that is unfavorable for DNA binding. Urate is produced by host xanthine oxidase in response to bacterial infection at levels that can exceed 200 μM [91], which suggests that MftR might regulate genes upon host colonization. Indeed, determination of the MftR regulon by using an *mftR* knockout mutant revealed differential expression of ~400 genes, such as genes that are involved in biosynthetic processes, metabolism, and pathogenesis [17]. A total of 331 genes were upregulated, while 70 genes were down-regulated in the Δ*mftR* strain, which suggests that MftR is a negative regulator of most genes directly or indirectly under its control. Notably, a number of large biosynthetic gene clusters encoding various secondary metabolites, which are not expressed under normal laboratory settings were upregulated in the Δ*mftR* strain as well as when *B. thailandensis* was grown in media containing urate. As urate attenuates MftR binding to DNA, this observation is consistent with MftR functioning as a global repressor of these gene clusters, and it identifies urate as a common signal for the production of secondary metabolites. A proteomics analysis of differential protein accumulation in host-associated *B. thailandensis* showed a correlation between overproduced proteins and genes that are upregulated on the addition of urate, in accord with this interpretation [82]. The gene encoding ScmR is among those upregulated (2–3-fold) in the Δ*mftR* strain, whereas the expression of *mftR* is unaltered on the deletion of *scmR*, which indicates that MftR acts upstream of ScmR [16,17].

As discussed above, secondary metabolite production might be controlled by quorum sensing via AHL-activated expression of *scmR*. By contrast, MftR may be important for regulation of gene expression under conditions of host infection that involve the activation of host xanthine oxidase and therefore increased urate production. However, gene expression analyses indicate that these regulatory networks are intricately intertwined. The expression of genes that are involved in quorum sensing (*btaI2-btaR2, btaI3-btaR3*) as well as AHL levels are elevated in the Δ*mftR* strain, which directly implicates MftR in the quorum sensing circuit [17]. Thus, *scmR* expression might be repressed either by direct binding of MftR to the *scmR* promoter or—more likely—indirectly via the MftR-mediated regulation of AHL synthesis (Figure 3). Genes encoding Bactobolin, including *btaR2*/*btaI2*, are upregulated ~15-fold on the deletion of MftR or on the addition of urate, and *scmR* expression is modestly increased. This suggests that the ScmR-mediated repression of *btaI2* (and in turn the repression of the *bta* gene cluster) inferred to occur during balanced growth is bypassed when MftR is absent or the inducing ligand for MftR (urate) is present, and it is consistent with the overproduction of a Bactobolin biosynthetic enzyme in host-associated *B. thailandensis*, even under conditions of increased ScmR levels [82]. As *scmR* expression is increased in Δ*mftR* cells, a viable interpretation is that an activator of *btaI2* becomes abundant in the absence of MftR and competes with ScmR for binding; this activator could potentially be BtaR2, the expression of which is markedly increased in Δ*mftR* cells [17].

ScmR represses the expression of the local activator BhcM that is required for expression of the *bhc* gene cluster. Both *scmR*, *bhcM* and the Burkholdac biosynthetic gene cluster are upregulated ~two-fold in the Δ*mftR* strain, an expression pattern that suggests a more complex mechanism for the control of this gene cluster. The modestly increased expression of the Burkholdac biosynthetic genes in Δ*mftR* cells may therefore also derive from the accumulation of an activator of *bhcM* that can compete with ScmR for binding.

Expression of the *mal* and *tha* gene clusters that are involved in production of Malleilactone and Thailandamide, respectively, are upregulated two- to four-fold in *B. thailandensis* deleted for *mftR*. Genes encoding the local regulators MalR and ThaA are similarly upregulated in the Δ*mftR* strain, which indicates the possibility of direct regulation of these regulators by MftR. Both of these secondary metabolites and their respective gene clusters are upregulated in a Δ*scmR* strain, the *mal* gene cluster in particular, but no upregulation of the respective local regulators was seen. Increased expression of the *mal* gene cluster in Δ*mftR* cells could be explained by derepression of *malR*, which encodes an activator, while the regulation by ScmR might entail a competition between ScmR and MalR for the *mal* promoter, as noted above. A similar mode of regulation may pertain in the case of *thaA*, with ScmR

more effectively competing with ThaA for the regulation of the *tha* gene cluster when *thaA* is repressed by MftR.

Several genes that were upregulated in *B. thailandensis* Δ*mftR* cells encode proteins that are involved in the production and transport of siderophores (Figure 3). This includes the gene clusters linked to production of Pyoverdine (BTH_II0229-0234) and the Ornibactin-like siderophore Malleobactin (BTH_I2414-2427). The mechanism of regulation of the Pyoverdine gene cluster remains unknown; specifically, we note that the convergent gene encoding a predicted LTTR activator of this gene cluster is not differentially expressed on the deletion of *mftR* [17]. The expression of genes in this gene cluster is modestly (<two-fold) lower in Δ*scmR* cells, which suggests moderate activation by ScmR. By contrast, the ECF sigma factor encoded as part of the Malleobactin gene cluster (BTH_I2427), which is predicted to activate expression based on comparison to the *P. aeruginosa* homolog PvdS, is upregulated in Δ*mftR* cells as well as in Δ*scmR* cells. Whether the gene encoding this sigma factor is directly or indirectly regulated by either MftR and/or ScmR has not been reported. This ECF is unlikely to participate in the activation of the Pyoverdine biosynthetic genes, as its upregulation in Δ*scmR* cells would have been expected to result in an increased expression of this gene cluster.

4. Trimethoprim as an Inducer of Cryptic Biosynthetic Gene Clusters

Genome mining has advanced the discovery of cryptic biosynthetic gene clusters and prompted efforts to optimize production of bioactive secondary metabolites. A variety of approaches, such as changes in fermentation conditions (media composition, temperature, and pH), co-cultivation, deletion of local regulators, and random insertional mutagenesis, have been successfully employed for the isolation and characterization of specific compounds in *Burkholderia* spp. For instance, production of Capistruin was greatly enhanced by growing *B. thailandensis* in M20 medium at 42 °C, while the isolation of Thailanstatin was optimized by growing cells in defined fermentation medium [35,36,73,74]. The production of siderophores was favored by growing the bacteria in iron deficient media or by cocultivation, and Malleilactone and Thailandamide were isolated as a result of an inducible promoter exchange strategy [15,44,71,92]. Such approaches have been inspired by the successful production of bioactive compounds from streptomycetes, which are well known as sources of clinically relevant compounds. In these species, strain development and metabolic engineering approaches have also been implemented for the improved production of select compounds [93]. While promising, metabolic engineering remains challenging, due to unintended consequences of competing pathways or the accumulation of toxic pathway intermediates.

In general, engineering approaches, including expression in heterologous hosts, have focused on the production of specific compounds. In contrast, chemical elicitors that create a stressful environment for the bacteria have the potential to induce the expression of multiple biosynthetic gene clusters. The activation of cryptic gene clusters by small molecules or ligands may not only increase the yield of individual compounds, but it is also a time and cost-saving technique by comparison to the more laborious culture optimization or systems metabolic engineering approaches [94,95]. Notably, a recent study that was focused on discovering novel elicitors of cryptic biosynthetic gene clusters in *B. thailandensis* found that, among the 640 candidates tested, a sub-lethal dosage of the antibiotic trimethoprim was the most potent elicitor [13]. It should be noted that a cocktail of trimethoprim and sulfamethoxazole (Co-trimoxazole) is a therapeutic drug prescribed for the treatment of *Burkholderia* infections [96,97]. Metabolomic profiling of cells that were treated with trimethoprim showed an upregulation of over 100 compounds, including molecules related to Capistruin, Malleilactone, Burkholdac, Thailandamide, and Bactobolin. Trimethoprim is a dihydrofolate reductase inhibitor, which prevents the conversion of dihydrofolate to tetrahydrofolate, a one-carbon donor that is essential in a variety of biosynthetic reactions, including the production of glycine, methionine, thymidine, and purines [98]. For trimethoprim to bind all of the structurally different, pathway-specific regulators of these cryptic biosynthetic gene clusters to induce gene expression would be unlikely. A more plausible scenario would be for trimethoprim or a metabolite that accumulates in cells treated with this antibiotic

to modulate the function of a global regulator. Indeed, a correlation exists between the compounds that were detected on the addition of trimethoprim and the gene clusters upregulated when *B. thailandensis* is grown in media with urate added. However, for MftR, it has already been shown that trimethoprim does not directly bind to modulate DNA binding, and neither does 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), an intermediate in de novo purine biosynthesis shown to accumulate in the presence of trimethoprim [13,17]. Thus, the inducing ligand, which accumulates in cells grown with trimethoprim, has yet to be discovered.

5. Conclusions and Future Outlook

Secondary metabolites confer a competitive advantage to bacteria in a hostile environment, including the inhospitable environment that is created by host defenses, yet the mechanisms that regulate their production have not been completely deciphered. The gene clusters that encode enzymes responsible for the production of these secondary metabolites, whether being harmful virulence factors or novel compounds with potential clinical activity, such as antibiotics, generally remain silent or cryptic to conserve cellular resources. Understanding how the expression of these gene clusters is elicited could shed light on mechanisms of pathogenicity as well as advance the discovery of novel beneficial compounds. The identification of global regulators, which control cryptic biosynthetic gene clusters, has opened a possible path towards achieving these goals.

Global regulators have previously been shown to be involved in the regulation of specialized genes, such as those that are involved in pathogenesis, quorum sensing, and biofilm formation. Increased biofilm formation, which also aids in bacterial antibiotic resistance, is a characteristic of the Δ*scmR* strain. In a similar vein, increased anaerobiosis has been reported for the Δ*mftR* strain, a metabolic state that is critical for the survival of bacterial species in an oxygen-deprived environment, such as the interior of a biofilm and abscesses that are caused by infection. A role for both ScmR and MftR in virulence is further supported by their regulation of genes encoding enzymes that are responsible for the production of siderophores, which become critical in the iron-deficient environment inside a host [16,17,99,100]. These findings not only suggest that global regulators can control bacterial fitness in a host environment and increase their antibiotic resistance, but they also suggest global regulators as suitable targets for drugs. A recent study in *P. aeruginosa* showed that the global regulator MvfR (also known as PqsR), an LTTR, is involved in biofilm formation. A drug (M64) targeting MvfR interfered with biofilm formation of P. *aeugronoisa* and increased the antibiofilm activity of other antibiotics when used in conjunction with M64 [101].

The secondary metabolites that are discussed above likely represent just the tip of the iceberg and additional, novel secondary metabolites isolated from *Burkholderia* spp. await characterization. Inactivation of the global regulators ScmR and MftR is associated with remarkable changes in secondary metabolite production and a corresponding induction of biosynthetic gene clusters. Based on these observations, we predict that identifying global regulators in other bacterial species and understanding their regulatory mechanisms through a combination of genome-wide transcriptomics, metabolomics, and ChIP-Seq may enhance our chances of discovering potentially bioactive compounds as well as novel drug targets for pathogenic strains.

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antibiotics

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Article

The Bactericidal Activity of Protein Extracts from *Loranthus europaeus* Berries: A Natural Resource of Bioactive Compounds

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Abstract: *Loranthus europaeus* is a well-known and important medicinal plant, with a long history of traditional medicine use. Several studies showed that it contains many bioactive compounds with a wide range of pharmacological effects. In light of these past researches, *L. europaeus* were chosen to consider its potential antimicrobial action. To this aim, different protocols were performed to selectively extract protein compounds, from *L. europaeus* yellow fruits, and evaluate the antimicrobial activity against four phytopathogenic fungi (*Aspergillus niger*, *Alternaria* spp., *Penicillium* spp., *Botritis cinereus*) and a number of foodborne bacterial pathogens (*Listeria monocytogenes*, *Staphylococcus aureus* strains, *Salmonella* Typhimurium and *Escherichia coli*) by using serial dilutions and colony formation assays. Results evidenced no antifungal activity but a notable bactericidal efficiency of a crude protein extract against two foodborne pathogens, with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values between 0.2 and 0.5 mg/mL, being *S. aureus* strains the most susceptible bacteria. Moreover, a strong bactericidal activity against *S. aureus* M7 was observed by two partially purified protein fractions of about 600 and 60 kDa molecular mass in native conditions. Therefore, these plant protein extracts could be used as natural alternative preventives to control food poisoning diseases and preserve foodstuff avoiding health hazards of chemically antimicrobial applications.

Keywords: *Loranthus europaeus*; Protein extract; Antibacterial agents; Natural compounds; *Staphilococcus aureus*

1. Introduction

In chapter XVI, 95 of the *Naturalis Historiae*, Pliny the Elder described the harvesting of the mistletoe growing on the oaks by Gaul druids, a cult related to the medicinal and magic properties attributed to mistletoes [1]. Indeed, these plants were highly reputed as a remedy for many ills, and specifically effective *contra venena* [2], but the traditions about magic and curative properties of mistletoes are not confined to Central-Northern Europe. The anthropologist James G. Frazer in his land-marking "The Golden Bush" [3] presented a detailed survey of the symbolic role of mistletoes in

the ancient Italic cult of Diana Nemorensis, and in the 1900s a growing evidence has been presented on their occurrence as sacred plants in folklore and mythology of several Indo-European cultures [4].

Mistletoe is a generic term encompassing all the obligate hemiparasitic species of Angiosperms, presently placed in five phylogenetically unrelated different families, within the order Santalales. Loranthaceae and Viscaceae are the families that include most of mistletoes worldwide diffused [5]. Archaeological findings support the use of *Viscum album* in the religious cults of druids in England, between the first and second century AD [6], but *V. album* is not the only mistletoe diffused in Europe. Indeed, magic and curative properties were attributed also to other mistletoe species *Loranthus europaeus*, the "true" golden bush, prevalently diffused in the Mediterranean territories of the Continent [7]. According to Liu et al. [8], the Loranthaceae originated in Australasia during Late Cretaceous, when these two Continents were connected or contiguous, and also the genus *Loranthus* has an Australasian origin, but dispersed also to North Asia and Europe. In the course of the last Century, the majority of *Loranthus* species has been transferred to different genera [9], and presently the genus is represented by about ten species [10]. European yellow mistletoe, *L. europaeus* Jacq., is the only species of the genus that migrated westward to Europe, and is presently diffused in Central Asia, Anatolia, South Russia, and South-Western Europe [11]. *L. europaeus* (European yellow mistletoe) is a small shrub with brown bark and deciduous leaves during the winter, with yellowish-green flowers. The fruit is a spheroidal golden-yellow berry, with a sticky liquid inside. As the other hemiparasitic Santalales, *L. europaeus* actively photosynthesizes, but gains from the host plants water, inorganic nutrients and also organic compounds, such as amino acids and sugars [12]. *L. europaeus* can establish a relation with different *Quercus* species, with *Q. pubescens* being by far the first choice host plant, although this mistletoe can also attack other trees such as chestnut [13]. *L. europaeus* is presently used to treat many numerous ailments in the folk medicine of different Asiatic and European Countries [14,15]. In several regions of Central Italy, the whole plant macerated in wine or grappa was used to cure atherosclerosis and hypertension [16], whereas in Calabria region (South Italy) leaves were also topically applied to cure wounds [17]. The therapeutic effects of *L. europaeus* have been attributed to the presence of a wide array of active substances: the mixture of flavonoids isolated from the plants has shown marked antioxidant properties [18,19], and a stimulatory effect on lymphocyte proliferation has been attributed to flavonoids and terpenoides isolated from the leaves [20]. Recently, an anti-leishmaniasis effect has been attributed to the presence a high concentrations of quercetin in the whole plant extract [14]. The antimicrobial and cytotoxic activities attributed to *L. europaeus* could be also due to the presence of the so-called defense peptides, more recently also known as plant defensins, largely occurring in different genera of parasitic members of Santalaceae and Loranthaceae [21]. Plant defensins have shown a specific activity not only against pathogenic fungi, but also to yeast models [22], and for this reason are considered a possible source of therapeutic compounds also against human fungal infections [23].

The insights gained from this work demonstrated, for the first time, the antimicrobial activity of a crude protein extract from the European medicinal plant *L. europaeus* against the Gram-positive bacteria *Staphylococcus aureus* and *Listeria monocytogenes*. The bactericidal effect of two partially purified protein compounds isolated from the yellow berries was also examined against the methicillin-resistant *S. aureus* M7, whose growth was completely inhibited already at a concentration of 0.01 mg·mL^{-1}. It should finally be noted that this study started thanks to the correlation of the anthropological and biogeographic data made by the coauthor of the manuscript Angelo Genovese.

2. Results

2.1. Samples Collection

In Italy, *Loranthus europaeus* is prevalently diffused in oak forests of Apennines, extending from Central to South Italy. For this study, the forest of Carpanzano (Calabria), located at an altitude of 610 m, was selected. Carpanzano is a continental territory of Calabria, far from the sea, with cold

winters and higher precipitations during spring and fall. Visible tufts of *L. europaeus* were scattered on numerous *Q. pubescens* trees and samples were collected during winter, when mistletoe twigs are leafless, and fruits acquire a bright yellow color (Figure 1).

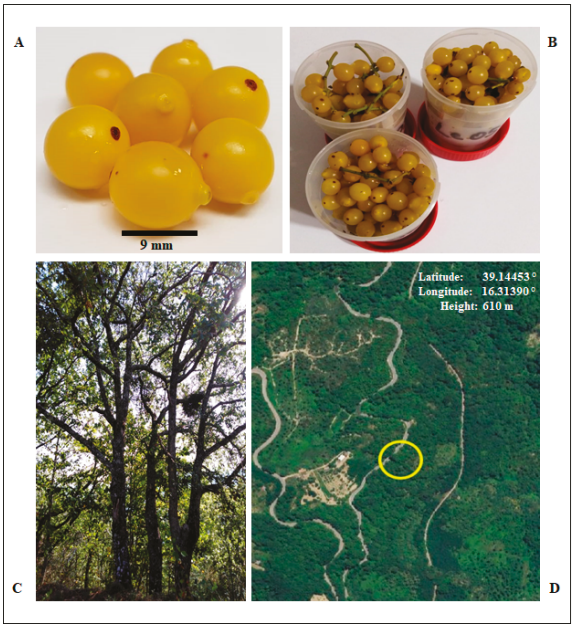


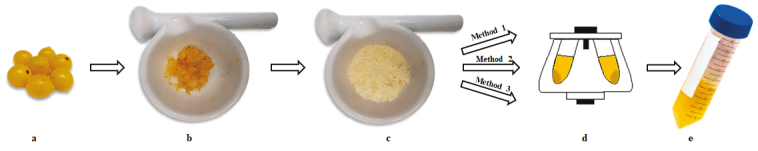
Figure 1. (**A**,**B**) Ripen berries of *L. europaeus*; (**C**) oak tree hosting *L. europaeus* twigs; (**D**) the sampling site (Carpanzano forest, Calabria, Italy).

2.2. *Preparation of Protein Extracts from* Loranthus europaeus *Berries and Plant Extracts Yield*

With the aim to recover and isolate new plant protein agents with antimicrobial activity, that can possibly be used as natural preservatives in the food and pharmaceutical industries, an efficient protein extraction method from *Loranthus europaeus* berries was developed. Indeed, the extraction efficiency is strongly affected by several factors such as the starting plant material, the buffer composition and the method used as well as the presence of interfering substances [24]. It is worth noting that at present there are relatively few reports on the extraction protocols of antibacterial proteins from plant berries respect to those on the wide variety of smaller molecules, obtained usually through ethanol or methanol extraction [25].

In this study, three of simply, fast and common extraction protocols used for proteins were carried out for berries with some modifications [26–29], taking into account both the pH and the presence of strong anionic detergents such as SDS (Figure 2A). As far as the protein recovery is concerned, the quantitative comparison among the different extracts showed that the highest yield was obtained with method 3, followed by method 2 and method 1 that gave the lowest protein yield (Figure 2B). However, it is worth noting that the protein quantitation assay on extracts from protocol 3 was affected by the presence of SDS-containing buffer that persisted even after extensive dialysis of the sample, thus interfering with the protein yield results [30].

A



B

Method	Extraction buffer	Protein yield (mg/g berry)
1	**Extract 1:** Tris-HCl 100 mM, pH 8.0; DTT 1 mM; PMSF 1 mM	1.46 ± 0.10
2	**Extract 2:** Sodium Acetate 100 mM, pH 5.0; DTT 1 mM; PMSF 1 mM	3.26 ± 0.15
3	**Extract 3:** Tris-HCl 500 mM, pH 8.0; DTT 10 mM ; EDTA 10 mM ; SDS 5%	3.89 ± 0.17

Figure 2. (**A**) Representative scheme of the different steps applied for the preparation of crude protein extracts from the yellows berries of *L. europaeus*. **a**: yellow berries; **b**: pitted berries; **c**: powdered berries in liquid nitrogen using a mortar and pestle; **d**: centrifugation of the mixture; e: crude protein extract. Finely ground powder of plant fruit was used as starting material in all three protocols. (**B**) Table reporting protein yields from berries of *L. europaeus* using three extraction protocols. Data are presented as means ± standard deviation (s.d.) of three different samples analyzed in triplicate.

The protein pattern of the three extracts was assessed by SDS-PAGE analysis and a representative Coomassie-stained gel is reported in Figure 3. The protein extracts 1 and 2 showed a similar electrophoretic profile in contrast to that associated with the extract 3, possibly resulting from the use of SDS in the extraction buffer, which is known to be extremely effective in the solubilization of membrane proteins [31].

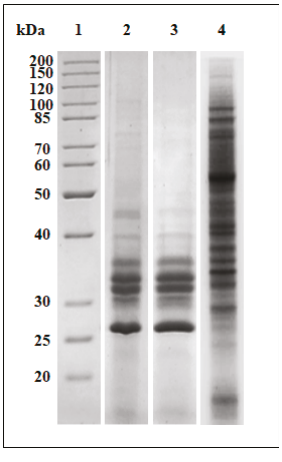


Figure 3. SDS-PAGE (10%) analysis of the total protein extracts from *L. europaeus* berries using the three methods. Lane 1: molecular weight markers (Thermo Scientific); crude protein extracts obtained by: method 1 (Lane 2); method 2 (Lane 3); and method 3 (Lane 4). Protein bands were detected by Coomassie blue staining. Equal amounts of proteins were loaded for each Lane. The gel is representative of three independent experiments on three different protein preparations.

2.3. Antifungal Activity

An initial in vitro screening was done to evaluate the antifungal activity of all the plant extracts against four of the most common phytopathogenic fungi. As depicted in Figure 4, none of the

two extracts at pH 8.0 and 5.0 (Method 1 and 2) showed antifungal activity against any of the test microorganisms, even at the highest amount investigated. In addition, the two protein samples seemed to promote the sporulation of *Aspergillus niger* (colony diameter of 3.5 ± 0.3 cm and 2.0 ± 0.8 cm for extract 2 and extract 1, respectively after 48 h of incubation), possibly due to the presence in the plant extracts of some additional nutrients which could further stimulate the fungal growth (Figure 4C). As far as the SDS-extract (Method 3) is concerned, it is worth noting that even after extensive dialysis of the samples, a residual amount of detergent persisted in the protein mixtures, resulting in a strong interference with the antifungal and antibacterial activity assays, which require a complete removal of this detergent. For these reasons, the SDS-extracts were not further considered for our investigations.

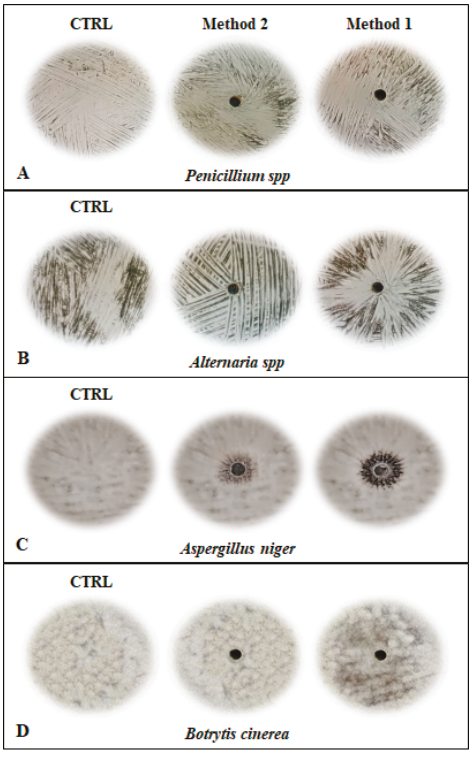


Figure 4. Antifungal activity assay of plant extract 1 (Method 1) and plant extract 2 (Method 2) against different phytopathogenic fungi: (**A**) *Penicillium* spp; (**B**) *Alternaria* spp; (**C**) *Aspergillus niger*; (**D**) *Botrytis cinerea*. CTRL: each tested fungus without treatment. The plates were incubated at 28 °C for 48 h. The pictures are representative of three independent experiments on three different protein preparations.

2.4. Antibacterial Activity

In order to explore the potential use of the protein samples as antimicrobial agents, the antibacterial activity of the extracts 1 and 2 was evaluated against a panel of bacteria, including 3 strains of Gram-positive (*L. monocytogenes*, *S. aureus* MSSA, and MRSA) and 2 strains of Gram-negative bacteria (*Salmonella* and *E. coli*), among those commonly associated with infectious diseases. Specifically, to compare the effect of the two extracts on the growth of the microorganisms under investigation, the MIC and MBC values were determined by using the serial dilution assay. It is known that sodium acetate can affect the bacterial growth [32,33], therefore preliminary experiments were performed in order to assess the effects of different concentrations of acetate on the growth of the foodborne

pathogens, considering that the extract 2 was obtained using acetate as extractant. The obtained results evidenced a linear decrease of bacterial growth rate with the increase in acetate concentration starting from 60 mM (data not shown). For this reason, all the subsequent experiments with extract 2 were performed only after dialysis of the sample in order to have a final concentration of 50 mM acetate that did not interfere with the antimicrobial assays. Interestingly, the tested microorganisms revealed a different sensitivity to the two types of extracts. Overall, the results demonstrated that the extract 1 was less effective in suppressing the microbial growth of all pathogens tested, exhibiting MIC values 2-fold higher than those observed for the acetate-extract. It can be hypothesized that the variation in MIC values between the two plant-fruit samples arose from a diverse nature of the proteins extracted by using the acetate respect to the Tris buffer. Hence, the extract 1 was not considered for any further study based on its weak antibacterial activity. As far as plant fruit extract 2 is concerned (Figure 5A), it exhibited an efficient and significant antimicrobial activity against *L. monocytogenes*, *S. aureus* MRSA, and *S.* Typhimurium, with MIC values ranging from 0.16 to 0.50 mg·mL^{-1} , being *S. aureus* MRSA the most sensitive bacterial species. Indeed, the protein sample was found to be ineffective against *E. coli* and *S. aureus* MSSA SA4 even at the highest amount (0.50 mg·mL^{-1}) assayed. To investigate further the antimicrobial effects of the extract 2, the MBC was evaluated revealing that it displayed a strong bactericidal activity against *L. monocytogenes* and S. aureus MRSA, with MBC values of 0.38 and 0.20 mg·mL^{-1}, respectively. These results clearly indicated that this protein extract was bacteriostatic at concentrations lower than those required to explain bactericidal activity against *L. monocytogenes*, being MBC value higher than the corresponding MICs. Instead, the MBC determined against *S. aureus* MRSA was on a par with the corresponding MIC (both at about 0.2 mg·mL^{-1}), thus demonstrating that the tested sample should be considered to have a strong bactericidal mode of action. On the other hand, *S.* Typhimurium needed protein concentrations higher than 1 mg mL^{-1} to be killed, indicating that the active substances were only bacteriostatic towards this strain. Therefore, according to the results obtained, the Gram-positive bacteria were more sensitive to the plant extract 2 than the Gram-negative microorganisms, presumably as consequence of the different bacterial membrane structures. Specifically, lipopolysaccharides layer and periplasmic space of Gram-negative bacteria could be the reasons of the relative resistance of this class of bacteria to the plant extract 2 treatment. However, this explanation represents a simplification as other mechanisms could play a role in this process. Interestingly, in relation to the antibacterial spectrum of the crude extract (Figure 5A), it is important to emphasize the strong growth inhibition of methicillin-resistance *S. aureus* M7 strain (Figure 5B,C), which is one of the most pathogenic bacterium resistant to multiple drugs, having acquired resistance to a variety of them.

Antibacterial studies were also performed against a no foodborne Gram-negative pathogen *Pseudomonas protegens* N, a widespread plant-protecting bacterium isolated from water samples of an irrigation well located in the region of Djebira in Bejaia, northern Algeria [34,35]. The obtained results clearly demonstrated that all the plant extracts under investigation were ineffective to inhibit the growth of the soil microorganism, confirming that the *L. europeaus*-antibacterial proteins appeared to be less potent both versus pathogenic and not pathogenic Gram-negative bacteria. In accordance with the reported findings concerning the screening of antimicrobial potentiality and taking into account the sensitivity of the tested bacteria, extract 2 and *S. aureus* MRSA M7 were chosen to perform the further analyses.

A

Bacteria	Plant fruit extract 2	
	MIC (mg mL^{-1})	MBC (mg mL^{-1})
L. monocytogenes 92	0.28±0.05*	0.38±0.02*
S. aureus MRSA M7	0.16±0.04*	0.20±0.01*
S. Typhimurium	0.50±0.03*	ND
S. aureus MSSA SA4	NI	NI
E. coli O157	NI	NI

ND = not defined.
NI = not inhibited

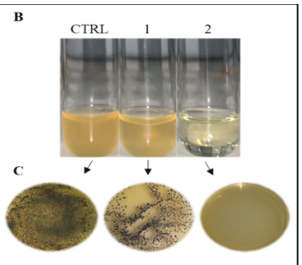


Figure 5. (**A**) Table of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of plant fruit extract 2 against different foodborne pathogens. (**B**) Antimicrobial test in vitro of plant fruit extract 2 against *S. aureus* MRSA M7. **CTRL**: *S. aureus* MRSA M7 control; (**1**) protein extract 2 at 0.08 mg·mL^{-1} concentration; (**2**) protein extract 2 at 0.16 mg·mL^{-1}concentration (MIC value). (**C**) MBC value (0.2 mg·mL^{-1}) determined by the standard plate count. Data are presented as means ± standard deviation (s.d.) of three different samples analyzed in triplicate. * Significant difference ($p < 0.05$) between the treated and the control samples.

2.5. Spectroscopic Analysis

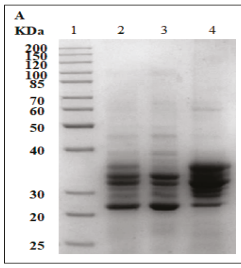
Many of the colors associated with higher plants are due to the presence of pigment molecules, such as chlorophylls and the carotenoids, which confer them a natural fluorescence. Therefore, the intense color of these pigments makes them ideal candidates for absorption spectroscopy studies, having a unique visible spectrum, which can provide a positive identification [36]. In this context, the pigment content in terms of chlorophyll a, chlorophyll b and carotenoids present in the plant extract 2 was determined by spectrofluorometric analysis, performing their extraction using ethyl acetate as solvent that is considered the best extractant for this class of molecules [37]. As shown in Figure S1, the photosynthetic fluorescence emission spectra obtained from the organic extracts evidenced the presence of three main bands: one of chlorophyll a at 650–684 nm, the second at 642–670 nm, characteristic to chlorophyll b, and the last one at 500–600 nm probably due to carotenoids. The same experiment was performed on the plant extract 2 after dialysis in bags with 10 kDa MWCO (Molecular weight cut-off), revealing that it was completely abolished the fluorescence emission peaks corresponding to the three pigment molecules, which were lost during dialysis (Figure S1). Therefore, it is reasonable to assess that the strong antibacterial activity measured in the extract 2, whose preparation includes dialysis, can be attributed to compounds with a molecular mass higher than 10 kDa.

2.6. Partial Purification of the Active Compounds

With the aim to gain insight into the protein component/s responsible for the antibacterial activity of the extract 2, a partially purification procedure was performed by a combination of ammonium sulphate fractionation and gel filtration chromatography. In the first step, precipitation experiments were conducted subjecting the extract 2 to precipitation using two sequential salt saturation levels (50% and 90%). The pellets resulting from the two precipitation steps were dissolved in 50 mM sodium acetate buffer pH 5.0, extensively dialyzed to remove the ammonium sulphate, tested for antibacterial activity and analyzed by SDS-PAGE (Figure 6A).

In vitro antibacterial assessment of the two precipitates (named pellet 50% and pellet 90%) was carried out at the MIC value (0.15 mg·mL^{-1}) determined with the total extract 2 against *S. aureus* MRSA (Figure 5A) and the results were reported in terms of the change in the Log CFU·mL^{-1} of viable colonies. The bactericidal activity was defined as being equal to 3 Log CFU·mL^{-1} or greater reduction in the viable colony count relative to the initial inoculum [38]. As shown in Figure 6B, a rapid reduction in the log of the viable cells counted (−4 Log CFU·mL^{-1}), was detected with both

samples. This acknowledged the fact that the bactericidal activity measured for the extract 2 resulted from the contribution of different protein components. However, given that the total protein yield in the 90% pellet was 5-fold lower than that obtained in 50% sample and taking into account the large amount of the starting material required to allow more detailed investigations, we firstly decided to proceed to the purification of 50% pellet. An important aspect to underline is the complete recovery of the proteins responsible of the antibacterial activity in the plant crude extract after precipitation by ammonium sulphate.



B

	S. aureus MRSA M7		
	CTRL Log_{10} CFU mL^{-1} at time 24 h	**Treated sample Log_{10} CFU mL^{-1} at time 24 h**	**Change in Log_{10} CFU mL^{-1} between CTRL and treated sample**
Pellet 50% (0.15 mg mL^{-1})	9	5	-4
Pellet 90% (0.15 mg mL^{-1})	9	5	-4
Plant extract 2 (0.15 mg mL^{-1})	9	0	-9

negative sign = extent of bacterial reductions as indicated by the Log_{10} (CFU mL^{-1}).

Figure 6. (**A**) SDS-PAGE analysis of protein fractions. Lane 1: molecular weight markers; Lane 2: plant extract 2; Lane 3: protein sample obtained by 50% ammonium sulphate precipitation; Lane 4: protein sample obtained by 90% ammonium sulphate precipitation. Equal amounts of total proteins were loaded for each lane. The gel is representative of three independent experiments on three different protein preparations. (**B**) antibacterial effect of pellet 50%, pellet 90% and plant extract 2 samples against *S. aureus* MRSA M7 reported in terms of change in the Log CFU·mL^{-1} of viable colonies observed between control and treated bacteria at 24 h. Data are representative of three independent experiments on three different protein preparations.

Additional purification step was conducted through the gel filtration chromatography on an SEC-4000 column. The elution profile (Figure 7) obtained from 50% pellet, showed five main protein fractions, which were assayed for the antibacterial activity against the *S aureus* MRSA (Figure 8A). A strong killing activity was exhibited by both protein fractions Fr 1 and Fr 2, with MIC values of 0.01 mg·mL^{-1} and 0.04 mg·mL^{-1}, respectively, which coincided with the MBCs. In contrast, no activity was observed with the remaining protein fractions Fr 3, Fr 4, and Fr 5. Based on the calibration curve of the gel filtration column, Fr 1 and Fr 2 displayed a molecular mass of approximately 600 kDa and 60 kDa, respectively.

On the other hand, the SDS-PAGE analysis of all the gel filtration fractions revealed not only an enrichment of the active compounds (Fr 1 and Fr 2) (Figure 8B) but also a possible oligomeric nature of the antibacterial proteins considering the molecular mass determined under native conditions (Figure 7).

However, it cannot be excluded that more than one active protein compound could cooperate and contribute to the intrinsic antibacterial activity of the *L. europaeus* plant fruits.

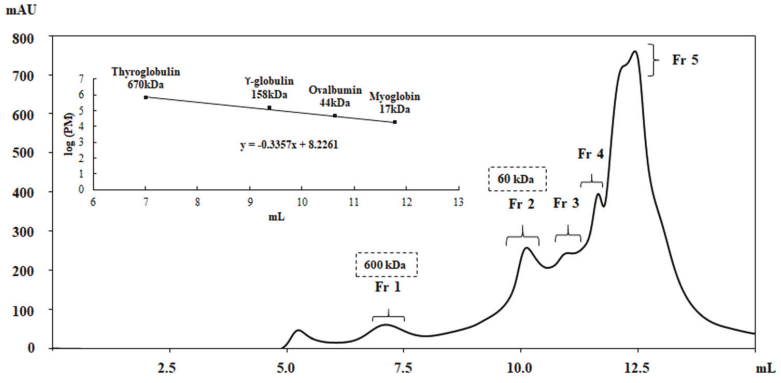


Figure 7. Elution profile of pellet 50% sample obtained by gel filtration chromatography performed on YARRA™ SEC-4000 column in 50 mM sodium acetate buffer pH 5.0 containing 50 mM NaCl. Insert: Calibration curve of the gel filtration YARRA™ SEC-4000 column using protein standards of known molecular masses. The collected fractions are indicated.

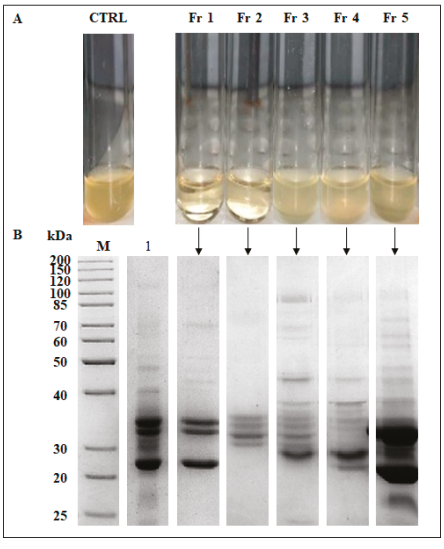


Figure 8. (**A**) Antimicrobial screening assay of gel filtration fractions against *S. aureus* MRSA M7. **CTRL**: *S. aureus* MRSA M7 control; **Fr 1, Fr 2, Fr 3, Fr 4 and Fr 5**: fractions obtained after gel filtration chromatography of the pellet 50% sample. (**B**) SDS-PAGE analysis of the protein fractions. M: molecular weight markers; Lane 1: pellet 50% sample. Equal amounts of total proteins were loaded for each Lane.

3. Materials and Methods

3.1. Collection of Plant Material

Shoots of berries of European mistletoe parasitic plant *Loranthus europaeus* were collected between December 2017 and March 2018 from infected oaks (*Quercus pubescens*) in the forest of Valle del Torrente, Savucchia, Carpanzano (Cs), Calabria, South Italy, Italy (39.14453° N–16.31390° E) at about 3–4 m from the ground level. All the picked samples were yellow-berried leafless aerial shoots. The berries were

rounded with a diameter of 0.5–1 cm and were stored at −80 °C until protein extraction. The yellow berries were gently collected by the Prof. Angelo Genovese of University of Naples "Federico II".

3.2. Preparation of Crude Extracts

Total protein extraction from yellow berries was carried out using three protocols. *L. europaeus* frozen berries were pitted and finely powdered in liquid nitrogen using a mortar and pestle and the pulverized mixtures were used for all the extraction protocols. Specifically, protein extracts were obtained by adding to the powdered materials a defined volume of each extraction buffer: 100 mM Tris-HCl pH 8.0, 1 mM DTT, 1 mM PMSF (extract 1); 100 mM Sodium Acetate pH 5.0, 1 mM DTT, 1 mM PMSF (extract 2); 100 mM Tris-HCl pH 8.0, 10 mM EDTA, 10 mM DTT, 5% SDS (extract 3). The extracts 1 and 2 were shaken on a rotatory shaker for 16 h at 4 °C and then centrifuged at 16,000× *g* for 40 min at 4 °C. The extract 3 was shaken on a rotatory shaker for 3 h at 4 °C and then centrifuged at the same conditions described above. All the resulting supernatants were collected and extensively dialyzed in bags with 10 kDa MWCO (Molecular weight cut-off) at 4 °C against 50 mM sodium acetate pH 5.0 for extract 2 and 50 mM Tris-HCl pH 8.0 for extract 1 and 3. The extracted proteins were stored at 4 °C in 5% glycerol until use. The protein concentration was determined according to Bradford's [39] method using bovine serum albumin as standard.

3.3. Antifungal Activity Assays

The antifungal activity of the three extracts was evaluated against four phytophatogenic fungi (*Aspergillus niger*, *Botrytis cinerea*, *Penicillium spp.*, and *Alternaria spp.*) as described in Agrillo et al. [34]. Before the antifungal testing, the protein extracts 1, 2 and 3 were sterilized by filtration through 0.22 μm sterile filters (Millex GV). Tests were performed pouring the extracts (300 μL) in wells (0.5 cm in diameter) aseptically punched on the PCA plates, previously scraped with fungi spores (2×10^4 conidia/mL) and by incubating the plates for 48 h at 28 °C. The antifungal activity was evaluated measuring the diameter of the inhibition zone on PCA plates.

3.4. Bacterial Culture and Inoculum Preparation

Methicillin-resistant *Staphylococcus aureus* (MRSA, M7), *Staphylococcus aureus* (MSSA, SA4), *Listeria monocytogenes* (92), no pathogenic *E. coli* (O157) and *Salmonella* Typhimurium isolated from different foods, were used in the microbiological assays. Bacterial cultures were stored at −80 °C. Before the experiments, the frozen stocks of each strain were plated on selective agar and incubated at 37 °C for 16 h to obtain single colonies. Working cultures were produced daily by transferring a loopful of culture to Tryptic Soy Broth (TSB, Biotec, Grosseto, Italy) and incubating for 16 h at 37 °C. To obtain the bacterial suspension, the density of the cell was assessed spectrophotometrically (OD_{600}) and the solution was adjusted to 0.1. Enumeration of the inoculum was completed by diluting to approximately 3.0 Log CFU·mL^{-1} and spread-plating 100 μL on selective plate agar. Plates were aerobically incubated at 37 °C for 48 h.

3.5. Antibacterial Activity Assay of Plant Fruit Extracts

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were carried out on berry extract 1 and 2 (after dialysis against sodium acetate at 50 mM) according to Clinical and Laboratory Standards Institute (CLSI, 2015), with some modifications [40]. The stock solution (0.50 mg·mL^{-1}) of the two berry extracts was diluted at different concentrations (from 0.41 to 0.01 mg·mL^{-1}.) in Tryptic Soy Broth (TSB; Biotec, Grosseto, Italy) to a total volume of 1 mL for each tube. 10 μL of each strain, 1.0×10^4 CFU·mL^{-1}, was inoculated. At the same time, equal volumes of sterile Tryptic Soy broth were inoculated as a control. The tubes were incubated for 20 ± 2 h at 37 °C and thereafter observed for turbidity. MIC is defined as the lowest concentration of the extract at which no bacterial growth was detected. MBC is defined as the lowest concentration of peptide at which more than 99.9% of the bacterial cells are killed. To determine the MBC, 100 μL of the bacterial

cell suspension was taken based on the MICs, cultivated on agar plate and then incubated for 24–48 h at 37 °C. At least six technical replicates were included for each group, and all experiments were performed in triplicate.

3.6. Antibacterial Activity Assay of Partially Purified Samples

The antimicrobial efficacy of the partially purified samples (pellets 50% and 90% and gel filtration fractions) was determined according to Palmieri et al. [41]. The pellets were tested at concentration of 0.15 mg·mL^{-1} *versus Staphylococcus aureus* (MRSA, M7). Gel filtration fractions were assayed at concentrations ranging from 0.01 mg·mL^{-1}to 0.04 mg·mL^{-1}. Under all the experimental conditions explored, the plate counting method was used to estimate the activities.

3.7. Partial Purification of the Active Components

Precipitation by ammonium sulphate, a method of protein purification, was performed on total protein extracts 2 followed by gel filtration chromatography to isolate the antibacterial compounds. Powdered ammonium sulphate was added in small portions under constant stirring at 4 °C to 50% and 90% saturation levels. Specifically, the precipitate by ammonium sulphate at 50% saturation was collected by centrifugation at 15,000× *g* for 30 at 4 °C, dissolved in 50 mM sodium acetate pH 5.0 and extensively dialyzed in bags with 10 kDa MWCO (Molecular weight cut-off) against the same buffer to completely remove the salt. The supernatant resulting from the precipitation by 50% was precipitated at 90% $(NH4)_2SO_4$ saturation at 4 °C. After centrifugation at 15,000× *g* for 30 at 4 °C, the resulting pellet was re-suspended in 50 mM sodium acetate pH 5.0 and extensively dialyzed against the same buffer in bags with 10 kDa MWCO (Molecular weight cut-off). All fractions obtained following ammonium sulphate precipitations were tested for antibacterial activity as described above. The sample obtained from salt precipitation which resulted active in the antibacterial tests were loaded on a gel filtration column Yarra 3µm Sec-4000 column (Pharmacia Biotech, Milan, Italy) connected to an HPLC system (Shimadzu, Milan, Italy) and pre-equilibrated in 50 mM sodium acetate containing 50 mM NaCl, pH 5.0. The samples were eluted at a flow rate of 0.5 mL·min^{-1}. Fractions were pooled, concentrated and tested for the antibacterial activity as previously described.

3.8. SDS-PAGE Analysis

In order to monitor purity, electrophoretic analyses were performed on 10% polyacrylamide gel under denaturing conditions (SDS-PAGE) according to the procedure described by Laemmli [42]. Standard proteins (Page rule Unstained ladder) were purchased from Thermo Scientific (Massachusetts, USA).

3.9. Spectroscopic Analyses

The fluorescence of plant fruit pigments was determined using a Jasco FP-8200 spectrofluorometer. The extraction of the main pigments were performed on the plant extract 2 before and after the dialysis using ethyl acetate as solvent. The extract obtained was centrifuged at 16,000× *g* for about 10 minutes. The supernatant was collected and the fluorescence emission spectra were collected at 25 °C in a 1 cm path length quartz cuvette using excitation and emission slit widths of 2.5 nm. The samples were excited at different λexc and the emission ranges used were: 500–800 nm (480-nm λexc), 600–800 nm (425-nm λexc), and 600–800 nm (470-nm λexc).

3.10. Statistical Analysis

All experiments were performed at least five times. Statistical analysis was carried out using the software GraphPad Prism®, version 6 (GraphPad, San Diego, California, USA). Statistical analysis of microbiological data was performed by using Student's t-test ($p < 0.05$) and the results were presented as mean ±standard deviation (s.d.).

4. Conclusions

Food spoilage is often caused by the growth of many pathogenic bacterial strains. As a rule, prevention of foodstuff is mainly based on the application of chemical preservatives, whose adverse effects on human health have increased the demand for finding effective, healthy safer and natural compounds [43]. In this context, the plants and their products are gaining a wide interest in the food industry for their potential as decontaminating agents, as they are Generally Recognized as Safe (GRAS) [44].

In the current in vitro study, antimicrobial activity of different protein extracts from *L. europeaus* yellow berries was examined against fungal phytopathogens, Gram-positive and Gram-negative bacteria. Among the investigated protein extracts, the findings clearly revealed that a protein sample containing bioactive constituents, exhibited a remarkable inhibitory activity against two Gram-positive bacteria, *L. monocytogenes* and the methicillin-resistant *S. aureus* M7 strain, being the latter the most susceptible. In addition, a partially purification of this plant fruit extract allowed to identify at least two protein compounds responsible for the efficient bactericidal activity against *S. aureus* M7. Moreover, to the best of our knowledge, this is the first study aimed at the identification of proteins present in the fruits of *L. europeaus* showing bactericidal activity. This work represents a pilot study and confirms that the traditional medicinal plants can be considered an important and rich source of naturally occurring products against common pathogenic microorganisms, thus representing an economic and safe alternative to treat human diseases.

Supplementary Materials: The following is available online at http://www.mdpi.com/2079-6382/9/2/47/s1, Figure S1: Fluorescence emission spectra of pigments extract from plant extract 2.

Author Contributions: conceptualization, G.P. and A.G.; methodology, L.G., R.L.A. and S.M.; investigation, L.G., R.L.A. and S.M; resources, A.G.; data curation, M.G., M.B. and G.P.; writing—original draft preparation, E.C., M.G. and G.P.; writing—review and editing, A.P., M.G. and M.B.; supervision, A.A.: and G.P.; funding acquisition, G.P. and E.C. All authors have read and agreed to the published version of the manuscript.

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Antibacterial Activities, Phytochemical Screening and Metal Analysis of Medicinal Plants: Traditional Recipes Used against Diarrhea

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Abstract: The aim of this study was to explore the phytochemical composition, heavy metals analysis and the antibacterial activity of six medicinal plants i.e., *Terminalia chebula* Retz (fruits), *Aegle marmelos* L., (fruits), *Curcuma longa* L., (rhizomes), *Syzygium aromaticum* L., (flower buds), *Piper nigrum* L., (seeds), *Cinnamomum cassia* L., (barks) and its two remedial recipes (recipe 1 and 2) used against diarrhea obtained from the local herbal practitioners (Hakeems). A preliminary phytochemical screening of the above-mentioned plants extract in methanol, chloroform, n-hexane and distilled water revealed the presence of various constituents such as alkaloids, flavonoids, tannins and saponins by using standard procedures. The quantitative phytochemical studies shows that alkaloids, flavonoid and saponins were in maximum amount in *Terminalia chebula*. The concentration of Cd, Ni, Pb, Fe, Cr, Cu and Zn were investigated by using an atomic absorption spectrometer. The obtained analysis shows that Cr, Fe and Pb were present in the highest concentration in medicinal plants and their recipes. The antibacterial activities of the crude extract found in the recipes of methanol, chloroform, n-hexane and distilled water were analyzed by using agar well disc diffusion assay and minimum inhibitory concentration (MIC) by broth dilution method against four bacterial strains, namely, *E. coli, Salmonella, Shigella* and *Methicillin-resistant Staphylococcus aureus (MRSA)*, respectively. The maximum zones of inhibition in methanol, water, chloroform and n-hexane extracts were seen in recipe 2 against *Shigella* (22.16 ± 0.47 mm), recipe 2 against *Shigella* (20.33 ± 0.24 mm), recipe 1 against *Shigella* (20.30 ± 0.29 mm) and recipe 2 against *E. coli* (30.23 ± 0.12 mm), respectively. Furthermore, the recipe extracts are more active against the tested bacterial strains than the extracts from individual plants. Therefore, it is concluded that the use of herbal plants and their recipes are the major source of drugs in a traditional medicinal system to cure different diseases.

Keywords: phytochemicals; heavy metals; recipes; MIC; *E-coli*; *Salmonella*; *Shigella and MRSA*

1. Introduction

The present study was conducted in district Karak, in the southern region of Khyber Pakhtunkhwa, Pakistan. The total population of district Karak is 706,299, with an area of about 3372 square Kms. Karak is situated between 31°15′ and 36°55′ latitude and between 70°05′ and 74°05′ longitude. Most of the people live in rural areas and depend on medicinal plants for the curing of different diseases. Medicinal plants can act as an indigenous source of new compounds possessing therapeutic value

and can also be used in drugs development. According to the World Health Organization (WHO), plants can provide different varieties of drugs for low-income nations to cope with their primary healthcare needs [1]. The plants are medicinally important due to the presence of biologically active secondary metabolites such as alkaloids, flavonoids, steroids, saponins and terpenoids, which exert their effects by interacting with human physiology. The antimicrobial activities of these phytochemicals are due to their chemical nature [2,3] and are a potential source of diarrheal disease [4]. For this reason, the WHO has encouraged the studies for the treatment and prevention of diarrheal diseases using traditional medicinal practices [5]. Presently, a large number of medicinal plants are being used in many countries of the world, including Pakistan, due to their anti-diarrheal properties [6]. In Bangladesh, over 250 floral species are used by the folk medicinal and tribal healers for the treatment of diarrhea [7]. The Indian Himalayan region also support approximately 1700 plant species of known medicinal value [8]. However, there are only few studies on the utility of medicinal plants in the treatment of specific diseases [9]. Various medications are used for diarrheal diseases which possess different adverse effects like nausea, headache, dry mouth and constipation. However, there are many medicinal plants that have anti-diarrheal activities with less, or even no, side-effects than the allopathic drugs. These plants show anti-diarrheal activity by reducing secretions and the gastrointestinal motility [10].

The medicinal plants are destroyed and contaminated by various factors, such as environmental pollution, soil harvesting, microbial growth and introduction of toxic metals. The ingredients of plants include metal ions which are responsible for nutritional as well as medicinal usage [11]. Heavy metals like zinc, manganese, cobalt, iron, copper, chromium and nickel are essential for proper body function and become toxic when they exceed the recommended level and cause various chronic and acute effects in the living organisms [12]; whereas, the metals like lead, mercury and cadmium are non-essential and are toxic in nature even in the trace amount [13].

Thus, due to the hazardous affects as well as antibiotic resistance to the synthetic drugs, researchers are trying to obtain the antimicrobial drugs from medicinal plants due to their non-toxic nature and less side effects [14,15]. In spite of all the progress in the field of allopathic drugs, the traditional medicines, particularly plant-based medicines, also have a key role. Many studies have shown that crude extracts of medicinal plants as well as the pure bioactive components can act as good therapeutic agents [16,17].

Apart from the individual medicinal plants used in this study, we also analyzed the two recipes and their MIC. These recipes were prepared and named according to the traditional Hakeems. Recipe 1 is named Akseer-e-Pechesh and recipe 2 is named Taryaq-e-tabkhir Balghami, respectively. We observed that recipe 1 contains *T. chebula* (fruits), *A. marmelos* (fruits) and *C. longa* (rhizomes), which are mixed in the ratio of 1:1:2, respectively. It is used in the form of powder which is relatively more effective against diarrhea and dysentery. Recipe 2 also contains *S. aromaticum (flower buds)*, *P. nigrum (seeds)* and *C. cassia* (barks), which are mixed in the ratio of 1:0.5:1 and ground to powder, which is also quite effective in the treatment of diarrhea and constipation. These plants, as well as their recipes, are substantially used by the local inhabitants and local herbal practitioner (Hakeems) to treat diarrhea caused by the pathogenic microorganisms like *E. coli, Salmonella, Shigella and MRSA.*

2. Results

Qualitative phytochemical screening shows that the alkaloids were present in chloroform and methanol extracts of *T. chebula* (fruits), *A. marmelos* (fruits), *S. aromaticum* (flower buds), *C. longa* (rhizomes), *C. cassia* (barks), *P. nigrum* (seeds), recipe 1 and recipe 2, while it was not detected in n-hexane extract of *A. marmelos* (fruits) and in aqueous extracts of *C. longa* (rhizomes), as shown in Table 1. Whereas, the quantitative amount of alkaloids determined in *T. chebula* (fruits), *A. marmelos* (fruits), *C. longa* (rhizomes), *S. aromaticum* (flower buds), *P. nigrum* (seeds), *C. cassia* (barks), recipe 1 and recipe 2 were 27.84%, 2.54%, 2.66%, 11.88%, 5.06%, 5.28%, 19.66% and 17.78% respectively, as predicted in Table 2. The Flavonoids are present in methanol extract of *A. marmelos* (fruits), *T. chebula* (fruits), *S. aromaticum* (flower buds), recipe 1 and recipe 2, while they were not detected in *P. nigrum (seeds), C. longa* (rhizomes) and *C. cassia* (barks). However, in the aqueous solution and chloroform extracts, the flavonoids were found in *A.*

marmelos (fruits) and in recipe 1, while in n-hexane extract the flavonoids were found in *S. aromaticum* and in recipe 2, as shown in Table 1. The quantity of flavonoids in *T. chebula* (fruits), *A. marmelos* (fruits), *C. longa* (rhizomes), *S. aromaticum* (flower buds), *P. nigrum* (seeds), *C. cassia* (barks), recipe 1 and recipe 2 were found to be 61.21%, 23.81%, 6.82%, 18.6%, 9%, 4.74%, 28.13% and 14.25% respectively, as shown in Table 2. Saponins were found preliminary in methanol, aqueous, chloroform and n-hexane extracts of all the medicinal plants and their recipes, as shown in Table 1. Quantitatively, the amount of saponins were determined in *T. chebula* (fruits), *A. marmelos* (fruits), *C. longa* (rhizomes), *S. aromaticum* (flower buds), *P. nigrum* (seeds), *C. cassia* (barks), recipe 1 and recipe 2 to be 6.32%, 0.24%, 0.36%, 1.10%, 0.19%, 0.23%, 1.19%, and 0.83% respectively, as shown in Table 2. Tannins were detected preliminarily in all the parts of plants as well as their recipes except *C. longa* (rhizomes) in crude methanol extracts. Tannins were also absent in the aqueous extracts of *C. cassia* (barks) and *C. longa* (rhizomes), while in the case of crude chloroform extracts, it was undetected in *P. nigrum* (seeds), *C. longa* (rhizomes) and *C. cassia* (barks). In the n-hexane crude extracts it was found in *S. aromaticum* (flower buds) and in recipe 2, as shown in Table 1. The results obtained in this study are highly consistent with the previously reported results from the study of medicinal plants of this region [18–23].

The results of seven different elements shown in Table 3 and Figure 1 indicate that there is no cadmium at all. The cadmium (Cd) is very toxic, non-essential and the accumulation of cadmium may damage the kidneys. According to the WHO, the recommended level of Cd is 0.3 mg/kg in medicinal plants [24]. The results show that in *T. chebula*, *C. cassia*, recipe 1 and recipe 2, the Ni is below the detection limit. The *S. aromaticum* (flower buds) contain 0.825 mg/kg, *A. marmelos* (fruits) 0.55 mg/kg, *P. nigrum* (seeds) 0.35 mg/kg and *C. longa* (rhizomes) 0.05 mg/kg for the mean concentration of Ni respectively, which are below the standard recommended level. According to the WHO, the maximum permissible limit of Nickel in medicinal plants is 1.5 mg/kg, while its recommended level for mankind is 1 mg/day [24]. The results illustrate that the maximum amount of iron present in the *S. aromaticum* (flower buds) 92.45 mg/kg, *T. chebula* (fruits) 66.775 mg/kg, *C. longa* (rhizomes) 55.9 mg/kg, *C. cassia* (barks) 48.475 mg/kg, *A. marmelos* (fruits) 48.1 mg/kg, recipe 2 44.475, recipe 1, 43.875 mg/kg and *P. nigrum* (seeds) 41.975 mg/kg, is beyond the maximum permissible value. According to the WHO, the maximum permissible limit of iron in medicinal plants is 20 mg/kg, while its daily requirement is 10 to 28 mg/day [20]. The results further show that the high concentration of Pb was found in recipe 1, 89.9 mg/kg, recipe 2, 74.45 mg/kg, *S. aromaticum* (flower buds) 70.675 mg/kg, *C. longa* (rhizomes) 70.1 mg/kg, *C. cassia* (barks) 65.875 mg/kg, *P. nigrum* (seeds) 61.925 mg/kg, *T. chebula* (fruits) 60.125 mg/kg and *A. marmelos* (fruits) 58 mg/kg, which are beyond the maximum permissible limit as recommended by the WHO. According to the WHO, the maximum permissible limit of Pb in medicinal plant is 10 mg/kg [24]. The results further predict that the concentration of Zn was found in *C. longa* (rhizomes) 61.375 mg/kg, recipe 1, 27.925 mg/kg, *S. aromaticum* (flower buds) 21.75 mg/kg, *T. chebula* (fruits) 21.075 mg/kg, *P. nigrum* (seeds) 16.9 mg/kg *A. marmelos* (fruits) 16.325 mg/kg, *C. cassia* (barks) 16.025 mg/kg and recipe 2 9.475 mg/kg, which are below the maximum permissible level, except for *C. longa* (rhizomes), as permitted by the WHO. According to the WHO, the maximum permissible limit of Zn in medicinal plants is 50 mg/kg, while its daily requirement in food is 11 mg/kg [25]. The results in Table 3 reveal that the concentration of Cu was found in *P. nigrum* (seeds) 10.9 mg/kg, *S. aromaticum* (flower buds) 8.3 mg/kg, *C. longa* (rhizomes) 8.225 mg/kg, *T. chebula* (fruits) 6.8 mg/kg, recipe 2 6.557 mg/kg, *C. cassia* (barks) 5.875 mg/kg, recipe 1 5.675 mg/kg and *A. marmelos* (fruits) 5.175 mg/kg, which is below or according to the maximum permissible level. According to the WHO, in medicinal plants, the maximum permissible amount of Cu is 10 mg/kg, while its daily requirement in food is 2–3 mg/day [24]. The results also indicated that the amount of Cr in recipe 1 was 143.9 mg/kg, recipe 2, 140.926 mg/kg, *C. cassia* (barks), 139.65 mg/kg, *A. marmelos* (fruits), 127.375 mg/kg, *S. aromaticum* (flower buds), 125.6 mg/kg, *C. longa* (rhizomes), 121.05 mg/kg, *T. chebula* (fruits), 119.475 mg/kg and *P. nigrum* (seeds), 114.325 mg/kg. The amount of Cr which exists in all these plant parts as well as their recipe is above the maximum permissible value. According to the WHO, the maximum permissible level of chromium in medicinal plants is 1.5 mg/kg and its daily requirement is 0.2 mg [24].

Table 1. Qualitative phytochemical screening of alkaloids, flavonoids, saponins and tannins of medicinal plant parts and their recipes.

S. No	Plant Parts	Alkaloids				Flavonoids				Saponins				Tannins			
		Meth ext	Aqe Ext	Chlor Ext	n-hex ext	Meth Ext	Aqe Ext	Chlo ext	n-hex ext	Meth ext	Aqe ext	Chlor ext	n-hex ext	Meth Ext	Aqe ext	Chlor ext	n-hex ext
1	*T. chebula* (fruits)	+	+	+	+	+	−	−	−	+	+	+	+	+	+	+	−
2	*A. marmelos* (fruits)	+	+	+	−	+	+	+	−	+	+	+	+	+	+	+	−
3	*C. longa* (rhizomes)	+	−	+	+	−	−	−	−	+	+	+	+	−	−	−	−
4	*S. aromaticum*	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+
5	*P. nigrum* (seeds)	+	+	+	+	−	−	−	−	+	+	+	+	+	+	−	−
6	*C. cassia* (barks)	+	+	+	+	−	−	−	−	+	+	+	+	+	−	−	−
7	Recipe (1)	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	−
8	Recipe (2)	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+

Phytochemical detection key: (−) = Absent, (+) = Present; Meth = Methanol, Aqe = Aqueous, Chlor = Chloroform, n-hex = n-hexane, ext = extract.

Table 2. Quantitative phytochemical screening of alkaloids, flavonoids and saponins of medicinal plant parts and their recipes.

S. NO	Recipes/Plant Parts	Alkaloids %	Flavonoids %	Saponins %
1	Recipe (1)	19.66	28.13	1.19
2	*T. chebula (fruits)*	27.84	61.21	6.32
3	*A. marmelos (fruits)*	2.54	23.81	0.24
4	*C. longa (rhizomes)*	2.66	6.82	0.36
5	Recipe (2)	17.78	14.25	0.83
6	*S. aromaticum*	11.88	18.6	1.10
7	*P. nigrum (seeds)*	5.06	9	0.19
8	*C. cassia (barks)*	5.28	4.74	0.23

Table 3. Metals in herbal plants and their recipes e.g., Cd, Ni, Fe, Pb, Zn, Cu and Cr.

S. NO	Name of Plants Part	Cd mg/kg	Ni mg/kg	Fe mg/kg	Pb mg/kg	Zn mg/kg	Cu mg/kg	Cr mg/kg
1	*T.chebula (fruits)*	BDL	BDL	66.775 ± 0.016	60.125 ± 0.250	21.075 ± 0.013	6.8 ± 0.008	119.475 ± 0.430
2	*A. marmelos (fruits)*	BDL	0.55 ± 0.019	48.1 ± 0.002	58 ± 0.184	16.325 ± 0.004	5.175 ± 0.008	127.375 ± 0.347
3	*C. longa (rhizomes)*	BDL	0.05 ± 0.013	55.9 ± 0.072	70.1 ± 0.348	61.375 ± 0.003	8.225 ± 0.022	121.05 ± 0.099
4	*S. Aromaticum*	BDL	0.825 ± 0.005	92.45 ± 0.039	70.675 ± 0.401	21.75 ± 0.030	8.3 ± 0.003	125.6 ± 0.302
5	*P. nigrum (seeds)*	BDL	0.35 ± 0.045	41.975 ± 0.034	61.925 ± 0.150	16.9 ± 0.006	10.9 ± 0.015	114.325 ± 0.402
6	*C. cassia (barks)*	BDL	BDL	48.475 ± 0.075	65.875 ± 0.179	16.025 ± 0.040	5.875 ± 0.064	139.65 ± 0.467
7	Recipe (1)	BDL	BDL	43.875 ± 0.045	89.9 ± 0.449	27.925 ± 0.022	5.675 ± 0.020	143.9 ± 0.326
8	Recipe (2)	BDL	BDL	44.475 ± 0.077	74.45 ± 0.438	9.475 ± 0.064	6.557 ± 0.016	140.926 ± 0.314

Key: Values are mean ± standard deviation (SD), BDL: below detection limit.

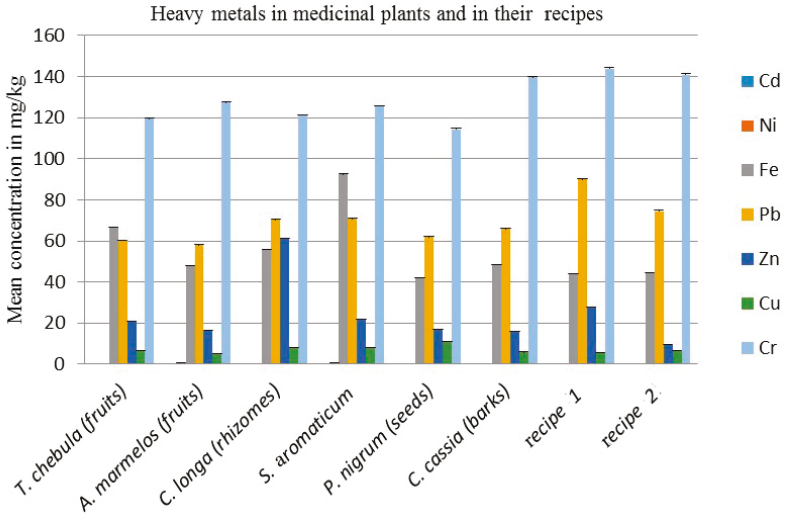

Figure 1. Mean concentration of heavy metals in mg/kg in medicinal plants and in their recipes. Each column represents the mean value of three independent replicates and the error bars indicate standard deviation.

The comparative antibacterial activities of aqueous, methanol, chloroform and n-hexane extracts of the *T. chebula* (fruits), *A. marmelos* (fruits), *C. longa* (rhizomes) and their recipe 1 are shown in Table 4 and Figure 2. The aqueous extracts of *A. marmelos* shows very good inhibition effects against all four bacterial strains. The aqueous extracts of *T. chebula* (fruits) and *C. longa* (rhizomes) displayed a significant zone of inhibition against *Salmonella* and *MRSA* respectively which possess moderate inhibitory effects against the remaining bacterial strains. Recipe 1 presents a very good inhibition zone as compared to its individual plant parts against all bacterial strains except *A. marmelos*, which produced a large inhibition zone against *E-coli* only.

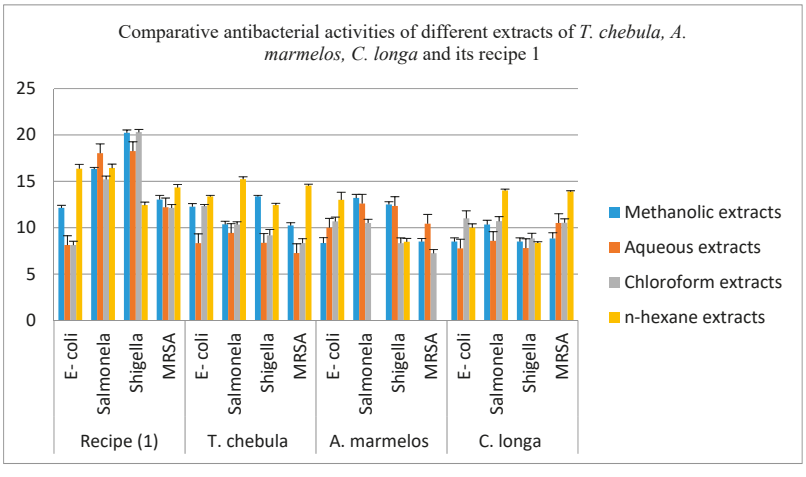

Figure 2. Comparative Antibacterial Activities of methanol, Aqueous, chloroform and n-hexane extracts of the *T. chebula* (fruits), *A. marmelos* (fruits), *C. longa* (rhizomes) and their recipe 1. Each column represents the mean value of three independent replicates and the error bars indicate standard deviation.

The methanol extracts of *T. chebula* (fruits) displayed a considerable inhibitory zone against all four bacterial strains. *A. marmelos* (fruits) and *C. longa* (rhizomes) also exhibit a strong inhibitory result except for *E-coli* and *MRSA*. Besides this, the *C. longa* also exhibited the moderate inhibitory effect against *Shigella.* Recipe 1 also revealed a very good inhibition zone as compared to individual plant parts against all four bacterial strains.

The chloroform extracts of *T. chebula*, *A. marmelos* and *C. longa* present good inhibition effects against *E-coli* and *Salmonella*. All these extracts exhibited a moderate inhibition zone against *Shigella*. The extract of *C. longa* shows a strong inhibitory effect against *MRSA*, while *T. chebula* and *A. marmelos* extracts show a moderate inhibition zone against *MRSA*. Recipe 1 revealed a significant inhibition effect against three bacterial strains, except for *E-coli*, as compared to its individual plant extract.

The n-hexane extracts of *T. chebula*, *A. marmelos* and *C. longa* displayed a marked effect against all four bacterial strains except the n-hexane extract of *A. marmelos*, which shows no inhibitory zone against *Salmonella* and *MRSA*. Recipe 1 of n-hexane indicates the better inhibition effect against all four bacterial strains as compared to all of its individual plant extracts.

The comparative antibacterial activities of aqueous, methanol, chloroform and n-hexane extracts of the *S. aromaticum* (flower buds), *P. nigrum* (seeds), *C. cassia* (barks) and their recipe 2 are shown in Table 5 and Figure 3. The aqueous extract of these three plants displayed a strong inhibition zone against *E-coli*, *Salmonella* and *MRSA*. Beside this, the *S. aromaticum* and *C. cassia* also show significant antibacterial activity against *Shigella* but the aqueous extract of *P. nigrum* indicated a moderate zone of inhibition against *Shigella*. The aqueous extract of recipe 2 revealed an excellent result, as compared to its individual plant extracts.

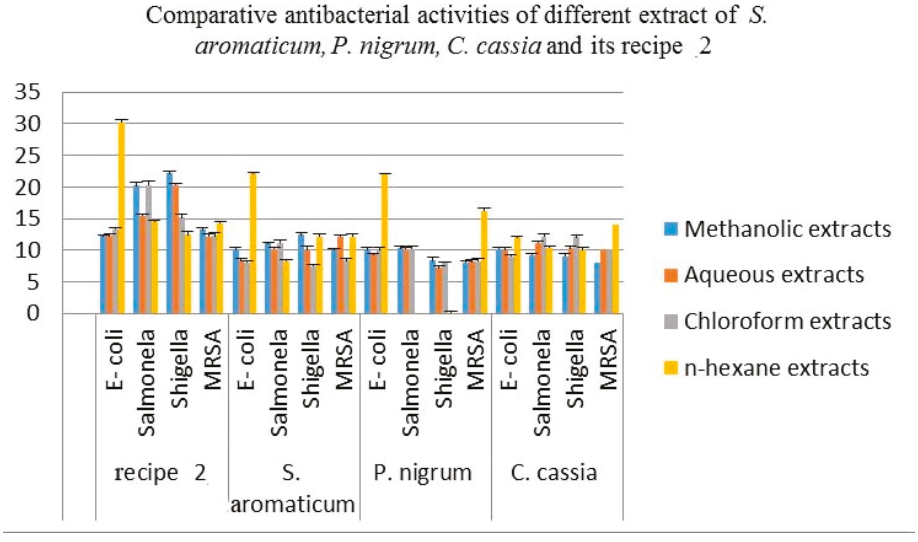

Figure 3. Antibacterial activities of methanol, aqueous chloroform and n-hexane extracts of the *S. aromaticum* (flower buds), *P. nigrum* (seeds), *C. cassia* and recipe 2. Each column represents the mean value of three independent replicates and the error bars indicate standard deviation.

Table 4. Comparative Antibacterial Activities of methanol, Aqueous, Chloroform and n-hexane extracts of the *T. chebula* (fruits), *A. marmelos* (fruits), *C. longa* (rhizomes) and their recipe 1.

Recipe/Plant Part	Bacteria	Methanol Extracts (mm)	Aqueous Extracts (mm)	Chloroform Extracts (mm)	n-Hexane Extracts (mm)	Cpx Mm	DMSO (mm)
Recipe (1)	*E-coli*	12.13 ± 0.28	8.13 ± 0.44	8.13 ± 0.41	16.36 ± 0.47	32	-
	Salmonella	16.33 ± 0.17	18.03 ± 0.70	15.2 ± 0.37	16.43 ± 0.43	33	-
	Shigella	20.23 ± 0.30	18.26 ± 0.32	20.3 ± 0.29	12.43 ± 0.33	31	-
	MRSA	13.03 ± 0.45	12.2 ± 0.24	12.13 ± 0.36	14.33 ± 0.32	23	-
T. chebula (fruits)	*E-coli*	12.26 ± 0.34	8.33 ± 0.48	12.33 ± 0.17	13.36 ± 0.12	32	-
	Salmonella	10.36 ± 0.33	9.43 ± 0.24	10.33 ± 0.30	15.23 ± 0.25	33	-
	Shigella	13.36 ± 0.12	8.36 ± 0.16	9.17 ± 0.63	12.43 ± 0.20	31	-
	MRSA	10.23 ± 0.30	7.26 ± 0.26	8.34 ± 0.47	14.53 ± 0.16	23	-
A. marmelos (fruits)	*E-coli*	8.34 ± 0.58	10 ± 0.81	10.67 ± 0.48	13 ± 0.82	32	-
	Salmonella	13.2 ± 0.39	12.6 ± 0.43	10.5 ± 0.41	NIL	33	-
	Shigella	12.5 ± 0.29	12.34 ± 0.47	8.33 ± 0.57	8.47 ± 0.37	31	-
	MRSA	8.5 ± 0.33	10.43 ± 0.42	7.24 ± 0.40	NIL	23	-
C. longa (rhizomes)	*E-coli*	8.5 ± 0.40	7.76 ± 0.20	11 ± 0.81	10 ± 0.41	32	-
	Salmonella	10.33 ± 0.47	8.57 ± 0.32	10.7 ± 0.49	14 ± 0.16	33	-
	Shigella	8.5 ± 0.40	7.8 ± 0.59	8.83 ± 0.57	8.36 ± 0.12	31	-
	MRSA	8.83 ± 0.62	10.5 ± 0.24	10.53 ± 0.42	13.9 ± 0.08	23	-

Cpx = Ceproflaxacene; MRSA = Methicillin Resistant Staphylococcus Aureus; Noted ANNOVA value ($p < 0.01$) for all samples.

Table 5. Comparative Antibacterial evaluation of methanol, aqueous, chloroform and n-hexane extracts of the *S. aromaticum, P. nigrum, C. cassia* and recipe 2.

Recipe/Plant Part	Bacteria	Methanol Extracts (mm)	Aqueous Extracts (mm)	Chloroform Extracts (mm)	n-Hexane Extracts (mm)	Cpx Mm	DMSO (mm)
recipe 2	*E-coli*	12.23 ± 0.49	12.26 ± 0.53	13.33 ± 0.12	24.23 ± 0.12	32	-
	Salmonella	20.23 ± 0.20	15.43 ± 0.33	20.33 ± 0.30	14.53 ± 0.44	33	-
	Shigella	22.16 ± 0.47	20.33 ± 0.24	15.16 ± 0.54	12.46 ± 0.26	31	-
	MRSA	13.16 ± 0.41	12.13 ± 0.28	12.23 ± 0.46	14.23 ± 0.45	23	-
S. aromaticum (flower buds)	*E-coli*	10.06 ± 0.41	8.3 ± 0.37	8.03 ± 0.45	22.2 ± 0.20	32	-
	Salmonella	11.03 ± 0.36	10.2 ± 0.33	11.13 ± 0.25	8.23 ± 0.12	33	-
	Shigella	12.46 ± 0.25	10.1 ± 0.29	7.5 ± 0.40	12.1 ± 0.29	31	-
	MRSA	10.03 ± 0.28	12.13 ± 0.49	8.36 ± 0.20	12.1 ± 0.53	23	-
P. nigrum (seeds)	*E-coli*	10.13 ± 0.28	9.3 ± 0.29	10.03 ± 0.37	22.16 ± 0.41	32	-
	Salmonella	10.33 ± 0.25	10.2 ± 0.24	10.16 ± 0.34	NIL	33	-
	Shigella	8.36 ± 0.32	7.2 ± 0.16	8.03 ± 0.41	NIL	31	-
	MRSA	8.03 ± 0.45	8.13 ± 0.37	8.2 ± 0.16	16.2 ± 0.24	23	-
C. cassia (barks)	*E-coli*	10.23 ± 0.20	10.03 ± 0.38	9.06 ± 0.41	12.03 ± 0.45	32	-
	Salmonella	9.2 ± 0.24	11.13 ± 0.36	12.13 ± 0.28	10.43 ± 0.24	33	-
	Shigella	9.06 ± 0.32	10.26 ± 0.34	12.03 ± 0.36	10.13 ± 0.28	31	-
	MRSA	8.03 ± 0.45	10.06 ± 0.41	10.13 ± 0.32	14.06 ± 0.32	23	-

NIL: No identified limit; Noted ANNOVA value ($p < 0.01$) for all samples.

The methanol extracts of all these plants perceive very influential inhibitory effects against *E-coli* and *Salmonella*. The methanol extracts of these medicinal plants indicated a desirable inhibition zone against *Shigella* and *MRSA* except for the methanol extract of *P. nigrum*, which shows a reasonable zone of inhibition against *MRSA*. The methanol extracts of *C. cassia* also displayed a moderate zone of inhibition against *MRSA*. The methanol extract of recipe 2 shows a significant zone of inhibition against all the four bacterial strains as compared to its individual plant extracts.

The chloroform extracts of *S. aromaticum* displayed a significant inhibition effect against *Salmonella*, while a moderate inhibition effect against *Shigella*, *E-coli* and *MRSA*. The chloroform extracts of *P. nigrum* shows strong inhibition effects against *Salmonella* and *E-coli*, which demonstrated the moderate inhibition effects against *Shigella* and *MRSA*. The chloroform extract of *C. cassia* has strong inhibition effects against all four bacterial strains. The extracts of recipe 2 showed a very strong inhibitory effect, as compared to the individual plant parts against all four bacterial strains.

The n-hexane extract of *S. aromaticum* perceived more pronounced effects against *Shigella*, *E-coli* and *MRSA* and exhibited moderate effect against *Salmonella*. The extracts of *P. nigrum* demonstrated good inhibitory effects against *E-coli, MRSA* and show no zone of inhibition against *Shigella* and *Salmonella*. The *C. cassia* had a strong inhibition effect against all four bacterial strains. The n-hexane extracts of recipe 2 exhibited an appreciable inhibition zone as compared to its individual plants against all these four strains.

Minimum inhibitory concentrations (MIC) of recipe 1 and recipe 2 are shown in Tables 6 and 7, respectively. Both of these recipes displayed minimum inhibitory concentrations (MIC) values against different bacterial strains. Recipe 1 shows MIC in methanol extract against *E-coli, Salmonella, Shigella* and *MRSA* at 11,000 mg/L, 12,000 mg/L 11,500 mg/L and 14,000 mg/L respectively, and similarly, in aqueous extracts against *E-coli, Salmonella, Shigella* and *MRSA* at 12,500 mg/L, 11500 mg/L, 12,000 mg/L and 14,500 mg/L, respectively. It shows MIC in chloroform extract against *E-coli, Salmonella, Shigella* and *MRSA* at 14,000 mg/L, 13,000 mg/L 14,000 mg/L and 15,000 mg/L respectively, and further, in n-hexane extract against *E-coli, Salmonella, Shigella* and *MRSA* at 15,000 mg/L, 15,000 mg/L 14,500 mg/L and 15,000 mg/L, respectively.

Table 6. Minimum inhibitory concentration (MIC) of recipe 1 against different bacterial strains.

S. No	Bacteria	Methanol Extracts (mg/L)	Aqueous Extracts (mg/L)	Chloroform Extracts (mg/L)	n-Hexane Extracts (mg/L)
1	*E-coli*	11,000	12,500	14,000	15,000
2	*Salmonella*	12,000	11,500	13,000	14,000
3	*Shigella*	11,500	12,000	14,000	14,500
4	MRSA	14,000	14,500	15,000	15,000

Table 7. Minimum inhibitory concentration (MIC) of recipe 2 against different bacterial strains.

S. No	Bacteria	Methanol Extracts (mg/L)	Aqueous Extracts (mg/L)	Chloroform Extracts (mg/L)	n-Hexane Extracts (mg/L)
1	*E-coli*	12,000	11,000	13,000	14,000
2	*Salmonella*	10,500	12,000	12,500	13,000
3	*Shigella*	11,500	12,500	13,000	14,000
4	MRSA	15,000	14,000	15,000	15,000

Recipe 2 displayed MIC in methanol extract against *E-coli, Salmonella, Shigella* and *MRSA* at 12,000 mg/L, 10,500 mg/L 11,500 mg/L and 15,000 mg/L, respectively. Similarly, it displayed MIC in aqueous extracts against *E-coli, Salmonella, Shigella* and *MRSA* at 11,000 mg/L, 12,000 mg/L 12,500 mg/L and 14,000 mg/L, respectively. It displayed MIC in chloroform extract against *E-coli, Salmonella, Shigella*

and *MRSA* at 13,000 mg/L, 12,500 mg/L 13,000 mg/L and 15,000 mg/L respectively, and in n-hexane extract against *E-coli, Salmonella, Shigella* and *MRSA* at 14,000 mg/L, 13,000 mg/L 14,000 mg/L and 15,000 mg/L, respectively.

3. Discussion

Medicinally important plants and their biologically active phytoconstituents are used globally for curing various human diseases including gastrointestinal infections, inflammation, heart disease, cancer and respiratory infection. The use of herbal products and their recipes have fewer side effects as compared to synthetic drugs. All over the world, mostly, people depend upon the herbal products for their healthcare needs. Phytochemicals are the real sources of medicinal value of plants which exert their effects by interacting with human physiology [26]. Preliminary phytochemical screening was performed to find the presence of alkaloids, saponins, flavonoids and tannins. Alkaloids vary greatly in chemical composition and play a vital role in drugs discovery. Typically, the antimicrobial properties of medicinal plants were found due to alkaloids. Typically, herbal species containing flavonoids are known to have therapeutic properties and a declining ratio of cancer has been reported by consuming fruits and vegetables containing flavonoids [27,28]. Saponins are very effective in gastrointestinal infection and have antitumor properties [29]. Tannins have antibacterial activity and are used against diarrhea and dysentery. Tannins play a very important role in the healing of wounds and in bleeding [30]. Many studies have reported the clinical importance of the medicinal plants based on their phytochemical screening, which greatly reinforces the idea of novelty in research in this area [31,32].

Human beings use different medicinal plants from the time immemorial in many aspects, as nutritional values, remedy for different diseases and as essential components in cosmetics. The usefulness of these plants as well as the toxicity is due to their chemical nature, particularly due to the presence of heavy metals like zinc, manganese, cobalt, iron, copper, chromium and nickel. The cadmium (Cd) is very toxic and non-essential and the accumulation of cadmium damages kidneys and liver. Nickel (Ni) is an essential element for all living organisms and is required in a very minute quantity for an individual [33]. Above the permissible level, it is toxic and causes heart failure, loss of vision, loss of body weight and skin irritation [34]. Iron (Fe) is an essential component of hemoglobin. Its deficiency causes nose bleeding, myocardial infractions and gastrointestinal infection [35]. Lead (Pb) is a non-essential trace heavy metal having no functions both in animals as well as in plants. High concentrations of lead causes oxidative stress, brain damage, colic, anemia, headaches and central nervous system disorders [36]. It accumulates in the spleen, kidney and liver through air (20%), food (65%) and water (15%). Zinc (Zn) is an essential trace heavy metal and plays an important role in various processes including bone formation, brain development, wound healing, normal growth and behavioral response. It is essential in protein as well as in DNA synthesis. It regulates structural and catalytic functions of different enzymes [37]. Copper (Cu) is an essential element for normal growth and development as well as for many enzymatic activities. The concentration of copper above the permissible level causes hair and skin discoloration, respiratory and some other lethal effects in human beings [38]. Its deficiency causes anemia and Wilson's diseases [13]. Chromium (Cr) is very essential for the metabolism of glucose, cholesterol and fat. Its concentration above the permissible level is carcinogenic and toxic in nature. The toxicity of Cr intake may appear in the form of a stomach ulcer, skin rash, kidney damage, lung cancer and nose irritation. Its deficiency may lead to elevated body fat and disturbance in proteins, lipids and glucose metabolism [39].

Due to harmful effects as well as antibiotic resistance to the synthetic drugs, researchers are trying to obtain antimicrobial drugs from medicinal plants due to their non-toxic nature. In this study, the crude extracts of *Terminalia chebula* (fruits), *Aegle marmelos* (fruits) and *Curcuma longa* (rhizomes) *Syzygium aromaticum* (flower buds), *Piper nigrum* (seeds) *Cinnamomum cassia* (barks) recipe 1 and recipe 2 showed a very good zone of inhibition against all four tested bacterial strains.

MIC of recipe 1 and recipe 2 were determined by using the broth dilution method against *E-coli*, *Salmonella*, *Shigella* and *MRSA* as shown in Tables 6 and 7, respectively. Both of these two recipes presented MIC against all the tested bacterial strains.

4. Materials and Methods

4.1. Collection of Medicinal Plants Parts and Their Identification

Medicinally effective parts of the selected plants including *Curcuma longa* L., (rhizomes), *Terminalia chebula* Retz., (fruits), *Aegle marmelos* L., (fruits), *Syzygium aromaticum* L., (flower buds), *Piper nigrum* L., (seeds) and *Cinnamomum cassia* L. (barks) were collected from the local herbal market (pansori shops) of Karak, Khyber Pakhtunkhwa. Plants parts were identified and used for further experimentation.

4.2. Plants Grinding and Recipe Formulation

The collected medicinal plant parts were initially washed using distilled water, dried and then sliced into small pieces, separately. Then, each part was mashed to form powder with the help of mortar and pestle, and these powdered samples were stored in a dirt-free separate closed glass container for further use. Apart from the individual plant parts, two recipes were also formed according to the Hakeem description [40–42]. Recipe 1 (Akseer-e-Pechesh) contains *T. chebula* (fruits), *A. marmelos* (fruits) and *C. longa* (rhizomes), which are mixed in the ratio of 1:1:2, respectively. Recipe 2 (Taryaq-e-tabkhir Balghami) contains *S. aromaticum (flower buds)*, *P. nigrum (seeds)* and *C. cassia* (barks), which are mixed in the ratio of 1:0.5:1, respectively.

4.3. Preparation of Plant Extracts

Extracts of each plant and its recipe were prepared by soaking 300 g of plant materials in 500 mL of four different solvents like methanol, distill water, chloroform and n-hexane. The mixtures were kept for 48 h stirring at room temperature, followed by the vacuum filtration. After that, the filtrate was rotary evaporated to obtain semi-solid extract. Then, the phytochemicals analysis in each sample were determined qualitatively and quantitatively [43,44].

4.4. Qualitative Phytochemical Screening

Qualitative phytochemical screening of medicinal plant parts and their recipes were carried out by means of some specific methods. Alkaloids, flavonoids, tannins and saponins were detected by the Tyler [45] and Harborne [46] method.

4.5. Quantitative Phytochemical Screening

Quantitative phytochemical analyses were carried by using the Harborne [46] and Obadoni [47] methods for the determination of alkaloids, the Boham [48] method for flavonoids and the Obadoni [47] method for saponins.

4.6. Heavy Metal Analysis of Medicinal Plants and Their Recipes

Heavy metals like Ni, Cd, Fe, Cr, Zn, Cu and Pb in the medicinal plants and its recipes were analyzed by an atomic absorption spectrophotometer (Perkin Elmer analyst 400, UK) using nitrous oxide (N_2O)-acetylene flame. For the calibration of equipment, the following sensitivity and detection limits were established, in the amounts of: Ni (0.5, 1 and 2 ppm), Cd (0.5, 1 and 1.5 ppm), Fe (2, 4 and 6 ppm), Cr (2, 4 and 6 ppm), Zn (0.5, 1 and 1.5 ppm), Cu (2, 4 and 6 ppm) and Pb (2, 10 and 20 ppm).

A homogeneous mixture of hydrogen peroxide (H_2O_2) 30% and nitric acid (HNO_3) 65% in 1:2 strength was prepared. One gram of each plant part in the form of dried powder was dissolved in this solution. The sample solutions were heated on a hot plate at 130 °C until the volume of each sample was reduced to 3 mL. Then, the solution was cool down, filtered and the volume was made up to 25 mL [49].

4.7. Antibacterial Activity

Collection of Bacterial strains and antibacterial activity.

Pure cultures of four bacterial strains e.g., *Shigella, Escherichia coli, Salmonella* and *MRSA* were obtained and selected for further experimentation. These four bacterial strains were further sub-cultured on nutrient agar. The agar well disc diffusion method was adopted for the evaluation of antibacterial activity. All the equipment was autoclaved and sterilized for 15 min at 120 °C before use. Then, 15 mL of the media was poured in each Petri plate and kept for cooling. The bacterial strains were applied on the Petri dishes by using a sterilized cotton swab. After that, by using the sterilized Cork borer of 6 mm diameter, each Petri plate was punched into five wells for DMSO, distilled water, chloroform, methanol and n-hexane crude extracts, respectively. After that, the stock solution of all crude extract in DMSO each of 30 mg/mL was prepared. Now, each well was filled with 100 µL stock solutions except one, which was filled with DMSO as a negative control. The standard disc of Ceproflaxacene (5 µg) was used as a positive control. All the processes were performed in the laminar flow hood in order to resist contamination. The plates were then incubated in the incubator at 37 °C for 24 h. At last, the zones of inhibition were measured in mm for each crude extract by using Digital Vernier Caliper and the obtained results were noted and recorded [50].

4.8. Determination of Minimum Inhibitory Concentrations (MICs) by Broth Dilution Method

MIC is the lowest concentration (in mg/L) of the antimicrobial agent that prevents visible growth of a microorganism under defined conditions. Broth dilution techniques (micro-dilution) were used to determine the minimal inhibitory concentration (MIC) of antimicrobial agents, including antibiotics that kill (bactericidal activity) or inhibit the growth (bacteriostatic activity) of bacteria. Broth dilution uses liquid growth medium containing geometrically increasing concentrations (typically a two-fold dilution series) of the antimicrobial agent, which is inoculated with a defined number of bacterial cells. The antibacterial agents were dissolved in DMSO. After incubation, the presence of turbidity or sediment indicates growth of the organism [51].

4.9. Statistical Analysis of the Data

The as-recorded data was analyzed and organized by means of Microsoft Excel. The entire experiments were performed three times, consecutively. Standard deviations as well as average zone of inhibitions were calculated by Microsoft Excel 2007. SPSS version 16 was applied to determine the phytoconstituent activities.

5. Conclusions

In summary, the overall results obtained show the medicinal values of the tested plant parts and their recipes, which are used against diarrhea. The qualitative phytochemical screening of these plants and their recipes exhibit the presence of alkaloids, flavonoids, saponins and tannins. The secondary metabolites are more potent in *T. chebula*, *S. aromaticum* and in their recipes. The atomic absorption study of the as-prepared plant samples predict the high concentration of Iron (Fe), Lead (Pb) and Chromium (Cr).

Therefore, it has been concluded that the present research work looking at the medicinal plants and their recipes used against diarrhea found that they are therapeutically active substances with enhanced activities. However, most of the crude extracts also show antibacterial activities. Furthermore, the recipes extracts are more active against the tested bacterial strains as compared to the extracts of individual plants. So, for this reason, the present research study is helpful to identify the bioactive compounds obtained from the medicinal plants which are used against different antimicrobial activities.

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antibiotics

MDPI

Article

In-Vitro Inhibition of Staphylococcal Pathogenesis by Witch-Hazel and Green Tea Extracts

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Abstract: whISOBAX (WH), an extract of the witch-hazel plant that is native to the Northeast coast of the United States, contains significant amounts of a phenolic compound, Hamamelitannin (HAMA). Green tea (GT) is a widely consumed plant that contains various catechins. Both plants have been associated with antimicrobial effects. In this study we test the effects of these two plant extracts on the pathogenesis of staphylococci, and evaluate their effects on bacterial growth, biofilm formation, and toxin production. Our observations show that both extracts have antimicrobial effects against both strains of *S. aureus* and *S. epidermidis* tested, and that this inhibitory effect is synergistic. Also, we confirmed that this inhibitory effect does not depend on HAMA, but rather on other phenolic compounds present in WH and GT. In terms of biofilm inhibition, only WH exhibited an effect and the observed anti-biofilm effect was HAMA-depended. Finally, among the tested extracts, only WH exhibited an effect against Staphylococcal Enterotoxin A (SEA) production and this effect correlated to the HAMA present in WH. Our results suggest that GT and WH in combination can enhance the antimicrobial effects against staphylococci. However, only WH can control biofilm development and SEA production, due to the presence of HAMA. This study provides the initial rationale for the development of natural antimicrobials, to protect from staphylococcal colonization, infection, or contamination.

Keywords: Staphylococcal Enterotoxin A (SEA); biofilm inhibition; Hamamelitannin (HAMA); *Staphylococcus aureus*; *Staphylococcus epidermidis*

1. Introduction

Staphylococci are gram-positive bacteria that can cause multiple diseases, from minor skin infections to severe device associated infections, sepsis, and death. Staphylococcal species like *S. aureus*, which are coagulase positive bacteria, cause diseases through the production of multiple toxins, and antibiotic resistant strains like MRSA (methicillin resistant *S. aureus*) are commonly found [1–3]. Staph species like *S. epidermidis* belong to the coagulase negative staphylococcal (CNS) group and cause disease mostly through the formation of biofilms that are highly resistant to antimicrobials and to the host's immune defenses [4,5].

Staphylococcal species, including *S. auerus* and *S. epidermidis*, are part of the healthy normal microflora of the skin and mucus membranes. However, they become pathogenic when their numbers increase, and they reach a certain quorum [2,6]. Quorum sensing systems are then activated, leading to virulence (e.g., production of toxins and/or formation of biofilms) [7,8]. These quorum-sensing systems

also allow the bacteria to survive under oxidative stress conditions that are part of the host's immune response, thus allowing the bacteria to better survive in the host [9,10].

There are multiple ways to prevent or treat bacterial infections. These include enhancing the host's immune response through vaccination, or interfering with bacterial survival by the use of antibiotics that e.g., disrupt bacterial cell walls or interfere with their replication. But even with the advancement of multiple therapeutic approaches now available, these bacteria cause millions of deaths around the world. The World Health Organization ranks bacterial resistance to antibiotics as one of the top three health care concerns worldwide, with staphylococcal bacterial infections being the largest contributors to this growing problem. In the US, according to the Center for Disease Control (CDC), of the estimated annual two million hospital-acquired infections in the US, approximately one half is due to staph bacteria. Annually, these infections result in over 90,000 deaths and over 100,000 amputations. Furthermore, antibiotic use can lead to disruption of the normal microflora, potentially giving rise to other health issues [11–15]. Alternatives to standard antibiotic treatment are thus needed, considering both genetic resistance as well as behavioral resistance (through formation of biofilms) [7,11,15].

Plants contain a large range of phenolic compounds of various polarities. These compounds are important because of their scavenging abilities due to their abundant levels of hydroxyl groups [16]. Based on some structural differences, polyphenols are subdivided into several major subclasses—phenolic acids, stilbenes, tannins, diferuloylmethanes, and flavonoids. These are potent antioxidants, and have multiple biological effects, including antimicrobial activity [17–19]. For example, green tea plant extracts containing high quantities of carvacrol, epicatechin, and epicatechin gallate polyphenols suppress bacterial growth in food [20]. The antibacterial activity of polyphenols has been attributed to their nonspecific hydrogen bonding and hydrophobic effects to microbial cell wall, membranes, adhesion molecules, enzymes, and cell envelope transport proteins [20–22]. For example, EGCG (epigallocatechin gallate) has been shown to bind peptidoglycan layers, thus disrupting their function as protective layers, making bacteria more vulnerable to environmental changes. Gallic Acid has been shown to interact with bacterial lipid bilayers (of both of gram-positive and gram-negative bacteria), thus disrupting cell function by increasing cell permeability, as well as interfering with cell adhesion, motility, sporulation, and spreading [21,23]. Tannic Acid was shown to interact with microbial enzymes, thus interfering with cellular metabolism and function. Gallotannins have been shown to chelate iron, which is vital for the survival of most pathogenic bacteria. Tannic acid and EGCG have been shown to specifically interfere with AHL-mediated quorum sensing signaling in gram-negative bacteria, leading to disruption of various quorum-sensing mediated functions like swarming motility [23,24]. Hamamelitannin has been shown to act as a quorum sensing inhibitor of staphylococci, inhibiting biofilms from forming and toxins from being produced [24–28].

Our aim is to use plant extracts rich in polyphenols that would limit the ability of the bacteria to evade host's immune response, while interfering with their ability to produce toxins or form biofilms. The extracts tested were witch-hazel and green tea, because of their known antibacterial properties. Green tea extracts are rich in flavanols and their gallic acid derivatives, like catechin, epicatechin (EC), gallocatechin (GC), epicatechin gallate (ECG), epigallocatechin (EGC), and the most abundant one, epigallocatechin gallate (EGCG) [29]. Both catechins and gallates have been shown to have antibacterial properties due to their interaction with the bacterial cell wall, proteins and/or membrane phospholipids, causing increased cell permeability, inhibiting respiration, and altering ion transport processes [30–33].

Witch-hazel (*Hamamelis virginiana*) bark contains high levels of phenolic compounds like Hamamelitannin (HAMA, 2',5-di-O-galloyl-d-hamamelose), which is a low molecular weight tannin. HAMA has been shown to act as a quorum sensing inhibitor in staphylococci, inhibiting bacterial virulence (toxin production and biofilm formation) [27]. Witch-hazel also contains other phenolic compounds, such as gallic acid, gallocatechin, and epigallocatechin, which cause bacterial cell disruption that result from binding to bacterial membranes [21,34].

In this paper, we tested the phenolic content of witch-hazel extract rich in HAMA (whISOBAX (WH), StaphOff Biotech Inc., Hopkinton, MA USA) and green tea extract (GT) (Naturex, Avignon, France) and compared their antimicrobial activity against planktonic and biofilm bacteria. The extracts were tested on *S. epidermidis*, a bacteria notorious for their ability to form biofilms and are common causes of device-associated infections. The extracts were also tested on *S. aureus*, a bacteria notorious for their antibiotic resistance and for their ability to cause sepsis due to the multiple toxins they can produce.

2. Results

2.1. Determination of Total Phenolic Content of WH and GT

The total phenolic content of WH and GT was tested. For WH (whISOBAX, a 50 mg/mL solution), the phenolic content was determined to be 12.66 mg/mL gallic acid equivalent (GAE). For a green tea 10 mg/mL solution the phenolic content was determined to be 10 mg/mL GAE, which is not surprising, since the extract purchased by Naturex has been standardized to a phenolic content > 98%. To understand how much of the WH phenolic content was due to HAMA, the total phenolic content (as GAE) of a 1 mg/mL of HAMA standard was determined to yield 0.544 mg/mL GAE. With this in mind, and knowing that the HAMA content in the extract is 17.3 mg/mL (as quantified by HPLC (High Pressure Liquid Chromatography) see below), we can expect that out of the 12.66 mg/mL GAE phenolic in the extract, 9.41 mg/mL GAE is due to HAMA (75%) and the remaining 3.25 mg/mL GAE is due to other phenolic compounds naturally present in the witch-hazel extract, like gallic acid, gallocatechin, and catechin [27].

2.2. Determination of HAMA Content in WH by HPLC

WH was analyzed by reverse phase HPLC and HAMA content was determined by comparison with a HAMA standard (Sigma-Aldrich). As shown in Figure 1, a single primary peak is evident at 210 nm, which was determined to be HAMA by comparison of absorbance profile to a HAMA standard and confirmed by liquid chromatography mass spectrometry (LCMS) analysis. The amount of HAMA in WH was calculated as 17.3 mg/mL.

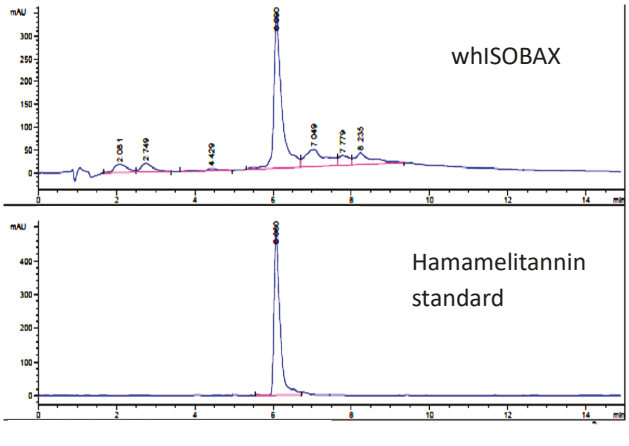

Figure 1. Determination of hamamelitannin in whISOBAX (WH) by reverse phase HPLC analysis. WH (StaphOff Biotech Inc) or HAMA (Sigma-Aldrich) were applied to Durashell reverse phase C18 column in water containing 0.1% trifluoroacetic acid (TFA). Bound material was eluted with an acetonitrile gradient. The amount of HAMA in WH was determined by comparing the retention time and absorbance spectrum with the HAMA standard.

2.3. Antibacterial Activity Against Planktonic Cells

To test for the antibacterial activity of the extracts, early exponential *S. epidermidis* cells were grown overnight with increasing extract concentrations, and the MIC (Minimal inhibitory concentration) and MBC (minimal bactericidal concentration) were determined using spectrophotometric and plating methods. The stock solutions of GT (10 mg/mL) and WH (50 mg/mL) that were used had a phenolic content of 10 mg/mL GAE and 12.66 mg/mL GAE, respectively. The stock solutions were evaluated at various dilutions (0 to 2000 times diluted). Table 1 shows the phenolic and dry weight content of GT and WH in the tested dilutions. As shown in Figure 2, the MBC of WH and GT was determined to be at 1:40 dilutions, which corresponds to 0.31 mg/mL GAE and 0.25 mg/mL GAE, respectively. The MIC was observed at 1:80 dilutions, which corresponds to phenolic contents of 0.125 mg/mL GAE for GT and 0.15 mg/mL GAE for WH. At the MBC level of WH, the amount of HAMA content is 0.23 mg/mL. As previously reported (e.g., [25]), when HAMA was tested alone, even at higher concentrations of over 50 times more than its content in effective WH concentrations, HAMA did not have any antibacterial effect (Figure 3), suggesting that the antibacterial effect of WH is due to other phenolic compounds present, like gallic acid, gallocatechin, and catechin [34].

Table 1. Phenolic content of GT and WH at tested dilutions.

Final Dilution of Tested Extracts	GT Phenolic Content (mg/mL GAE)	WH Phenolic Content (mg/mL GAE)
1:2000	0.005	0.006
1:800	0.012	0.015
1:400	0.025	0.031
1:200	0.050	0.063
1:80	0.125	0.157
1:40	0.250	0.315
1:26	0.375	0.471
1:20	0.500	0.630
1:16	0.625	0.790

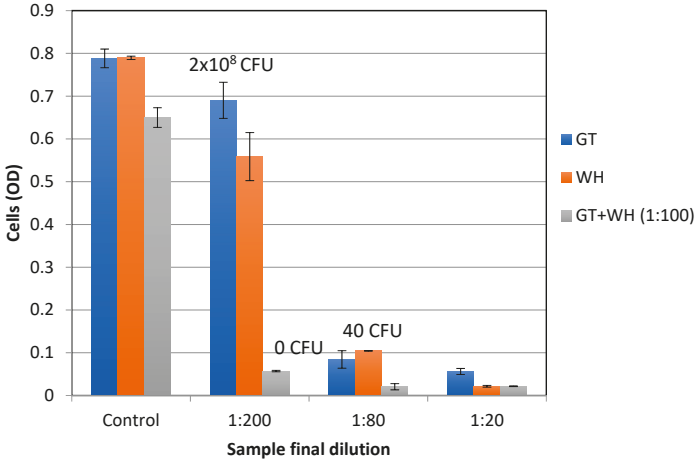

Figure 2. The effect of GT and WH on the growth of *S. epidermidis*. Bacteria were grown overnight at 37 °C with increasing concentrations of GT, WH, or GT with WH diluted 1:100, and cell density determined spectrophotometrically at OD_{630}. The control solution for GT or WH was TSB (Tryptic Soy Broth) alone, while control solution for GT+WH 1:100 was WH 1:100. Cells were plated and CFU (colony-forming units) indicated.

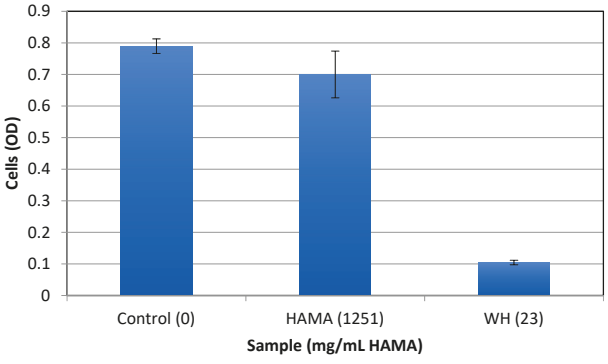

Figure 3. The effect of HAMA on the growth of *S. epidermidis*. Bacteria were grown overnight at 37 °C with HAMA (Sigma-Aldrich, 1251 mg/mL) or with WH (StaphOff Biotech Inc) diluted 1:80 (containing 23 mg/mL HAMA), and cell density determined spectrophotometrically at OD_{630}. As a control, cells were grown in TSB only.

To evaluate the possible synergistic antibacterial effect of GT and WH on the growth of *S. epidermidis*, GT was tested at the same doses with or without a single sub-inhibitory dose of WH (1:100 dilution that corresponds to 0.126 mg/mL GAE). As shown in Figure 2, in the presence of WH diluted 1:100, the MBC of GT significantly decreased ($p < 0.01$) by 5-fold, from 1:40 to 1:200 (from 0.25 to 0.05 mg/mL GAE).

The antibacterial effect of GT and WH were tested also on *S. aureus*. Bacteria were grown overnight with increasing concentrations of GT or WH. As shown in Figure 4, the MIC for both GT and WH was observed at 1:80 dilutions, which correspond to a phenolic content of 0.125 mg/mL GAE and 0.157 mg/mL for GT and WH, respectively. The MBC for both treatments was observed at the 1:40 dilution, that corresponds to a phenolic content of 0.25 mg/mL GAE and 0.214 mg/mL GAE for GT and WH, respectively. Importantly, GT and WH were also shown to inhibit the growth of the MRSA strain *S. aureus* ATCC 43300, where their MICs were ~0.03 mg/ml GAE [35].

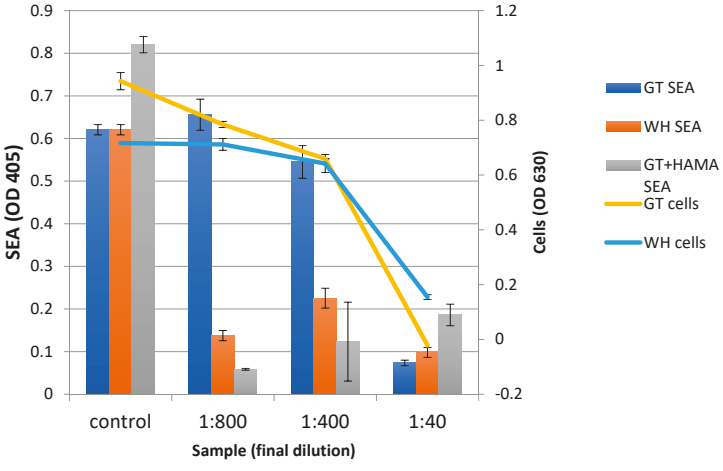

Figure 4. The effect of GT, WH, or HAMA on *S. aureus* growth and toxin production; *S. aureus* cells were grown overnight with increasing concentrations of GT or WH, or with increasing concentrations of GT+0.043 mg/mL HAMA. Cell density was measured (Cells), cells removed by centrifugation, and the presence of SEA was determined in cell supernatants by enzyme-linked immunosorbent assay (ELISA) (SEA).

2.4. The Effect of WH and GT on Staphylococcal Pathogenesis (Biofilm Formation and Toxin Production)

The hallmark of *S. aureus* pathogenesis is the production of multiple toxins that are highly regulated by quorum sensing systems and are produced only when the bacteria reaches a certain cell density. One of those toxins is Staphylococcal Enterotoxin A (SEA), which belongs to a family of heat stable enterotoxins that act as super-antigens and are a leading cause of gastroenteritis resulting from consumption of contaminated food [36].

The dose-dependent effect of WH and GT was tested on *S. aureus* SEA production by ELISA (Figure 4). In the presence of GT, the amount of SEA produced paralleled the cell growth pattern (Figure 4), i.e., more toxins were produced as more cells were present, suggesting that GT had no effect on SEA production. More specifically, we only observed a reduction of SEA production at the 1:40 dilution of GT, which is its MBC against *S. aureus* (Figure 4). On the other hand, SEA production was inhibited in the presence of WH, even at concentrations that did not inhibit cell growth. More specifically, with WH, we observed a reduced SEA production at the 1:800 dilution of WH (0.015 mg/mL GAE) while the MBC of WH was observed at the 1:40 dilution (0.214 mg/mL GAE). The inhibitory effect of WH on SEA production is most likely due to its high HAMA content, as HAMA has been shown to suppress toxin production in *S. aureus* [27]. Indeed, when HAMA and GT were combined, SEA production diminished, and their inhibition profile was similar to that of WH (Figure 4). Of note is that the amount of HAMA (0.043 mg/mL) was tested because it represented a concentration of HAMA that is found in WH 1:400, without having any effect on cell growth.

The hallmark of *S. epidermidis* pathogenesis is the formation of biofilms, which are highly resistant to antibiotic therapy. The effect of WH and GT on eradicating *S. epidermidis* biofilms was tested by first forming biofilms, consisting of about 1×10^7 CFU. These biofilms were then exposed to various doses of GT, WH, or GT+WH for 12 h (to about 1×10^9 CFU if untreated). We observed that GT had a minimal inhibitory effect on *S. epidermidis* biofilms (Figure 5), even when tested at higher concentrations of the observed MBC against *S. epidermidis*. WH, on the other hand, was more effective against biofilm bacteria, reducing biofilm load to almost 50% when at the 1:26 dilution (0.47 mg/mL GAE). The inhibitory effect of WH on bacterial biofilms is likely due to its high HAMA content [24,27].

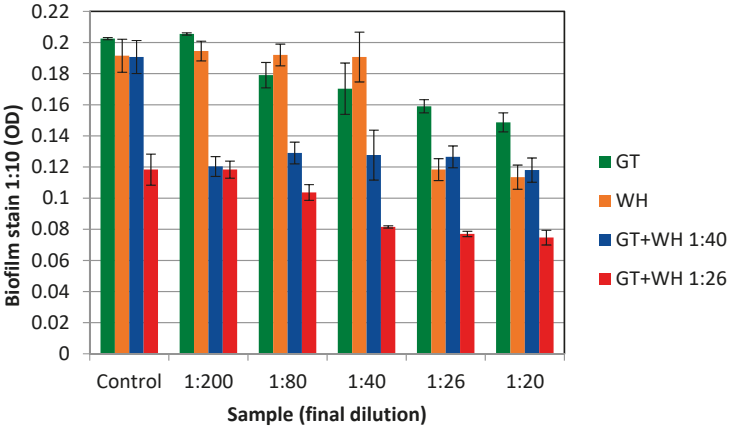

Figure 5. The effect of GT and WH on pre-formed *S. epidermidis* biofilm; Cells were grown in microtiter plates with slight shaking for 4 hrs. Unbound cells were removed and bound cells (biofilm cells) were further incubated overnight with increasing concentrations of GT, WH, or a combination of the two extracts. Unbound cells were removed. Remaining attached (biofilm) bacteria were washed and stained with crystal violet, and their OD determined. As a control for single extract treatment of GT or WH, cells were grown in TSB alone. As a control for combination treatments of GT+WH, cells were grown with no GT but with WH at 1:26 or 1:40 dilutions.

To test for the possible synergistic effect of the two extracts, increasing concentrations of GT were mixed with two WH dilutions, 1:40 (0.31 mg/mL GAE) and 1:26 (0.47 mg/mL GAE). These two WH doses were selected because when WH was tested alone we observed no biofilm inhibition at the 1:40 dilution, and the 1:26 dilution was the first dilution that an observed effect (Figure 5). As shown in Figure 5, when GT was mixed with the two WH dilutions, an enhanced biofilm inhibitory effect was observed. More specifically, even the 1:40 WH dilution, when combined with GT, resulted in a significant biofilm reduction also at the lowest GT doses tested (Figure 5). Higher (1:26) WH dilutions in combination with various concentrations of GT resulted in an even greater biofilm reduction (Figure 5) ($p < 0.01$).

3. Discussion

The results presented here indicate that GT suppresses staphylococcal growth while WH suppresses both staphylococcal growth and pathogenesis (Biofilm formation and toxin production). These factors are important in acute infections (planktonic-associated) and in chronic infections (biofilm-associated). The results presented here also show that WH and GT are synergistic to one another and enhance their respective antibacterial activities.

The inhibitory effect of WH on the growth of both *S. epidermidis* and *S. aureus* was tested and the MBC/MIC against planktonic cells was determined to be at 0.31/0.15 mg/mL GAE (Figures 2 and 4). The MIC of WH needed to inhibit a biofilm was 3× higher, at 0.47 mg/mL GAE (Figure 5). This is not surprising, considering that biofilms are known for their enhanced tolerance to antibacterial treatments. While biofilm cells were more tolerant to the inhibitory effect of WH, tolerance was reduced when the two extracts were combined. WH is very effective at inhibiting S. aureus from producing SEA, and is shown to suppress toxin production even at low concentrations of 0.015 mg/mL GAE (Figure 4).

The effect of GT against growth of planktonic cells (*S. aureus* or *S. epidermidis*) was tested, and its MBC/MIC was shown to be 0.125/0.25 mg/mL GAE (Figures 2 and 4). However, GT had a minimal effect on biofilm reduction, even at the highest tested dose of 0.5 mg/mL GAE (Figure 5). GT also had no effect on *S. aureus* SEA production, since any observed reduction in SEA production was only due to the direct inhibition of GT on *S. aureus* growth (Figure 4).

WH or GT alone did not have any significant effect on biofilm growth while the same concentration caused a reduction in SEA production. This further indicates that while some phenolic compounds in both GT and WH affect cell growth, other phenolic compounds that are only present in WH affects toxin production. This compound was shown to be Hamamelitannin; At the MBC level of WH against planktonic cells (0.31 mg/mL GAE), the amount of HAMA content is 0.23 mg/mL. HAMA alone, even at 20-fold higher concentrations, had no effect on bacterial growth, suggesting that the antibacterial effect of WH is not due to its HAMA content but due to other phenolic compounds present, like gallic acid, gallocatechin, and catechin [34]. On the other hand, HAMA was an important factor in suppressing bacterial virulence (biofilm by *S. epidermidis* and toxin production by *S. aureus*), as GT alone had no effect on bacterial virulence, but inhibitory activity was observed when mixed with WH or HAMA (Figures 4 and 5). While we could not show any effect of GT alone on staphylococcal quorum-sensing (QS) mediated functions such as biofilm formation or toxin production, its anti-QS activity had been demonstrated in gram-negative bacteria [37].

Studies on the molecular mechanism of HAMA indicate that it inhibits bacterial pathogenesis (biofilm formation and toxin production) by interfering with QS systems that are necessary for the bacteria to survive within the host [6,9,10]. QS is a communication system between bacteria, which are activated by chemicals secreted by the bacteria itself that in turn activate signal transduction pathways, leading to regulation of genes that are necessary for bacterial survival once their numbers increase and a quorum is reached. In *S. aureus*, those include activation genes encoding for toxins, like surface proteins that promote colonization of host tissues, invasins (leukocidin, proteases, hyaluronidase) that promote the spread of bacteria in tissues; membrane-damaging toxins (hemolysins, leukotoxin, leukocidin) that puncture human cell membranes, thereby causing cell damage and/or death; and exotoxins

staphylococcal enterotoxins, toxin shock syndrome toxins (SEs, TSST) that damage host tissues and cause symptoms of disease like fever, inflammation, low blood pressure, circulatory collapse, and death [2]. Collectively inhibiting the production of the many toxins by HAMA-rich WH would greatly benefit the host [2,27].

HAMA inhibits staphylococcal *agr/TraP* quorum sensing regulatory systems, leading to a change in the expression of multiple genes important for cell survival and virulence (stress response, toxin production, and biofilm formation) [10,27]. HAMA has also been shown to affect *S. aureus* biofilm susceptibility to different classes of antibiotics (through the TraP receptor), by affecting cell wall synthesis [24]. Bacteria are then unable to overcome the stressors they encounter during infections, and thus become more vulnerable to the host's immune response and to antibiotics.

The antibacterial activity of witch-hazel can be seen as a two-throng approach, where some of the phenolic compounds act to disrupt bacterial cells, reducing their number. At the same time, HAMA disrupts residual biofilm cells while also preventing toxin production, thus inhibiting cells from causing harm to the host. Collectively, these specific phenolic compounds hinder bacterial survival in the host, allowing eradication of both acute and chronic (biofilm-based) infections. The addition of green tea, with its strong antibacterial activity, complements that of WH, further enhancing their respective antibacterial activity.

In conclusion, the results presented here clearly indicate that WH is very effective in suppressing both growth and virulence of coagulase negative and coagulase positive staphylococci, while GT is very effective in suppressing only planktonic cell growth. Our results also indicate the benefit of using a combination of WH and GT for the suppression of staphylococcal pathogenesis, with the synergist effects of the anti-bacterial properties exhibited by GT and WH, along with the strong anti-biofilm and anti-toxin production exhibited by WH. Findings from this work provide the basic biochemical rationale for the further evaluation of witch-hazel and green tea for the development of natural remedies to staph-associated infections and contaminations.

4. Materials and Methods

4.1. Bacteria

S. epidermidis strain ATCC 35984 (RP62A), a biofilm producing strain and *S. aureus* USDA strain, an enterotoxin A producer., were used for this study. The bacteria were grown in Tryptic Soy Broth (TSB) with shaking (220 RPM) at 37 °C overnight, diluted 1:500 in TSB, and grown for about two more hours to the early exponential phase of growth of about 0.1 OD_{630}.

4.2. Test Formulations

whISOBAX (WH) was supplied by StaphOff Biotech Inc. (Hopkinton, MA, USA) and a polyphenol standardized (> 98% phenolic content) green tea extract (GT) was supplied by Naturex (Avignon, France). Unless noted, all chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

4.3. Total Phenolic Content Determination for whISOBAX

The total phenolic content was determined essentially as described [31]. Briefly, 0.5 mL of the sample (WH, GT, HAMA, or increasing concentrations of Gallic Acid standard) was mixed with 0.5 mL distilled water, 1 mL 95% ethanol, 5 mL distilled water, and 0.5 mL 50% (v/v) Folin–Ciocalteu reagent, and incubated at 22 °C for 5 min. One milliliter of 5% Na_2CO_3 was added, and the mixtures were kept in the dark at 22 °C for 1hr. The solution was mixed by vortexing, and the absorbance was determined spectophotometrically at 725 nm. The results were expressed as mg of gallic acid equivalents (GAE) per gram of sample of dried extract weight (DW) or per sample volume. The data presented are an average of three measurements.

4.4. Hamamelitannin Content in WH (HPLC Determination)

WH was analyzed by High Pressure Liquid Chromatography (HPLC) and the HAMA content was determined by comparison to a standard HAMA sample, according to Wang et al. [34] with some modifications to provide a faster method that is less susceptible to solvent composition, and is compatible with LC requirements. The column used was the Durashell reverse phase C18 (Agilent Technologies, Santa Clara, CA, USA) 3 μm, 100 Å, 4.6 x 50 mm column. The solvents used were acetonitrile/water (both containing 0.1% TFA) gradient. HPLC (Agilent 1200 System, Agilent Technology, Santa Clara, CA, USA) was used with a variable wavelength Detector. The amount of HAMA in WH was confirmed by comparing the retention time and absorbance spectrum with the HAMA standard. LCMS analysis (Agilent 1100 System, Agilent Technology, Santa Clara, CA, USA was carried out using the detector stmospheric pressure chemical ionization in mass spectrometry (APCI-MS), in positive mode (carried out by Organix Inc. Woburn, MA, USA).

4.5. Antibacterial Testing on Planktonic Cells

The minimal inhibitory concentration (MIC) was determined using a microbroth dilution method with an initial inoculum of early exponential bacteria. All test dilutions were made in TSB, to obtain a similar GAE content between test extracts (Table 1). Specifically, cells were grown to the early exponential phase of growth in TSB and cells (20 μL, approximately 2×10^4 CFU per well) were incubated with increasing dilutions of test solutions in a final volume of 200 μL per well (Polystyrene 96-well plates (Falcon, Corning, NY, USA)) for about 18 h at 37 °C in air. The cell density was determined using a microtiter plate reader (BioTek, Winooski, VT, USA) at an optical density of 600 nm or 630 nm. The cell number was determined by plating samples on Tryptic Soy Agar (TSA) plates, incubating overnight at 37 °C, and colony-forming units (CFU) counted the next day. The MIC was taken as the lowest drug concentration resulting in observable colonies. The minimal bactericidal concentration (MBC) was taken as the lowest drug concentration that resulted in no observable colonies. All experiments were performed in triplicates. The optical density (OD) of test solutions in TSB (no cells) were determined and used as background values. The positive controls included growing cells in TBS alone or TSB with relevant solvents.

4.6. Antibacterial Testing on Biofilm Cells

The biofilm assays were carried out essentially as described [17,38,39]. Bacteria were grown in TSB to their early exponential phase of growth (OD_{630} of about 0.045, which was about 1000 CFU/μL). To develop a biofilm, 200 μL were placed in 96 polystyrene well plates (Falcon, Corning, NY, USA), and grown for 4–5 h with gentle agitation (~50 RPM) at 37 °C. Unbound cells were removed, and bound cells were rinsed two times with sterile Phosphate Buffer Saline (PBS) under aseptic conditions. (Sample wells were fixed with ethanol to determine initial biofilm by staining (see below)). To adherent cells (approximately 6×10^6 CFU), 200 μL test solutions (in TSB) were added, and the microtiter plates were incubated for ~18 h at 37 °C with gentle agitation (50 RPM). The cell density was determined spectophotometrically at OD_{630}. Non-adherent cells ("cells") were removed to another microtiter plate and the cell density was determined. CFU was determined by plating a sample on TSA plates.

To evaluate the formation of a biofilm, the remaining attached bacteria ("biofilm") were washed three times with PBS, fixed with ethanol, ethanol was then removed, and the cells were air-dried. Biofilm cells were then stained for 5 min with filtered 0.2% crystal violet in 20% ethanol. The unbound stain was rinsed off with water. The plates were air-dried and the dye bound to adherent cells was solubilized with 200 μL 0.1% sodium dodecyl sulfate (SDS). The OD of each well was determined at 630 nm (BioTek Microplate Reader, Winooski, VT, USA). The tests were performed in triplicates.

4.7. Staphylococcal Enterotoxin A (SEA) Production

"Sandwich" ELISA testing was used to determine the amount of SEA produced by *S. aureus* as described [36]. Specifically, sheep anti-SEA IgG (Toxin Technology, Sarasota, FL, USA) was used as the capture antibody, and sheep anti-SEA Horse Radish Peroxidase (HRPO) (Toxin Technology, Sarasota, FL, USA) was used as the detection antibody. The capture antibody was diluted in a coating buffer (0.01 M $NaHCO_3$, 0.1 M Na_2CO_3) at a final concentration of 10 µg/mL and 100 µL/well was added to microtiter 96-well plates (Costar, Washington D.C., USA) and incubated for 1hr at 37 °C or overnight at 4 °C. The plates were washed three times with PBST (PBS containing 0.05% Tween-20), and the same solution (100 µL/well) was used for blocking unbound sites for 15 min at room temperature (RT). To prepare test samples, the treated cells were removed by centrifugation, and the supernatants were collected. One hundred microliters of each sample were added (in triplicate wells) and the plates were incubated for 2 h at 37 °C. The plates were washed three times with PBST. A detection antibody, diluted 1:300 in PBST, was added (100 µL/well), and incubated for 1 h at 37 °C. The plates were washed five times with PBST. One hundred microliters of 3,3′,5,5;-tetramethylbenzidine chromogen solution (Invitrogen, Carlsbad, CA, USA) substrate was added, and 0.3 HCl (50 µL/well) was added to stop the reaction. The absorbance was measured at 450 nm in a microplate reader (BioTek, Winooski, VT, USA) and expressed as 10X OD measured. All tests were performed in triplicate. Increasing amounts of SEA (1 µg/mL to 10 ng/mL) was used as a standard curve.

4.8. Statistical Analysis

All experiments were carried out in triplicates and the averages were presented. The standard deviation was calculated using the "unbiased" n-1 method by Microsoft Excel. The significance of differences between treatment groups was calculated using a two-tailed Student's t-test. $p < 0.05$ was considered significant.

5. Practical Applications

The high content of phenolic compounds in Green Tea (GT) and the high content of hamamelitannin in whISOBAX (WH) make these products ideal for restoring oral and digestive health, and enhancing food safety and stability. The synergist effects of the anti-bacterial properties exhibited by GT and WH, along with the strong anti-biofilm and anti-toxin production exhibited by WH, support the development of nutraceutical alternatives to antibiotics, to enhance food-safety and health.

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antibiotics

MDPI

Article

Hibiscus Acid and Chromatographic Fractions from *Hibiscus Sabdariffa* Calyces: Antimicrobial Activity against Multidrug-Resistant Pathogenic Bacteria

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Abstract: The anti-microbial properties of acetone extracts from *Hibiscus sabdariffa* calyces, fractions isolated by silica gel chromatography and hibiscus acid purified from some of these fractions and additionally identified by nuclear magnetic resonance spectroscopy, mid-infrared spectroscopy and X-ray diffraction, were studied against both multidrug-resistant *Salmonella* strains and pathogenic *Escherichia coli* bacteria. Gel diffusion was used to determine the anti-microbial effects. The mode of action of hibiscus acid was determined by crystal violet assay. Hibiscus acid and 17 of the 25 chromatographic fractions obtained, displayed an anti-microbial effect against all bacterial strains tested. Hibiscus acid showed a greater anti-microbial effect than the acetone extract against most of the bacteria strains, while chromatographic fractions IX–XIV exerted the greatest anti-microbial effect against all bacteria. The minimum inhibitory concentration of the acetone extract was 7 mg/mL, and the minimum bactericidal concentration was 10 mg/mL, while the corresponding values for hibiscus acid were 4–7 and 7 mg/mL, respectively. The results of the crystal violet assay indicate that hibiscus acid alters membrane permeability. Hibiscus acid is a potential alternative to control multidrug-resistant bacteria. Due to its ready availability and easy extraction from *H. sabdariffa*, hibiscus acid is potentially useful in the food industries.

Keywords: anti-microbial; Hibiscus acid; *Hibiscus sabdariffa*; multidrug-resistant; antibiotic

1. Introduction

Salmonella is one of the leading causes of foodborne diseases, and its infection (salmonellosis) is spread worldwide. Due to its prevalence, salmonellosis has become a public health burden, representing significant costs in many countries. A range of fresh fruit and vegetables, especially those eaten raw (lettuce, sprouts, melon and tomatoes), are implicated in *Salmonella* infection [1]. *S. enterica* subspecies *enterica* is composed of more than 1500 serotypes with some of great importance, such as S. Typhimurium and *S*. Enteritidis. *S. enterica* subsp. *enterica* is responsible for more than 99% of human salmonellosis and therefore it is widely studied [2].

Another relevant group of foodborne bacteria is the diarrheagenic *Escherichia coli* pathotypes, including enterotoxigenic, enteroinvasive, enteroaggregative, diffuse adherent and Shiga

toxin-producing strains [3]. Some studies documented the importance of diarrheagenic *Escherichia coli* pathotypes as agents associated with acute and persistent diarrhoea in Mexican children [4–7]. These *E. coli* strains circulating in Mexico were identified in various food and beverages and diarrheic faecal stool samples [5,8–11].

The emergence of multidrug-resistant *Salmonella* strains and *E. coli* pathotypes are related to the use of antibiotics in animals. Resistant bacteria can be transmitted to humans through foods, especially those consumed raw or of animal origin [12]. The presence of multidrug-resistant pathogenic bacteria in food is an important public health issue [13].

The increased resistance of pathogenic bacteria to antibiotics has intensified the demand for safe and natural alternative anti-microbial agents in food products. Plant species used for medicinal purpose and human consumption are currently being studied since they may constitute a source of anti-bacterial compounds. It was reported that extracts obtained from calyces of roselle (*Hibiscus sabdariffa*) have an anti-microbial effect on antibiotic-resistant and non-resistant pathogenic microorganisms [14–24]. The extracts from *H. sabdariffa* calyces are a possible alternative to control antibiotic-resistant pathogenic bacteria.

Calyces of *H. sabdariffa* are known to contain chemical compounds, such as organic acids, phytosterols, polyphenols and anthocyanins [25]. It was suggested that different compounds such as anthocyanins, polyphenol or protocatechuic acid are responsible for the anti-microbial activity of *H. sabdariffa* [21,25–27]. However, no prior studies fully demonstrated the anti-microbial effect of the specific chemical compounds in *H. sabdariffa* or reported the isolation of specific anti-microbial constituents from its calyces, which are used in many regions of the world in hot and cold beverages. It is possible that in the *H. sabdariffa* calyces there are other compounds other than those suggested and that they are primarily responsible for the anti-microbial activity.

Hydroxycitric acid, hibiscus acid and it derivatives as the major organic acids in the leaves and calyces extracts of *H. sabdariffa* [28].

Hibiscus acid was demonstrated to have an inhibitory effect on pancreatic α-amylase and intestinal *α*-glucosidase, resulting in reduction of carbohydrate metabolism and blood insulin levels [29]. Furthermore, hibiscus acid was demonstrated to have a vasorelaxant effect on the rat's aorta [30].

Hibiscus acid is not commercially available; however, it is a chiral compound and its diastereomer, garcinia acid from (*Garcinia cambogia*) is commercially available.

The present study sought to isolate and identify at least one chemical compound with anti-microbial activity from *H. sabdariffa* calyces and evaluate its anti-microbial activity against multidrug-resistant foodborne bacteria.

2. Materials and Methods

2.1. Preparation of Hibiscus Sabdariffa Extract

Ten kilograms of dehydrated calyces of *H. sabdariffa* ("Criolla de Oaxaca" variety) grown in Oaxaca, Mexico were used in the study. The calyces were stored in a closed polyethylene container at room temperature until use. The acetonic extract from calyces of *H. sabdariffa* was obtained exactly as we previously described. Briefly, samples (100 g) of dehydrated calyces were placed in glass flasks and 900 mL of acetone were added (Sigma-Aldrich, Toluca, Mexico). The flasks were hermetically sealed and stored at room temperature for 7 days with manual shaking for 1 min once a day. After, the liquid phase was filtered through filter paper (Whatman Grade 4). The filtered extracts were concentrated in a rotary evaporator (V-800, Vacuum Controller, BÜCHI, Switzerland). The acetone was completely removed from the rotaevaporated concentrate by placing it in an air recirculation oven (Ambi-Hi-Low Chamber, Lab-Line, Jefferson, MO, USA) at 45 °C for 24 h [22,31].

2.2. Chromatographic Fractionation of Acetone Extract

Two hundred and thirty grams of dry acetone extract of *H. sabdariffa* calyces was separated by column chromatography. The dried extract was mixed with silica gel (Sigma-Aldrich, Toluca, México), previously activated at 120 °C for 1 h in a drying recirculation oven (Ambi-Hi-Low Chamber, Lab-Line, Jefferson, USA), at a ratio of 1:2. A glass chromatography column was filled with the silica gel–acetonic extract mixture. Different solvents (hexane, hexane–ethyl acetate, ethyl acetate, ethyl acetate–methanol and methanol) were used as the mobile phase to recover consecutive 100 mL fractions from the packed column. The fractions were concentrated on a rotary evaporator, placed in glass vials and analysed by thin-layer chromatography. The fractions whose components showed the same level of displacement in the plate were pooled and placed in an air recirculation oven at 40 °C to evaporate solvent residues. The anti-microbial activity of the chromatographic collections against multidrug-resistant pathogenic bacteria was determined.

2.3. Extraction of Hibiscus Acid

Two hundred and thirty grams of dry acetone extract of *H. sabdariffa* calyces was packed with silica gel in a chromatographic column, as described in Section 2.2. Hexane was used as the mobile phase to separate the oils in the extract, and 600 mL fractions were recovered in glass flasks. All the chromatographic fractions obtained were rotary-evaporated to remove the solvents and concentrate the separated compounds. After discarding most of the oils from the extract, the solvent mixture hexane–ethyl acetate (9:1 *v/v*) was used as the mobile phase to remove all residual oils. The mobile phase (8:2 *v/v*) passed through the packed column until some small crystals were observed in the rotary-evaporated fractions and it was then used at a ratio of 7:3 (*v/v*) to obtain well-defined crystals in the rotary-evaporated fractions. The crystals were analysed by thin-layer chromatography to determine their purity, re-crystallised using 7:3 (*v/v*) acetone–ethyl acetate in a separatory funnel and then stored for 24 h. Once the formation of crystals on the wall of the separation funnel was observed, the liquid was decanted, and the crystals were recovered. Finally, the residual acetone was removed in an air recirculation oven at 45 °C for 2 h.

2.4. Structural Identification of Hibiscus Acid

2.4.1. Nuclear Magnetic Resonance Spectroscopy

The crystals (Section 2.3) were examined by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy, using deuterated acetone (acetone-d_6; Sigma-Aldrich, Toluca, Mexico) to solubilize the crystals, in a 400 MHz NMR spectrometer (Jeol, Tokyo, Japan). The acquired spectra were analysed using MestReNova 2009 software (version 6.0.2-5475; Mestrelab Research S.L., Santiago de Compostela, Spain).

2.4.2. Infrared Spectroscopy with Attenuated Total Reflection

The crystals (Section 2.3) were ground in a mortar to reduce the particle size and analysed using a diamond-accessorised attenuated total reflection infrared spectrometer (Frontier, Perkin Elmer, Norwalk, CT, USA) at 25 ± 2 °C. Infrared spectra were recorded between 4000 and 400 cm^{-1} at a resolution of 4 cm^{-1}, and 64 spectra per sample were co-added to improve the sample-to-noise ratio.

2.4.3. X-ray Crystallography

The crystals were grown in aqueous acetone by slow evaporation. Diffraction data were measured on a Gemini CCD diffractometer (Oxford Diffraction Ltd., Abingdon, Oxfordshire, England) at room temperature using graphite-monochromated CuKα radiation (λ = 1.54184 Å) and processed using the CrysAlis program (version 1.171.33.31, 2009; Oxford Diffraction Ltd., Abingdon, UK). The structure was solved using Olex2 [32]) and SHELXT [33] structure solution program using intrinsic phasing

or direct methods and refined with the crystal structure refinement program SHELXL [34] using least-squares minimization.

2.4.4. Differential Scanning Calorimetry

The melting point of hibiscus acid was measured using a differential scanning calorimeter (Q2000, TA Instruments, New Castle, NJ, USA), previously calibrated with indium (onset temperature T_o = 156.6 °C, transition enthalpy ΔH = 28.4 J/g, respectively. Five milligrams of hibiscus acid crystals were placed in an aluminum crucible, which was then sealed and heated from 25 to 250 °C at a rate of 5 °C/min. The transition temperatures and ΔH values were obtained directly using Universal Analysis software version 4.4A (TA Instruments).

2.5. Determination of the Anti-microbial Effect of Acetone Extract, Chromatographic Collections and Hibiscus Acid

2.5.1. Preparation of Test Solutions

Solutions of acetone extract, chromatographic collections and hibiscus acid were prepared at final concentrations of 100 mg/mL. Only distilled water was used to prepare the solutions of acetone extract and hibiscus acid. To obtain the solutions from the fraction collections, a mixture of distilled water and 20% Tween 80 (Sigma-Aldrich, Toluca, Mexico) was used.

2.5.2. Bacterial Strains

Eight multidrug-resistant bacteria strains were isolated from food as follows: *Salmonella* Montevideo C1 and *S*. Typhimurium C65 from cilantro [24], *S*. Typhimurium C63 from carrots [18], enteroinvasive *E. coli* MAC B from nopalitos [16], enteropathogenic *E. coli* MAC A from coriander [35], and enterohemorrhagic *E. coli* EHEC A and two strains of Shiga toxin-producing *E. coli* C558 and C636 from raw beef, in our laboratory. All bacteria were resistant to the same 10 antibiotics (kanamycin, neomycin, streptomycin, amikacin, tetracycline, erythromycin, chloramphenicol, ceftriaxone, nalidixic acid and trimethoprim/sulphamethoxazole) according to the Clinical and Laboratory Standards Institute (CLSI) criterion [36].

2.5.3. Preparation of Bacterial Strains

The eight antibiotic-resistant strains were inoculated in 3 mL of tryptic soy broth (TSB; Bioxon, Becton Dickinson, Ciudad de México, Mexico) and incubated at 35 ± 2 °C for 18 h. The cultures were washed twice in sterile isotonic saline (0.85% NaCl; ISS) by centrifugation at 3500 rpm for 20 min, and the pellet was resuspended in ISS at approximately 10^9 colony forming units/mL (CFU/mL). Finally, a decimal dilution of these washed cultures was done with ISS to produce a final approximate concentration of 8 log CFU/mL [22,31].

2.5.4. Anti-microbial Activity of Acetone Extract, Chromatographic Collections and Hibiscus Acid

The gel diffusion technique with paper discs was used as follows: 100 µL washed bacterial cultures, from a concentration of 1×10^8 CFU/mL, were inoculated onto trypticase soy agar plates (Bioxon, Becton Dickinson) and distributed over the agar by the streak plate method. Sterilized paper discs (Whatman Grade 5, 6-mm diameter) were placed on the surface of the inoculated agar. Then, 20 µL aliquots containing acetonic extract, chromatographic collections and hibiscus acid, respectively, were placed on the paper disks (final dose per disk: 2 mg extract, chromatographic collection or hibiscus acid). ISS was used as a negative control. Treatments were performed in triplicate. The plates were incubated at 35 ± 2 °C for 24 h. For each treatment, the diameters (mm) of the resulting inhibition zones were measured and expressed as the average [31].

2.6. Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The broth macrodilution method [37] was used to obtain the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Tubes were prepared with TSB containing acetonic extract or hibiscus acid at concentrations of 1–100 mg/mL. The tubes were inoculated with a final suspension of microorganisms at 1×10^5 CFU/mL (from the culture washed in ISS at a concentration of 1×10^9 CFU/mL, 2 decimal dilutions were made in TSB, and from the last dilution 10 µL were taken and inoculated in a tube containing 990 µL to have a final concentration of 1×10^5 CFU/mL) and incubated at 37 °C for 24 h. The MIC was the lowest concentration of acetone extract or hibiscus acid to inhibit bacterial growth without turbidity in the tubes. To assess the MBC, TSB tubes containing the lowest concentrate ions of extract or hibiscus acid and no turbidity were inoculated into trypticase soy agar using the pour plate technique and incubated at 35 °C for 24–48 h. The MBC was defined as the lowest concentration of acetone extract or hibiscus acid that showed no colony growth in TSB.

2.7. Measurement of Permeability with Crystal Violet

Alteration of membrane permeability was detected by crystal violet assay exactly as described by Devil et al. 2010 [38]. Briefly, one hundred µL of *S*. Typhimurium C65 and enterohemorrhagic *E. coli* EHEC A were inoculated in TSB and incubated at 37 °C for 6 h. The bacterial suspensions were centrifuged at 10,000 rpm for 20 min. The supernatant was discarded and the cell pellets were washed twice with 0.5 mM potassium phosphate buffer solution (PBS). The bacterial cell suspension was prepared by re-suspending the cell pellet in PBS. The washed bacterial cell suspensions were incubated with different concentration of hibiscus acid at minimum sub-inhibitory concentration (MSIC; 1.25 mg/mL and 1.75 mg/mL for enterohemorrhagic *E. coli* EHEC A and *S*. Typhimurium C65, respectively), MIC (5 mg/mL and 7 mg/mL for enterohemorrhagic *E. coli* EHEC A and *S*. Typhimurium C65, respectively), 10× MIC (50 mg/mL and 70 mg/mL, for enterohemorrhagic *E. coli* EHEC A and *S*. Typhimurium C65, respectively), MBC (7 mg/mL) and ethylenediaminetetraacetic acid (EDTA, positive control, 0.25 M) at 37 °C for 60 min. Control samples were prepared similarly without treatment and EDTA (0.25 M) was used as a positive control. The cells were harvested (10,000 rpm for 5 min) and suspended in PBS containing crystal violet (10 µg/mL). The cell suspension was then incubated (10 min at 37 °C) and centrifuged (10,000 rpm for 5 min). The optical density (OD) 590 of the supernatant was measured using a UV-VIS spectrophotometer (Thermo Scientific, Nanodrop, Verona, Wisconsin, USA). The OD value of crystal violet solution was considered to be 100% excluded. The OD of the supernatant of the normal untreated cell was used as blank. The percentage of crystal violet uptake for all samples was calculated using the following formula:

$$\% \text{ uptake of crystal violet} = (\text{OD Value of sample})/(\text{OD Value of CV solution}) \times 100 \quad (1)$$

2.8. Statistical Analysis

Significant differences ($p < 0.05$) between treatments were calculated by analysis of variance and Tukey's test using SPSS Statistics 20 (IBM Corp., Armonk, NY, USA).

3. Results and Discussion

3.1. Anti-microbial Activity of Acetonic Extract of Hibiscus Sabdariffa

A total of 4.6 g of dry acetonic extract was obtained per 100 g of dehydrated *H. sabdariffa* calyces. The dry extract had anti-microbial activity against the eight multidrug-resistant *Salmonella* and pathogenic *E. coli* strains, while the radial inhibition zone on the culture medium varied from 9.8 to 12.6 mm. These results agree with those previously reported on the anti-microbial effect of extracts obtained from *H. sabdariffa* calyces [18].

3.2. Anti-microbial Activity of Chromatographic Collections against Pathogenic Bacteria

The acetone extract was separated by column chromatography into 903 fractions using different solvent mixtures (Table 1). Fractions displaying the same or similar thin-layer chromatogram were pooled together. Among these 25 collections (I–XXV; Table 2), 14 were anti-microbial against all multidrug-resistant *Salmonella* and pathogenic *E. coli* strains tested (Table 3), while three showed an effect against some of the *Salmonella* and pathogenic *E. coli* strains (Table 3). Collection VI was only effective against *Salmonella* C1 and C65, and collection VII, only against *Salmonella* C65, respectively. In contrast, collection XXII was not anti-microbial against *E. coli* C558, *E. coli* C636 and enteroinvasive *E. coli* MAC B (Table 3). Finally, collections I, II, III, IV, V, VIII, XXIV and XXV had no anti-microbial effect against any pathogenic bacteria. In general, statistically significant differences were observed between the effects produced by some collections and within collections against different pathogenic strains (Table 3).

Table 1. Chromatography fractions obtained from *Hibiscus sabdariffa* calyx acetonic extract.

Fraction Number	Solvent Ratio Used in Chromatography Column as Mobile Phase
1–37	Hexane
38–59	90–10% Hexane–ethyl acetate
60–131	80–20% Hexane–ethyl acetate
132–277	70–30% Hexane–ethyl acetate
278–348	60–40% Hexane–ethyl acetate
349–396	50–50% Hexane–ethyl acetate
397–441	40–60% Hexane–ethyl acetate
442–486	30–70% Hexane–ethyl acetate
487–535	20–80% Hexane–ethyl acetate
536–572	10–90% Hexane–ethyl acetate
573–616	Ethyl acetate
617–660	90–10% Ethyl acetate–methanol
661–693	80–20% Ethyl acetate–methanol
694–731	70–30% Ethyl acetate–methanol
732–771	60–40% Ethyl acetate—methanol
772–794	50–50% Ethyl acetate–methanol
795–810	40–60% Ethyl acetate–methanol
811–842	30–70% Ethyl acetate–methanol
843–868	20–80% ethyl acetate–methanol
869–886	10–90% ethyl acetate–methanol
887–903	Methanol

Table 2. Fraction collections from *Hibiscus sabdariffa* calyx acetonic extract classified according to thin-layer chromatography.

Collection	Fraction	Collection	Fraction
I	1–42	XIV	285–379
II	43–46	XV	380–407
III	47–59	XVI	408–447
IV	60–62	XVII	448–473
V	63–68	XVIII	474–564
VI	69–107	XIX	565–584
VII	108–116	XX	585–620
VIII	117–132	XXI	621–695
IX	133–155	XXII	696–740
X	156–176	XXIII	741–792
XI	180–200	XXIV	793–867
XII	201–256	XXV	868–903
XIII	257–284		

Table 3. Anti-microbial effect of chromatographic fraction collections from *Hibiscus sabdariffa* calyx acetonic extract against eight multidrug-resistant *Salmonella* and pathogenic *Escherichia coli* bacteria.

Collection	*Salmonella* C1	*Salmonella* C65	*Salmonella* C63	EHEC A	EIEC MAC B	*E. coli* C558	*E. coli* C636	EPEC MAC A
VI [1]	7.0 ± 0.2 ^{b,2}	7.2 ± 0.4 ^{a}	- ^{a}	- ^{a}	- ^{a}	- ^{a}	- ^{a}	- ^{a}
VII	- ^{a}	7.5 ± 0.1 ^{ab}	- ^{a}	- ^{a}	- ^{a}	- ^{a}	- ^{a}	- ^{a}
IX	10.8 ± 0.2 ^{g}	12.6 ± 0.2 ^{gh}	13.3 ± 0.2 ^{i}	11.9 ± 0.2 ^{gh}	12.2 ± 0.3 ^{h}	9.3 ± 0.2 ^{bcd}	10.1 ± 0.5 ^{efg}	12.0 ± 0.4 ^{h}
X	13.5 ± 0.4 ^{h}	11.5 ± 0.4 ^{f}	13.3 ± 0.2 ^{i}	12.3 ± 0.2 ^{h}	11.6 ± 0.6 ^{fgh}	11.5 ± 0.2 ^{g}	11.6 ± 0.7 ^{h}	12.4 ± 0.2 ^{h}
XI	13.5 ± 0.2 ^{h}	11.6 ± 0.2 ^{fg}	15.2 ± 0.1 ^{j}	14.2 ± 0.2 ^{i}	12.5 ± 0.4 ^{h}	11.5 ± 0.3 ^{g}	13.2 ± 0.3 ^{i}	11.5 ± 0.2 ^{gh}
XII	11.1 ± 0.6 ^{g}	12.6 ± 0.2 ^{gh}	10.1 ± 0.1 ^{defgh}	9.6 ± 0.5 ^{ef}	10.5 ± 0.6 ^{de}	11.8 ± 0.3 ^{g}	10.2 ± 0.2 ^{efg}	9.5 ± 0.5 ^{cde}
XIII	10.5 ± 0.3 ^{fg}	15.2 ± 0.5 ^{i}	11.0 ± 0.1 ^{h}	11.5 ± 0.3 ^{g}	11.8 ± 0.5 ^{gh}	11.4 ± 0.3 ^{fg}	10.9 ± 0.1 ^{gh}	11.0 ± 0.3 ^{fg}
XIV	10.9 ± 0.4 ^{g}	11.9 ± 0.5 ^{fg}	10.7 ± 0.6 ^{gh}	10.2 ± 0.3 ^{f}	10.5 ± 0.1 ^{def}	11.5 ± 0.4 ^{g}	9.8 ± 0.4 ^{def}	10.3 ± 0.5 ^{def}
XV	10.8 ± 0.4 ^{g}	13.5 ± 0.2 ^{h}	10.5 ± 0.3 ^{fgh}	9.5 ± 0.3 ^{ef}	11.1 ± 0.5 ^{efg}	9.6 ± 0.5 ^{cde}	10.6 ± 0.4 ^{fg}	9.6 ± 0.1 ^{cde}
XVI	9.5 ± 0.1 ^{def}	9.7 ± 0.4 ^{e}	9.8 ± 0.4 ^{defg}	9.9 ± 0.1 ^{f}	10.1 ± 0.1 ^{bcde}	10.4 ± 0.3 ^{ef}	9.6 ± 0.4 ^{de}	10.4 ± 0.1 ^{ef}
XVII	9.1 ± 0.2 ^{cde}	9.7 ± 0.3 ^{e}	9.5 ± 0.4 ^{de}	10.0 ± 0.4 ^{f}	10.3 ± 0.7 ^{cde}	9.7 ± 0.1 ^{cde}	9.8 ± 0.4 ^{def}	9.4 ± 0.7 ^{cd}
XVIII	9.4 ± 0.6 ^{de}	9.6 ± 0.3 ^{e}	9.6 ± 0.5 ^{def}	9.1 ± 0.2 ^{de}	9.9 ± 0.6 ^{bcd}	10.1 ± 0.7 ^{de}	9.5 ± 0.1 ^{de}	8.8 ± 0.2 ^{c}
XIX	8.7 ± 0.2 ^{cde}	8.5 ± 0.7 ^{bcd}	9.3 ± 0.3 ^{cd}	8.4 ± 0.3 ^{cd}	9.5 ± 0.4 ^{bcd}	9.3 ± 0.2 ^{bcd}	7.4 ± 0.5 ^{b}	7.8 ± 0.1 ^{b}
XX	8.9 ± 0.4 ^{cde}	9.5 ± 0.0 ^{de}	9.9 ± 0.1 ^{defg}	9.0 ± 0.2 ^{de}	9.3 ± 0.1 ^{bc}	9.2 ± 0.3 ^{bcd}	8.2 ± 0.1 ^{bc}	8.7 ± 0.1 ^{bc}
XXI	8.1 ± 0.1 ^{c}	8.8 ± 0.3 ^{cde}	8.3 ± 0.2 ^{b}	7.8 ± 0.2 ^{bc}	9.0 ± 0.1 ^{b}	8.3 ± 0.3 ^{b}	8.5 ± 0.4 ^{c}	8.9 ± 0.4 ^{c}
XXII	8.5 ± 0.4 ^{cd}	8.3 ± 0.2 ^{bc}	8.6 ± 0.2 ^{bc}	7.5 ± 0.2 ^{b}	- ^{a}	- ^{a}	- ^{a}	10.0 ± 0.2 ^{de}
XXIII	9.6 ± 0.5 ^{ef}	9.1 ± 0.4 ^{cde}	10.3 ± 0.2 ^{efgh}	8.6 ± 0.1 ^{d}	9.0 ± 0.7 ^{b}	8.9 ± 0.5 ^{bc}	9.1 ± 0.1 ^{cd}	10.1 ± 0.3 ^{def}

[1] Chromatographic collections showing no effect against any microorganism are not in the table. [2] Mean ± standard deviation of three replicas of zone of inhibition diameter (mm). - No anti-microbial effect, values with different letters in the same column per pathogen express significant difference at $\alpha = 0.05$ by Tukey's test. *Salmonella* C1 = *S.* Montevideo, *Salmonella* C65 = *S.* Typhimurium, *Salmonella* C63 = *S.* Typhimurium, EHEC A = enterohemorrhagic *E. coli*, EIEC MAC B = enteroinvasive *E. coli*, *E. coli* C558 and C636 = Shiga toxin-producing *E. coli*, EPEC MAC A = enteropathogenic *E. coli*.

In a study on anti-microbial chromatographic collections from plants with solvents of different polarities, Avila-Sosa et al. [39] obtained and fractionated the chloroform extract from Mexican oregano (*Lippia berlandieri* Schauer) using chloroform and mixtures of chloroform–acetone (70:30, *v/v*), chloroform–acetone (30:70, *v/v*), acetone–methanol (70:30, *v/v*) and acetone–methanol (30:70, *v/v*) as mobile phases. Afterwards, they evaluated the anti-microbial activity of the chromatographic fraction collections obtained against *E. coli*. While most of the chromatographic collections showed anti-microbial activity against *E. coli*, the collections higher in polarity were less potent. Consistent with that observation, the low and intermediate polarity collections displayed the greatest anti-microbial effect in the current work (Tables 1–3). Furthermore, Kuete et al. [40] determined the methanolic extract of *Ficus polita* (FP) was anti-microbial against *S.* Typhi (ATCC 6539) and two strains of *E. coli* (ATCC 8739 and AG100). In addition, the researchers tested five chromatographic fractions from FP (FPR1–FPR5), obtained using different mobile phases: hexane (FPR1); 75:25 (*v/v*) hexane–ethyl acetate (FPR2); 50:50 (*v/v*) hexane–ethyl acetate (FPR3); ethyl acetate (FPR4); methanol (FPR5). Of the five fractions, only those of low polarity (FPR1 and FPR2) exhibited anti-microbial activity against the studied strains.

Do et al. [41] also investigated the anti-microbial effect of five chromatographic fraction collections obtained from the methanolic extract of *H. sabdariffa*, using different solvent mixtures as mobile phases: 50% hexane–50% ethyl acetate (CF1); 30% hexane–70% ethyl acetate (CF2); 90% ethyl acetate–10% methanol (CF3); 60% ethyl acetate–40% methanol (CF4); 70% ethyl acetate–30% methanol (CF5). Among them, only CF3, which was active against *E. coli*, *Staphylococcus aureus*., *Bacillus cereus* and *B. subtilis*, and CF4 and CF5, which were active against *S. aureus* and *B. subtilis*, possessed anti-microbial properties. Moreover, only CF3, which showed the greatest anti-microbial action, contained the flavonoid quercetin, among other unidentified compounds. However, further studies would be required to conclusively identify the anti-microbial molecules in CF3–CF5 and the methanolic extract, and any interactions responsible for the activity.

In the current study, collections IX, X, XI, XIII and XIV showed greater anti-microbial activity than the rest. These collections were obtained with the polarities of the following mixtures: 70% hexane–30% ethyl acetate, 60% hexane–40% ethyl acetate and 50% hexane–50% ethyl acetate (Tables 1 and 2). It is important to note that defined crystals were formed in the pooled fractions IX, X and XI, which were collected using 70% hexane–30% ethyl acetate as the mobile phase (Tables 1 and 2).

Since the preliminary NMR analysis of the crystals suggested the presence of hibiscus acid and other compounds, a second chromatographic separation was completed using another sample of dry acetonic extract (230 g) from *H. sabdariffa* to obtain a higher concentration of crystals for purification. The aim was to confirm the presence and anti-microbial activity of hibiscus acid by different structural analysis techniques. The second column chromatography separating the acetonic extract yielded presumptive crystals of hibiscus acid.

3.3. Obtaining Presumptive Crystals of Hibiscus Acid from the Acetonic Extract

Following the procedure described in Section 2.3, a total of 413 fractions were grouped into four collections, according to the mobile phase used: hexane (17 fractions), 90% hexane–10% ethyl acetate (52 fractions), 80% hexane–20% ethyl acetate (61 fractions) and 70% hexane–30% ethyl acetate (283 fractions). After purification, 65 g presumptive crystals of hibiscus acid (collection IV, fractions 113–413) were obtained from 230 g acetonic extract of *H. sabdariffa* (1.3% crystals from 5 kg dehydrated calyces) and, additionally, characterized by NMR, infrared spectroscopy and X-ray crystallography, as described in Sections 3.4.1–3.4.3, respectively, to identify the structure of hibiscus acid.

3.4. Structural Identification of Hibiscus Acid

3.4.1. ^{1}H NMR Spectrum

The ^{1}H NMR spectrum of presumptive crystals of hibiscus acid corresponded to that of the molecular structure of hibiscus acid (Figure 1). Most of the proton signals (^{1}H) appeared between δ_H 0 and 12. The signal observed at δ_H 2.05 corresponds to the acetone-d_6 used to dissolve the presumptive crystals of hibiscus acid. The other signals were δ_H: 5.34 (^{1}H, singlet [s], C<u>H</u>-COOH), 4.16 (^{1}H, s, C<u>O</u>H-COOH), 3.25 (^{1}H, doublet [d], J = 17.2 Hz, <u>H$_a$</u>H$_b$C-C=O), 2.77 (^{1}H, d, J = 17.2 Hz, H$_a$<u>H$_b$</u>C-C=O), where J is the coupling constant. Accordingly, the signals of the spectrogram shown in Figure 1 correspond to the molecular structure of hibiscus acid and the deuterated solvent used as the vehicle.

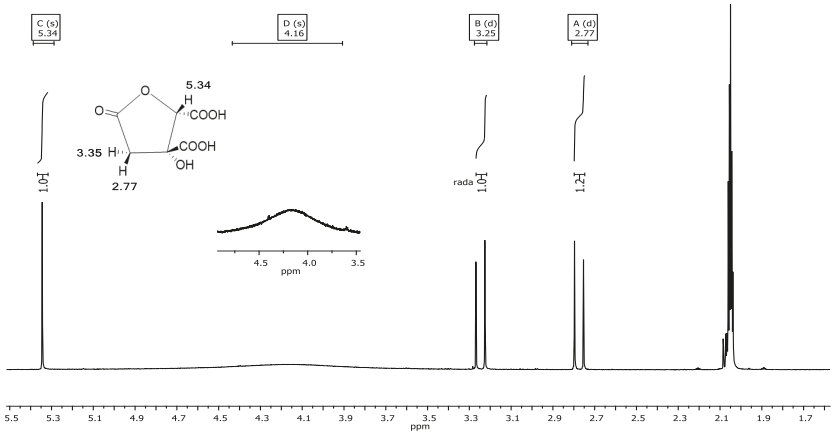

Figure 1. ^{1}H NMR spectrum at 400 MHz in acetone-d_6 of purified crystals obtained from *Hibiscus sabdariffa* calyces acetonic extract.

In an NMR (400 MHz) analysis of the structure of hibiscus acid determined using acetone-d_6 to dissolve the crystals, Ibnusaud et al. [42] detected signals at δ_H 5.38 (^{1}H, s, C<u>H</u>-COOH), 3.30 (^{1}H, d, J = 17.1 Hz, <u>H$_a$</u>H$_b$C-C=O) and 2.80 (^{1}H, d, J = 17.1 Hz, H$_a$<u>H$_b$</u>C-C=O). In previous ^{1}H NMR analysis of hibiscus acid prepared from *H. sabdariffa* calyces extracts, the crystals were dissolved in deuterated water [43], deuterated dimethylsulphoxide [39] and deuterated methanol [40]. The resulting spectrograms showed two doublets at δ_H 2.88 and 3.41, respectively (J = 18.4 Hz) [38] signals at δ_H 5.31 (^{1}H, s), 3.23 (^{1}H, d, J = 17.19 Hz) and 2.77 (^{1}H, d, J = 17.18 Hz) [43]; and signals at δ_H 5.25 (^{1}H, s), 3.20 (^{1}H, d, J = 17.3 Hz) and 2.69 (^{1}H, d, J = 17.3 Hz) [44]. In this context, the parameters published by Ibnusaud et al. [42] and Rasheed et al. [45] are most similar to those obtained in the present work (Figure 1).

3.4.2. Infrared Spectroscopy

The infrared spectrum of the crystals prepared from the *H. sabdariffa* acetone extract contained signals at 3410 cm^{-1} (OH groups), 1797 cm^{-1} (ester groups) and 1742 cm^{-1} (C=O stretching) (Figure 2). These values corresponded strongly to those reported by Ibnusaud et al. [42], which were 3400, 1790 and 1735 cm^{-1}.

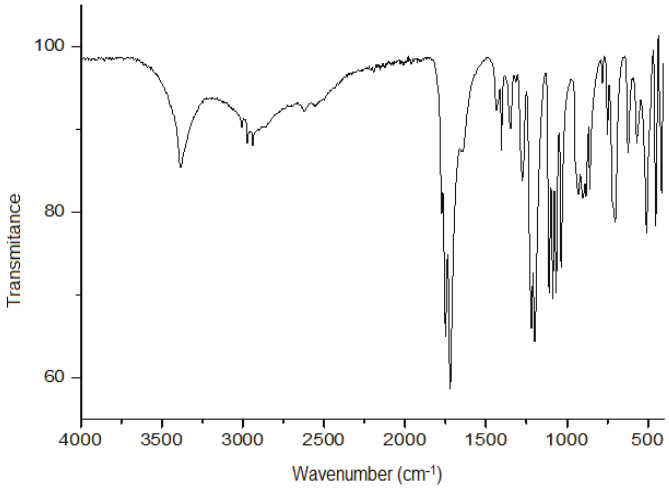

Figure 2. Infrared spectrum of purified crystals obtained from *Hibiscus sabdariffa* calyces acetonic extract.

3.4.3. X-ray Crystallography

The molecular structure of hibiscus acid (Figure 3), was confirmed by X-ray diffraction which was solvated with a water molecule through a hydrogen bond (O6-H6●●●O8, distance = 1.841 Å, ∠ O6-H6●●●O8 = 162.62°) (Figure 3). Hibiscus acid is a five-membered lactone ring, with four carbon atoms and one oxygen atom. C3 (sp2) has a double-bonded oxygen atom, C1 an OH group and a COOH group, and C2 a COOH group, respectively.

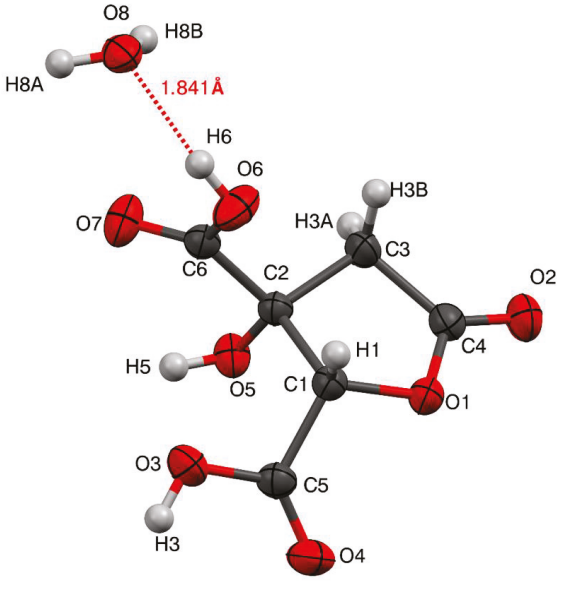

Figure 3. Molecular structure of hibiscus acid isolated from *Hibiscus sabdariffa* calyces acetonic extract determined by X-ray diffraction. Ellipsoids shown at 50% of probability.

The crystallographic details and refined structure of hibiscus acid are provided in Table 4 and Tables S1–S5. Analogous X-ray crystallographic data of hibiscus acid, albeit attached to a dimethylsulphoxide molecule, were presented by Zheoat et al. [44]. Figure 4 shows the possible hydrogen bond donors of the hibiscus acid molecule, in which the interactions that can be established with other molecules of hibiscus acid and water molecules are observed.

Table 4. X-ray spectroscopy details of crystal data and structure refinement parameters of hibiscus acid isolated from Hibiscus sabdariffa calyx acetonic extract.

Experimental Data	
Empirical Formula	$C_6H_6O_7 \bullet H_2O$
Molecular weight	208.12
Temperature (K)	293(2)
Crystal system, space group	orthorhombic, $P2_12_12_1$
Unit cell dimensions (Å, °)	
a	8.2069(2)
b	9.9228(2)
c	10.1747(2)
$\alpha(^\circ)$	90
$\beta(^\circ)$	90
$\gamma(^\circ)$	90
Volume (Å^3)	828.58(3)
Z	5
Radiation type	CuKα (λ = 1.54184 Å)
μ (mm^{-1})	1.797
ρ_{calc} (g cm^{-3})	2.096
F (000)	545.00
2θ range for data collection	12.46–155.038
Index Ranges	$-10 \le h \le 10$, $-11 \le k \le 12$, $-11 \le l \le 12$
Absorption Correction	Multi-scan
Collected Reflections	11147
Independent Reflections	1754 (R_{int} = 0.0293)
Data/Restraints/Parameters	1754/0/133
Goodness-of-fit on F^2	1.077
R_1, wR_2 [$I \ge \sigma 2s(I)$]	0.0309, 0.0859
R_1, wR_2 [all data]	0.0314, 0.0864
Largest Difference Peak/Hole (e Å^{-3})	0.25 and −0.21
Flack and Hooft Parameters	0.05(6) and 0.07(5)
Inverted Flack and Hooft Parameters	0.95(6) and 0.93(5)

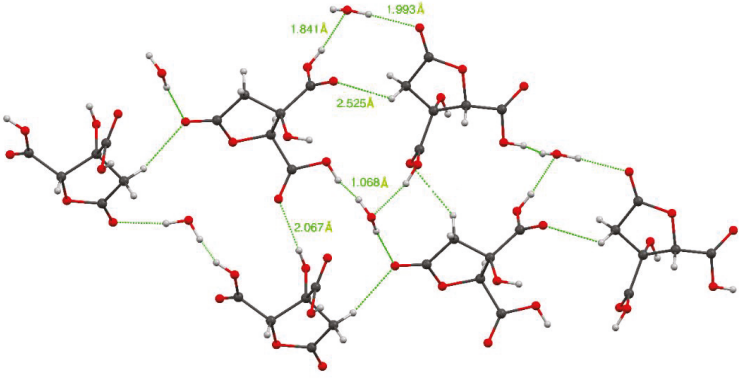

Figure 4. Hydrogen bond contacts of hibiscus acid isolated from *Hibiscus sabdariffa*.

3.4.4. Hibiscus Acid Melting Point by Differential Scanning Calorimetry

The differential scanning calorimetry of the crystals of hibiscus acid showed a T_o of 186.87 °C, peak temperature of 190.61 °C and a final temperature of 194.15 °C, while the fusion enthalpy (ΔH) was 146.7 J/g. Contrastingly, Ibnusaud et al. [37] reported a melting point of 182 ºC for hibiscus acid, which differs from the current results, possibly because of either the equipment or the technique used by the authors.

3.5. Anti-microbial Effect of Hibiscus Acid

Hibiscus acid demonstrated an anti-microbial effect against all multidrug-resistant *Salmonella* and pathogenic *E. coli* strains (Table 5). In general, the anti-microbial effect of hibiscus acid was higher ($p < 0.05$) than that of the acetonic extract (Table 5). Previous reports attributed the anti-microbial activity of *H. sabdariffa* calyces to compounds, such as protocatechuic acid and anthocyanins, in the plant [14,21,23,46]. However, no information regarding the isolation and identification of anti-microbial compounds obtained directly from *H. sabdariffa* calyces has been published until now.

Table 5. Zone of inhibition diameter of *Hibiscus sabdariffa* calyx acetonic extract and hibiscus acid against multidrug-resistant *Salmonella* and pathogenic *Escherichia coli* strains.

Bacteria	Treatment	
	Acetone extract	**Hibiscus Acid**
Salmonella C1 [1]	12.6 ± 0.1 [a]	16.0 ± 0.4 [b]
Salmonella C65	10.8 ± 0.3 [a]	14.5 ± 0.1 [b]
Salmonella C63	10.3 ± 0.3 [a]	11.6 ± 0.2 [b]
EHEC A	10.7 ± 0.4 [a]	10.0 ± 0.3 [a]
EIEC MAC B	11.5 ± 0.1 [a]	13.4 ± 0.6 [b]
E. coli C558	11.8 ± 0.1 [a]	11.6 ± 0.4 [a]
E. coli C636	10.4 ± 0.5 [a]	11.1 ± 0.2 [a]
EPEC MAC A	9.8 ± 0.1 [a]	10.5 ± 0.3 [b]

[1] Mean ± standard deviation of three replicas of zone of inhibition diameter (mm). Values with different letters in the same row per pathogen express significant difference at $\alpha = 0.05$ by Tukey's test. *Salmonella* C1 = *S.* Montevideo, *Salmonella* C65 = *S.* Typhimurium, *Salmonella* C63 = *S.* Typhimurium, EHEC A = enterohemorrhagic *E. coli*, EIEC MAC B = enteroinvasive *E. coli*, *E. coli* C558 and C636 = Shiga toxin-producing *E. coli*, EPEC MAC A = enteropathogenic *E. coli*. Final dose per disk: 2 mg.

It should be noted that while several publications described the anti-microbial effect of *H. sabdariffa* calyx extracts obtained with solvents of varying polarities (including acetonic extract) when tested against different pathogenic bacteria, no published article describes or suggests that hibiscus acid or its derivatives are anti-microbial. Furthermore, there is no proof of their efficacy in the control and elimination of multidrug-resistant pathogenic bacteria. In other words, this document constitutes the first report on the anti-microbial activity of hibiscus acid, even against multidrug-resistant pathogenic bacteria. The MIC and the MBC of hibiscus acid were determined to assess its potential use as an anti-microbial agent in the industry.

3.6. Determination of the MIC and MBC of the Acetone Extract and Hibiscus Acid

The MICs and MBCs of the acetonic extract and hibiscus acid obtained from *H. sabdariffa* calyces were determined using the eight multidrug-resistant pathogenic strains. For hibiscus acid, the MIC values were 4–7 mg/mL, while the MBC range was 5–7 mg/mL (Table 6). The MIC of the acetonic extract was 7 mg/mL for all the pathogenic strains, while the MBC was 10 mg/mL for most of the pathogenic bacteria (Table 6). Abdallah [42] evaluated the MIC and MBC of the *H. sabdariffa* calyces methanolic extract by broth dilution using five multidrug-resistant *Acinetobacter baumannii* strains and obtained MIC and MBC values of 25–50 and 50–100 mg/mL, respectively. These MIC and MBC values are higher than those obtained with the acetonic extract of *H. sabdariffa* (Table 6).

Table 6. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and MBC/MIC ratio of *Hibiscus sabdariffa* calyx acetonic extract and hibiscus acid on multidrug-resistant *Salmonella* and pathogenic *Escherichia coli* strains.

Bacteria	Acetone Extract			Hibiscus Acid		
	MIC (mg/mL)	MBC (mg/mL)	MIC/MBC	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC
Salmonella C1	7	10	1.4	4	5	1.3
Salmonella C65	7	7	1.0	7	7	1.0
Salmonella C63	7	10	1.4	5	7	1.4
EHEC A	7	10	1.4	5	7	1.4
EIEC MAC B	7	10	1.4	5	7	1.4
E. coli C558	7	10	1.4	5	7	1.4
E. coli C636	7	10	1.4	5	5	1.0
EPEC MAC A	7	10	1.4	4	7	1.8

Salmonella C1 = *S*. Montevideo, *Salmonella* C65 = *S*. Typhimurium, *Salmonella* C63 = *S*. Typhimurium, EHEC A = enterohemorrhagic *E. coli*, EIEC MAC B = enteroinvasive *E. coli*, *E. coli* C558 and C636 = Shiga toxin-producing *E. coli*, EPEC MAC A = enteropathogenic *E. coli*.

Yin and Chao [26] tested aqueous and ethanolic extracts of *H. sabdariffa* calyces, obtaining MIC values of 0.112–0.144 and 0.072–0.096 mg/mL, respectively, for *S*. Typhimurium, *E. coli*, *Listeria monocytogenes*, *S. aureus* and *B. cereus*. The MIC of the aqueous extract against *Campylobacter* strains susceptible to antibiotics varied between 0.096 and 0.152 mg/mL [27].

As mentioned above, protocatechuic acid is a compound that was reported to be present in the calyces of *H. sabdariffa* and is likely to be anti-microbial [21,26,27]. However, there is no information available in the literature regarding the isolation, characterization or concentration of the protocatechuic acid in *H. sabdariffa* calyces. Protocatechuic acid is widely distributed in a variety of plants [46]. Commercial protocatechuic acid showed anti-microbial activity against *S. aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *A. baumannii*, with MIC values of 8, 16, 24 and 16 mg/mL, respectively [21]. In comparison, however, Chao and Yin [26] recorded much lower MIC values (24–44 μg/mL) for the protocatechuic acid against *S*. Typhimurium, *E. coli*, *L. monocytogenes*, *S. aureus* and *B. cereus*. Since both studies used a pure commercial compound, the difference in MIC values was expected to be small, especially because they included a bacterial strain of the same genus and species (*S. aureus*). In this study, hibiscus acid showed MIC values (Table 6) within the limits of those reported for protocatechuic acid [26,47]).

Finally, the anti-microbial effect of the acetone extract and hibiscus acid from *H. sabdariffa* calyces was determined as bactericidal or bacteriostatic. A compound is considered bactericidal when the MBC/MIC ratio is ≤4 and bacteriostatic when this ratio is >4 [48]. Both the acetone extract and the hibiscus acid were bactericidal against all eight multidrug-resistant pathogenic strains (Table 6). Abdallah [49] also reported the bactericidal activity of methanolic extract from *H. sabdariffa* calyces (MBC/MIC 1–2 mg/mL) against *A. baumannii* strains.

3.7. Measurement of Permeability with Crystal Violet

Hydrophobic crystal violet is known to display weak penetration of the outer membrane but on the contrary, it is found to penetrate cells with impaired cell membranes thus, crystal violet assay may be employed for the detection of membrane damage [38,50].

The uptake of crystal violet by enterohemorrhagic *E. coli* EHEC A was 9% in the absence of hibiscus acid, but increased to 40%, 57% and 90% after MIC, MBC, 10× MIC hibiscus acid treatments, respectively (Figure 5). The uptake of crystal violet by *Salmonella* C65 was 8% in the absence of hibiscus acid, but increased to 66%, 68% and 82% after MIC, MBC, 10× MIC hibiscus acid treatments, respectively (Figure 6). Minimum sub-inhibitory concentration (MSIC) of hibiscus acid showed no effect, which reveals that it did not alter the membrane permeability in both pathogenic bacteria (Figures 5 and 6).

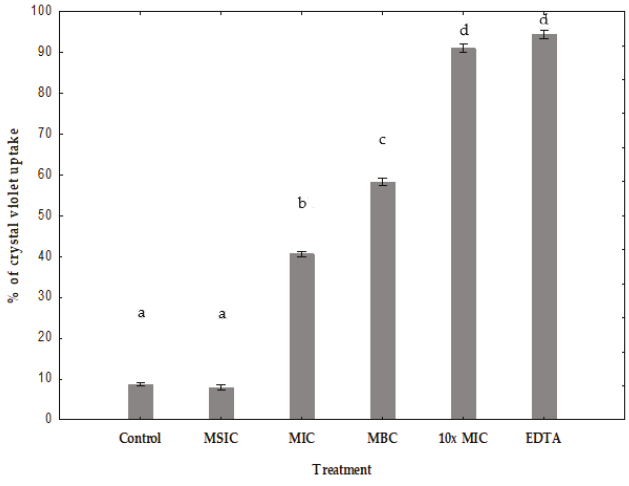

Figure 5. Change in bacterial membrane permeability of EHEC A (assayed by crystal violet uptake) in presence of different concentrations of hibiscus acid and EDTA. Percentage of crystal violet uptake was plotted against the concentration of the treatment. The mean ± standard deviation for three replicates are illustrated. Values with different letters express significant difference at $\alpha = 0.05$ by Tukey's test. MSIC: minimum sub-inhibitory concentration, MIC: minimum inhibitory concentration, MBC: minimum bactericidal concentration, 10× MIC: 10× minimum inhibitory concentration, EDTA: ethylenediaminetetraacetic acid.

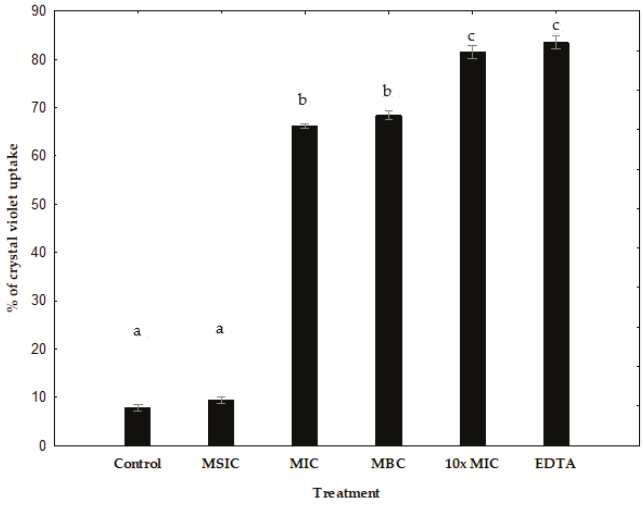

Figure 6. Change in bacterial membrane permeability of *Salmonella* C65 (assayed by crystal violet uptake) in presence of different concentrations of hibiscus acid and EDTA. Percentage of crystal violet uptake was plotted against the concentration of the treatment. The mean ± standard deviation for three replicates are illustrated. Values with different letters express significant difference at $\alpha = 0.05$ by Tukey's test. MSIC: minimum sub-inhibitory concentration, MIC: minimum inhibitory concentration, MBC: minimum bactericidal concentration, 10× MIC: 10× minimum inhibitory concentration, EDTA: ethylenediaminetetraacetic acid.

The results of the crystal violet absorption assay indicate that hibiscus acid alters membrane permeability of enterohemorrhagic *E. coli* EHEC A and *Salmonella* C65.

The effect of hibiscus acid on outer membrane permeability was shown by the uptake of the dye crystal violet. Crystal violet penetrates the outer membrane poorly, but it easily enters when the membrane is damaged. A significant enhancement in the uptake of crystal violet was observed in enterohemorrhagic *E. coli* EHEC A and *Salmonella* C65 treated with hibiscus acid when compared to control cells. This shows that hibiscus acid alters membrane permeability and makes the cells perpermeable to solutes. Furthermore, EDTA also significantly increased the uptake of crystal violet into the cells (Figures 5 and 6). In Gram-negative bacteria, EDTA induces outer membrane permeabilization and cell lysis [51].

4. Conclusions

The present work showed that hibiscus acid is one of the compounds responsible for the anti-microbial effect of *H. sabdariffa* calyces. It was found at a level of 1.3% (13 g/kg) in dried *H. sabdariffa* calyces, and 28.3% in the dry acetonic extract from *H. sabdariffa* calyces, respectively. Due to its relatively high concentration, hibiscus acid is likely one of the main bactericidal compounds in *H. sabdariffa* calyces, although other anti-microbial compounds yet to be reported may also contribute to this effect. Both the hibiscus acid and the acetonic extract from *H. sabdariffa* constitute a potential alternative in the control of multidrug-resistant pathogenic bacteria, such as *Salmonella* and *E. coli* pathotypes. In addition, the hibiscus acid from *H. sabdariffa* calyces is potentially useful in the food industries given its relative abundance and availability. Finally, further research is needed to identify other anti-microbial compounds in *H. sabdariffa* and their mechanisms of action against bacteria. In addition, hibiscus acid affected membrane permeability of enterohemorrhagic *E. coli* EHEC A and *Salmonella* C65.

Supplementary Materials: The following are available online at http://www.mdpi.com/2079-6382/8/4/218/s1, Table S1: Fractional Atomic Coordinates (×104) and Equivalent Isotropic Displacement Parameters (Å2 × 103) for hibiscus acid, Table S2: Anisotropic Displacement Parameters (Å2 × 103) for hibiscus acid, Table S3: Bond Lengths for hibiscus acid, Table S4: Bond Angles for hibiscus acid, Table S5: Hydrogen Atom Coordinates (Å × 104) and Isotropic Displacement Parameters (Å2 × 103) for hibiscus acid.

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Effects of Dietary Chicory (*Chicorium intybus* L.) and Probiotic Blend as Natural Feed Additives on Performance Traits, Blood Biochemistry, and Gut Microbiota of Broiler Chickens

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Abstract: The experiment was designed to determine the effect of different levels of chicory (*Chicorium intybus* L.) powder and a probiotic blend (PrimaLac®) on productive performance, blood biochemical parameters, and ileal microbiota in broiler chickens. A total of 225 one-day-old broilers (Ross 308) were used in a completely randomized design with five experimental diets as follows: 1—basal-diet without supplements (control-group); 2—basal-diet including probiotic blend; 3—basal-diet including 0.10% chicory; 4—basal-diet including 0.15% chicory; 5—basal-diet including 0.20% chicory. At 42 days of age, representative birds per replicate were randomly selected for blood samples and carcass measurements. Results showed that the body weight gain of broilers fed the probiotic blend or 0.10% chicory was significantly ($P < 0.05$) higher than those fed on the other treatments. The abdominal fat pad was significantly ($P < 0.05$) lower in birds fed diets including chicory compared with control or probiotic. Blood triglycerides and LDL levels were reduced ($P < 0.05$) and HDL increased ($P < 0.05$) when fed probiotic or chicory whereas no significant effect on the other serum parameters was found. Broiler ileal microflora from the control group had significantly ($P < 0.05$) higher count of *E. coli* and lower *Lactobacillus* than those from the other groups. From findings, it is possible to conclude that dietary chicory powder supported positively growth performance and improved gut microbiota in broiler chickens. However, more research is needed on this subject to better understand the mode of action of feed additives used.

Keywords: feed additive; growth promoter; medicinal plant; broiler

1. Introduction

Antibiotics are usually used in poultry for therapeutic, preventive, and nutritional purposes [1–3]. Using antibiotics for the growth promotion or prevention and treatment of infectious diseases can result in resistance among both resident bacterial pathogens and commensal organisms [4,5]. Therefore, there is increasing interest in using medicinal plants or probiotics and as natural alternatives to antibiotics for poultry production because they have the potential of inhibition against organisms improving animal performance [6–11].

Probiotics as live microorganisms can also improve the intestinal balance of the host livestock [12]. The mechanism of action of probiotics is mainly related to the competition for attachment sites of the intestinal mucosa, to prevent the pathogenic bacteria attachment by forming a physical obstacle [13], and also to stimulate epithelial and immune cells activities; therefore, supplementing diet with natural additives can improve poultry production.

Chicory (*Cicorium intybus* L.) is an important medicinally perennial herb plant of the *Asteraceae* family [14], containing valuable levels of fructooligosaccharides, inulin, coumarins, flavonoids, and many vitamins [15,16]. Chicory was used as an anti-hepatotoxic, antiulcerogenic, anti-inflammatory, digestive, diuretic, depurative, alexiteric, and tonic agent [17]. In particular, inulin is one of the beneficial components in chicory that can regulate appetite and lipid-to-glucose metabolism [18]. It has also been shown that chicory can promote the growth of useful microbes [19] and inhibit gut pathogenic bacteria growth [20]. Thus, chicory could be supplemented to poultry diets to manipulate the gut microflora composition and to enhance its integrity [20], improving also broiler performance and health status by modulating lipid metabolism with hypolipidemic effects [21–23].

Therefore, the aim of this study was to evaluate the effects of chicory powder and a commercial probiotic blend as natural feed additives on the productive performance, blood biochemical parameters, and ileal microflora of broiler chickens.

2. Results and Discussion

2.1. Growth Performance

Feed intake, body weight gain, and feed conversion ratio of broilers during the experimental period are summarized in Table 1, Table 2, and Table 3, respectively. No significant ($P > 0.05$) differences due to dietary treatment effects were observed on feed intake in the first and second growing periods, however, birds receiving diet supplemented with 0.10% chicory powder during the whole period of the experiment (1–42 days) had a higher ($P < 0.05$) feed intake than those receiving the basal or basal-diet supplemented with probiotics or 0.15–0.20% chicory. Similar results were reported by Faramarzzadeh et al. [24], who supplemented the broiler diet with 4.5% chicory powder. Live body weight gain of chickens was significantly affected by supplementing 0.10% chicory powder or probiotic blend during all rearing periods ($P < 0.05$).

Table 1. Feed intake (g) in broilers fed experimental diets.

Item	1-21 DOA	22-42 DOA	1-42 DOA
Control	1019.5	3272.2	4293.7 ^{b}
Probiotic	1025.7	3228.4	4254.1 ^{b}
Chicory (0.10%)	1036.3	3279.3	4392.3 ^{a}
Chicory (0.15%)	1041.0	3289.4	4330.4 ^{ab}
Chicory (0.20%)	1049.1	3290.5	4255.6 ^{b}
P-value	0.290	0.366	0.009
SEM	9.89	23.21	23.36

Means within each column with different superscript differ significantly at $P < 0.05$; DOA: days of age.

Table 2. Bodyweight gain (g) in broilers fed experimental diets.

Item	1-21 DOA	22-42 DOA	1-42 DOA
Control	688.7 ^{c}	1331.5 ^{d}	2020.2 ^{c}
Probiotic	770.3 ^{a}	1555.6 ^{a}	2326.0 ^{a}
Chicory (0.10%)	768.2 ^{a}	1549.3 ^{a}	2317.5 ^{a}
Chicory (0.15%)	728.0 ^{b}	1492.0 ^{b}	2220.0 ^{b}
Chicory (0.20%)	750.0 ^{ab}	1439.0 ^{c}	2189.1 ^{b}
P-value	0.0002	0.0001	0.0001
SEM	8.46	13.74	17.66

Means within each column with different superscript differ significantly at $P < 0.05$; DOA: days of age.

Table 3. Feed conversion ratio (g/g) in broilers fed experimental diets.

Item	1-21 DOA	22-42 DOA	1-42 DOA
Control	1.48 [a]	2.46 [a]	2.12 [a]
Probiotic	1.33 [c]	2.07 [c]	1.82 [c]
Chicory (0.10%)	1.33 [c]	2.16 [b]	1.89 [c]
Chicory (0.15%)	1.43 [ab]	2.20 [b]	1.95 [b]
Chicory (0.20%)	1.40 [bc]	2.22 [b]	1.94 [b]
P-value	0.005	0.0001	0.0001
SEM	0.022	0.025	0.018

Means within each column with different superscript differ significantly at $P < 0.05$; DOA: days of age.

Overall, the current study revealed that birds receiving diet supplemented with a probiotic blend or different levels of chicory powder during the experimental period improved significantly ($P < 0.05$) their feed conversion ratio than the other treatments. In line with the current findings, it was found that chickens fed diet supplemented with chicory powder had significantly lower feed efficiency compared to the unsupplemented group [24]. Similarly, Cabuk et al. [25] observed significant improvement in feed conversion ratio in broilers fed diet supplemented with herbal plant mixture. Further, Liu et al. [26] also reported that adding a basal diet with chicory powder significantly improved the feed conversion ratio for the first 13 days of the feeding period, which agrees with the present study. Improvement in broiler growth performance fed diet supplemented with chicory powder can be attributed to the enhancements of length, number, and surface area of intestinal villi that are paralleled with an increased digestive and absorptive capacity of jejunum [27,28]. Moreover, broiler chickens at a younger age will get more benefits than older ages by adding chicory powder to diets due to villi growth stimulation [26].

2.2. Carcass Traits

The effects of treatments on the relative weight of broiler carcass and non-carcass components are reported in Table 4. Apart from abdominal fat, there were no significant differences in carcass traits among dietary treatments. In particular, the abdominal fat percentage was significantly ($P < 0.05$) lower in broilers fed diet supplemented with three different levels of chicory powder than control and probiotic blend groups.

Table 4. Carcass characteristics (%) in broilers fed different experimental diets.

Item	Eviscerated Carcass	Breast	Drumsticks	Liver	Gizzard	Abdominal Fat
Control	70.65	32.11	26.59	2.68	1.55	0.96[a]
Probiotic	70.83	31.94	26.71	2.55	1.62	0.84 [a]
Chicory (0.10%)	70.86	31.98	26.90	2.42	1.60	0.74 [b]
Chicory (0.15%)	70.59	32.19	26.81	2.54	1.59	0.63 [b]
Chicory (0.20%)	70.90	32.10	26.62	2.59	1.66	0.72 [b]
P-value	0.487	0.425	0.250	0.434	0.892	0.008
SEM	0.14	0.09	0.10	0.09	0.07	0.11

Means within each column with different superscript differ significantly at $P < 0.05$.

Accordingly, Pournazari et al. [10] found no effect of probiotics on carcass characteristics of broiler chickens. Furthermore, Aminzade et al. [29], Norbakht [30], Ocak et al. [31], and Mansoub [32] reported similar findings. In contrast, Panda et al. [33] and Faramarzzadeh et al. [24] found significant improvement in dressing percentage by supplementing chicory powder (3.0%) to broiler diet. The current results are also in agreement with Yusrizal and Chen [21] who found that broiler chickens fed diets supplemented with chicory fructans (1%) were heavier compared to controls. The abdominal fat accumulation in broiler chicken may be due to fat synthesis (lipogenesis) and fat catabolism via

β-oxidation (lipolysis) [34]. Moreover, a positive relationship was found between adding chicory supplementation and carcass characteristics in poultry species [35–37]. The effect of adding chicory powder or probiotic blend to broiler diet may be due to an overall improvement of the intestinal microenvironment and to a reduction of endogenous nitrogen loss, leading to lower abdominal fat deposition in broiler chickens [21,33].

2.3. Blood Parameters

Table 5 summarized the effect of experimental diets on biochemical serum parameters of broilers at 42 days of age. Apart from serum triglycerides, LDL and HDL, dietary treatments did not induce any significant effect. Blood serum of broiler chicken fed control diet had significantly ($P < 0.05$) higher triglycerides and HDL concentrations than those fed chicory powder, whereas LDL level was lower by feeding experimental supplemented diets. The current findings are in agreement with those of Faramarzzadeh et al. [24], Yusrizal and Chen [21], Safamehr et al. [34] and Agazadehh et al. [38] who found that adding chicory-based fructans to broiler diet decreased also the serum total cholesterol, in addition to triglycerides and LDL in broilers.

Table 5. Blood biochemical constitutes (mg/dl) in broilers fed experimental diets.

Item.	Total Protein	Albumin	Glucose	Total Cholesterol	Triglycerides	HDL	LDL	Uric Acid
Control	3.78	2.18	191.32	159.53	87.08 ^{a}	79.33 ^{a}	68.17 ^{a}	5.43
Probiotic	3.83	2.30	209.89	161.11	67.49 ^{b}	84.01 ^{ab}	63.61 ^{a}	5.64
Chicory (0.10%)	3.68	2.08	199.19	134.92	55.14 ^{b}	91.66 ^{b}	32.23 ^{b}	5.46
Chicory (0.15%)	3.59	2.10	210.40	158.73	60.08 ^{b}	93.01 ^{b}	45.81 ^{b}	5.69
Chicory (0.20%)	3.91	2.18	178.80	154.76	74.07 ^{ab}	88.33 ^{b}	51.62 ^{b}	5.72
P-value	0.381	0.503	0.224	0.688	0.021	0.032	0.025	0.311
SEM	0.11	0.09	10.22	13.84	8.87	5.33	12.17	0.10

Means within each column with different superscript differ significantly at $P < 0.05$.

2.4. Ileal Microflora

Data showed that adding probiotic blend and chicory powder to broiler diet had an antibacterial effect against *E. coli* while increasing *Lactobacillus* bacteria count (Table 6). The three supplementation levels of chicory powder and probiotic blend showed pronounced inhibition against *E. coli*.

Table 6. Ileal microflora (log CFU/g) in broilers fed experimental diets.

Item	*E. coli*	*Lactobacillus*
Control	6.85 ^{a}	6.79 ^{b}
Probiotic	5.64 ^{b}	7.65 ^{a}
Chicory (0.10%)	5.73 ^{b}	7.52 ^{a}
Chicory (0.15%)	5.80 ^{b}	7.70 ^{a}
Chicory (0.20%)	5.99 ^{b}	7.68 ^{a}
P-value	0.0002	0.0001
SEM	0.12	0.07

Means within each column with different superscripts differ significantly at $P < 0.05$.

In a previous study, Farrukh et al. [39] stated that chicory powder was effective against pathogenic bacteria, which in agreement with the present study. Furthermore, the antibacterial activity of chicory, and other herbal plants, was reported to counteract the growth of harmful bacteria [17,29]. The current study confirmed that chicory can be used as a natural antibacterial feed supplement, which may be due to the presence of inulin, bitter sesquiterpene lactones, coumarins, and other compounds. It has been

also reported that herbs and plant extracts stimulate the growth of beneficial bacteria and minimize pathogenic bacteria activity in the poultry gut [40,41].

The antibacterial activity of chicory mainly depended on strains and it is dose-dependent. The present study also showed that the feed additives used contributed to reducing the growth of pathogenic bacteria such as *E. coli*, and increased the beneficial bacteria, such as lactobacilli, and similar conclusions were reported by Jain et al. [42], Ocak et al. [31], and Nobakht et al. [30]. In this respect, Lee et al. [43] reported that the presence of pathogenic bacteria population in the gut may cause the breakdown of amino acids, and thereby reduce their availability for absorption. Herbs, such as chicory, might have the ability to stimulate the production of secretions in the small intestinal mucosa, pancreas, and liver to enhance nutrients digestion and increase their availability at the intestinal brush border [34,44]. It has been also demonstrated that the main beneficial effect of probiotic blend or chicory is to induce changes in the intestinal microbiota by selective stimulation of health-promoting bacteria [45–47].

3. Materials and Methods

3.1. Birds and Management

All procedures used in this study were approved by the Animal Ethics Committee of the Islamic Azad University, Rasht Branch, Iran, in agreement with the Directive 2010/63/EU. A total of 225 one-day-old broiler chicks (Ross 308) with similar initial body weight were randomly divided into 15 experiment units of 15 birds, each with three replicates per treatment for a total of five experimental diets. The experiment was conducted at a commercial poultry farm (Khomam city, Guilan, Iran). The broilers were kept in floor pens (1.0 × 1.5 × 0.5 m) and treatment groups were equally distributed among pens. Temperature and relative humidity were maintained within the optimum range. The lighting program was 23 h light and 1 h darkness. Each pen was equipped with an individual feeder and a nipple drinker. Broilers in a pen were not able to consume feed assigned to the adjoining pen. All experiments were carried out from day 1 to day 42 of age. Birds were vaccinated against infectious bronchitis (1 and 18 days of age), Newcastle disease (1 and 18 days of age), avian influenza (1 day of age), and Gumboro disease (14 and 24 days of age).

3.2. Feed Formulation

A two-phase feeding program was used; a starter diet was used from 1–21 days and a finisher diet from 22–42 days. The ingredients and nutrient composition of diets are shown in Table 7. Feed and water were supplied ad libitum throughout the experimental period. Diets were formulated to meet or exceed the National Research Council (NRC) nutrient requirements for broiler chickens. Two types of natural feed supplements were used: a multi-strain probiotic (PrimaLac®, Star-Labs/Forage Research Inc. Clarksdale, USA) as a lyophilized mixture containing 1×10^8 CFU/g of *Lactobacillus casei*, *Lactobacillus acidophilus*, *Bifidobacterium thermophilum*, and *Enterococcus faecium* (the microbial blends and concentrations are proprietary); chicory powder purchased from a local producer (Rasht, Iran). The following dietary treatments were formulated: Treatment 1—basal-diet with no supplementation as control; Treatment 2—basal-diet supplemented with probiotic blend [following manufacturer's instructions level: 0.900 g/kg (0.09%) from 1–14 days, 0.454 g/kg (0.0454%) from 15–28 days, 0.225 g/kg (0.0225%) from 29–42 days]; Treatment 3—basal-diet supplemented with chicory powder at 0.10%; Treatment 4—basal-diet supplemented with chicory powder at 0.15%; Treatment 5—basal-diet supplemented with chicory powder 0.20%.

Table 7. Ingredients and chemical composition of the basal diet.

Item	Starter (1–21 Days)	Finisher (22–42 Days)
Ingredient (%)		
Corn	56.90	58.70
Soybean meal (44% CP)	33.10	30.00
Fish meal	3.40	3.50
Soybean oil	2.00	3.50
Dicalcium phosphate	1.55	1.10
Oyster shell	1.03	1.18
DL-methionine 98%	0.01	0.01
Vitamin mixture [1]	0.50	0.50
Mineral mixture [2]	0.50	0.50
NaCl	0.26	0.26
Sand	0.75	0.75
Calculated chemical composition		
Metabolizable energy (kcal/kg)	2910	3030
Crude protein (%)	20.1	19.0
Fat (%)	4.60	6.14
Calcium (%)	0.95	0.90
Total phosphorus (%)	1.23	1.06
Available phosphorus (%)	0.45	0.36
Methionine (%)	0.50	0.38
Lysine (%)	1.01	1.00
Methionine + Cystine (%)	0.83	0.71

[1] Supplied per kg of mixture: 3,600,000 IU vitamin A; 800,000 IU vitamin D3; 7200 IU vitamin E; 710 mg vitamin B1; 2640 mg vitamin B2; 1176 mg vitamin B6; 400 mg vitamin B9; 6 mg vitamin B12; 800 mg vitamin K3; 3920 mg pantothenate acid; 12,000 mg niacin; 40 mg biotin; 200,000 mg choline chloride. [2] Supplied per kg of mixture: 40,000 mg manganese; 20,000 mg iron; 33,900 mg zinc; 4000 mg copper; 400 mg iodine; 80 mg selenium.

3.3. Growth and Carcass Measurements

Mortality rate and growth performance, as feed intake, feed conversion ratio, and body weight gain, were recorded per pen at 21 and 42 days of age. After 12 h of fasting, five birds per treatment were randomly selected for carcass characteristics measurements at 42 days of age. Birds were sacrificed, plucked, and eviscerated, then the weight of the whole carcass, carcass components (drumsticks and breast), liver, gizzard, and abdominal fat were excised and individually weighed. Carcass traits components were calculated as a percentage of the preslaughter live body weight.

3.4. Serum Biochemical Analysis

At the end of the trial (42 days), ten broilers per group (30 birds/treatment) were selected for blood sampling. Blood samples were collected from the wing veins and centrifuged at 3000 $g \times 10$ min to obtain serum. Samples were stored at −20 °C until analyzed for total protein, albumin, glucose, total cholesterol, triglycerides, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and uric acid, using commercial laboratory kits (TeifAzmoon Pars Co, Tehran, Iran).

3.5. Ileal Microflora

For the ileal microbial count, at 42 days of age, 30 birds (two birds from each replicate) were randomly selected and killed by cervical dislocation. One gram of ileum content was sampled and transferred into sterile tubes containing 9 ml of sterile PBS and homogenized and serially diluted. A small sample (0.1 ml) from each dilution was inoculated on MRS agar (Man Rogosa Sharpe agar, Merck, 1.10660.500) was used to culture *Lactobacilli*, and Eosin Metilan-Blue (EMB, Merck, 1.01347.0500) was used for *E. coli*, then incubated under anaerobic conditions at 37 °C for 72 h. Bacterial units were counted by a colony counter and adjusted to 1 g sample. The bacteria counts were reported as log10 CFU/g of ileal digesta.

3.6. Statistical Analysis

The experiment was carried out in a complete randomized design with dietary feed additives as the main effects. The model assumptions of normality and homogeneity of variance were tested using Shapiro–Wilk's and Levene's tests, respectively. Data were subjected to analysis of variance (ANOVA) according to the General Linear Model (GLM) procedure of SAS/STAT (SAS software version 8, Institute Inc., Cary, NC, USA). The statistical model used was: $Y_{ijk} = \mu + T_i + R_{ij} + \varepsilon_{ijk}$, where: Y_{ijk} = response variables from each individual replication or pen; μ = the overall mean; T_i = the effect of dietary additive; R_{ij} = the inter-experimental unit (replications) error term; ε_{ijk} = the intra-experimental unit error term. Means were compared for significant differences using the LSMEANS option of SAS/STAT (SAS software version 8, Institute Inc., Cary, NC, USA). Statistical significance was established at $P < 0.05$.

4. Conclusions

Chicory powder and probiotic blend as natural feed supplements in broiler diet supported positively the growth and feed efficiency of birds, without affecting most of the blood biochemical parameters. Moreover, it is important to underline that probiotics and chicory supplementation showed a significant effect in inhibiting the growth of potentially pathogenic *E. coli*, and in enhancing the growth of beneficial *Lactobacillus* gut bacteria. Thus, this study demonstrated that natural feed additives can be successfully used as an alternative to antibiotics as growth promoters for broiler chickens.

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Article

Effects on Intestinal Mucosal Morphology, Productive Parameters and Microbiota Composition after Supplementation with Fermented Defatted *Alperujo* (FDA) in Laying Hens

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Abstract: The olive oil sector is currently adapting its traditional function to also become a supplier of high-value by-products that possess antioxidant, anti-inflammatory and antimicrobial properties. In this study, we evaluated the effect of the fermented defatted *alperujo* (FDA) on the intestinal health of laying hens. The morphology of the duodenal and cecal mucosa, the composition of the intestinal microbiota and the productivity of a batch of laying hens were evaluated after FDA supplementation. At early life stages, significant differences ($p < 0.001$) were observed in duodenal villi height and in crypt depth of both the duodenum and the cecum in the FDA-supplemented group, indicating improved intestinal health in this group. Microbiota composition in the hatchery group supplemented with FDA had a higher abundance of Actinobacteria, Firmicutes and Proteobacteria, and higher bacterial diversity. During the production period, significant differences ($p < 0.05$) were observed in the number of broken eggs from the supplemented group. We conclude that FDA supplementation improves the absorption capacity of the intestinal mucosa and modifies the intestinal microbiota to favor a greater immune response, leading to an increase in egg production.

Keywords: fermented defatted *alperujo* (FDA); olive oil by-products; intestinal health; laying hens; histomorphology; microbiota

1. Introduction

The European Union, particularly the Mediterranean countries, is the main producer of olive oil worldwide [1]. Spain is by far the largest manufacturer: a total of 1,260,000 tons were produced in the 2017–2018 campaign [1,2], with a mean worth of about 1.9 billion euros per year [3]. The olive oil industry has a major economic impact in the producing countries, accounting for around 46 million jobs per production cycle (from seed to final product). This industry also has cultural and environmental implications [3,4].

The centrifugation process used in olive oil production generates by-product wastes, which is of great concern due to their polluting activity and, thus, significant environmental impact [4,5]. Nowadays, the olive oil production process consists of two centrifugation phases, which has resulted

in a reduction of waste products. As a result of this process, a solid by-product called two-phase olive mill waste, sometimes referred to as olive cake or olive pulp but commonly known as *alperujo*, is obtained [6]. The production of approximately one ton of olive oil generates four tons of this by-product [4,6].

Despite its pollutant nature, olive-oil by-products have been described as having antioxidant, anti-inflammatory, antimicrobial and anti-tumoral proprieties [7]. Furthermore, a significant proportion of this by-product is comprised of non-soluble fibers, carbohydrates, high-quality fats and proteins [6]. Some of these bioactive compounds are polyphenols, primarily hydroxytyrosol and tyrosol [8]. These molecules act as antioxidants by inhibiting oxidative reactions thus protecting the cell from oxidative damage and as anti-inflammatories by mediating a reduction in cytokine secretion [8,9]. In addition, the phenols and polyphenols contained in olive oil by-products have antibacterial activity against Gram-positive bacteria like *Staphylococcus aureus* and Gram-negative bacteria like *Campylobacter* spp. [8,10–12].

Consequently, the olive oil industry is currently adapting to also become suppliers of high-value by-products, which may reduce the industry's environmental impact [13]. One of the proposed uses of olive oil by-products is in animal feed supplementation, and several studies have tested their effects in various animal species including poultry [13]. In broiler chickens, feed supplemented with *alperujo* have been demonstrated to improve productive performance parameters [14,15], and enhance redox status in tissues [16,17]. In addition, *alperujo* has been shown to possess anti-coccidial proprieties [18]. In laying hens, dried olive pomace supplementation has been demonstrated to modulate inflammation and cholesterol content in eggs through affecting gene expression [19].

Supplementation with *alperujo*, however, has not been as extensively studied in laying hens. *Alperujo* supplementation does not appear to affect production performance in laying hens [20–24].

Alperujo, like other olive oil by-products, is a fat-rich compound. However, although possess beneficial effects, fat-rich compounds should be limited in the feed formulation to avoid their counterproductive effects in high percentages [14]. For this reason, in this study *alperujo* had first undergone a fermentation process to stabilize the raw material, a hydrolysis to decrease the total fat content, and desiccation and grinding process in order to adapt it to animal feed, obtaining fermented defatted *alperujo* (FDA).

To our knowledge, the impact of *alperujo* or their derived products on intestinal health has not been previously evaluated in laying hens. Intestinal health depends mainly on gut environment and diet [25]. This environment is, in turn, defined by the intestinal mucosa and microbiota, which constitute the main components of the gut barrier. The intestinal mucosa is the most extensive surface in the organism: it absorbs nutrients and is equipped with numerous mechanisms that constitute a first line of defense against potential hazards [26]. Likewise, an adequate microbiota composition seems to limit pathogenic bacterial colonization [27].

Improvement of the intestinal health in laying hens may optimize nutrient absorption and, thus, production performance. It may also induce changes in microbiota that could protect against pathogenic colonization and disease, decreasing the use of antimicrobial agents. Our study assesses changes in the intestinal mucosa and microbiota, as well as productivity, of FDA-supplemented laying hens compared with controls during their productive life in a commercial farm.

2. Results

2.1. Histological Study

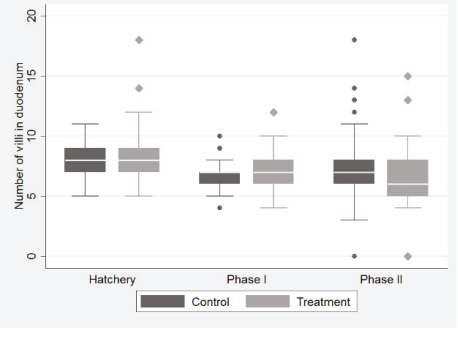
The results of the histomorphometric analysis of the duodenal and cecal mucosa are shown in Table 1 and Figure 1. In hatchery-staged hens, duodenal villi height and crypt depth and cecal crypt number and depth were significantly higher in the FDA-supplemented group ($p < 0.05$). In hens at the production phase I stage, the only significant difference observed was an increase in duodenal villi

number in the FDA-supplemented group. In production phase II hens, only cecal crypt depth was significantly higher in the supplemented hens.

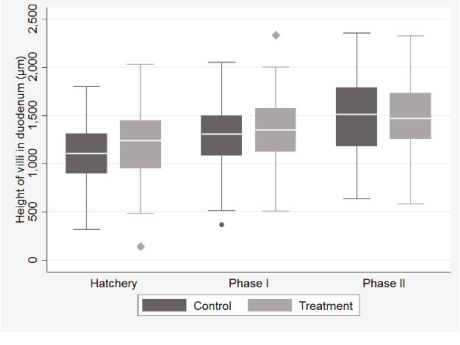
Table 1. Statistical results as mean and interquartile range (IQR) for villi number and height and crypt depth in the duodenum and crypt number and depth in the cecum in control and fermented defatted *alperujo* (FDA)-supplemented hens at three phases of production.

Histomorphometric Parameters	Control Group		FDA-Supplemented Group		*p*-Value [1]
	Mean	[IQR]	Mean	[IQR]	
Hatchery					
Duodenum					
Number of villi	8.00	[2.00]	8.00	[2.00]	0.311
Height of villi (μm)	1109.95	[410.00]	1237.85	[492.10]	<0.001
Depth of crypts (μm)	161.05	[62.53]	188.10	[90.55]	<0.001
Cecum					
Number of crypts	14.00	[5.00]	15.00	[9.00]	0.028
Depth of crypts (μm)	325.65	[374.20]	358.15	[356.60]	<0.001
Production phase I					
Phase I					
Duodenum					
Number of villi	7.00	[1.00]	7.00	[2.00]	<0.001
Height of villi (μm)	1309.27	[411.03]	1353.32	[449.94]	0.157
Depth of crypts (μm)	249.66	[81.83]	255.15	[107.59]	0.135
Cecum					
Number of crypts	16.50	[16.00]	18.00	[17.00]	0.821
Depth of crypts (μm)	359.12	[411.14]	280.74	[440.91]	0.150
Production phase II					
Phase II					
Duodenum					
Number of villi	7.00	[2.00]	6.00	[3.00]	0.230
Height of villi (μm)	1511.99	[605.57]	1471.92	[476.69]	0.537
Depth of crypts (μm)	272.65	[138.47]	268.46	[102.79]	0.160
Cecum					
Number of crypts	14.00	[16.00]	14.00	[12.00]	0.883
Depth of crypts (μm)	402.68	[606.61]	518.50	[576.58]	<0.001

[1] The Mann-Whitney test was used to assess significant differences ($p < 0.05$) between supplemented ($n = 43$) and control animals ($n = 47$).

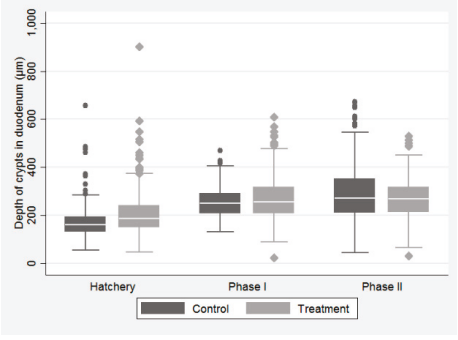


(**A**)

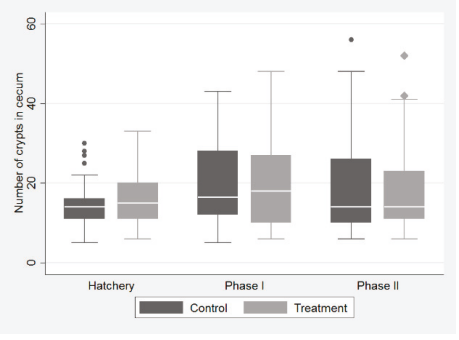


(**B**)

Figure 1. *Cont.*



(**C**)



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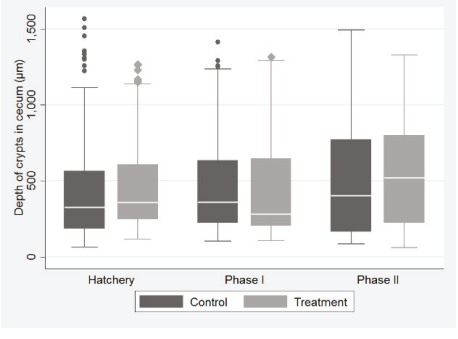


(**E**)

Figure 1. Graphs showing changes in histomorphometric parameters of the duodenal and the cecal mucosa from hatchery to phases I and II: (**A**) number of villi in the duodenum was significantly increased in phase I in the FDA-supplemented group (treatment); (**B**) height of villi in the duodenum was significantly increased in the hatchery in the FDA-supplemented group (treatment); (**C**) depth of crypts in the duodenum was significantly increased in the hatchery in the FDA-supplemented group (treatment); (**D**) number of crypts in the cecum: no significant differences were observed between the control and treatment group; (**E**) depth of crypts in the cecum was significantly increased in the hatchery and phase I in the FDA-supplemented group (treatment).

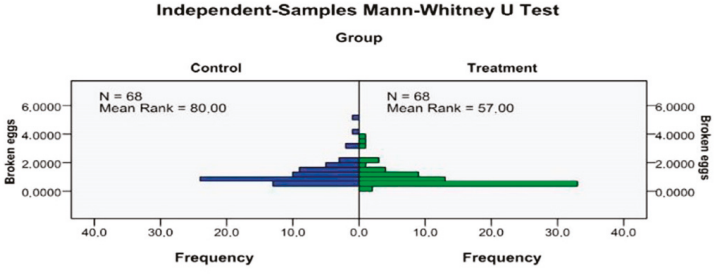
2.2. Production Performance

The effects of FDA on production performance of laying hens are shown in Table 2. Hen production percentages, total eggs (in terms of number and weight) and feed consumption improved with FDA supplementation; however, the differences observed between the two groups was not significant ($p > 0.05$). Mortality rate was higher in the treatment group; however, the differences observed between the two groups were not significant ($p > 0.05$). The economic impact of the difference in production performance between the two groups was a 1.7% increase in egg sale profits and a 1.5% decrease in feed costs for the treated group. The percentage of cracked or broken eggs eliminated from production was significantly lower in the treated group ($p < 0.05$) (Figure 2).

Table 2. Production performance of control and FDA-supplemented hens.

Productive Parameter	Control Group	Treatment Group	*p*-Value [1]
Mortality (%)	0.15	0.16	0.299
Laying (%)	78.00	79.00	0.970
Feed/hens (g)	115.37	113.37	0.124
Egg weight (g)	62.58	62.59	0.720
Egg mass (g/d)	48.04	49.28	0.730
Extra-large eggs (%)	5.20	5.40	0.989
Large eggs (%)	44.25	43.94	0.467
Medium eggs (%)	39.04	40.39	0.917
Small eggs (%)	5.8	5.4	0.084
Dirty eggs (%)	2.3	2.1	0.424
Broken eggs (%)	3.47	2.83	0.001
Total eggs (number)	21,526,722	21,892,058	0.808
Total eggs (Kg)	1,342,571.52	1,363,916.15	0.931
CI (Conversion Index)	0.37	0.35	-

[1] The Mann-Whitney test was used to assess significant differences ($p < 0.05$) between supplemented ($n = 43$) and control animals ($n = 47$).



Total N	136
Mann-Whitney U	1,530.000
Wilcoxon W	3,876.000
Test Statistic	1,530.000
Standard Error	229.733
Standardized Test Statistic	-3.404
Asymptotic Sig. (2-sided test)	.001

Figure 2. According to the Mann-Whitney U test, there is a statistically significant difference based on the *p*-value (Asymptotic Significance (two-sided test) = 0.001). The FDA-supplemented group (Mean Rank of 57.00) reported a lower number of broken eggs compared to control group (Mean Rank of 80.00).

2.3. Metagenomics

Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria were the main bacterial phyla identified in all samples (Figure 3). The other phyla detected at lower levels were grouped together and classified as "Rest". The relative abundance (RA) of Bacteroidetes (43.72 versus 43.42% in treated versus control, respectively), Actinobacteria (10.72 versus 6.82%) and Proteobacteria (4.30 versus 2.58%) were higher in the treated group, while the relative abundance of Firmicutes was higher in the control group (38.67 versus 43.67%). Hens at the hatchery stage showed a higher relative abundance of Actinobacteria (28.39 versus 14.31%), Firmicutes (45.92 versus 39%) and Proteobacteria (8.29 versus 1.64%) in the

treated group compared to the control one, whereas the relative abundance of Bacteroidetes was higher in the latter (17.37 versus 44.60%). In phase-I hens, only Actinobacteria (5.72 versus 4.58%) and Bacteroidetes (50.19 versus 16.86%) were more abundant in the treated group. In phase-II hens, Bacteroidetes (55.29 versus 49.47%) was still more abundant in the treated compared to control group, as was Proteobacteria (2.25 versus 1.98%).

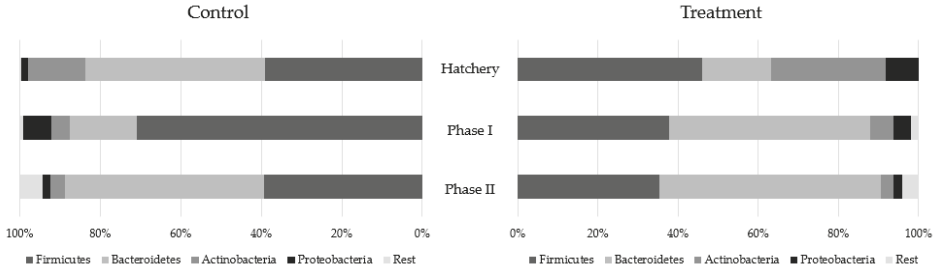


Figure 3. Metagenomic results. Phyla distribution by groups over time. Hens showed a higher relative abundance (RA) of Actinobacteria, Firmicutes and Proteobacteria in FDA-supplemented group (treatment) compared to control in hatchery. In phase I, the RA of Actinobacteria and Bacteroidetes was higher in the FDA group. In phase II, the RA of Bacteroidetes and Proteobacteria was higher in the treated group.

3. Discussion

Olive oil industry wastes have emerged as highly valuable products due to their potential beneficial properties such as being antimicrobial and anti-inflammatory [7]. One modified by-product, the fermented defatted *alperujo* (FDA), contains molecules like polyphenols, which make it interesting as a potential supplement in animal feed [8], as demonstrated by the capacity of polyphenols-containing olive oil by-products to modulate gene expression in vivo [19]. FDA supplementation has not been previously used for animal feed, although *alperujo* supplementation has been tested in several species with the objective of improving performance or exploiting the product's antioxidant capacity [28]. Iannaccone et al. [19] demonstrated that olive oil pomace supplementation in laying hens affects gene expression and thus enhance oxidative status and improves inflammatory response, which suggests that *alperujo* may contribute to hens welfare and health. However, the effects of this natural compound on intestinal health have not been studied extensively, at least in laying hens.

Nutrient absorption and innate immune response depend on the mucosal structure of the intestine. Therefore, we performed a histomorphometric analysis to evaluate the effects of FDA on the morphology of the intestinal mucosa. Although no previous studies have focused on the impact of olive oil by-products on the intestinal morphology of laying hens, several have assessed the effects of other plant-derived compounds, whose fiber and bioactive compounds content has shown to improve intestinal morphology in broilers, particularly after oligosaccharides supplementation [29]. Therefore, the high fiber, high-quality fat and phenolic content of FDA may enhance intestinal health in supplemented hens.

The majority of these studies focused on the small intestine, mainly the jejunum mucosa, with only a few evaluating the duodenum [30–37], despite its very high absorption potential [38]. In one case, supplementation with bamboo vinegar or polyunsaturated fatty acid from an extruded flax product resulted in a significant increase in duodenal villi height in aged hens, suggesting that diet composition affects the duodenal mucosal structure [29,36]. In another study, an increase in duodenal crypt depth was observed in 33-week-old hens supplemented with rapeseed expeller cake [34]. In these studies, changes induced by different substances on the intestinal mucosa were evaluated during the productive phase. In contrast, in our study, the impact of FDA was assessed during the production cycle of laying hens, from hatchery to late phases. The significant increase in duodenal villi height that we observed

in supplemented pullets supposes a concomitant increase in absorptive area, as has been proposed by other authors [36], which, in turn, leads to increased productivity. Changes in intestinal morphology during the initial phases, which are thought to influence digestion and performance, are critical for later intestinal functions during the productive phase [39].

The large intestine, particularly the cecum, has been rarely assessed in intestinal morphometric studies. In a recent study on the effects of essential oils and organic acids, no significant differences were reported in these segments [31]. In our study, however, cecal crypt depth proved to be a relevant parameter in our assessment of the impact of FDA on mucosal morphology. The increase in cecal crypt depth in pullets and aged hens appears to not only improve nutrient absorption but also influence a non-specific immune response by better responding to potential superficial epithelial damage. Due to the function of intestinal crypts in epithelial renewal [38,40], an increase in crypt depth could favor fermentation, digestion and water absorption capacity, prevent the emergence of disease in pullets and extend productive life in aged hens.

The goals of livestock feed supplementation are species-dependent. However, for all species, production performance must be maintained at an acceptable level [12], and egg production is not an exception. Our analysis of production performance indicates that FDA-supplemented hens performed better than control-fed hens. Feed is one of the most important costs in animal production [25]; therefore, the increase in profit from the sale of eggs from the supplemented group may be, in part, attributed to a decrease in feed consumption. To our knowledge, shell hardness has not been previously correlated with any olive oil by-product feed supplement. The intestine has a central role on calcium absorption, and thus, influences calcium metabolism [41]. The increase of villi height and crypt depth in the intestine increases duodenal and cecal absorption capacity, and therefore, the positive impact on eggshell hardness observed here may be in relation to the augmented mineral and absorption in the intestine as previously suggested [30,42]. In addition, other authors have shown that oligosaccharides present in dietary fiber may increase mineral uptake, and thus, the high fiber content of FDA may be in relation with an improvement of calcium absorption in the intestine [29]. The smaller percentage of broken eggs observed in the supplemented group may have also contributed to the economic increase. However, further studies are needed to determine whether FDA directly or indirectly improves shell hardness.

It is important to study the molecular and metabolic mechanisms behind the productive parameters in order to correctly manage animal feed [19]. In poultry, as in other species, the intestinal microbiota collectively acts as a metabolic organ, facilitating nutrient absorption and the immune response against pathogens [31,43]. Microbiota is determined by host genes and the environment, with diet being one of the most important factors. Modifications in dietary composition may, therefore, induce changes in microbiota [31]. Microbiota composition in poultry, especially in laying hens, has become an important area of research in veterinary medicine [27]. Although some studies have focused on microbiota variation in laying hens at different phases [44–47] and under diverse production systems [47,48], analyses of microbiota in laying hens fed with plant-derived supplements is limited [49,50]. Given the main role of microbiota in fermentation and digestive processes in laying hens [47], we focused on its composition in the cecum in our analyses.

According to the literature, cecal microbiota variations in laying hens are expected during the production cycle [44,47]. Consistent with this, we observed phyla fluctuations in our analyses. The metagenomics results showed the predominance of Bacteroidetes and Firmicutes, as has been previously reported in other studies [27,40,44,46,47]. In addition, our results suggest a decrease in bacterial diversity over time in both groups. For instance, we observed a progressive and proportional increase in Bacteroidetes compared to the other phyla in treated adult laying hens. Similar results have been reported by other studies [44,46]. Decreased microbial diversity has been related to a reduction in the production of short chain fatty acids, which leads to reduced intestinal barrier function [48]. According to some studies and in contrast to adult hens, Firmicutes abundance in pullets is expected to be higher than that of Bacteroidetes [44,46]. Our results are in agreement with these previous

studies. Moreover, we observed that the relative abundance of Firmicutes was higher than that of Bacteroidetes in treated animals at the early hatchery phase, suggesting greater bacterial diversity in animals supplemented with FDA. Finally, we observed that Actinobacteria comprised a significant proportion of the microbiota at the hatchery stage but progressively decrease over time. Our results contrast with other studies in which Actinobacteria was considered a minority phylum, accounting for less than 1% of the total bacterial community [44,49]. In our study, the proportion of Actinobacteria was higher in treated animals, up to 25 weeks-old, compared with the control group. Similarly, the abundance of Proteobacteria, which progressively decreased over time, was present at higher levels in the treated group during the first post-hatching weeks. Although the relative abundance of Proteobacteria was higher in pullets, it was far from the 50% reported in the cecum of one-week-old laying hens [44].

4. Materials and Methods

4.1. Ethical Approval

This project has been carried out in accordance with animal welfare standards for the species with ethical approval from the Complutense University of Madrid and the Community of Madrid (PROEX 152/19).

4.2. Animals and Rearing Conditions

The study was performed in a commercial farm using a whole batch of Hy-Line 2015 laying hens (122,250 animals in total). The animals were randomly divided into two groups, control and treated, and raised from the hatchery stage to the end of the production cycle. The hens were kept in an intensive housing system in the same place and under the same controlled environmental conditions (24–32 °C, depending on the phase, and 50–70% humidity). Feed and water were supplied ad libitum.

4.3. Experimental Diets

Both groups were fed the same commercial formulation, according to the production phase, as routinely used at the farm. Feed of the treated group was supplemented with fermented defatted *alperujo* (FDA), which had first undergone a controlled anaerobic bacterial fermentation and was then defatted with chemical solvents (fat hydrolysis). Finally, it was desiccated at 80 °C in a low oxygen content atmosphere and then suffered a grinding process. FDA composition was determined by Labocor S.L. (Colmenar Viejo, Spain) (Table 3).

Table 3. Fermented defatted *alperujo* (FDA) composition.

Determination	Results
Moisture 103° (%w.w.)	12.2
Crude protein (Kjeldahl) (%w.w.)	6.4
Brute fat (%w.w.)	3.0
Ash content (%w.w.)	7.7
Lignin (%w.w.)	23.3
Acid detergent fiber (%w.w.)	39.2
Neutral detergent fiber (%w.w.)	49.3
Tannins (%w.w.)	0.06
Oleic acidity index (%w.w.)	46.1
Peroxide value (%w.w.)	7.9
Total polyphenols (meq/kg)	0.89
Crude fiber (%w.w.)	27.7

4.4. Production Performance

Productive parameters were registered for all production phases using the business management enterprise resource planning software Navision from Microsoft. The conversion rate was expressed as kg of feed consumed in relation to the number of eggs produced. Egg production and mortality were recorded daily; feed consumption was measured weekly. Eggs were collected over a 24 h period and weighed for determination of egg weight and grade.

4.5. Samplings and Necropsy

A total of 11 samplings across the three phases of the production cycle were performed during the study: hatchery (1–16 weeks old, four samplings), phase I (laying hens until peak laying, 16–23 weeks old, two samplings) and phase II (from 24-weeks old to the end of production, five samplings).

For all samplings, 15 hens of each group were sacrificed by the gas-stunning method. A complete necropsy was carried out for each animal. During the necropsy, a gross survey was performed, and duodenal and cecal samples were collected and fixed in a 4% formaldehyde-buffered solution for 48 h (Panreac Química SLU, Barcelona, Spain). In addition, fresh cecal feces were collected from each animal and preserved at −80 °C for the metagenomics analysis.

4.6. Histology

After fixation, intestinal tissues were dehydrated through an ethanol series and xylene substitute (Citadel 2000 Tissue Processor, Thermo Fisher Scientific, Waltham, MA, USA), then embedded in synthetic paraffin. After paraffin block formation (Histo Star Embedding Workstation, Thermo Fisher Scientific), 4-µm sections were cut (Finesse ME+ Microtome, Thermo Fisher Scientific), stained with hematoxylin and eosin (Panreac Química SLU) and mounted and examined under a light microscope (Leica, Wetzlar, Germany).

A histomorphometric study was performed using an image analyzer (Leica Application Suite, Leica), which measured different parameters of the duodenum and the cecum in each animal in five fields at 40× magnification. In each case, the number of duodenal villi and duodenal and cecal crypts were counted. Duodenal villi height was measured from the top of the villus to the villus–crypt junction; duodenal and cecal crypt depth was measured from the villus–crypt junction to the muscularis mucosae. A minimum of seven well-oriented villi and 14 crypts were measured from different sections of each hen.

4.7. Metagenomics

DNA was isolated from cecal samples using the QIAamp DNA Stool Mini Kit (Qiagen NV, Hilden, Germany). The next-generation sequencing and bioinformatics analyses of the bacterial 16S rRNA gene were performed by Stab Vida (Caparica, Portugal) and Era7 Bioinformatics (Granada, Spain). Regions V3 and V4 of 16S rRNA were sequenced on the Illumina Miseq platform using 300 bp paired-end sequencing.

4.8. Statistical Analysis

IBM SPSS Statistics Software (IBM; Armonk, NY, USA) was used for statistical analysis. Differences in production performance and histomorphological parameters between control and treated groups were assessed using Mann-Whitney test and statistical significance was considered at $p < 0.05$.

5. Conclusions

Dietary fermented defatted *alperujo* (FDA) supplementation in laying hens significantly improved duodenal villi height in pullets, which may enhance intestinal function during their productive life, as suggested by the significant decrease of broken eggs eliminated from production. Additionally, the increase of cecal crypt depth in pullets and aged hens appears to improves nutrient absorption.

It may also influence non-specific immune responses by being able to better respond to potential harmful events, contributing to the intestinal health of laying hens. Our findings also suggest that diet composition can modulate intestinal microbiota at early life stages. Specifically, FDA supplementation seems to increase intestinal bacterial diversity by increasing the relative abundances of Firmicutes and Proteobacteria. Establishing a diverse microbiota early in life may enhance intestinal health by providing metabolic substances, improving immune response and competing with pathogenic bacteria, thus potentially reducing antimicrobial usage.

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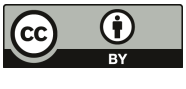

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Article

Short Chain Fatty Acids Commonly Produced by Gut Microbiota Influence *Salmonella enterica* Motility, Biofilm Formation, and Gene Expression

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Abstract: Short chain fatty acids (SCFAs) are commonly produced by healthy gut microbiota and they have a protective role against enteric pathogens. SCFAs also have direct antimicrobial activity against bacterial pathogens by diffusion across the bacterial membrane and reduction of intracellular pH. Due to this antimicrobial activity, SCFAs have promising applications in human health and food safety. In this study, the minimum inhibitory concentrations (MICs) of four SCFAs (acetic acid, butyric acid, propionic acid, and valeric acid) in *Salmonella* strains isolated from poultry were determined. The effect of subinhibitory concentrations of SCFAs in *Salmonella* biofilm formation, motility, and gene expression was also evaluated. Butyric acid, propionic acid, and valeric acid showed a MIC of 3750 μg/mL in all strains tested, while the MIC of acetic acid was between 1875 and 3750 μg/mL. Subinhibitory concentrations of SCFAs significantly ($p < 0.05$) reduced the motility of all *Salmonella* strains, especially in the presence of acetic acid. Biofilm formation was also significantly ($p < 0.05$) lower in the presence of SCFAs in some of the *Salmonella* strains. *Salmonella* strain. *Salmonella* Typhimurium T7 showed significant ($p < 0.05$) upregulation of important virulence genes, such as *invA* and *hilA*, especially in the presence of butyric acid. Therefore, SCFAs are promising substances for the inhibition of the growth of foodborne pathogens. However, it is important to avoid the use of subinhibitory concentrations that could increase the virulence of foodborne pathogen *Salmonella*.

Keywords: *Salmonella*; short chain fatty acids; antimicrobial activity; biofilm; motility; gene expression

1. Introduction

Short chain fatty acids (SCFAs) are end metabolites produced by microbial fermentation of undigested carbohydrates and dietary fibers. Butyrate, acetate, and propionate are the main SCFAs, but others, such as lactate and valerate, are also produced by microbiota [1–3]. SCFAs have important roles in human gut homeostasis by exerting several effects on the host and its own microbiota. SCFAs are used as a source of energy by intestinal epithelial cells. They also modulate the absorption of electrolytes and increase the production of anti-inflammatory cytokines. SCFAs also reduce the production of molecules that act as pro-inflammatory substances, such as nitrous oxide, interleukins, and tumor necrosis factor. SCFAs also have a protective effect against bacterial pathogens by maintaining the integrity of the epithelial barrier [4,5]. In addition, it has been observed that SCFAs induce the production of antimicrobial peptides by enterocytes [6]. In the same way, it has been observed that macrophages that differentiate in the presence of butyrate show increased antimicrobial activity even in the absence of an increased inflammatory cytokine response [6].

In adequate concentrations, SCFAs also have direct antimicrobial activity against pathogenic bacteria. SCFAs can reduce microbial growth by modifying the intracellular pH and the metabolism.

At lower pH, SCFAs are commonly present in nonionized forms that can diffuse across the bacterial membrane into the bacterial cytoplasm. Once in the cytoplasm, SCFAs dissociate, increasing the anion and proton concentrations and lowering the intracellular pH [7–9].

The production of adequate and balanced SCFAs by a healthy gut microbiota is an important factor that prevents infection by common foodborne pathogens [10]. It was found that *Bacteriodes* spp. mediates resistance to *Salmonella* colonization by producing propionate [9]. Diverse studies have observed total SFCAs concentrations ranging between 60 and 85 mM with levels of acetate between 40 and 50 mM, propionate around 15 mM, and butyrate around 10 mM [11,12]. A dysregulation of SCFAs levels can facilitate the colonization of intestine by pathogens. In this sense, decreased concentrations of butyrate cause upregulation of virulence genes in enterohemorrhagic *Escherichia coli* (EHEC), and different spatial gradients of SCFAs regulate the expression of virulence and commensal genes in *Campylobacter jejuni* [13,14]. Propionate decreases the expression of *Salmonella* genes located in *Salmonella* Pathogenicity Island 1 (SPI-1) [15]. In addition, pre-incubation of *Salmonella enteritidis* with propionate and butyrate results in a reduction in host cell invasion [16]. It is noteworthy that SCFA concentrations similar to those found in the distal ileum cause upregulation of *Salmonella* virulence genes, while concentrations similar to those found in the colon have the opposite effect [17]. Therefore, the protective and antimicrobial effects of SCFAs are concentration dependent.

Understanding the inhibitory effects of SCFAs on enteric pathogens is not only important from a gut health point-of-view, but this knowledge can also be important from a food safety point-of-view. In the last years, different researchers have been evaluating alternatives to inhibit the growth of foodborne pathogens in the food industry as antimicrobial peptides [18,19]. In the same way, SCFAs can be added to food and feed as preservatives, avoiding the growth of bacterial pathogens. The SCFAs ingested through can also have positive effects in gut balance with inhibitory effects in enteric pathogens. For this purpose, the aim of this study was to determine the minimum inhibitory and biocidal concentrations of four SCFAs (acetic acid, butyric acid, propionic acid, and valeric acid) in *Salmonella* and to determine the effect of subinhibitory concentrations of these SCFAs in biofilm formation, motility, and gene expression. As poultry products are mainly responsible of human salmonellosis [20], the authors decided to use a total of 12 *Salmonella* strains isolated from poultry. These *Salmonella* strains also belonged to seven different serotypes and two *Salmonella* subspecies in order to observe if they have a similar response to the presence of SCFAs.

2. Results and Discussion

2.1. Antimicrobial Activity of Short Chain Fatty Acids

Until now, there have been limited studies evaluating the MIC and minimum biocidal concentration (MBC) values of SCFAs in food-borne pathogens [21]. In this study, the MICs of the four SCFAs were determined. Butyric acid, propionic acid, and valeric acid had the same MIC value (3750 μg/mL) in all *Salmonella* strains tested in this study. In addition, all strains showed the same MBC (3750 μg/mL) with all the SFCAs tested. However, in the case of acetic acid, five of the twelve strains had a MIC value of 1875 μg/mL, while the other strains had the same MIC as those with the others SCFAs (3750 μg/mL). The results observed with acetic acid were similar to those observed in previous work where the MIC_{50} was 1650 μg/mL and the MIC_{90} was 3280 μg/mL in 88 multidrug resistant *Salmonella* isolates [22]. In the same way, another study that evaluated the antimicrobial activity of acetic acid in different pathogens, such as *E. coli*, *Staphylococcus aureus*, or *Acinetobacter baumannii*, found that the MIC values were between 0.16% and 0.31% [23]. However, another study that determined the antimicrobial activity of acetic acid in *E. coli* and *Salmonella* sp. strains found MIC values of 1.5% and 1%, respectively [24]. *Campylobacter coli* strains isolated from pigs had MIC values of 2048 μg/mL in most of the strains tested in the presence of butyric acid and propionic acid [25], lower than the MIC values observed in this study. Conversely, in others works with *Salmonella* Typhimurium ATCC 14028 or *Vibrio harveyi*, the inhibition values and propionic acid of butyric acid, respectively, were between 500 and 100 μg/mL [26,27].

The antimicrobial activity of SCFAs is due to their diffusion across the bacterial membrane into the bacterial cytoplasm, modifying the intracellular pH and the metabolism [7,8].

2.2. *Effect of Short Chain Fatty Acids in Salmonella enterica Motility*

Once the MICs were calculated, the effect of SCFAs on *Salmonella* motility was determined. For this purpose, the highest subinhibitory concentrations of the SCFAs were used. The motility was significantly reduced in the presence of SCFAs in comparison to the control semisolid agar in all strains tested in this study (Table 1). There were also significant differences ($p < 0.05$) between the different SCFAs included in the study. The higher reduction in motility was observed in the presence of acetic acid, while valeric acid caused a lower reduction in motility in comparison to the control agar. In accordance with this study, a previous study also observed that organic acids, including acetic acid, decreased the motility of *E. coli* and *Salmonella* strains isolated from fresh fruit and vegetables [24]. It was observed that *Salmonella* Typhimurium ATCC 14,028 motility decreased as the subinhibitory concentrations of propionic acid increased [27]. Deepening in these effects, it has been observed that the fermentation products of *Clostridium ramosum* and SCFAs reduce the motility in enterohemorrhagic *E. coli* (EHEC), disturb the flagellar rotation, and the flagella length is lower in comparison to control conditions [28]. However, the decrease in intracellular pH could be responsible for the slow flagella motor rotation and the reduced motility [29].

Table 1. Influence of the four short chain fatty acids tested in this study (acetic acid, propionic acid, butyric acid, and valeric acid) in the motility of *Salmonella* strains at 37 °C expressed in millimeters. Different letters in the same row represent statistically significant differences ($p < 0.05$) between the different agar media.

		Motility (mm)				
Strain	**Code**	**Control (n = 3) Mean ± SD**	**Acetic Acid (n = 3) Mean ± SD**	**Butyric Acid (n = 3) Mean ± SD**	**Propionic Acid (n = 3) Mean ± SD**	**Valeric Acid (n = 3) Mean ± SD**
S. enterica subsp. *arizonae*	AZ3	16.00 ± 1.73 ^{a}	2.33 ± 1.53 ^{c}	6.00 ± 1.73 ^{b,c}	5.00 ± 1.00 ^{b,c}	8.67 ± 1.15 ^{b}
S. enterica subsp. *arizonae*	AZ26	17.00 ± 2.00 ^{a}	2.00 ± 1.00 ^{c}	5.33 ± 1.53 ^{b,c}	5.67 ± 2.10 ^{b,c}	8.00 ± 2.00 ^{b}
S. Bredeney	BR1	15.33 ± 1.15 ^{a}	2.00 ± 1.73 ^{c}	4.67 ± 1.15 ^{c}	3.00 ± 1.00 ^{c}	8.33 ± 1.53 ^{b}
S. Infantis	I18	15.33 ± 2.31 ^{a}	1.67 ± 1.15 ^{c}	3.67 ± 1.15 ^{c}	3.67 ± 0.57 ^{c}	8.67 ± 1.15 ^{b}
S. Infantis	I4	15.33 ± 1.53 ^{a}	3.00 ± 1.73 ^{c}	5.67 ± 1.15 ^{b,c}	2.67 ± 1.53 ^{c}	7.33 ± 2.08 ^{b}
S. Isangi	IG1	15.67 ± 2.08 ^{a}	1.67 ± 0.58 ^{c}	4.00 ± 1.00 ^{c}	4.67 ± 1.15 ^{c}	9.00 ± 1.73 ^{b}
S. Montevideo	M1	20.00 ± 2.00 ^{a}	1.33 ± 0.58 ^{c}	6.33 ± 2.08 ^{b}	5.33 ± 0.58 ^{b}	8.33 ± 0.58 ^{b}
S. Newport	N6	18.33 ± 1.53 ^{a}	2.67 ± 1.53 ^{d}	7.00 ± 1.73 ^{b,c}	4.00 ± 2.00 ^{c,d}	10.00 ± 2.00 ^{b}
S. Typhimurium	T1	20.00 ± 1.00 ^{a}	3.67 ± 1.15 ^{c}	4.67 ± 1.53 ^{c}	6.00 ± 1.73 ^{c}	10.00 ± 1.00 ^{b}
S. Typhimurium	T3	17.77 ± 2.52 ^{a}	2.67 ± 0.58 ^{c}	4.33 ± 1.53 ^{b,c}	4.00 ± 2.65 ^{b,c}	8.00 ± 1.00 ^{b}
S. Typhimurium	T7	19.33 ± 2.08 ^{a}	3.33 ± 1.15 ^{c}	5.33 ± 1.53 ^{c}	3.33 ± 1.15 ^{c}	10.67 ± 1.53 ^{b}
S. Typhimurium	T23	19.67 ± 2.10 ^{a}	2.33 ± 2.31 ^{c}	6.00 ± 1.00 ^{b,c}	3.67 ± 2.10 ^{c}	9.33 ± 2.52 ^{b}
Total	-	17.36 ± 2.41 ^{a}	2.43 ± 1.36 ^{d}	4.12 ± 1.60 ^{c}	4.24 ± 1.69 ^{c}	8.45 ± 1.92 ^{b}

2.3. Effect of Short Chain Fatty Acids on Biofilm Formation

The presence of subinhibitory concentrations of SCFAs influence biofilm formation in some of the *Salmonella* strains included in this study (Figure 1). There were no significant differences in the reduction of biofilm formation between the different SCFAs tested in eight of the nine strains. However, in *Salmonella* Typhimurium T23, the reduction in biofilm formation caused by propionic acid was significantly lower ($p < 0.05$) than the reduction caused by acetic acid and butyric acid. It is also worth noting that the strains in which biofilm formation was not influenced by the SFCAs were *Salmonella* strains with a low ability to produce a biofilm at 37 °C.

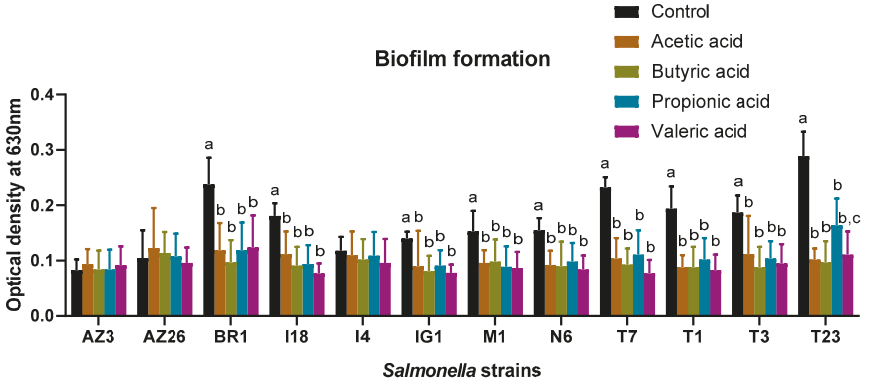

Figure 1. Influence of the four short chain fatty acids tested in this study (acetic acid, propionic acid, butyric acid, and valeric acid) in the biofilm formation of *Salmonella* strains at 37 °C expressed as optical density. Results are expressed as the mean of three different experiments ($n = 3$) and error bars represent the standard deviation. Different letters in the same *Salmonella* strain represent statistically significant differences ($p < 0.05$) between the different growth media.

Consistent with the above-mentioned, previous studies have also observed a reduction in biofilm formation by *Salmonella* strains under the presence of subinhibitory concentrations of acetic acid [23,24,27]. The presence of acetic acid causes inhibition of extracellular polysaccharides production in foodborne pathogens such as *E. coli* or *S.* Typhimurium, although to a lesser extent in the latter [24]. Acetic acid also showed anti-quorum sensing activity in *E. coli* and *S.* Typhimurium [24]. Quorum sensing is a cell-to-cell communication mechanism that has great importance in biofilm formation, and inhibition of this mechanism can result in lower biofilm formation [30]. Flagella also have important roles in the initial adhesion of bacterial cells to a surface in biofilm formation [31]. In this sense, it has been observed that propionic acid causes changes in type 1 fimbriae, with a brittle and broken appearance [27]. The authors hypothesize that these effects of SCFAs, in combination with the short flagella that can be synthetized in the presence of intracellular acid [29], could be responsible for the lower biofilm formation observed. The results of this study should be considered as preliminary and only exploratory in nature and might not have external validity. There are some biofilm formation assays that are more labor-intensive and more accurate than using 96-well microplates in recovering the sessile cells using sonication or the beads/vortex method [32,33]. Therefore, the preliminary results of this study should be confirmed in the future by using more accurate assays and including different surfaces of great importance in the food industry, such as stainless steel.

2.4. Effect of Short Chain Fatty Acids on Salmonella enterica Gene Expression

The results of this and previous studies have demonstrated that SCFAs are able to inhibit the growth of foodborne pathogens such as *Salmonella* [9,22,24,27]. However, previous studies also

observed that subinhibitory concentrations of SCFAs can induce the expression of virulence genes in *E. coli* [14], *Salmonella* [17], and *C. jejuni* [13]. In this study, the expression of 14 genes related to virulence, stress response, and carbon storage was determined in two *Salmonella* strains (*S.* Typhimurium T7 and *S.* Infantis I4).

The expression profiles of the two strains were different. *Salmonella* Typhimurium T7 showed overexpression of all genes tested in this study in presence of SCFAs (Figure 2), especially acetic acid and butyric acid, in comparison to control growth media. Butyric acid caused significant upregulation of all genes evaluated in this study in comparison to propionic acid and valeric acid. For gene *invA*, the upregulation in the presence of butyric acid was also significantly higher than in the presence of the other three SCFAs. Only the transcription of *rpoS* was not influenced by SCFAs. *Salmonella* Infantis I4 (Figure 3) showed different expression profiles according to the gene and the SFCAs. It is remarkable that nine genes showed no significant differences in their regulation between the different SCFAs and in comparison to the control sample. While gene *fliC* was significantly upregulated in the presence of valeric acid, gene *invA* was significantly downregulated in comparison to the other SCFAs. The expression of *hilA* and *spiA* was also significantly upregulated in the presence of acetic acid and butyric acid.

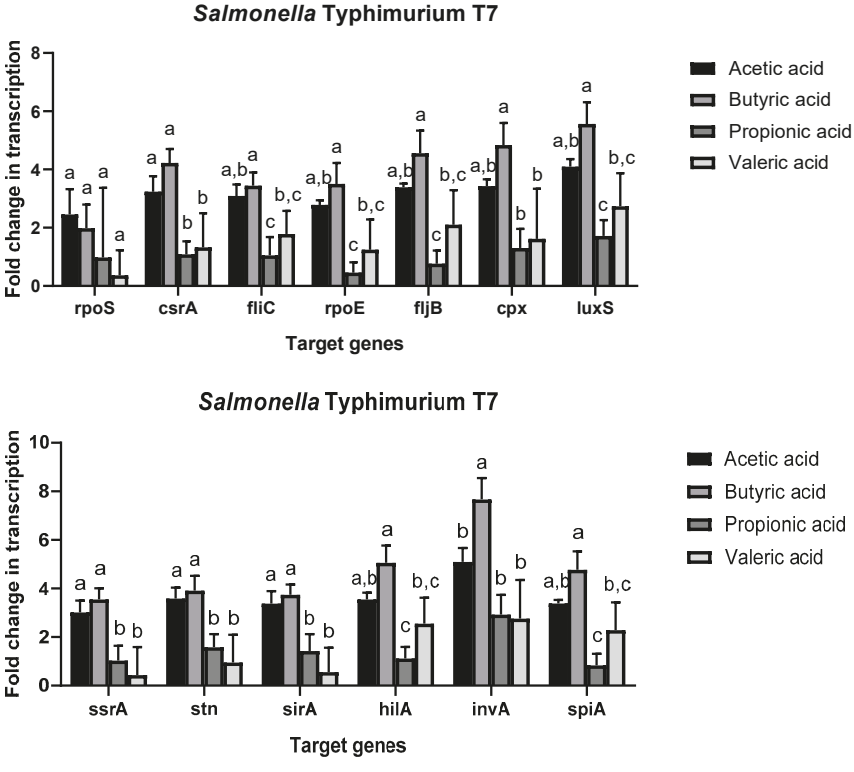

Figure 2. Fold change normalized to control gene *16s rRNA* in the transcription of genes related to virulence and stress in the presence of short chain fatty acids in comparison to control TSB growth media in *Salmonella* Typhimurium T7. Only target genes with different letters between the different growth media present statistically significant differences after analysis of variance (one-way ANOVA) and the Tukey's honestly significant difference test ($p < 0.05$).

Previous studies have shown that acetate can be used by *Salmonella* as a signal for invasion gene expression by upregulation of genes such as *hilA* and *sirA* [17]. On the other side, it has been observed that propionate downregulates invasion genes located in SPI-1 [15]. The results of this study showed that upregulation of *hilA* or *spiA* genes caused by propionate was lower in the presence of propionic acid than in the presence of other SCFAs, such as acetic acid or butyric acid. In this regard, it is worth mentioning that most of the genes tested in this study were significantly upregulated in *S.* Typhimurium T7 in the presence of acetic acid and especially butyric acid (Figure 2). In concordance with these results, it was observed that enterohemorrhagic *E. coli* increased the expression of virulence genes in the presence of subinhibitory concentrations of SFCAs and especially in the presence of butyric acid [14]. Consequently, subinhibitory concentrations of SCFAs can produce an overexpression of virulence genes in common enteric pathogens, such as *Salmonella* and *E. coli*, resulting in increased virulence of these pathogens.

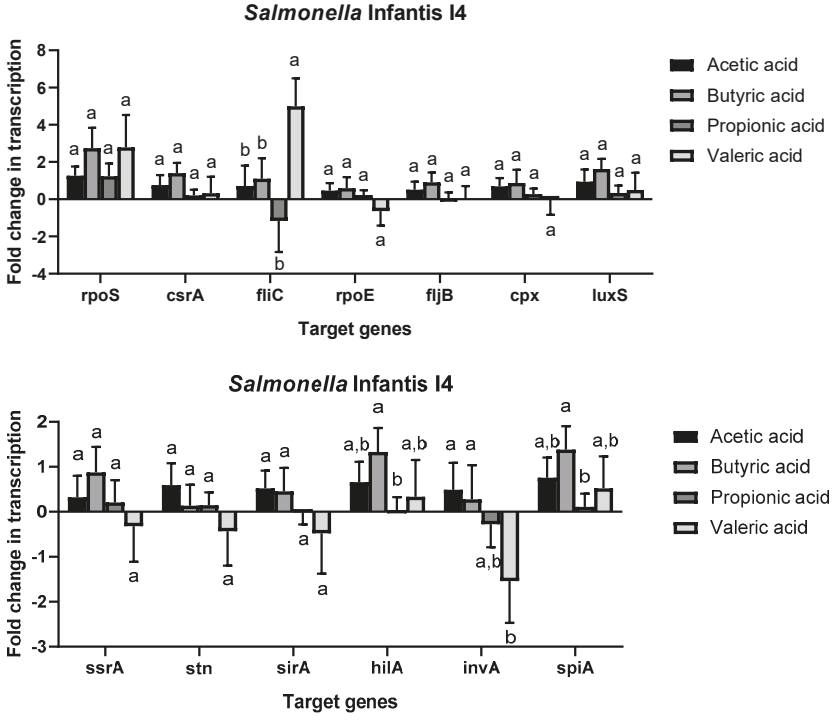

Figure 3. Fold change normalized to control gene *16s rRNA* in the transcription of genes related to virulence and stress in presence of short chain fatty acids in comparison to control TSB growth media in *Salmonella* Infantis I4. Only target genes with different letters between the different growth media present statistically significant differences after analysis of variance (one-way ANOVA) and the Tukey's honestly significant difference test ($p < 0.05$).

In previous work, organic acids, such as acetic acid, showed anti-quorum sensing activity in *Salmonella* and *E. coli* [24]. Quorum sensing is an important mechanism that regulates the expression of virulence determinants to effectively colonize the host [34]. In this study, the expression of *luxS*, which codifies the Autoinducer-2 (AI-2), was significantly upregulated in *S.* Typhimurium T7 in the presence of SCFAs. The authors consider that SCFAs can inhibit the quorum sensing mechanism by blocking AI-2 instead of downregulating the genes implicated in the production of the signal molecule. Finally, it is necessary to re-emphasize the different transcriptional profiles observed in

S. Typhimurium T7 and *S.* Infantis I4. This is due to the strain variability in the behavior of foodborne pathogens [35]. It is important to consider that *S.* Typhimurium is one of the most pathogenic serotypes of *S. enterica* [36]. As a consequence, strains of this serotype could have an enhanced virulence response in the presence of subinhibitory concentrations of SCFAs.

3. Materials and Methods

3.1. Salmonella enterica Strains and Short Chain Fatty Acids

A total of twelve *Salmonella* strains belonging to seven different serotypes and two *Salmonella* subspecies were included in this study (Table 2). All the strains were previously isolated from poultry houses and chicken meat in our laboratory according to ISO 6579:2003 [37]. The strains were kept at −20 °C in tryptic soy broth (TSB, Oxoid, UK) supplemented with 20% glycerol until use. *Salmonella* strains were previously grown in nutrient agar (Applichem Panreac, Barcelona, Spain) and incubated for 24 h at 37 °C before use. The following four different SCFAs were tested in this study: acetic acid (≥99%, Sigma Aldrich, Schnelldorf, Germany), butyric acid (≥99%, Alfa Aesar, ThermoFisher Scientific, Massachusetts, Waltham, MA, USA), propionic acid (≥99%, Sigma Aldrich), and valeric acid (≥99%, Sigma Aldrich).

Table 2. List of *Salmonella* strains included in this study and its source.

Strain	Code	Source
S. enterica subsp. *arizonae*	AZ3	Poultry farm
S. enterica subsp. *arizonae*	AZ26	Poultry farm
S. Bredeney	BR1	Poultry farm
S. Infantis	I18	Poultry farm
S. Infantis	I4	Poultry farm
S. Isangi	IG1	Poultry farm
S. Montevideo	M1	Poultry farm
S. Newport	N6	Poultry farm
S. Typhimurium	T1	Chicken meat
S. Typhimurium	T3	Chicken meat
S. Typhimurium	T7	Poultry farm
S. Typhimurium	T23	Poultry farm

3.2. Minimum Inhibitory Concentration and Minimum Biocidal Concentration of Short Chain Fatty Acids

Minimum inhibitory concentrations (MICs) of SCFAs were determined according to the microdilution broth method described by the Clinical and Laboratory Standards Institute (CLSI) guidelines. An initial concentration of SCFAs at 20% in Mueller–Hinton broth was used to perform serial dilutions of the tested SCFAs. *Salmonella* strains were initially grown in nutrient agar for 24 h at 37 °C, and isolated colonies were used to prepare a saline suspension with a turbidity equivalent to a 0.5 McFarland standard. Two serial dilutions of the initial inoculum in Mueller–Hinton broth were performed to obtain a final concentration of 1×10^6 CFU/mL. In a 96-well microtiter plate, 100 µL of each dilution was mixed with 100 µL of the final inoculum, and microplates were incubated for 24 h at 37 °C. The MIC was defined as the lowest concentration in which no visual bacterial growth was observed. The liquid of wells with no visual bacterial growth observed was transferred to nutrient agar plates to determine the minimum bactericidal concentration (MBC). Plates were incubated for 24 h at 37 °C.

3.3. Motility Assays

Motility assays were carried out in nutrient semisolid agar plates composed of 8 g/L of nutrient broth (AppliChem, Panreac, Barcelona, Spain), 4 g/L of agar (Liofilchem, Abruzzi TE, Italy), and 0.05 g/L of 2,3,5-triphenyltetrazolium chloride (Sigma Aldrich). Five different types of agar were used: a control

agar and four semisolid agars, each supplemented with one SCFA at the highest subinhibitory concentration observed in this study. A saline suspension with a turbidity equivalent to 0.5 McFarland was prepared for each strain from the isolated colonies of nutrient agar plates incubated for 24 h at 37 °C. An inoculating loop was immersed in the *Salmonella* saline suspension, and then the strains were inoculated in the semisolid agar plates by stabbing. Motility agar plates were incubated at 37 °C for 48 h. After incubation, the ratio between the stabbing point and the end of the growth circle was measured. The experiments were carried out in triplicate.

3.4. Biofilm Formation on Polystyrene

The effect of SCFAs on the ability to produce a biofilm in polystyrene of *Salmonella* strains included in this study was evaluated according to the method described by Stepanović et al. [38]. Five different growth media were used: control TSB media and four growth media, each supplemented with one of the SCFAs tested in this study to a final concentration of the highest subinhibitory concentration observed the MICs evaluation. Polystyrene, 96-well microplates were filled with 200 μL of the corresponding growth media and then inoculated with 20 μL of saline solution with a *Salmonella* concentration of 10^4 CFU/mL. Microplates were incubated for 24 h at 37 °C and then washed three times with 250 μL of distilled water. *Salmonella* cells that were adhered to the surface of the wells were fixed with 250 μL of methanol for 15 min. Then, wells were emptied, air dried, and filled with 250 μL of 0.1% crystal violet solution (Panreac, Barcelona, Spain) for 5 min. Excess crystal violet was removed under tap water, and the crystal violet that was adhered to the wells was resolubilized with 250 μL of 33% acetic acid solution. The absorbance of the microplates was read at 630 nm using a plate reader (das, Palombara Sabina, Italy). The experiments were carried out in triplicate.

3.5. RNA Isolation and RT-qPCR

The transcription of 14 *Salmonella* genes (Table 3) in the presence of subinhibitory concentrations of SCFAs was evaluated in two *Salmonella* strains (*S.* Typhimurium T7 and *S.* Infantis I4). A total of five growth media were used: TSB media that was used as control media and four growth media, each supplemented with the highest subinhibitory concentrations of one of the SFCAs tested in this study. Fifteen-milliliter plastic tubes were filled with 10 mL of the corresponding growth media and 100 μL of a saline solution with a concentration of 10^4 CFU/mL of the corresponding *Salmonella* strains. The tubes were incubated for 12 h at 37 °C and then centrifuged at 2000× *g* for 10 min. The supernatant was discarded, and the pellet was resuspended in 1 mL of NZYol (nzytech, Lisboa, Portugal), and the RNA was isolated according to the manufacturer's recommendations. Immediately, RNA was quantified using a fluorometer (Qubit, Invitrogen, Carlsbad, CA, USA, Thermofisher Scientific) and reversed transcribed using the NZY First-Strand cDNA Synthesis kit (nzytech) following the manufacturer's protocol. The cDNA was stored at −20 °C until use.

The 10 μL RT-qPCR reactions, composed of 5 μL of NZYSpeedy qPCR green master mix 2× ROX (nzytech), 0.4 μL of each primer, 1 μL of the sample, and 3.2 μL of RNase-free water, were carried out in a QuantStudio 12 k Flex real-time PCR system (Applied Biosystems, ThermoFisher Scientific, Foster, CA, USA). The conditions were as follows: initial denaturation at 95 °C for 20 s, followed by 40 cycles of denaturation at 95 °C for 1 s, annealing at 60 °C for 20 s, and a final melting curve program of 15 s at 95 °C, 60 s at 60 °C, and followed by a dissociation step for 15 s at 95 °C. The expression of target genes in comparison to the control gene 16 s rRNA was evaluated using the $2^{\Delta\Delta Ct}$ method, where $\Delta\Delta C_t = (Ct_{\text{target genes}} - Ct_{\text{16s rRNA}})_{\text{treatment}} - (Ct_{\text{target genes}} - Ct_{\text{16s rRNA}})_{\text{control.}}$

Table 3. Primers sequences of genes tested in this study.

Target Genes	Sequence (5′–3′)	Reference
16s rRNA	F: AGGCCTTCGGGTTGTAAAGT R: GTTAGCCGGTGCTTCTTCTG	[39]
luxS	F: ATGCCATTATTAGATAGCTT R: GAGATGGTCGCGCATAAAGCCAGC	[40]
hilA	F: AATGGTCACAGGCTGAGGTG R: ACATCGTCGCGACTTGTGAA	[41]
invA	F: CGCGCTTGATGAGCTTTACC R: CTCGTAATTCGCCGCCATTG	[41]
rpoS	F: CAAGGGGAAATCCGTAAACCC R: GCCAATGGTGCCGAGTATC	[42]
csrA	F: CTGGACTGCTGGGATTTTTC R: CATGATTGGCGATGAGGTC	[43]
fliC	F: CTCGGCTACTGGTCTTGGTG R: CCGTAACGGTAACTTTGGCG	[44]
ssrA	F: CGGCTGGTATTCTTGTAAGGGT R: AAGCAGACACAAATTCGCAAG	[45]
stn	F: CAACCAGATAGTAAAGACCG R: ATTAGCGTAGAGGCAAAAGA	[46]
fadA	F: ATCTCTCCGCCCACTTAATGCGTA R: AGCCTTGCTCCAGCGTTTGTTGTA	[42]
sirA	F: CCAGCTACTTTCGCAGCAA R: AACACGTTGTAACGCGGTTG	[41]
spiA	F: AGGCGCTTGATATGTGC R: GCAGGCTCCGGAATTTTAGG	[46]
cpx	F: CATTTAACGACCGCGAGCTG R: ACCCGGATTAAGGCTTAGCG	[44]
rpoE	F: CACCTTACGGGAGCTGGATG R: GAAGATACGTGAACGCACCG	[44]
flijB	F: ATGGTACTACACTGGATGTATCG R: GTAAAGCCACCAATAGTAAC	[44]

3.6. Statistical Analysis

GraphPad Prism 8 (GraphPad, CA, USA) was used for the statistical analyses. Analysis of variance (one-way ANOVA) and the Tukey's honestly significant difference test ($p < 0.05$) were used to determine the influence of SCFAs on *Salmonella* motility, biofilm formation, and transcription profiles.

4. Conclusions

The results of this study showed that SCFAs are promising antimicrobial substances that can be potentially used for human health and food safety purposes. A SCFA concentration of 3750 μg/mL is enough to inhibit the growth of *Salmonella* strains. In addition, subinhibitory concentrations of SCFAs caused a reduction in motility and biofilm formation in *Salmonella* strains. This study demonstrated that subinhibitory concentrations of SCFAs can enhance the expression of virulence genes in *Salmonella* strains, resulting in a higher virulence of strains. Thus, for future applications in human health and food safety, it is important to assess the appropriate concentrations of the SCFAs used.

Author Contributions: Conceptualization, A.L. and C.M.F.; methodology, A.L.; software, A.L., B.V. and P.R.; formal analysis, A.L. and P.R.; investigation, A.L.; data curation, A.L., P.R. and C.M.F.; writing—original draft preparation, A.L.; writing—A.C., B.V., P.R. and C.M.F.; supervision, A.C. and C.M.F.

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Honey as a Strategy to Fight *Candida tropicalis* in Mixed-Biofilms with *Pseudomonas aeruginosa*

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Abstract: Fungal contaminations with *Candida* species are commonly responsible for several infections, especially when associated to bacteria. The therapeutic approach commonly used is being compromised due to microbial resistances of these microorganisms to antimicrobial agents, especially in biofilm. The use of honey as an antimicrobial agent has been emerging as a valuable solution and proving its potential in planktonic and in biofilm cells. This work aims to assess the effect of different honeys on biofilms of *Candida tropicalis* and *Pseudomonas aeruginosa*. The effect of Portuguese heather (PH) and manuka honeys on planktonic growth of *Candida* was initially evaluated by determination of the minimum inhibitory concentrations (MIC). Then, the same effect was evaluated in mixed biofilms, by colony-forming units numeration and fluorescence microscopy. The combinations of honey plus fluconazole and gentamicin were also tested. The results showed that the honeys tested enabled a great reduction of *C. tropicalis*, both in planktonic (12.5% and 25% of MIC for PH and manuka) and in biofilm. In polymicrobial biofilms, the use of PH and manuka honeys was revealed to be a promising choice and an alternative treatment, since they were able to reduce cells from both species. No synergistic effect was observed in antimicrobial combinations assays against polymicrobial biofilms.

Keywords: *Candida tropicalis*; *Pseudomonas aeruginosa*; biofilms; honey therapy; antifungal agents

1. Introduction

Fungal infections are widely recognized as one of the main causes of morbidity and mortality, particularly those infections caused by opportunistic pathogenic fungi, such as the *Candida* species [1]. The incidence of *Candida* infections (Candidosis) has increased remarkably in the last years [1].

On human pathology, *Candida* species have an important role as colonizers of the mucosal membranes of the oral cavity and gastrointestinal tract, as well as normal components of the skin and vaginal flora; so, in normal conditions, *Candida* are non-pathogenic commensal microorganisms in humans [2,3]. However, modifications in human host defenses may lead to a disproportional growth of *Candida* and consequently to a pathogenic colonization by these species [4,5]. In general, transition from commensal to pathogen in *Candida* is facilitated by a series of virulence factors, such as hemolytic activity, secretion of extracellular hydrolytic enzymes (coagulase, phospholipase, and proteases), and producing specific adhesins (for example, fibrinogen and fibronectin), which appear to play an important role in adhesion, penetration, invasion, and destruction of human tissues. Also, the ability of *Candida* to adhere to medical devices or host tissues with the formation of more resistant structures (biofilms) is a particularly important virulence factor [6,7]. In normal environments, biofilm formation represents the most predominant type of microbial growth and is frequently associated to persistent clinical infections [8,9]. While each *Candida* species possesses unique features, biofilms in nature are formed by more than one microbial species, both bacteria and fungi, which confers an even higher resistant phenotype to the biofilm [10]. Although the study of the structure and properties of single-species

biofilms is an important step for understanding infectious diseases, the elucidation of communal behavior of microorganisms in biofilms composed of different species may have a high impact for understanding infectious diseases and to develop new therapeutic strategies [10]. Indeed, *Candida* species and *Pseudomonas aeruginosa* microorganisms tend to form polymicrobial biofilms and, as such, they are often responsible for nosocomial infections in immunocompromised individuals [11,12].

Candida albicans remains as the most prevalent species of these infections, but a clear rise in the proportion of non-*Candida albicans Candida* (NCAC) species has been noted [6,7,13]. According to some epidemiological studies, *Candida tropicalis* stands out in the NCAC species group and is correlated with other forms of *Candida* mortality [14–16]. For example, a predominance of NCACs species was observed in the south of America, where *C. albicans* accounted for 40.9% of cases, followed by *C. tropicalis* (20.9%), *C. parapsilosis* (20.5%), and *C. glabrata* (4.9%) [7,17,18]. A study by Kontoyiannis et al. has shown that *C. tropicalis* is more persistent, leading this process to situations of uselessness in cases of infection [14]. This may imply increased virulence and resistance to antifungals compared to *C. albicans*, for example, particularly in the oral cavity [19]. As a consequence of the lack of knowledge on NCAC species virulence, namely in *C. tropicalis*, the rising levels of resistance to the traditional antifungal therapies, and the association of Candidosis to high levels of mortality, there is an urgent need to develop new strategies to fight these infections [1]. In this sense, an alternative approach for the treatment of Candidosis is the use of natural compounds as antifungal agents, among which is honey. Honey is a natural product which has been known for its biological and pharmacological properties for centuries. It has been extensively used in traditional medicine and also complementary medicine because of its antibacterial, antifungal, antimycobacterial, and antiviral activities [20]. These antimicrobial activities may be caused by honey's acidity (low pH), osmotic effect, high sugar concentration, presence of bacteriostatic and bactericidal factors, increase in cytokine release, as well as immune modulating and anti-inflammatory properties [21,22]. Besides that, one of the most important factors is the presence of hydrogen peroxide (H_2O_2), the primary antimicrobial component in most honeys, produced by the enzyme glucose oxidase, and its action consists in the destruction of the essential components of the cells [21,23]. Some types of honey contain additional antimicrobial activity by methylglyoxal (MGO), bee defensin-1, and other bee-derived compounds, such as phenolic compounds of floral origin and lysozyme, among other compounds [23].

The present work aims to evaluate the antimicrobial effect of two different honeys (Portuguese Heather (PH) and manuka) alone, or in combination with a commercial antimicrobial agent, in mixed biofilms of *C. tropicalis* with *P. aeruginosa*.

2. Results

2.1. Susceptibility Testing of Planktonic Populations of C. tropicalis

The minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) of PH and manuka honey against planktonic populations of *C. tropicalis* were determined. A lower MIC was observed for PH (12.5% (w/v)), in comparison to manuka honey (25% (w/v)). Similarly, the MFC recorded for the PH was lower (50% (w/v)) than for manuka honey (>50% (w/v)).

2.2. Effect of Honey in C. tropicalis and P. aeruginosa Biofilms

The therapeutic effect of PH and manuka honey (25% (w/v), 50% (w/v), and 75% (w/v)) was assessed on 24 h-old *C. tropicalis* and *P. aeruginosa* single (Figure 1A,B, respectively) and dual-species (Figure 1C,D, respectively) biofilms, by determining biofilm viable cells. The effect of both honeys was monitored at 24 h (Figure 1). The range of honey concentrations tested is in accordance with the results obtained previously for MIC and MFC.

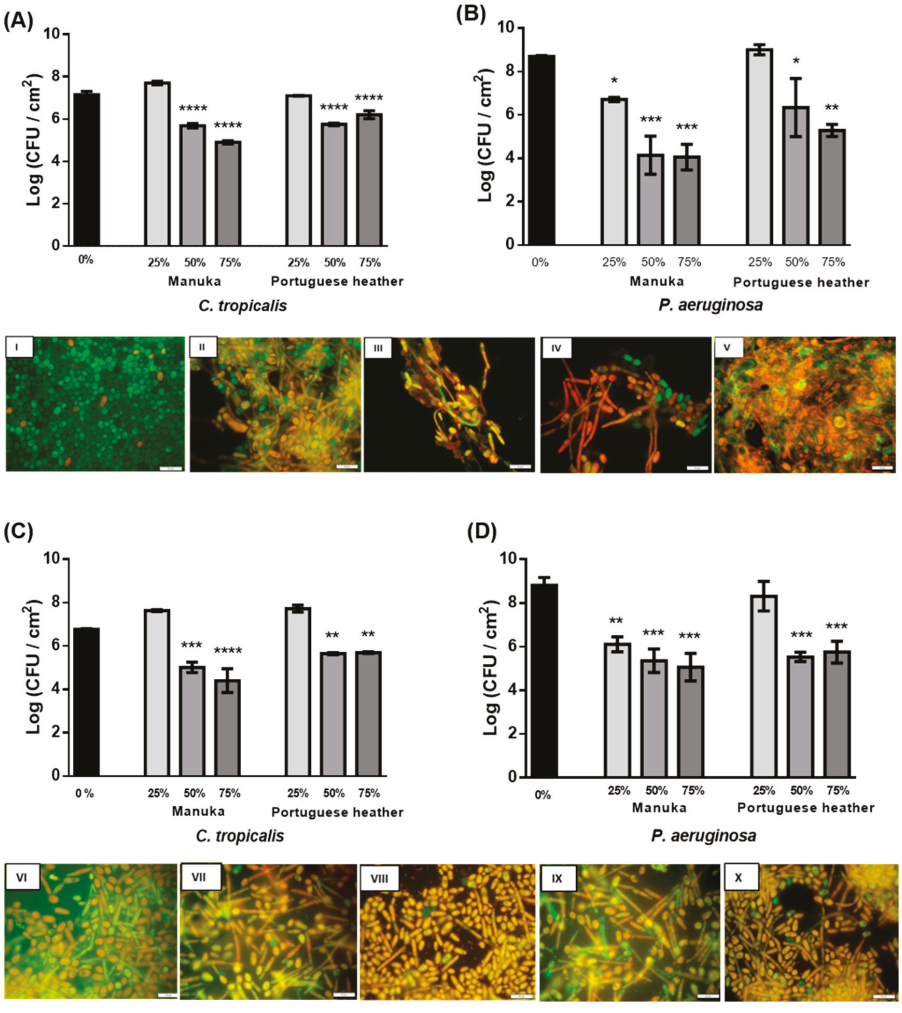

Figure 1. Therapeutic effect of manuka honey and Portuguese heather (PH) on 24 h-old (**A**,**B**) single- and (**C**,**D**) dual-species biofilms formed by (**A**,**C**) *C. tropicalis* and (**B**,**D**) *P. aeruginosa* after 24 h. * $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ indicates a statistically different reduction in comparison with the respective control. Fluorescence microscopy images of 24 h-old *C. tropicalis* biofilms treated with 0% honey (I), 50% manuka honey (II), 75% manuka honey (III), 50% PH (IV), and 75% PH (V), and images of dual-species (*C. tropicalis* and *P. aeruginosa*) biofilms treated with 0% honey (VI), 50% manuka (VII), 75% manuka (VIII), 50% PH (IX), and 75% PH (X). Live cells were green-stained and dead cells were red-stained. The bar represents 10 μm.

Analyzing the results for 24 h *C. tropicalis* biofilms, a reduction of about 1.5 Log (CFU/cm^2) relative to the control was observed with both honeys at 50% (w/v). Manuka honey caused the highest cell reduction of about 2 Log (CFU/cm^2) when used at 75% (w/v) (Figure 1A). For a better understanding of the changes that occurred in these biofilms, after application of honey, they were observed under a fluorescent microscope. By comparing the untreated biofilm image (Figure 1I) with the images after honey administration, an increase in the number of damaged cells (stained in red) is evident. In fact, in the control (Figure 1I), cells were mostly viable (stained in green) and in the yeast form. Comparatively,

after the action of both honeys, at 50% (w/v) (Figure 1II,IV) and 75% (w/v) (Figure 1III,V), the cells became clearly damaged, which could be interpreted as a positive action of honey in the infection treatment.

After treatment, it was observed that 75% (w/v) manuka honey had a similar effect of 2 Log (CFU/cm^2) reduction in *C. tropicalis*, regarding single (Figure 1A) and mixed (Figure 1C) biofilms ($p < 0.0001$). For PH, similar results were obtained with concentrations of 50% (w/v) and 75% (w/v), with a significantly higher reduction in single species biofilms (1 Log (CFU/cm^2)) ($p < 0.0001$) (Figure 1A) than in mixed biofilms ($p < 0.01$) (Figure 1C).

In relation to *P. aeruginosa*, significant viable cell reductions were also observed after the action of both honeys; nevertheless, the higher reduction ($p < 0.001$) was obtained with manuka honey: 4 and 3 Log (CFU/cm^2) at 50% (w/v) and 75% (w/v), respectively, in single (Figure 1B) and mixed (Figure 1D) biofilms.

After observation of the mixed biofilms under a fluorescence microscope, it was found that the untreated biofilms had already damaged cells of *C. tropicalis* (Figure 1VI). However, *P. aeruginosa* cells remained viable. It was also found that with 50% (w/v) manuka (Figure 1VII) or PH (Figure 1IX) honeys, the *Candida* cells began to form hyphae. In the treatment with 75% (w/v) manuka (Figure 1VIII) or PH (Figure 1X) honeys, the biofilm was highly damaged in both species.

2.3. Effect of Antimicrobial Combinations Against C. tropicalis and P. aeruginosa Biofilms

The antimicrobial effect of the combination of the different honeys (manuka or PH) at different concentrations (25% (w/v) and 50% (w/v)) with a commercial antifungal agent (100 $mg{\cdot}L^{-1}$ of fluconazole (FLU)) on single and dual-species 24 h-old biofilms (*C. tropicalis* and *P. aeruginosa*) was monitored at 24 h (Figure 2). Since *P. aeruginosa* has been demonstrated to be susceptible to honey after 6 h of treatment, those concentrations were selected in order to reduce the higher concentrations of honey and the conventional concentrations of FLU. The control assay of 24 h-old single species biofilms is presented in Figure 2A (*C. tropicalis*) and Figure 2B (*P. aeruginosa*).

Regarding the results of Figure 2, it was noted that honey combined with FLU caused higher cell reductions comparatively to treatment with honey at 25% (w/v) (1 Log reduction for manuka honey, $p < 0.0001$; 0.5 Log for PH, $p < 0.1$). Nevertheless, while the combination of 50% (w/v) honey with FLU had no advantage over honey treatment alone, an increased reduction was observed when compared to the effect of FLU alone ($p < 0.0001$ for manuka honey and $p < 0.01$ for heather honey).

Regarding *C. tropicalis* in mixed biofilms (Figure 2C), and similar to results observed for single biofilms (Figure 2A), less viable cells were recovered after 24 h of combined therapy comparatively to the treatment with manuka honey alone, at 25% (w/v) ($p < 0.1$). Similar results were also observed for *P. aeruginosa*, in single (Figure 2B) and mixed (Figure 2D) biofilms, i.e., when FLU was added, honey substantially increased the number of viable cells in mixed biofilms compared with treatment with honey alone.

The efficacy of the honey–antifungal–antibacterial combination was further inspected in preformed dual-species biofilms of *C. tropicalis* (Figure 3A) and *P. aeruginosa* (Figure 3B). The therapeutic effect of the combined action of both honeys at 50% (w/v) with a commercial antifungal (100 mg L^{-1} of FLU) and with a commercial antibiotic (20 $mg{\cdot}L^{-1}$ of gentamicine (GEN)) was assessed.

Considering the results obtained with a third antimicrobial agent added (GEN), it is possible to verify more viable cells in biofilms compared to treatment with honey alone, for all conditions tested (Figure 3).

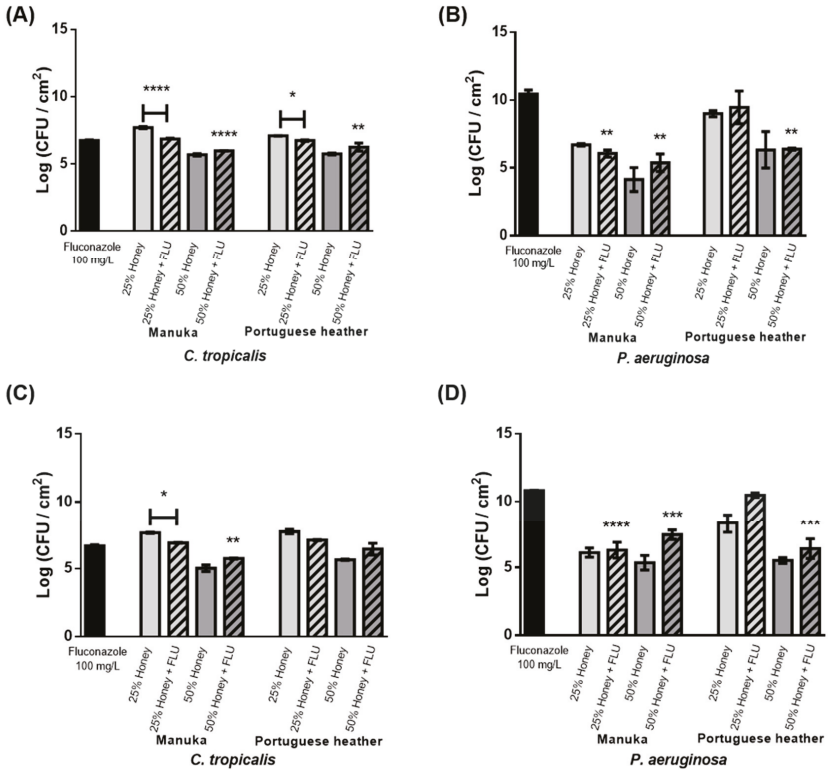

Figure 2. Therapeutic effect of manuka or PH honeys combined with 100 mg L^{-1} of FLU on 24 h-old (**A**,**B**) single- and (**C**,**D**) dual-species biofilms formed by (**A**,**C**) *C. tropicalis* and (**B**,**D**) *P. aeruginosa* at 24 h. * $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ indicates a statistically different reduction in comparison with the respective control.

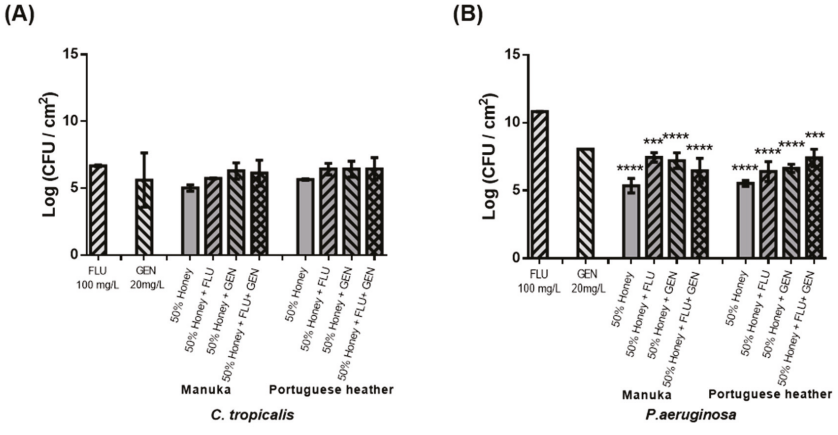

Figure 3. Therapeutic effect of manuka or PH honeys combined with 100 mg L^{-1} of FLU and 20 mg L^{-1} of GEN on 24 h-old dual-species biofilms formed by *C. tropicalis* (**A**) and *P. aeruginosa* (**B**) at 24 h. * $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ indicates a statistically different reduction in comparison with the FLU.

3. Discussion

Analyzing the MIC and MFC values of the two honeys under study for *C. tropicalis*, it was verified that this strain is susceptible to both honeys, especially the PH. Similar results were obtained by Khosravi et al. for *C. tropicalis* (MIC of 38.5% and MFC of 43.7%) [24].

The antimicrobial effect of PH and manuka honeys in 24 h biofilms of *C. tropicalis* and *P. aeruginosa* was determined through quantification of viable cells (CFU) per unit of area (cm^2) (Figure 1). Regarding *C. tropicalis* single biofilms, after 24 h of treatment, none of the honeys at 25% (w/v) caused cell reduction (Figure 1A). This may be a consequence of the fungal cells taking advantage of the lower sugar concentration present in honey at 25% to promote their own growth. Still, significant reductions were obtained with both honeys at 50% (w/v) (about 1.5 Log-reduction (CFU/cm^2)) and with manuka honey at 75% (w/v) ($p < 0.0001$) (about 2 Log (CFU/cm^2) (Figure 1A)). A significant reduction was only observed with 75% (w/v) PH ($p < 0.01$). Some properties of honey may be involved in the antimicrobial effect, particularly in the antifungal effect [21,22], such as the presence of metilglioxal (MGO) [25]. an effective antimicrobial agent against planktonic and biofilm cells [26]. The presence of aromatic acids or special proteins with antifungal activity, such as flavonoids, polyphenois, and defensin-1 [25], and the production of H_2O_2 by the enzyme glucose oxidase [26]. Indeed, in a previous study, the authors have explored the analysis of these different characteristics of honeys, and the results showed that PH honey was one of the most interesting values indicating the possible high antimicrobial potential (data not shown).

In *C. tropicalis* single and mixed biofilms, after 24 h of treatment, 75% (w/v) manuka honey significantly reduced viable cells in 2 Log (CFU/cm^2) ($p < 0.0001$) (Figure 1A,C). For PH, similar results were obtained with 50% (w/v) and 75% (w/v) honey, with a significant reduction of 1 Log (CFU/cm^2) ($p < 0.0001$) (Figure 1A–C). These data suggest that, after 24 h of treatment, manuka honey exerts an antifungal effect both in the presence and absence of *P. aeruginosa*, while PH exerts a slightly higher effect in single species biofilms of *C. tropicalis*.

The images obtained by fluorescence microscopy (Figure 1I–X), which are only representative of the cell form and structure, revealed a direct relationship between the presence of honey or *P. aeruginosa* and the hyphae. In *Candida* species, the morphological transition involving the formation of hyphae is an important virulence factor, which is associated with cell stress, which could be caused by the presence of *P. aeruginosa* or the several compounds of honey (such as H_2O_2 and defensin-1) [27]. Also, from the microscopy images (Figure 1I–X), it was observed that, curiously, the untreated mixed biofilm presented several damaged cells of *C. tropicalis* (Figure 1VI), compared to single biofilms (Figure 1I). This is supported by the experiments carried out by Bandara et al. that investigated the interactions between *Candida species* and *P. aeruginosa*, showing a reduction of 88% (w/v) of *C. tropicalis* after a 24 h-incubation [10]. It was confirmed in the same study that, in general, *Candida* species and *P. aeruginosa* have mutually suppressive effects at all stages of biofilm formation. However, most of the previous studies on interactions between *Candida* species and bacteria in mixed biofilms are concentrated in *C. albicans* and only a few are related to NCAC [10].

Regarding the results obtained for *P. aeruginosa* in single and mixed biofilms, significant reductions could be observed after 6 h of treatment for both honeys at all concentrations tested. In this period of time, the manuka honey allowed for a reduction of the single biofilm to half of the viable cells ($p < 0.0001$) and the PH managed to reduce in average 2–3 Log (CFU/cm^2). In mixed biofilms, there was also a significant reduction, but it was not as pronounced as in single biofilms ($p < 0.001$). With the results obtained, it can be observed that the honey has a faster and superior antibacterial effect compared to the results obtained for *C. tropicalis*. Comparing the two honeys tested, it was observed that the highest reductions were obtained with manuka honey: 4 and 3 Log (CFU/cm^2) with 50% (w/v) and 75% (w/v) ($p < 0.001$) in single (Figure 1B) and mixed (Figure 1D) biofilms, respectively. However, the reduction occurred from 25% (w/v) of manuka honey, both in single ($p < 0.1$) (Figure 1B) and in mixed biofilms ($p < 0.01$) (Figure 1D). The effect of lower concentrations of manuka on *P. aeruginosa* was already reported by Cooper and Molan [28]. According to the literature, honey has broad-spectrum

antibacterial activity. Regarding the effect of manuka honey on bacteria, a study by Roberts, Moddocks, and Cooper found that honey acts by inhibiting the flogging of bacteria, which limits their mobility and prevents the formation of biofilms [29]. In summary, after 24 h of treatment, honey promoted cell reduction in biofilms of both species simultaneously, suggesting honey as a promising agent for the treatment of polymicrobial infections of *C. tropicalis* and *P. aeruginosa*. Very few treatments were reported as being able to reduce more than one species in a mixed biofilm.

The effect of the combination of honey (PH or manuka honeys at 25% (w/v) and 50% (w/v)) with FLU (100 mg L^{-1}) was then evaluated in single and mixed biofilms (Figure 2). For *C. tropicalis*, the cell reduction obtained with the combination (honey and FLU) was higher than with only honey at 25% (w/v) (1 Log reduction for manuka honey, $p < 0.0001$; 0.5 Log for PH, $p < 0.1$) (Figure 2A). Conversely, combinations of honey at 50% (w/v) and FLU had no advantage over honey treatment alone; nevertheless, an improved reduction was observed when compared to the effect of FLU alone ($p < 0.0001$ for manuka honey and $p < 0.01$ for heather honey). Comparing results obtained for honey combined with an antifungal agent, it was observed that it had advantages over single honey or antifungal treatment. The combined treatments allowed for a 50% reduction of the dosage of antifungal typically required in clinical settings, i.e., 200 mg L^{-1}.

In mixed biofilms, a slight reduction in biofilm was observed after 12 h compared to the individual treatment with either honey or FLU. After 24 h of combinational therapy, a significant reduction was only obtained in comparison to treatment with manuka honey alone at 25% (w/v) ($p < 0.1$) (Figure 2C), similar to what was observed for single biofilms (Figure 2A). This demonstrates that the combination of *C. tropicalis* and *P. aeruginosa* is indifferent to treatment with honey and FLU, and that *C. tropicalis* remains less tolerant to FLU. Furthermore, adding FLU to honey substantially increased the number of *P. aeruginosa* viable cells in mixed biofilms (Figure 2D) compared with treatment with only honey.

In summary, honey by itself allows for a superior reduction in comparison with the combination of honey and FLU in all conditions tested in mixed and single biofilms, with the exception of 25% (w/v) honey. *C. tropicalis* remains highly resistant to FLU, even when combined with honey, both in single and mixed biofilms. This is in accordance with several studies stating that resistance to FLU is increasing in clinical isolates of this species [29].

Considering the results previously obtained, a third antimicrobial agent was added (GEN) to the combinations tested. So, combinational therapy was evaluated using honey 50% (w/v), FLU 100 mg L^{-1}, and GEN 20 mg L^{-1}. However, this resulted in an increased number of viable cells in biofilms compared to treatment with honey alone, for all conditions tested (Figure 3). Indeed, the combination of these three elements had no advantage over treatment with honey alone, both for the fungal and bacterial species. The interaction between FLU and GEN was investigated by Thomas et al. as a treatment of pre-formed biofilms of 4, 8, and 12 h of *C. albicans*, where synergism was observed only against pre-formed biofilms of 4 and 8 h, with no synergism observed at 24 h [30].

4. Material and Methods

4.1. Microorganisms and Culture Conditions

C. tropicalis ATCC 750 and *P. aeruginosa* DSM 22644 were stored at –80 ± 2 °C in broth medium with 20% (v/v) glycerol. Prior to each assay, *C. tropicalis* and *P. aeruginosa* strains were subcultured from the frozen stock preparations onto Sabouraud Dextrose Agar (SDA) and Tryptic Soy Agar (TSA) plates, respectively. SDA and TSA were prepared from Sabouraud Dextrose Broth (SDB; Liofilchem, Roseto degli Abruzzi, Italy) or Tryptic Soy Broth (TSB; Liofilchem, Roseto degli Abruzz, Italy), supplemented with 2% (w/v) agar (Liofilchem, Roseto degli Abruzzi, Italy). The plates were then incubated aerobically at 37 °C for 18–24 h.

Pure liquid cultures (pre-inocula) of *C. tropicalis* were maintained in SDB, whereas *P. aeruginosa* was grown overnight in TSB. For planktonic and biofilm assays, 0.22 µm filter-sterilized Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco® by Life Technologies ™, Grand Island, NY, USA)

at pH 7.0 was used. Unless otherwise stated, all rinse steps were performed either by using 0.9% (w/v) saline solution (NaCl; J.T. Baker, Deventer, The Netherlands) or ultrapure (UP) sterile water.

4.2. Antimicrobial Agents

Stock solutions of two commercial antimicrobial agents, Fluconazole (FLU, Sigma-Aldrich, St. Louis, MO, USA) and Gentamicin sulfate (GEN, Sigma-Aldrich, St. Louis, MO, USA) were prepared and stored according to the manufacturer's instructions. Also, two different honeys, Portuguese heather honey (raw dark amber honey whose main plant nectar is heather (30%, APISMaia company (Porto Portugal)) collected by a beekeeper in the North of Portugal) and manuka (commercial Medihoney®) were stored at 4 °C, and the dilutions were prepared with RPMI 6420 medium. All the concentrations of different antimicrobial agents (natural and commercial) tested in this work are presented in Table 1.

Table 1. Tested concentrations of honey (Portuguese heather (PH) and manuka), commercial antifungal agent (FLU) and commercial antibiotic agent (GEN).

Antimicrobial Agents		Concentration
Honeys	**Manuka PH**	6% (w/v) 12.5% (w/v) 25% (w/v) 50% (w/v) 75% (w/v)
FLU		100 mg L^{-1} *
GEN		20 mg L^{-1} *

* Values of concentrations selected in a previous study [31].

4.3. Planktonic Antimicrobial Susceptibilities

Susceptibilities of *C. tropicalis* planktonic-cell cultures were evaluated by determining the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC). The MIC values were determined according to standard European Committee on Antimicrobial Susceptibility Testing (EUCAST), through the broth microdilution method [32]. Briefly, the initial cell concentration for both microorganisms was adjusted for 1×10^6 CFU/mL and dispensed into 96-well plates in a proportion of 1:2 (the final inoculum concentration was 5×10^5 CFU/mL) with the working antimicrobial solutions (previously diluted in RPMI 1640 broth with double of the desired final concentration). Wells containing only broth medium (antimicrobial-free medium) were used as negative controls and wells containing *C. tropicalis* culture without antimicrobial agent were used as positive controls. Plates were incubated overnight at 37 °C. MIC was obtained by visual observation of the turbidity gradient. The minimum concentration where growth inhibition occurs is equivalent to the MIC value.

For the determination of MFC values, 10 µL was removed from each well of the microdilution trays, after incubation, and plated onto SDA plates and incubated at 37 °C. The lowest antimicrobial concentration that yielded no colony growth after 12–24 h was considered as the MFC.

4.4. Biofilms Antimicrobial Susceptibilities

Biofilms were developed according to the modified microtiter plate test proposed by Stepanović et al., with some modifications [33]. Briefly, different cultures were centrifuged twice (3000 *g*, 4 °C, 10 min) and the pellet was resuspended in RPMI 1640, until reaching 1×10^7 cells/mL. Yeast cells (*C. tropicalis*) were enumerated by microscopy using a Neubauer counting chamber. Bacteria (*P. aeruginosa*) concentration was adjusted to 0.13 (corresponding between 2–3 $\times$ 10^{-8} CFU·mL^{-1}), using an ELISA microtiter plate reader with a wavelength of 640 nm (Sunrise-Basic

Tecan, Männedorf, Switzerland). For mixed-species cultures, a combination of 50% of the suspended inoculum of each species was used.

The cellular suspensions were transferred, under aseptic conditions, to 96-well flat tissue culture plates (polystyrene, Orange Scientific, Braine-L'Alleud, Belgium) (200 μL per well). To promote biofilm formation, microtiter plates were incubated aerobically for 24 h on a horizontal shaker at 120 rpm and 37 °C.

The effect of each honey (PH or manuka) alone was evaluated in single and mixed-species biofilms of *C. tropicalis* and *P. aeruginosa*. For this, 24 h-old biofilms were exposed to increasing concentrations of each agent (25% (w/v), 50% (w/v), and 75% (w/v)). Briefly, after biofilm formation, 200 μL of cell suspension were replaced by the antimicrobial solutions prepared at 2-fold the desired concentration. Plates were then incubated aerobically at 37 °C for 24 h. After 6, 12, and 24 h, the treated biofilms were removed to assess biofilm-cells cultivability through CFU enumeration.

After biofilm formation, the wells were washed twice with saline solution after discarding the planktonic fraction. In order to estimate the number of cultivable biofilm-entrapped cells in single- and mixed-species, the microdrop technique was used. Briefly, 200 μL of fresh saline solution was added to each well and the biofilms were scraped. The resulting biofilm-cells suspensions were then serially diluted in saline solution and plated onto non-selective agar (SDA for *C. tropicalis* and TSA for *P. aeruginosa* pure cultures) plates. Selective agar was also used for colony-forming units (CFU) determination of *Candida* species (SDA supplemented with 20 mg L^{-1} gentamycin (GEN), to suppress the growth of *P. aeruginosa*) and *P. aeruginosa* (*Pseudomonas* Isolation Agar, (PIA)). Agar plates were incubated aerobically at 37 °C for 24 h for cultivable cell counting. Values of cultivable sessile cells were expressed as Log CFU per area (cm^2). All negative (wells containing only broth medium) and positive (wells containing *C. tropicalis*, *P. aeruginosa*, *C. tropicalis*, and *P. aeruginosa* cultures without antimicrobial agents) controls were performed.

4.5. Combinatorial Effect of Antimicrobial Agents (Honey and Commercial Agents) on Biofilms

The combinatorial effect of honeys (25% (w/v) and 50% (w/v) of PH or manuka honey with 100 mg L^{-1} FLU was assessed against 24 h-old *C. tropicalis* biofilms, following a procedure similar to the individual application of the antimicrobials, and against 24 h-old dual-species (*C. tropicalis* and *P. aeruginosa*) biofilms. Also, the triple combinatorial effect of antimicrobial agent (100 mg L^{-1} FLU and 20 mg L^{-1} GEN) and 50% (w/v) of honey (PH or manuka) was assessed against 24 h-old dual-species (*C. tropicalis* and *P. aeruginosa*) biofilms. Biofilm cells were removed after 6, 12, and 24 h to assess biofilm-cells cultivability through CFU enumeration. Also, all negative (wells containing only broth medium) and positive (wells containing *C. tropicalis*, *P. aeruginosa*, *C. tropicalis*, and *P. aeruginosa* cultures without antimicrobial agents) controls were performed.

4.6. Cell Viability Assessment of Biofilm-Embedded Cells

In order to evaluate the cell morphology and viability of polymicrobial biofilms after treatment in a qualitative way, the Live/Dead® BacLight™ Bacterial Viability Kit (Molecular Probes, Leiden, The Netherlands) was employed. Basically, biofilms were formed on polystyrene coupons, as described above, and were then stained for 15 min in the dark with a mixture of the SYTO 9 and Propidium Iodide, both prepared at 3 μL/mL in saline solution. For microscopic observation, an Olympus BX51 microscope fitted with fluorescence illumination was used. The optical filter combination consisted of 470 to 490 nm in combination with 530 to 550 nm excitation filters.

4.7. Statistical Analysis

Data were analyzed using the Prism software package (GraphPad Software version 6.01 for Macintosh). One-way analysis of variance (ANOVA) tests were performed, and means were compared by applying Tukey's multiple comparison test. The statistical analyses performed were

considered significant when $p < 0.1$. For all assays, at least three independent experiments were carried out in triplicate.

5. Conclusions

The increasing incidence of *Candida*-associated infections requires the discovery of more efficient new antifungal therapies, with less adverse effects. In this scope, honey emerges as a potential antifungal agent. Here, the antifungal effect of different concentrations of PH and manuka honeys was evaluated in *C. tropicalis*. A reduction of *C. tropicalis* cell growth in both planktonic and biofilm state was observed with honey treatment. Comparing both honeys tested, for biofilm culture, manuka had a higher effect than PH.

However, understanding the behavior of *Candida* species in polymicrobial biofilms is an important step in the clinical context and for the selection of the most efficient treatment. Because of this, the effect of both honeys was assessed on mixed biofilms of *C. tropicalis* and *P. aeruginosa*. The honeys were able to reduce both species in the mixed biofilm and were demonstrated to be a promising alternative for the treatment of infections caused by mixed species biofilms. The combinations, honey–antifungal and honey–antifungal–antibiotic, were also tested but without positive results. Other antifungal and antimicrobial agents need to be tested to understand the feasibility of using these combinations in therapy.

Overall, the results obtained here highlight the potential of honey as an alternative therapy for controlling infections induced by *C. tropicalis*, especially when associated to bacteria. The use of a natural product such as PH honey may be used in clinical practice, especially in skin applications, to prevent or even treat *C. tropicalis* and *P. aeruginosa* infections.

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antibiotics

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Article

Prevention of *Pseudomonas aeruginosa* Biofilm Formation on Soft Contact Lenses by *Allium sativum* Fermented Extract (BGE) and Cannabinol Oil Extract (CBD)

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Abstract: Two natural mixtures, *Allium sativum* fermented extract (BGE) and cannabinol oil extract (CBD), were assessed for their ability to inhibit and remove *Pseudomonas aeruginosa* biofilms on soft contact lenses in comparison to a multipurpose Soft Contact Lens-care solution present on the Italian market. *Pseudomonas aeruginosa* (ATCC 9027 strain) and *Pseudomonas aeruginosa* clinical strains isolated from ocular swabs were tested. Quantification of the biofilm was done using the microtiter plate assay and the fractional inhibitory concentration index was calculated. Both forms of *Pseudomonas aeruginosa* generated biofilms. BGE at minimal inhibitory concentration (MIC) showed inhibition percentages higher than 55% for both strains, and CBD inhibited biofilm formation by about 70%. The care solution at MIC inhibited biofilm formation by about 50% for both strains tested. The effect of BGE on the eradication of the microbial biofilm on soft contact lenses at MIC was 45% eradication for *P. aeruginosa* ATCC 9027 and 36% for *P. aeruginosa* clinical strain. For CBD, we observed 24% biofilm eradication for both strains. For the care solution, the eradication MICs were 43% eradication for *P. aeruginosa* ATCC 9027 and 41% for *P. aeruginosa* clinical strain. It was observed that both the test soft contact lenses solution/BGE (fractional inhibitory concentration index: 0.450) and the test soft contact lenses solution/CBD (fractional inhibitory concentration index: 0.153) combinations exhibited synergistic antibiofilm activity against most of the studied bacteria. The study showed that BGE and CBD have good effect on inhibition of biofilm formation and removal of preformed biofilms, which makes them promising agents that could be exploited to develop more effective care solutions.

Keywords: biofilm; soft contact lens; *Pseudomonas aeruginosa*; *Allium sativum* fermented extract; cannabinol oil extract

1. Introduction

Diseases related to the eye are frequently observed in clinical practice. Soft Contact lenses have a great impact on improving vision, but their use can often be associated with a risk of infections [1]. Eye infections related to the use of soft contact lenses are linked to various risk factors such as falling

asleep with contact lenses, wetting the lenses with water, not replacing soft contact lenses periodically and reusing the disinfectant solution [2].

Several studies have reported that adolescent and young adult soft contact lens wearers present greater risks of contracting eye infections compared to adult or elderly wearers likely because the former have incorrect hygienic practices for maintenance of their soft contact lenses [3,4].

The Center for Disease Control and Prevention established that there were about 41 million soft contact lens wearers aged ≥18 years in the United States in 2015, and most of them behaved in a manner that put them at risk of contracting eye infections. In 2016, in the United States, it was estimated that one in seven adolescent and one in six adult soft contact lens wearers stated that they had at least one risky episode of eye infection. They reported falling asleep with soft contact lenses, swimming with soft contact lenses, and replacing the containers and storage solution at intervals longer than recommended [5].

Common ocular pathogens include *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and other organisms [6]. Previous epidemiological studies identified *Pseudomonas aeruginosa* as the primary causative agent in soft contact lens-related corneal infection [7].

The solutions must be able to inhibit the growth of pathogens to protect users from infections [8] and thus to decrease the risk of soft contact lens-related infections [9]. Furthermore, because the formation of bacterial biofilms on soft contact lenses increases infectious eye diseases likelihood, and as biofilms are highly resistant to antibiotics, it is necessary for soft contact lens care solutions to have the ability to reduce or prevent biofilm formation [10,11].

Bacterial cells that colonize a surface within a biofilm show greater resistance to antimicrobial substances than free cells. This phenomenon is attributed to both the lower speed of diffusion of biocides through the biofilm matrix, and the lower levels of oxygen and nutrients that the cells receive compared to the planktonic ones. This results in a lower growth rate, but also less sensitivity to antibiotics and disinfectants [12].

Preventing, reducing or eliminating microbial biofilms from soft contact lenses is now a necessity for improving eye health. Therefore, anti-biofilm coatings and development of anti-biofilm therapies are the most promising goals for reducing the risk of eye infections associated with biofilms [13].

The inherent biofilm resistance to common disinfectants makes the use of natural compounds as "anti-biofilm agents" challenging. Many natural compounds have been used to kill infectious pathogens, and others have been used for eye remedies [14]. Since ancient times, garlic (*Allium sativum*) and onion (*Allium cepa*), have represented important components of typical recipes and traditional healing systems [15]. Mohsenipour and Hassanshahian studied the effects of *Allium sativum* extracts on biofilm formation and activities on six pathogenic bacteria. The abilities of *A. sativum* alcoholic extracts in inhibition of biofilm formation of *S. pneumoniae*, *P. aeruginosa* and *K. pneumoniae* were more than their ability to destroy the biofilm of these bacteria. This study confirmed the ability of garlic extracts to inhibit the attachment of *Staphylococcus* spp., and therefore, their ability to inhibit the biofilm formation of these bacteria. According to the results of this research and other studies performed on extracts and essential oils of *A. sativum*, the antimicrobial potential of this plant was confirmed and the extracts of this plant were shown to be suitable choices against pathogenic microorganisms [16].

Recently, the antibacterial activity of *Cannabis sativa* was also studied. Several researchers noted its activity against various microorganisms and its anti-biofilm ability [17].

To reduce the risk of eye infections associated with biofilms, several studies have been devoted to the development of anti-biofilm coatings and therapies [13].

In the current study, two natural compounds, *Allium sativum* fermented extract (BGE) and cannabinol oil extract (CBD), *Cannabis sativa* metabolite, were assessed for their activity on inhibition and removal of *P. aeruginosa* biofilms on soft contact lenses in comparison to a multi-purpose Soft Contact Lens-care solution found in the Italian market.

2. Results

2.1. Assessment of Biofilm Formation

Two strains were tested for biofilm production, *P. aeruginosa* (*P. aeruginosa*; ATCC 9027 strain) and *Pseudomonas aeruginosa* clinical strain. Figure 1 shows the total biomass of microbial biofilms on soft contact lenses. The graph highlights that both microbes are capable of forming biofilms on the surface of soft contact lenses and they are classified as strongly biofilm-forming (4.ODc < OD).

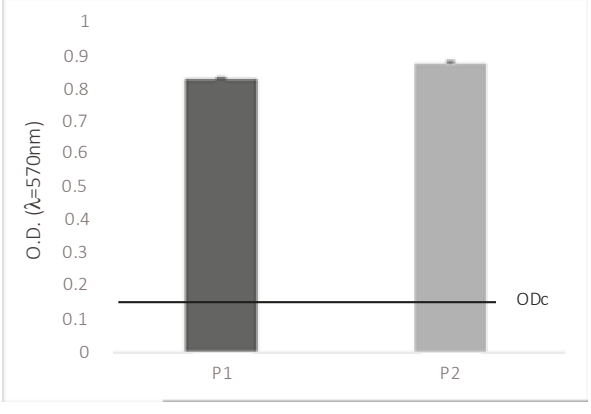

Figure 1. Total biomass of microbial biofilms on soft contact lenses. Negative (OD ≤ ODc), weak (ODc ≤ OD ≤ 2.ODc), moderate (2.ODc < OD ≤ 4.ODc), and strong biofilm production (4.ODc < OD). OD = optical density; P1 = *P. aeruginosa* ATCC 9027; P2 = *P. aeruginosa* clinical strain.

2.2. Effectiveness of Disinfectant Solution and Natural Compounds on the Inhibition of Biofilms

The MIC for Soft Contact Lens-care solution was (50%) of the original concentration. While the MIC for BGE and CBD were 20% and 2%, respectively (Table 1).

Table 1. Minimum inhibitory concentration (MIC) of BGE, CBD and soft CL-care solutions against tested stains (S.D. = Standard deviation).

Tested Substances	*P. aeruginosa* ATCC 9027	S.D.	*P. aeruginosa* Clinical Strain	S.D.
BGE	4%	0.13	4%	0.38
CBD	2%	0.31	>2%	-
CL-care solutions	50%	0.16	50%	0.23

2.3. Prevention of Biofilm Formation

The MIC and sub-MIC of disinfectant solutions were tested for biofilm inhibition capacity.

For both *Allium sativum* fermented and CBD, $\frac{3}{4}$, $\frac{1}{2}$ and $\frac{1}{4}$ MIC were tested on biofilm-forming *Pseudomonas aeruginosa* (*P. aeruginosa*; ATCC 9027 strain) and *P. aeruginosa* clinical strains, while for the care solution, $\frac{4}{5}$ (40%) and $\frac{3}{5}$ (30%) MIC were tested (Figure 2).

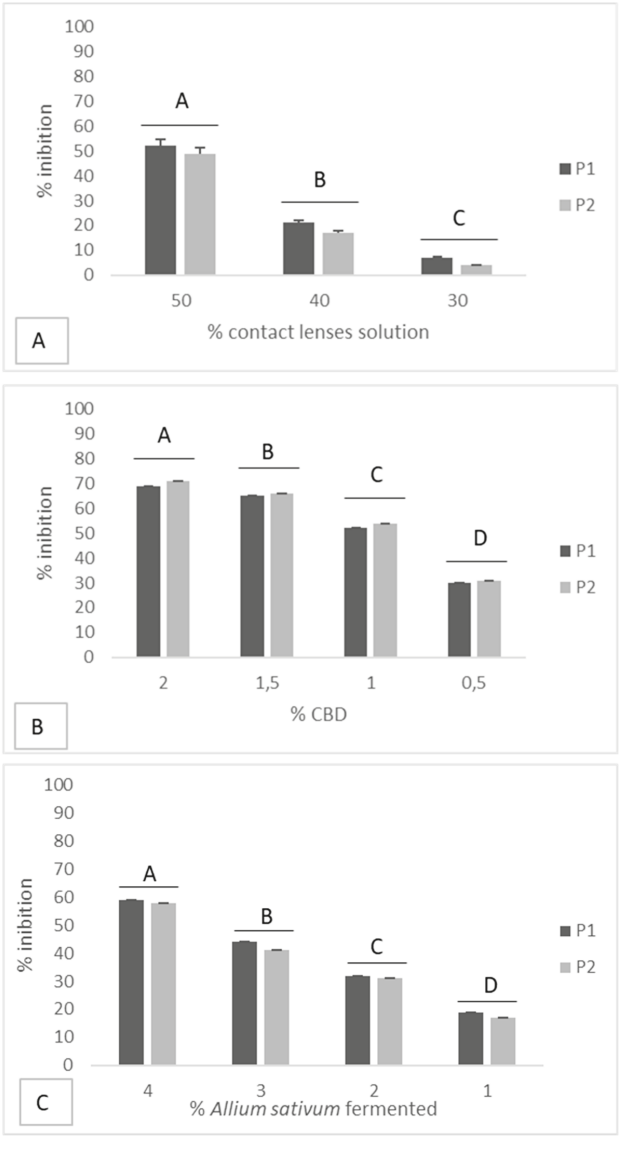

Figure 2. Effect of *Allium sativum* fermented, CBD and Soft CL-care solution on the inhibition of microbial biofilms on soft contact lenses; data with different letters (A–D) are significantly different ($p < 0.05$, T- Test). P1 = *P. aeruginosa* ATCC 9027; P2 = *P. aeruginosa* clinical strain.

Allium sativum extracts at a concentration of 20% (MIC) showed an inhibition percentage higher than 55%, while at a concentration of 10% ($\frac{1}{2}$ MIC), they inhibited biofilm formation by about 35%, for both strains tested.

CBD at a concentration of 2% (MIC) inhibited biofilm formation by about 70%, while at a concentration of 1% ($\frac{1}{2}$ MIC), it inhibited biofilm formation by about 50%, for both strains tested.

Soft Contact Lens-care solution at a concentration of $\frac{1}{2}$ (50%) of the original concentration inhibited biofilm formation by about 50%, while at a concentration of $\frac{4}{5}$ (40%) and $\frac{3}{5}$ (30%) MIC, it inhibited biofilm formation by about 20% and less than 10%, respectively, for both strains tested.

2.4. Eradication of Biofilm Formation

Different concentrations of each compound were tested for their biofilm removal effect.

The effect of *Allium sativum* fermented on the eradication of microbial biofilms on soft contact lenses at MIC was 45% eradication for *P. aeruginosa* ATCC 9027 and 36% for *P. aeruginosa* clinical strain. At a concentration of $\frac{3}{4}$ MIC, it was 17% eradication for *P. aeruginosa* ATCC 9027 and 10% for *P. aeruginosa* clinical strain (Figure 3).

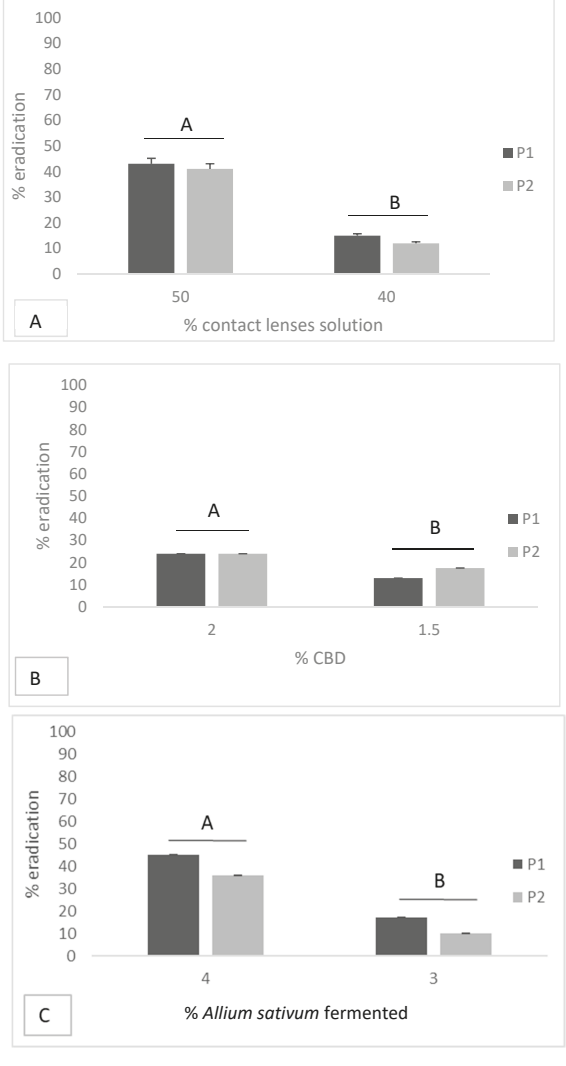

Figure 3. Effect of *Allium sativum* fermented, CBD and Soft CL-care solution on the eradication of microbial biofilms on soft contact lenses; data with different letters (A–B) are significantly different ($p < 0.05$, T-Test). P1 = *P. aeruginosa* ATCC 9027; P2 = *P. aeruginosa* clinical strain.

For CBD, 24% eradication of biofilm formed by both strains was observed at MIC, while at a concentration of $\frac{3}{4}$ MIC, there was 13% eradication for *P. aeruginosa* ATCC 9027 and 18% for *P. aeruginosa* clinical strain (Figure 3).

The effect of care solution on the eradication of microbial biofilms on soft contact lenses at MIC concentration was 43% eradication for *P. aeruginosa* ATCC 9027 and 41% for *P. aeruginosa* clinical strain. At a concentration of 4/5 MIC, it was 15% eradication for *P. aeruginosa* ATCC 9027 and 12% for *P. aeruginosa* clinical strain (Figure 3).

2.5. Determination of Fractional Inhibitory Concentration Index

Table 2 shows the fractional inhibitory concentration index values of test soft contact lenses solution/BGE and test soft contact lenses solution/CBD combinations on biofilms formed by the studied bacteria. It was observed that both test soft contact lenses solution/BGE (fractional inhibitory concentration index: 0.450) and test soft contact lenses solution/CBD (fractional inhibitory concentration index: 0.153) combinations exhibited synergistic antibiofilm activity against the two strains studied.

Table 2. Fractional inhibitory concentration values (FICI) of test soft contact lenses solution in combination with *Allium sativum* fermented extract (BGE) and cannabinol oil extract (CBD) against *Pseudomonas aeruginosa* (*P. aeruginosa*; ATCC 9027) and *Pseudomonas aeruginosa* clinical strain biofilms.

Microorganism.	Combinations					
	Soft Contact Lenses Solution + BGE (CLS+B)			Soft Contact Lenses Solution + CBD (CLCS+C)		
	FIC	*FICI*		*FIC*	*FICI*	
P. aeruginosa ATCC 9027	0.250 (CLS) / 0.200 (B)	0.450	Synergistic	0.150 (CLCS) / 0.030 (C)	0.153	Synergistic
P. aeruginosa clinical strain	0.250 (CLS) / 0.200 (B)	0.450	Synergistic	0.150 (CLCS) / 0.030 (C)	0.153	Synergistic

3. Discussion

Soft contact lens wearers are exposed to an increased risk of developing eye infections on a daily basis, especially when their lenses are not cleaned properly. Soft contact lenses, particularly the soft variety, can provide the ideal reproduction conditions for different pathogens; thus, it is essential that disinfectant solutions are effective against contaminating pathogens to ensure the health of the patient's eyes [18].

In vivo and in vitro studies have suggested that persistent microbial contaminations of soft contact lenses may be associated with biofilm formation and microbial resistance. A bacterial biofilm can be defined as a structured community of bacterial cells [19]. It is possible that during lens insertion and removal, bacteria may be transferred into the lens storage cases via fingers.

Bacterial biofilms forming in soft contact lens storage cases has been well documented [20].

Although soft contact lens multipurpose solutions meet the international ISO 14729 and FDA 510(k) standard for adequate antimicrobial efficacy, they are only subjected to assessment against selected reference strains of planktonic bacteria and fungi. Antimicrobial activity against attenuated laboratory strains does not ensure efficacy against clinical strains. In addition, commercially available disinfecting solutions may be ineffective against biofilms [21].

The effectiveness of disinfection systems was experimentally tested by Wilson and collaborators. The most effective system for biofilm prevention was 3% hydrogen peroxide. The chlorhexidine base systems were shown to be less effective than peroxide but more effective than some quaternary ammonium derivatives or polyamine polypropylene biguanide [20].

In another study, the effectiveness of disinfectants was tested on the prevention of biofilm formation for long and continuous storage times (6 weeks). The results showed that the contamination rate on the walls of the container is 40% if the storage takes place in solution with polyhexamethylene

biguanide, 45% if storage is solution with polyquad, 0% with hydrogen peroxide storage, and 3% if neutralized with a metal catalyst [22].

However, in other studies, the use of hydrogen peroxide was instead associated with a higher degree of container contamination [23,24].

The current study aimed to screen for the biofilm forming from *P. aeruginosa* (*P. aeruginosa*; ATCC 9027 strain) and *P. aeruginosa* clinical strain, and to evaluate the anti-biofilm activity of some natural compounds in comparison to CL-care solution.

Our results showed that the disinfectant solution on the market has moderate activity in inhibiting biofilm formation at MIC concentration (about 50%) without the rubbing step (recommended by the manufacturer but not complied with by some consumers). At the considered sub-minimal inhibitory concentrations, the percentage of inhibition dropped drastically, never exceeding 21%.

We also evaluated the ability of the solution to eradicate the biofilm. The results show a percentage of about 40% for both strains with MIC, but already a percentage lower than 15% at the first tested sub-MIC.

On the contrary, a previous study attributed great efficacy against planktonic bacterial growth to all the solutions tested for the care of soft contact lenses, but a poor activity against bacterial biofilms in vitro [19].

The tested organisms were also exposed to minimal inhibitory concentrations and sub-minimal inhibitory concentrations of two natural compounds to evaluate their ability to inhibit and remove biofilms: *Allium sativum* fermented extract and cannabinol oil extract, *Cannabis sativa* metabolite.

Several studies showed the antimicrobial activity of garlic [16,25] and of *Cannabis sativa* [26].

CBD showed the highest activity in inhibiting biofilm formation. Inhibition rates were above 50%, even at sub-minimal inhibitory concentrations.

Instead, *Allium sativum* fermented extract showed higher eradication rates to MICs, while the results are superimposable for sub-minimal inhibitory concentrations.

In the ophthalmological field, *Allium sativum* extracts seem to give good results in solving eye problems and are well tolerated by the eye [27].

There has been a resurgence in interest and use of the cannabis plant for medical purposes. The use of cannabis for therapeutic purposes was increasingly limited up to the prohibition of its use with the Single Convention on Narcotic Drugs of 1961. Only several decades later, cannabis has been readmitted as a pharmacological active drug and "medical cannabis" is used and legalized for therapeutic purposes in many countries [28].

Different possibilities of medical use of cannabis have been reported in the literature and, among these, are antitumor effects and treatment of glaucoma [29].

Recently, synergistic combinations of antimicrobials have been proposed to eradicate infections due to multi-resistant pathogens. Some studies indicate the choice of synergistic combination therapy as a preferential treatment in biofilm-associated infections [30]. To evaluate the type of antibiofilm interactions (synergistic, additive or antagonistic), fractional inhibitory concentration index values of test antimicrobials were determined. From the fractional inhibitory concentration index values (Table 2), it was observed that the test soft contact lenses solution/BGE combination showed synergistic antibiofilm efficacy against 69% of test bacterial isolates, whereas this value for test soft contact lenses solution/CBD combination was 75% (data not shown).

Possible mechanisms behind the synergistic interactions of test soft contact lenses solution in combination with BGE and CBD is not clear right now. Studies have shown that two different anti-biofilm mechanisms are able to modulate biofilm formation: inhibition of bacterial surface attachment and interruption of quorum sensing [31].

Such scientific evidence led us to test these two natural compounds against biofilms.

4. Materials and Methods

4.1. Bacterial Culture

Pseudomonas aeruginosa (*P. aeruginosa*; ATCC 9027) and *Pseudomonas aeruginosa* clinical strain isolated from ocular swabs were maintained in glycerol stock cultures at −80 °C prior to use and cultured onto Tryptone soy agar (TSA) (Becton Dickinson and Company). Single colonies of bacteria from the overnight cultures were inoculated into tryptone soy broth (TSB) (Becton Dickinson and Company) and incubated in a shaking incubator at 37 °C.

4.2. Screened Compounds

One care solution available in the Italian market was tested. It is a sterile isotonic solution, containing polyhexamethylene biguanide at 0.00005% as a preservative active ingredient. Two natural compounds were also tested: BGE (stock solution 175 mg/mL) and CBD (stock solution 3%), *Cannabis sativa* metabolite.

BGE was prepared in the Food Engineering Lab of the Department of Chemical, Material and Production Engineering, University of Naples Federico II. Fresh garlic, bought locally, was fermented for 7 days at a high temperature and high relative humidity (90 °C and RH 70%). The fermented garlic was then pulverized and mixed with distilled water in a 1:1 ratio. Subsequently the aqueous fraction of this mix, the BGE, was separated by a patented extraction process using gaseous norflurane in subcritical condition as a solvent [32]. Cannabinol oil extract (CBD) was purchased from Enecta B.V. (Amsterdam, Holland) (300 mg, 3% CBD).

4.3. Biofilm Production

Overnight cultures of isolates from TSA were inoculated in 5 mL TSB and incubated for 24 h at 37 °C. The suspension was diluted 1:100 in TSB to obtain a density of 10^6 cells/mL. Then, 100 µL of the suspension was added into individual wells, containing silicone hydrogel contact lens (Soft15 energy by Salmoiraghi and Viganò), of polystyrene 24-well plates and incubated at 37 °C for 24 h to allow the develop of the biofilm; media alone was the negative control included.

The total biomass of the biofilm was analyzed using the crystal violet (CV) staining method [33], as described elsewhere [34]. The content of each well was aspirated and then washed three times with phosphate buffered saline to remove any non-adherent bacteria. The soft contact lens were placed in new 24-well plates at 44 °C for 60 min to allow fixation.

Then, 150 µL of CV (0.2% p/v) was added to each well and incubated for 15 min. After washing the wells with deionized water, excess stain was gently rinsed off by tap water. Crystal violet bound to the biofilm was detached using 150 µL of 30% v/v acetic acid for 30 min at room temperature, and the absorbance at 570 nm was detected with a spectrophotometer (DR5000, HACH). The test was done in duplicates. Based on the measured optical density, *Pseudomonas aeruginosa* (*P. aeruginosa*; ATCC 9027) and *Pseudomonas aeruginosa* clinical strain were classified into four categories; non-adherent, weakly adherent, moderately adherent, and strongly adherent [35].

4.4. Determination of Minimum Inhibitory Concentration of Screened Compounds

Minimum inhibitory concentrations of screened compounds were determined with a microbroth dilution technique as described by the Clinical and Laboratory Standards Institute (CLSI, 2006 M7-A6) with some modifications, using tryptone soy broth [36].

Two fold serial dilutions of each disinfectant agent were prepared using microtiter plates; 100 µL of each dilution were placed in adjacent wells. Then, 100 µL of prepared inoculum was added to each dilution, control wells were included and experiments were made in triplicate. Plates were incubated at 37 °C for 24 h and examined. Wells without the test molecule served as control. The minimum inhibitory concentration was defined as the lowest concentration of compound that completely inhibited visible growth analyzed at 590 nm using a microplate reader (Synergy H4 BioTek) [37].

4.5. Effectiveness of Screened Compounds on Inhibition and Eradication of Biofilm Formation

Strains were grown overnight at 37 °C in tryptone soy broth, washed twice in phosphate buffered saline, and suspended to obtain a suspension equivalent to 1×10^5 cells/ml (OD600). Then, 100 µl of each inoculum was dispensed into wells of 24-well microtiter plates.

To prevent cell adherence at the intermediate stage (24 h biofilms) (MBIC), the plates were incubated at 37 °C for 24 h with the soft contact lens solution at 50%, 40% and 30% of its original concentration; with BGE at concentrations of 40 mg/mL, 30 mg/mL, 20 mg/mL, and 10 mg/mL, and with CBD at concentrations of 20 mg/mL (2%), 15 mg/mL (1.5%), 10 mg/mL (1%), and 5 mg/mL (0.5%).

To eradicate preformed biofilm at the maturation stage (48 h biofilms) (MBEC), the plates were incubated for 48 h, the medium was renewed after 24 h, and disinfectants at the same concentrations were added at the last 24 h. Biofilms formed by bacteria that did not undergo any treatment were used as controls for comparison with the means of the treatments.

The effect of disinfectants on biofilm inhibition and eradication was quantified by using the XTT assay that analyzed the density of the adhered cells, measuring the relative metabolic activity using the XTT (2,3-bis (2-methoxy-4-nitro-5-sulfo phenyl)-5-(phenylamino) carbonyl)-2H-tetrazolium hydroxide) colorimetric assay kit (Sigma) following manufacturer's instructions as described elsewhere [38].

Continuous variables were compared using the Student t-test.

4.6. Determination of Fractional Inhibitory Concentration Index

The synergistic activity of the test care solution and the two natural compounds was evaluated by calculation of the fractional inhibitory concentration index (FICI) using the method of Ramage et al. [39].

Biofilm formation of the test bacterial strains was achieved following the same protocol as described above. After biofilm formation, the medium was aspirated gently and non-adherent cells were removed by washing the biofilms three times with sterile phosphate buffered saline. Then, 100 µL of 2-fold serial dilutions (1/32× minimum inhibitory concentration to 4× minimum inhibitory concentration) of the test care solution and natural compounds were added to each biofilm. The two antimicrobial agents and the test care solution were mixed in the plate crosswise in such a way that the resulting checkerboard contained each combination of the substances in eight doubly increasing concentrations, with wells containing the highest concentration of each substance at opposite corners.

The MBEC of compound combinations, defined as the lowest concentration of substance required to eradicate the biofilm was determined by the XTT reduction assay following the method of Ramage et al. [39].

Fractional inhibitory concentration indices were calculated using the formula: fractional inhibitory concentration index = (minimum biofilm eradication concentration of natural compound in the presence of test care solution/minimum biofilm eradication concentration of natural compound alone) + (minimum biofilm eradication concentration of test care solution in the presence of natural compound/minimum biofilm eradication concentration of test care solution alone). The results were interpreted according to fractional inhibitory concentration indices as follows: 'synergy' (fractional inhibitory concentration index ≤ 0.5), 'additive' (fractional inhibitory concentration index > 0.5–4) and 'antagonism' (fractional inhibitory concentration index > 4) [40]. All the experiments were repeated twice.

5. Conclusions

The results of this study are supported by previous studies according to which natural compounds could be used as substances that prevented eye infections, especially those caused by the reckless use of soft contact lenses. The current study is the first to assess the anti-biofilm activity of both BGE and CBD on soft contact lens. It showed that BGE and CBD have an excellent effect on inhibition of biofilm formation and removal of preformed biofilms, which make them promising agents that could be added to new more effective care solutions.

The results provide evidence that the test soft contact lenses solution alone and in combination with BGE and CBD may serve as a potential source for treatment of biofilm-associated soft contact lens, hoping for less negative effects on eye health and less problems related to drug resistance.

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Microbial Growth and Biogenic Amine Production in a Balkan-Style Fresh Sausage during Refrigerated Storage under a CO_2-Containing Anaerobic Atmosphere: Effect of the Addition of *Zataria multiflora* Essential Oil and Hops Extract

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Abstract: Fresh sausages are highly perishable, and the preservatives allowed in these types of meat preparations are limited. Balkan-style fresh sausages were prepared in triplicate without antimicrobials (Control), with an aqueous hops extract (30 mL/kg), with *Zataria multiflora* Boiss essential oil (1 mL/kg), or a combination of both (15 and 0.5 mL/kg, respectively), and refrigerator-stored under a 20% CO_2 and 80% N_2 atmosphere. The spoilage microbial growth, i.e., lactic acid bacteria (LAB), *Brochothrix thermosphacta*, *Enterobacteriaceae*, *Micrococcaceae*, molds and yeasts, the pH value, and the production of biogenic amines in the sausages were monitored weekly and compared with a control sausage during a 35-day storage period. Furthermore, 349 colonies of presumptive LAB (isolated from the De Mann, Rogose-Sharpe agar plates) were identified using a MALDI-TOF-based method. Growth levels to ≈ 9 Log colony forming units (CFU) per g were reached by LAB, with a predominance of *Lactobacillus sakei*. *Enterobacteriaceae* and *B. thermosphacta* also showed significant growth (up to 6 Log CFU/g). Biogenic amine levels increased, and tyramine values overcame 250 mg/kg. The study could not demonstrate a significant effect of antimicrobial source treatments in any of the characteristics studied, and thus, the shelf-life of sausages.

Keywords: lamb sausage; lactic acid bacteria; shelf-life; natural antimicrobials; meat preparations; modified atmosphere packaging

1. Introduction

Fresh sausages are produced with comminuted meat, salt, species, and condiments and a limited number of allowed additives. Their formulation, preparation, and dimensions strongly depend on local preparation. Fresh sausages must be refrigerator-stored and cooked before consumption. They are considered to be highly perishable, with pH values >5.5 and water activity (a_w) ≥0.97 [1]. To retard microbial growth, fresh sausages are commonly stored at low temperatures under anaerobic

CO_2-containing modified atmosphere packaging (MAP). The spoilage microbiota of fresh sausages on these conditions consists of facultative anaerobic microorganisms such as lactic acid bacteria (LAB), *Brochothrix thermosphacta*, and *Enterobacteriaceae*, with LAB being observed as the predominant group [2,3]. The shelf-life of fresh sausages refrigerator-stored under anaerobiosis depends on the hygienic quality of raw materials, pH, a_w, storage temperature, atmosphere, etc. [4]. Some authors, based on the appearance of off-odors and discoloration, have found a shelf-life for these sausages slightly longer than 10 days [3] and others of more than 20 days [5–7].

During refrigerated storage of fresh sausages under vacuum or anaerobic MAP, and most probably due to the growth of LAB and *Enterobacteriaceae*, a significant production of biogenic amines (BA) such as tyramine, putrescine, and cadaverine occurs [4,8]. In a previous study [2], levels of tyramine higher than 100 mg/kg were found in a Mexican fresh sausage stored in anaerobic MAP for more than two weeks, which represents a health risk and corroborates the need to control the production of BA in fresh sausages.

A current approach to extend the shelf-life of fresh sausages is the use of natural antimicrobials [9]. Hops, the strobiles (female flowers) of the *Humulus lupulus* L. plant, which are commonly used in brewery and have found application in other foods [10], appear to be a potentially suitable ingredient for this purpose. Hops contains antimicrobial compounds, such as prenylated acylphloroglucinols, bitter acids or xanthohumol, among others, which have been probed to inhibit Gram-positive bacteria [11,12]. The Food Safety and Inspection Service from the USA has approved the use of hops α-acids as antimicrobials for cooked meat and casings [13]. Moreover, Kramer et al. [11] found hops extract to inhibit total aerobic microbial growth in marinated pork. However, the effect of hops in fresh sausages packaged under anaerobic conditions seems to have been rarely studied. Hops could interfere in the growth of Gram-positive spoilage microorganisms such as LAB or *Brochothrix thermosphacta*, thus extending the sausage shelf-life.

Plant-derived essential oils (EO) obtained from aromatic and medical plant materials have proved wide antimicrobial spectra against bacteria, yeasts, and molds [14]. Nonetheless, among bacteria, the Gram-positive are more susceptible than the Gram-negative [15]. The effectiveness of EO at levels up to 2% in extending the lag phase or reducing the final population of spoilage microbiota in minced meat and meat products during refrigerated storage has been reported [16,17]. EO has been claimed to be one of the best alternatives to synthetic preservatives in meat and meat products [17]. However, the use of EO in meat as well as in other foods as natural preservatives present relevant limitations regarding deleterious effects in sensory quality due to their strong flavor, loss of antimicrobial activity due to interactions with food components, and regulatory or safety issues [18]. In this context, the use of EO combined with other synergistic or complementary natural antimicrobials has been suggested as a viable approach to using lower amounts of EO, thus not affecting the sensory acceptation, while achieving a significant antimicrobial effect.

Among the EO, that obtained from *Zataria multiflora* Boiss (ZM), which contains carvacrol and thymol as its main components, has shown a significant antimicrobial effect, this effect being greater on Gram-negative bacteria [19–21]. *Zataria multiflora* Boiss, belonging to the *Laminaceae* family, is cultivated in warm parts of the Middle East, where it is popularly used in traditional medicine and as food flavoring and preservative [22]. Regarding processed meat, it has been reported that the addition of ZM's EO at levels up to 0.1% reduced the growth of total viable microbiota, *Pseudomonas* spp., and LAB in buffalo burgers during aerobic storage [23,24]. Moreover, a chitosan film containing this EO (5–10 g/kg) also reduced the counts of total viable microbiota at the surface of mortadella-type slices packaged in oxygen permeable polyethylene bags during refrigerated storage [25]. The above-mentioned studies were carried out using aerobic storage; however, no study has been found in the literature addressing the antimicrobial effect of ZM's EO on meat or meat products stored under anaerobic MAP.

This study has aimed to evaluate the growth of spoilage microorganisms and BA production in a typical fresh sausage during refrigerated storage under CO_2 plus N_2 MAP, and to assess the effect of two natural antimicrobials: hops and ZM's EO, alone or combined. The study focused on LAB

population considering them as the predominant spoilage microorganisms in fresh sausages packaged under this type of atmosphere and responsible for the BA formation.

2. Results and Discussion

2.1. Water Activity, pH, Microbial Contents, and Biogenic Amine Production

The mean (standard deviation) a_w and pH values of the sausages from the three batches at day 0 were 0.987 (±0.004) and 6.01 (±0.02), respectively. During storage, the pH values decreased steadily ($P < 0.05$) from day 7 to day 28 for all the treatments, with the effect of either treatment or treatment x storage time interaction being non-significant. The mean values of pH in sausages (the four treatments) at days 7, 14, 28, and 35 were 6.05 (±0.01), 5.89 (±0.01), 5.66 (±0.02) and 5.61 (±0.03), respectively (data not shown in tables for brevity).

As is shown in Figure 1, the counts of LAB, *B. thermosphacta*, *Enterobacteriaceae*, and *Micrococcaceae* were not significantly affected by antimicrobial treatment. The mean counts of the Gram-positive LAB and *B. thermosphacta* tended to be higher in control (C) sausages, although the *P* values from the analysis of variance (ANOVA) were not significant, i.e., 0.263 and 0.397, respectively (not shown in figures). ZM's EO contains high amounts of antimicrobial molecules, i.e., thymol, carvacrol, α-terpinene, and a contrasted antimicrobial effect in in vitro experiments [19,21]. In this study, the lack of effect of ZM's EO on microbial growth in the fresh sausages could be explained by a loss of inhibitory efficacy due to interactions between the antimicrobials and sausage matrix compounds such as fat or specific proteins, which could be influenced by the sausage pH and a_w [15,18].

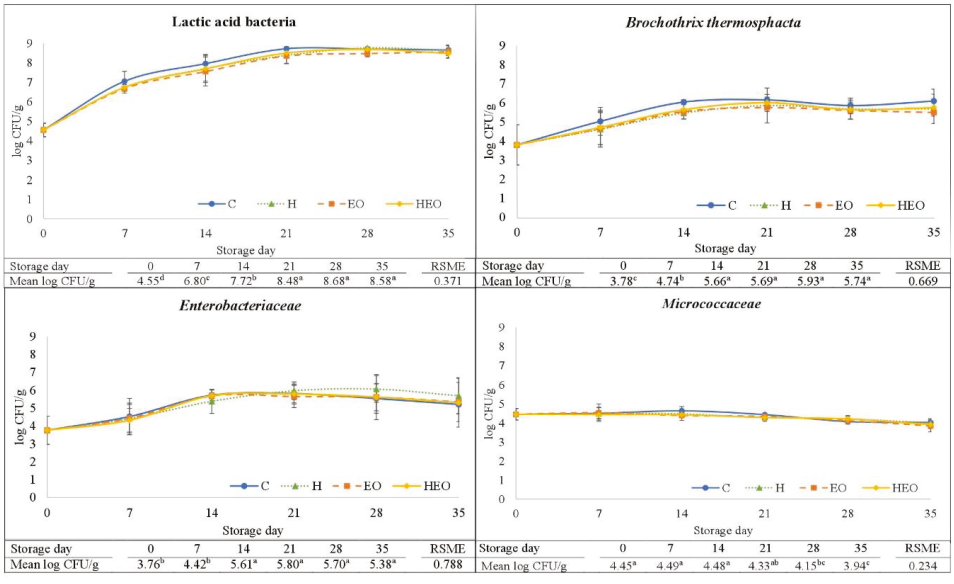

Figure 1. Effect of the addition of different natural antimicrobial sources on lactic acid bacteria: *Brochothrix thermosphacta*, *Enterobacteriaceae*, and *Micrococcaceae* counts (mean values, $n = 3$, and standard deviation, vertical bars) in fresh lamb sausages packaged under modified atmosphere (80% N_2, 20% CO_2) during refrigerated storage (4 °C). CFU: Colony forming units. RSME: Root square mean error. C: Control sausages; H: hops; EO essential oil (*Zataria multiflora* Boiss); HEO: hops and essential oil. ^{abcd}: Total means ($n = 12$) with different superscripts time-related indicate statistical differences (Tukey test, $P < 0.05$).

In contrast with our results, the addition of different EO, i.e., bay leaf, cassia, clove, holy basil, lemon, thyme, or sage, to fresh sausages at levels between 0.01% and 0.25% has shown significant reducing effects on the growth of spoilage microflora during refrigerated storage of fresh sausages packaged under aerobic atmosphere [26–29]. Moreover, the ZM's EO at levels up to 0.1% also significantly decreased the growth of total viable microbiota, *Pseudomonas* spp. and LAB in burgers during aerobic refrigerated storage [23,24]. Nevertheless, a clear difference between those studies and this one is that in the formers, the atmosphere was aerobic and in this study it was anaerobic. This suggests that the antimicrobial effect of EO in fresh comminuted meat products might be higher on the microbiota growing in fresh minced meat products with O_2 than in that growing when the presence of O_2 is restricted.

Hops extracts have been demonstrated to be useful as antimicrobials in casings, cooked ready-to-eat meat, and marinated meat products [11,13]. However, no study has been found investigating their antimicrobial effect in fresh comminuted meat products. In this study, hops extract given alone or combined with ZM's EO did not reduce the growth of spoilage bacteria. Again, chemical interactions between the hops antimicrobials and the food matrix would be the reasons for the lack of significant antimicrobial activity. In order to achieve a positive antimicrobial effect due to the use of hops extracts in fresh sausages stored under anaerobiosis, it is suggested to use a higher amount of hops antimicrobials or reduce the sausage pH, due to the reported higher effect of hops in food matrix with pH close to 5 [11].

Regarding the changes on microbial growth (Figure 1), LAB became the dominant microbial group from day 7 onwards, reaching final values slightly higher than 8 Log colony forming units (CFU) per g at day 21, which can be considered as the onset of the stationary growth phase. Psychrotrophic LAB have been found to become the major microorganisms in fresh sausages refrigerator-stored under anaerobic CO_2-containing MAP over the third week of storage [3,30], with the maximum LAB levels being comparable to those from this study. Lactic acid bacteria are considered as the principal spoilage-specific microorganisms in meat and fresh sausages stored under vacuum or CO_2-containing anaerobic MAP [31]. Thus, the appearance of off-flavors, i.e., sour or putrid, in fresh pork sausage has been related to LAB counts over 7–8 Log CFU/g [5,32].

Both *B. thermosphacta* and *Enterobacteriaceae* showed similar growth patterns between them, i.e., starting with counts near to 4 Log CFU/g at day 0 and reaching the stationary phase at day 14 with counts of 5–6 Log CFU/g (Figure 1). In both cases, the growth phase was slower and shorter than that for LAB, which suggests a competitive effect of LAB, probably due to a higher ability of LAB for the consumption of limiting nutrients under the anaerobic MAP fresh sausage conditions [33].

Other studies also described how *B. thermosphacta* steadily increases its levels in fresh meats during refrigerated storage under vacuum and anaerobic MAP, becoming one of the dominant spoilage species and originating cheesy, buttery, or sour odors [34]. According to Samelis [35], the levels of *B. thermosphacta* associated to fresh meat spoilage are around 7 Log CFU/g.

The control of *Enterobacteriaceae* in fresh sausages seems to be desirable since levels of 4–5 Log CFU/g [36] have been associated with meat spoilage—counts higher than this level were overcome in this study at day 14. The growth pattern of *Enterobacteriaceae* in fresh sausages stored under CO_2- and N_2-containing MAP has shown variability among studies. In agreement with our results, Benson et al. [32] reported an exponential growth of *Enterobacteriaceae* during the first two weeks of storage of a fresh pork sausage, reaching counts around 6 Log CFU/g; however, Ruíz-Capillas and Jiménez Colmenero [3] found the levels of *Enterobacteriaceae* in fresh pork sausages to decrease after 10 days of refrigerated storage. These differences might be explained by variations among studies in spice mixtures, sausage pH, or bacterial communities and their competence.

Micrococcaceae counts were stable up to day 21 of storage and then decreased slightly until day 35 (Figure 1). A decrease after some weeks of storage has been described in other studies on fresh sausages during anaerobic refrigerated storage [37,38], and attributed to both pH decrease and low O_2 and nutrients availability. No differences were found due either to treatment nor storage time in the molds and yeast counts (the mean values considering all the treatments and days were 2.99 ± 0.17 Log CFU/g; n = 24; data not shown in tables for brevity), which is probably due to their low growing ability under diminishing O_2 levels [38].

Changes in BA production in sausages are shown in Table 1. The levels of BA were not affected by antimicrobial treatment except for spermine (P = 0.037), with slightly higher amounts in the hops extract and essential oil (HEO) sausages than in the C sausages. Mono and diamines in fresh sausages are presumably produced from microbial enzymatic decarboxylation of free amino acids. In ripened sausages, this is mainly carried out by LAB and *Enterococci*, this ability being strain-dependent [39]. *Enterobacteriaceae* and *B. thermosphacta* can also contribute to the production of BA in LAB-fermented meats, with the first being more active in cadaverine and putrescine formation and the latter in histamine and tyramine [40–42]. The lack of effect of treatment on BA in the fresh sausage is thus coherent with the absence of a significant effect on microbial growth. However, the levels of tryptamine, putrescine, and histamine were significantly different between the experimental batches (data not shown in tables). Thus, the levels of putrescine and histamine in the second batch were respectively more than 5 times and 20 times higher than in the other two batches, which would corroborate the dependence of BA production on microbial strains.

In contrast with our results, Lu et al. [43] reported a reduction in biogenic mono and diamine production in Chinese smoked sausages as a result of the addition of a mixture of essential oils (from cinnamon, cloves, ginger, and anise; 0.12% in total) and tea polyphenols (0.19%) to the sausage mix. The discrepancy between both studies could be attributed to differences in the antimicrobial source used, the making process, and storage conditions (i.e., 50 °C smoking, 20–22 °C fermentation and 10–12 °C ripening-drying steps versus continuous refrigerated storage under CO_2-containing MAP).

Storage time affected the amounts of all mono and diamines in the fresh sausage, which increased steadily, indicating a continuous formation of those BA by the active microbiota during storage. However, time did not affect the content of polyamines originated from de novo synthesis in animal tissues [44]. Overall, the time-related changes in the content of BA in this study have been observed in other studies on fresh sausages stored under anaerobic MAP [2,8]. Biogenic monoamines can produce toxic effects on the consumers resulting in migraine, hypertensive crisis, or allergy [45]. Among them, histamine and tyramine present the highest health concern [46]. The maximum recommended levels for both amines in fermented sausages are over 100 mg/kg—although their toxicity depends not only on their levels in food but also on dietary factors and consumers' susceptibility [47]. Tyramine content in the fresh sausages from this study exceeded that limit (100 mg/kg) at day 14. On the other hand, although the diamines putrescine and cadaverine are not considered toxic per se, they can enhance the toxic effect of histamine and tyramine [48].

Table 1. Biogenic amine contents (mg/kg) in fresh sausages stored at 2 °C under anaerobic modified atmosphere storage as a function of antimicrobial treatment (Treat) and storage day (Time).

Biogenic Amine	Treat				Time						RMSE	*P*-Value		
	C	H	EO	HEO	0	7	14	21	28	35		Treat	Time	Treat × Time
Monoamines														
Tryptamine	17.66	20.48	17.52	19.08	5.58 ^{b}	6.28^{b}	16.85 ^{ab}	18.80 ^{ab}	29.61 ^{a}	34.99 ^{a}	16.33	0.942	0.000	1.000
Histamine	6.90	7.58	4.53	8.24	0.53 ^{b}	0.37 ^{b}	2.40 ^{b}	4.31 ^{b}	13.96 ^{ab}	19.29 ^{a}	17.48	0.926	0.046	1.000
Tyramine	143.04	152.28	150.89	150.40	19.88 ^{e}	99.04 ^{d}	138.37 ^{cd}	176.22 ^{bc}	206.08 ^{ab}	255.31 ^{a}	44.25	0.924	0.000	1.000
Diamines														
Putrescine	8.36	6.97	6.04	6.99	1.36 ^{b}	1.86 ^{b}	3.06 ^{ab}	7.03 ^{ab}	12.23 ^{ab}	17.00 ^{a}	12.35	0.955	0.015	1.000
Cadaverine	98.03	121.74	126.37	114.29	2.14 ^{c}	14.33 ^{c}	66.69 ^{c}	148.73 ^{b}	203.61 ^{ab}	255.14 ^{a}	54.56	0.432	0.000	0.996
Poliamines														
Spermine	25.52 ^{b}	28.88 ^{ab}	29.22 ^{ab}	29.89 ^{a}	30.12	30.17	26.57	26.88	27.16	29.34	4.72	0.037	0.200	0.506
Spermidine	5.83	5.80	6.06	6.22	5.56	6.02	5.97	6.06	5.87	6.37	1.24	0.715	0.738	0.587

C: Control sausages; H: hops; EO essential oil (*Zataria multiflora* Boiss); HEO: hops and essential oil. $^{a\text{-}e}$: Means in the same row within treatment or time showing different superscripts are significantly different ($P < 0.05$; Tukey test).

2.2. Identification of Lactic Acid Bacteria

Only eight out of the 346 isolates from the DeMan-Rogosa-Sharpe (MRS) agar plates were not positively identified. Among the identified isolates, 90% corresponded to LAB (70% of isolates were identified as LAB at day 0 and ≥90% at the other sampling days). Among the non-LAB bacteria, the genus identified in order of abundance were *Staphylococcus* spp., *Enterobacter* spp., *Serratia* spp., *Filifactor* spp., *Escherichia* spp., and *Macrococcus* spp. (not shown in tables). Table 2 shows the frequency (%) of the LAB species identified at different storage days considering the isolates in sausages from the four antimicrobial treatments. The genus *Lactobacillus* was the most abundant (84% of the LAB isolates) regardless of the storage time. *Lactobacillus sakei* was the predominant LAB, with its frequency overcoming 50% from day 7 onwards—when LAB counts showed significant growth (counts higher than 7 Log CFU/g; Figure 1). Among the isolates identified as *Lb. sakei*, 59% were identified as *Lb. sakei* subsp. *carnosus* (not shown in tables) and the others were identified as *Lb. sakei* (only species level). On the other hand, approximately 40% of the isolates ascribed to the *Lactobacillus* genus were not positively identified at species level (provided as *Lactobacillus* spp. in Table 2). Comparing between the first weeks (especially day 0) and the last weeks of storage, the diversity (number) of LAB species showed a tendency to decrease. On days 28 and 35, there was a clear dominance of *Lactobacillus*, and among them, *Lb. sakei* (<90% and ≤70% of total LAB, respectively).

Table 2. Lactic acid bacteria (LAB) species in the sausages [#] at the different storage days (expressed in % of total isolates identified as LAB).

Species	0 (n = 33)	7 (n = 50)	14 (n = 63)	21 (n = 52)	28 (n = 56)	35 (n = 53)
Aerococcus viridans	12	-	-	-	-	-
Carnobacterium maltraromaticum	-	-	5	2	-	4
Lactobacillus casei	3	-	-	-	-	-
Lactobacillus curvatus	-	2	2	-	-	2
Lactobacillus sakei	33	62	51	56	70	72
Lactobacillus spp.	12	14	38	37	30	23
Lactococcus lactis	24	2	-	-	-	-
Leuconostoc mesenteroides	12	14	3	6	-	-
Enterococcus faecalis	-	6	2	-	-	-
Streptococcus salivarius	3	-	-	-	-	-

n = number of isolates identified as lactic acid bacteria; [#] Results on each day include the isolates from the four antimicrobial treatments.

Figure 2 depicts the frequency of LAB species or genus, as identified by the matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis, obtained for each of the experimental treatments from day 7 to 35. *Lb. sakei* was predominant for each of the treatment-day combinations except for H-day 14 (ranging from 40% to 85%). The contingency chi-square test showed no significant effect of treatment either on the frequency of *Lb. sakei* or on *Lactobacillus* spp. (P = 0.419 and P = 0.729, respectively).

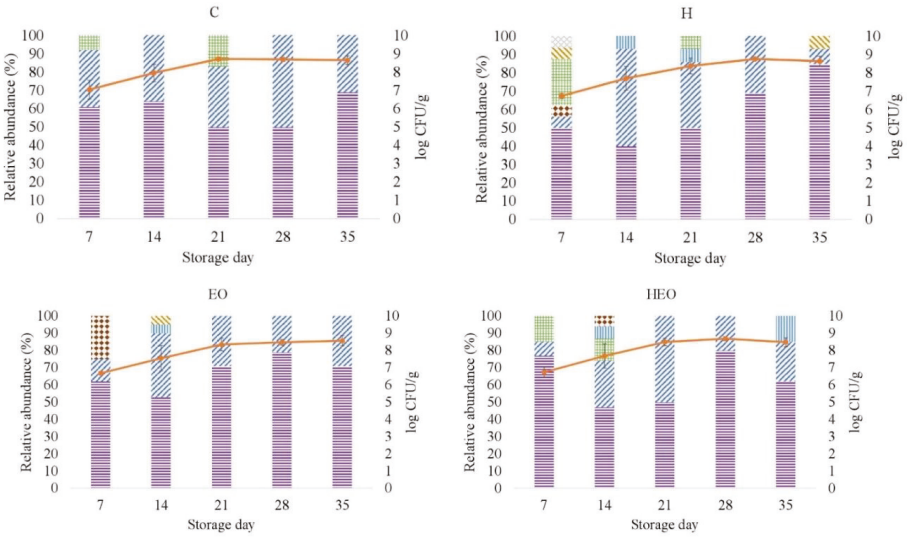

Figure 2. Relative abundance (%) and growth curve (mean and standard deviation, vertical bars; $n = 3$) of the isolates identified as lactic acid bacteria in fresh lamb sausages packaged under modified atmosphere (80% N_2, 20 %CO_2) during refrigerated storage (4 °C). CFU: Colony forming unit. C: Control sausages; H: hops; EO essential oil (*Zataria multiflora* Boiss); HEO: hops and essential oil. *Lactobacillus sakei* (), *Lactobacillus* spp. (), *Leuconostoc mesenteroides* (), *Enterococcus faecalis* (), *Carnobacterium maltromaticum* (), *Lactobacillus curvatus* (), *Lactococcus lactis* ().

Most of the studies on the succession of microbial population in fresh sausages during refrigerated storage have been carried out with the sausages stored under aerobic normal or modified atmosphere [1,4,6,32]. In these studies, LAB species have been one of the major microorganisms detected together with species belonging to *Enterobacteriaceae*, *Micrococcaceae*, *Pseudomonas* spp., and *B. thermosphacta*. However, LAB (*Lb. sakei*, *Lb. curvatus*/*graminis*) and *B. thermosphacta* tended to be the most predominant groups at the end of storage. The degree of abundance of LAB would directly depend not only on storage time but also on the reduction degree of redox potential during storage [49]. On the other hand, in the study by Fougy et al. [7], the sausages were packaged under vacuum and anaerobic 50% CO_2-containing atmosphere. The authors, in agreement with the results of the present study, found, using metagenetic 16S rRNA pyrosequencing, *Lb. sakei* to be the most abundant species in spoiled sausages (after 21 days of storage). Moreover, they also reported the presence of *Lactococcus piscium*, *Carnobacterium divergens*, *Carnobacterium maltaromaticum*, *Serratia proteamaculans*, and *B. thermosphacta*.

Lb. sakei together with *Lb. curvatus* appears to be the dominant LAB species in fermented sausages produced by spontaneous fermentation, and *Lb. sakei* is the most used LAB species as a starter culture for these sausages [50]. This species has been demonstrated to have an effective metabolic adaptation to the meat environment, and to cold temperature and high NaCl concentration. These abilities explain its growth in refrigerator-stored fresh sausages. This LAB have been demonstrated to be resistant to the presence of the antimicrobials tested in the present study at the levels used.

In contrast with traditional fermented sausages, fresh sausages became spoiled after fermentation, which would be due to differences in the LAB metabolism in a meat environment with higher a_w and the concomitant activity of other spoilage microorganisms, i.e., *B. thermosphacta* and *Enterobacteriaceae*.

3. Materials and Methods

3.1. Experimental Plan

Three batches of ćevapi, a Bosnian-style fresh sausage, were prepared using lamb lean meat at the pilot plant at the Faculty of Veterinary Medicine, University of León (Spain). Each batch was composed of four treatments: control (C), with no antimicrobial additives in the formulation; hops extract (H), including an aqueous hops extract; essential oil (EO), with ZM's EO, and with both hops extract and essential oil (HEO). Sausages were packaged in bags under a modified atmosphere (20% of CO_2 and 80% N_2) and then stored under refrigeration at 2 °C for 35 days. Water activity (a_w) was determined at the day of packaging (day 0), and pH, the presence of relevant microbial groups, i.e., lactic acid bacteria (LAB), *Enterobacteriaceae, Brochothrix thermosphacta, Micrococcaceae* and yeast and mold, and the concentration BA were analyzed weekly (storage days 0, 7, 14, 21, 28, and 35). Moreover, 346 colonies (4–5 per batch, day, and treatment) were picked from De Man Rogosa agar plates used for LAB counts and identified by MALDI-TOF mass spectrometry.

3.2. Lamb Meat, Hops Extract, and Essential Oil

Lamb meat came from the legs of six male Assaf lambs reared at the Instituto de Ganadería de Montaña (CSIC; Grulleros, León, Spain). The lambs were weaned with 14 ± 2 kg of weight and fattened to 50 ± 4 kg of weight ad libitum on a pelleted complete diet based on straw (150 g/kg), cereals (barley, corn and soybean meal; 810 g/kg), molasses (10 g/kg), a mineral-vitamin premix (25 g/kg), and sodium bicarbonate (5 g/kg). The animals were then slaughtered in a local abattoir, and their legs were separated from the right-hand carcasses after 24 h post-mortem and then deboned. The meat was then cut into approximately 3 cm cubes, which were trimmed of visible fat. The lean meat from each leg was packaged under vacuum and frozen (−20 °C) until being used (up to 3 months).

The aqueous hops extract used in the study was obtained from recently cropped Nugget variety hop, with α-acid, β-acid, and co-humulone composition of 4.8–5.3%, 12–16%, and 22–28%, respectively. The hops was provided by a local producer (Orbigo Valley S.L., Madrid, Spain). An amount of 50 g of hops was boiled into 1 L of water for 30 min and the final volume was filled up to 1 L, which was filtered through a Whatman number 1 filter paper (GE Healthcare Europe, Barcelona, Spain) and frozen at −18 °C until further use. ZM's EO was obtained from the Faculty of Agriculture, University of Tehran, Iran. Crushed dried leaves of ZM plant were transferred to an all-glass Clevenger-type apparatus and steam distilled for 2.5 h. The essential oil was then dried over anhydrous Na_2SO_4 and stored in opaque glass bottles until further use.

3.3. Sausage Manufacture

Three batches of sausages were produced on different days using the meat from the legs of 2 among the 6 lambs for each of the batches. The sausage-making process was based on a Balkans-style ćevapi recipe. Lamb meat was thawed at 5 °C for 24 h and minced using a butcher's mincer equipped with a 5 mm diameter sieve. A total of 3.8 kg of minced meat was mixed with salt (80 g) for 10 min and placed into a bowl covered with cling film and stored at 4 °C until the next day (24 h). A mixture of finely cut fresh garlic and pepper was boiled in water for 2 min. The mixture (spices solution) was cooled, filtered, and then stored (4 °C) until the next day. The salted minced meat was divided into four parts of 950 g each, one for each of the four above-mentioned treatments (C, H, EO, and HEO) using the ingredients provided in Table 3. All the portions were mixed (for 5 min) with the spice solution (20 mL/kg) and 3 g/kg of sodium bicarbonate. C, EO, and HEO were also mixed with an amount of water, H and HEO treatment with hops extract, and EO and HEO with essential oil (see Table 3). The amount of hops extract added to the H sausage was equivalent to 1.5 g of hops per kg of sausage (i.e., 30 mL of the solution obtained from boiling 50 g of hops per L), which is that commonly used in brewery. The amount of ZM's EO used (1 mL/kg) was within the concentration

ranges reported for antimicrobial activity of EOs in food [16], i.e., around 0.5–20 mL of EO per kg. When both antimicrobial sources were added, their amounts were halved.

Table 3. Ingredients and amounts (expressed in g or mL, solid or liquids, respectively) used in the sausage preparation for the experimental treatments.

Ingredients	Treatments			
	C	H	EO	HEO
Lamb meat	980	980	980	980
Salt	20	20	20	20
Sodium bicarbonate	3	3	3	3
Spice infusion [a]	20	20	20	20
Water	30	-	30	15
Hops extract	-	30	-	15
Essential oil	-	-	1	0.5

C: Control sausages; H: hops; EO essential oil (*Zataria multiflora* Boiss); HEO: hops and essential oil. [a]: Filtered solution obtained by boiling garlic and pepper in water.

The sausage mixtures were stuffed into lamb casings (20/22 cm diameter) and drained for 3 h at 12 °C. The sausages were then cut into 100 g portions, which were individually packaged in bags (150 μm plastic film, oxygen permeability of 30 $cm^3/(m^2 \times bar \times 24\ h)$ at 23 °C and 0% relative humidity) under a 20% CO_2 and 80% N_2 atmosphere at 750 mbars, and refrigerator-stored (2 °C). One C portion was used for analysis at day 0 and one packaged portion for each of the treatments was sampled after 7, 14, 21, 28, and 35 days of storage for subsequent analysis.

3.4. Analysis of Water Activity, pH, Microbial Content, and Biogenic Amine Production

Water activity (a_w) was determined in duplicate at 25 °C using a CX-2 hygrometer (Decagon Devices Inc., Pullman, WA, USA) following the manufacturer's instructions, and pH using a pHmeter (Model 507; Crison, Barcelona, Spain) according to the International Organization for Standardization (ISO) guideline 2917 [51]. For microbiological analysis, samples of 25 ± 0.1 g of sausages were homogenized with 225 mL of peptone water (0.1% peptone) for 2 min in sterile bags using a Stomacher-400 circulator (Seward, West Sussex, UK). Serial decimal dilutions were prepared, and aliquots of the appropriate dilutions were cultured in duplicate on the corresponding media and incubated, according to the procedure described by the culture media manufacturer, as follows: 1 mL on the De Man-Rogosa-Sharpe agar (Oxoid) with double agar layer at 30 °C for 72 h for LAB; 1 mL in Mannitol Salt Agar (Oxoid) at 35 °C for 48 h for *Micrococcaceae*; 1 mL in Violet Red Bile Glucose Agar (VRBGA; Oxoid) with double agar layer at 35 °C for 48 h for *Enterobacteriaceae*; 1 mL in Oxytetracycline Glucose Yeast extract agar (Oxoid) at 22 °C for 5 days for molds and yeast, and 0.1 mL onto the surface of STAA Agar Base (CM 0881; Oxoid) plates containing STA Selective Supplement (0.4 mL/100 mL) and sterilized glycerol (1.5 g/100 mL), at 22 °C for 48 h (only the straw colored oxidase-negative colonies were considered).

Biogenic amine contents were analyzed following the Eerola, Hinkkanen, Lindfors, and Hirvi [52] procedure using a high performance liquid chromatograph (HPLC) Alliance (Waters 2695) equipped with a double wavelength detector (Waters 2996, Waters Corporation, Milford, MA, USA) and a Spherisorb ODS2 column (125 × 4 mm ID; 5 μm; Waters). The standards used for detection and quantification were tryptamine cadaverine dihydrochloride, histamine dihydrochloride, putrescine dihydrochloride, spermidine, spermine, tryptamine hydrochloride, and tyramine hydrochloride (Sigma-Aldrich Química, Madrid, Spain).

3.5. Identification of Lactic Acid Bacteria

From the growth in the MRS plates, 4–5 colonies were picked for each experimental treatment (4), sampling day (7) and batch (3), giving 346 colonies in total. These isolates were then grown in Tryptone

Soy Broth (TSB; Bacto, Mt Printchard, Australia) with 0.5% (*w*/*v*) of yeast extract (YE; Difco, Leeuwarden, The Netherlands) (TSB-YE) at 37 °C for 24 h. One mL aliquot was centrifuged (12,000 rpm, 3 min) in Eppendorf tubes (Eppendorf Ibérica, San Sebastián de los Reyes, Madrid). The supernatants were discarded, and the pellets were suspended in 1 mL of MRS broth with 50% (*v*/*v*) of glycerol. The isolates were maintained at −40 °C for storage purposes. Isolates were recovered for their identification as follows: they were grown at 30 °C on MRS broth (Oxoid) with 0.5% (*w*/*v*) of YE (Difco) at 37 °C for 24 h, and then a loopful of bacteria was sub-cultured in MRS agar (Oxoid).

The analysis was carried out at the Laboratory for Instrumental Analysis, University of Valladolid (Valladolid, Spain). For the analysis, one colony from the MRS plate was picked using a sterilized toothpick and smeared gently onto a MALDI-TOF target plate (Bruker Daltonik GmbH, Leipzig, Germany). After air-drying, 1 μL of formic acid was added. The dried sample was overlaid with 1 μL matrix solution containing 10 mg/mL α-cyano-4-hydroxycinnamic acid (HCCA) in a mixture of acetonitrile, deionized water, and trifluoracetic acid (50/47.5/2.5, *v*/*v*/*v*). The target plate with samples were introduce in the MALDI-TOF equipment for analysis. Not all the bacteria were amenable to analysis: approximately 30% of the isolates were not accurately identified, i.e., identification score at genus level <1.7. For these isolates, the analysis was repeated including an ethanol extraction tube protocol before analysis to extract ribosomal proteins according to the manufacturer's instructions (Bruker Daltonik). Briefly, one colony from MRS plates was sub-cultured in TSB + 0.5% (*w*/*v*) of yeast extract at 35 °C overnight. One mL aliquot of the isolate was transferred into an Eppendorf tube and centrifuged at 12,000 rpm for 2 min. The supernatant was discarded, and the pellet was mixed thoroughly with 1 mL of deionized water and centrifuged at the same speed and time. This stage was performed twice. Afterward, 900 μL of absolute ethanol and 300 μL deionized water were added, mixed for 2 min, and the tube was centrifuged at 15,000 rpm for 5 min. The supernatant was discarded, and the pellet was air-dried for a minimum of 30 min until dryness. The pellet was re-suspended with 15 μL of formic acid (70%) and mixed thoroughly. Moreover, the mix was kept for 5 min at room temperature and then 15 μL of acetonitrile was added and mixed. The mixture was centrifuged at 15,000 rpm for 3 min and subsequently, 1 mL of the supernatant was spotted onto a MALDI-TOF target plate. After being air-dried, the sample was overlaid with 1 μL of matrix solution (HCCA).

For identification, each series of measurements was preceded by a calibration step with a bacterial test standard (BTS 155 255343; Bruker Daltonik) to validate the run. Mass spectra were generated by a Flex Analysis MALDI-TOF mass spectrometer (Bruker Daltonik) equipped with a nitrogen laser (l1⁄4337 nm) operating in linear positive ion detection mode under the Bruker Flex Control software (Bruker Daltonik). The Autoflex LT Speed was periodically calibrated by using the Bruker Daltonik *Escherichia coli* bacterial test standard DH5. Automated analysis of the raw spectral data was performed by the MALDI BioTyper automation (version 3.1) software (Bruker Daltonik) using a library of 5627 main spectra (MSPs; database update of 7/15/2015). Identifications at species or genus level were considered if scores were above 2.0 and 1.7 respectively, according to the report generated by Bruker Compass [53,54].

3.6. Statistical Analysis

Data on microbial counts and BA levels were analyzed by two-way analysis of variance (ANOVA) with treatment and storage day as fixed factors. When the fixed factors or their interaction showed significant differences ($P < 0.05$), the ANOVA was followed by the Tukey's post-hoc test. For the results of the LAB identification, a contingency table (4 by 2; treatment by positive or negative) chi-square analysis was used to test the eventual dependence between treatment and the frequency of the presence in the sausages of the main genus or species identified, considering the entire storage period. The statistical analysis was performed using the SPSS Statistics software (version 24; IBM, Somers, NY, USA).

4. Conclusions

The results from this study demonstrate that Balkan-style fresh sausages stored under anaerobic atmosphere are already fermented in the first week of storage and the predominant responsible species are *Lactobacillus* spp., specifically *Lb. sakei*. The fermentation was compatible with a controlled growth of *B. thermosphacta* and *Enterobacteriaceae* and resulted in BA production to a concerning level, thus suggesting that the contents of BA in anaerobic MAP fresh sausages should be controlled. The use of *Zataria multiflora* Boiss EO, hops extract, or the combination of both at the levels used did not significantly affect the microbial development in the sausages. More studies using higher amounts of these antimicrobial sources, different combinations with other antimicrobials, extracts with higher concentrations of active compounds, or previous encapsulation, would be needed to achieve their effectiveness in fresh sausage preservation.

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Article

Antifungal Agents Based on Chitosan Oligomers, ε-polylysine and *Streptomyces* spp. Secondary Metabolites against Three Botryosphaeriaceae Species

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Abstract: Grapevine trunk diseases (GTDs) are a major threat to the wine and grape industry. The aim of the study was to investigate the antifungal activity against *Neofusicoccum parvum*, *Diplodia seriata*, and *Botryosphaeria dothidea* of ε-polylysine, chitosan oligomers, their conjugates, *Streptomyces rochei* and *S. lavendofoliae* culture filtrates, and their binary mixtures with chitosan oligomers. In vitro mycelial growth inhibition tests suggest that the efficacy of these treatments, in particular those based on ε-polylysine and ε-polylysine:chitosan oligomers 1:1 *w*/*w* conjugate, against the three Botryosphaeriaceae species would be comparable to or higher than that of conventional synthetic fungicides. In the case of ε-polylysine, EC_{90} values as low as 227, 26.9, and 22.5 μg·mL^{-1} were obtained for *N. parvum*, *D. seriata*, and *B. dothidea*, respectively. Although the efficacy of the conjugate was slightly lower, with EC_{90} values of 507.5, 580.2, and 497.4 μg·mL^{-1}, respectively, it may represent a more cost-effective option to the utilization of pure ε-polylysine. The proposed treatments may offer a viable and sustainable alternative for controlling GTDs.

Keywords: *Botryosphaeria dothidea*; conjugate complexes; *Diplodia seriata*; grapevine trunk diseases; *Neofusicoccum parvum*

1. Introduction

Grapevine trunk diseases (GTDs) have been reported in most grapevine producing regions worldwide, causing a serious decline and loss of productivity. These diseases include black dead arm, caused by *Botryosphaeria dothidea*; esca, which includes vascular symptoms and internal white rot in the trunk; eutypiosis, caused by *Eutypa lata*; Petri disease; black foot; and Phomopsis dieback, being the esca complex the most frequent and increasing syndrome in almost all European countries [1]. A recent International Organization of Vine and Wine (OIV) publication reported that incidence of GTDs was 10% in Spain, 13% in France, and between 8% and 19% in Italy, and that the losses in California were at least 260 M$ per year [2].

A thorough and up-to-date panorama of the state-of-the-art of chemicals (including synthetic organic compounds, inorganic compounds, natural compounds, and plant-defense stimulating compounds) and biocontrol agents that have been tested towards GTDs can be found in the recent review paper by Mondello et al. [2].

Unfortunately, chemical fungicides traditionally used to control aforementioned fungal crop infections, such as sodium arsenite, carbendazim, or tecobunazole, have several drawbacks in terms of toxicity and efficacy, and, in recent years, public pressure to reduce their use has increased. In fact, concerns have been raised about both their environmental impact and the potential associated health risks. In this context, the use of natural antifungals as a feasible alternative is receiving increasing attention.

Among the tested natural compounds, Nascimento et al. [3] reported the antifungal effect of chitosan on several fungal species involved in grapevine decline. Greenhouse experiments using foliar sprays of chitosan on potted grapevine plants growing in a substrate artificially infected with *Phaeomoniella chlamydospora* or *Ilyonectria liriodendri* demonstrated that chitosan significantly improved plant growth and decreased disease incidence. More recently, Cobos et al. [4] reported that chitosan oligosaccharides, garlic extract, and vanillin were able to significantly reduce infection in pruning wounds by *Diplodia seriata*. Galarneau et al. [5] also examined the potential role of antimicrobial phenolic compounds on *Neofusicoccum parvum* and *D. seriata*, two causal fungi of Botryospheria dieback.

ε-polylysine (EPL), a natural antimicrobial produced from aerobic bacterial fermentation by *Streptomyces albulus*, widely used in Japan and USA as an antimicrobial agent in food products, could also be a promising antifungal agent [6]. Although it has been reported to have a strong activity against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis* [7], either alone or in chitosan conjugate compounds, its efficacy has not been assayed against GTDs.

In a similar fashion, even though beneficial bacteria inhabiting the rhizosphere and/or the endosphere of plants and their secondary metabolites have been put forward by some authors to reduce grapevine pathogen diseases [6], information reported in the literature is limited [8–12]. These biocontrol agents, such as *Streptomyces* spp., would affect pathogen performance by antibiosis, competition for niches and nutrients, interference with pathogen signaling, or by stimulation of host plant defenses.

The aim of the study presented herein has been to assess the in vitro antifungal activity of EPL, EPL:chitosan oligomers (EPL:COS) conjugates, and secondary metabolites from two beneficial actinobacteria (*Streptomyces rochei* and *S. lavendofoliae*) to control *N. parvum*, *D. seriata*, and *B. dothidea*, three of the most frequently isolated fungal pathogens in GTDs.

2. Results

2.1. Vibrational Analysis of the ε-polylysine: Chitosan Conjugates

The vibrational spectra of conjugates prepared with six different EPL:COS mass ratios were examined in order to confirm their secondary structure and to determine the most suitable proportion (Figure 1).

The absorption bands at 1150 cm^{-1} and 1018 cm^{-1} were assigned to asymmetric stretching of the C−O−C bridge and to the skeletal vibration of C−O stretching, respectively [13–15]. The absorption band at 895 cm^{-1} could be assigned to the β-D-configuration. There was a shift of amide/amino bands in the reaction products, indicating the progress of Maillard reaction: the absorption peaks at 1659 cm^{-1} and 1597 cm^{-1} (associated with amino groups characteristic of chitosan oligomers) disappeared, and new bands at 1665 cm^{-1} and 1565 cm^{-1} were observed. The appearance of these bands suggest that a Schiff base (C=N bond) was formed between the reducing end of chitosan and the amino groups [16]. Thus, the Fourier-Transform Infrared (FTIR) results showed that ε-polylysine had actually attached to chitosan.

An interesting feature was that the absorbance of the bands associated with Schiff base formation were stronger in the 1:1 EPL:COS conjugate than in the spectra of conjugates prepared with other EPL:COS ratios. Thus, the Schiff base for the 1:1 conjugate seems to feature the desired balance of components to undergo the Amadori rearrangement with formation of ketosamines, but avoiding their subsequent decomposition observed in more COS-rich conjugates. This result was in good agreement with the findings of Liang et al. [7] for EPL:chitosan, who concluded that the conjugate with EPL and chitosan ratio of 1:1 exhibited the strongest antibacterial and antifungal activity. Consequently, the 1:1 EPL:COS conjugate was chosen for the mycelial growth inhibition tests in this study.

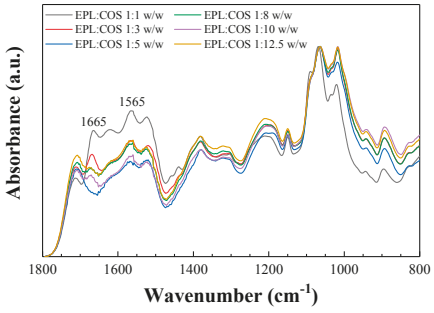

Figure 1. Comparison of the attenuated total reflection (ATR)-Fourier-Transform Infrared (FTIR) spectra of ε-polylysine:chitosan oligomers conjugates prepared with different ε-polylysine:chitosan oligomers mass ratios. Only the fingerprint region is shown.

2.2. Mycelial Growth Inhibition Tests

The in vitro radial growth inhibition attained by each of the treatments against *N. parvum* is depicted in Figure 2, showing only for one replicate per treatment and dose. Those attained against *D. seriata* and *B. dothidea* are depicted in Figures S1 and S2, respectively. The values across the three replicates for the three Botryosphaeriaceae species are summarized in Figure 3.

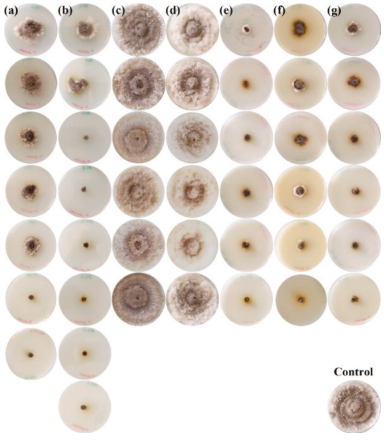

Figure 2. *N. parvum* mycelial growth inhibition assays for: (**a**) chitosan oligomers; (**b**) ε-polylysine; (**c**) *S. rochei* secondary metabolites; (**d**) *S. lavendofoliae* secondary metabolites; (**e**) ε-polylysine:chitosan (1:1 *w/w*) conjugates; (**f**) *S. rochei* secondary metabolites + chitosan oligomers (1:1 *w/w*); and (**g**) *S. lavendofoliae* secondary metabolites + chitosan oligomers (1:1 *w/w*). The concentration of the treatments decreases from top to bottom (doses for each treatment are indicated in Table 3). The petri dish in the bottom right corner shows the PDA control. Only one replicate per each treatment and dose is shown.

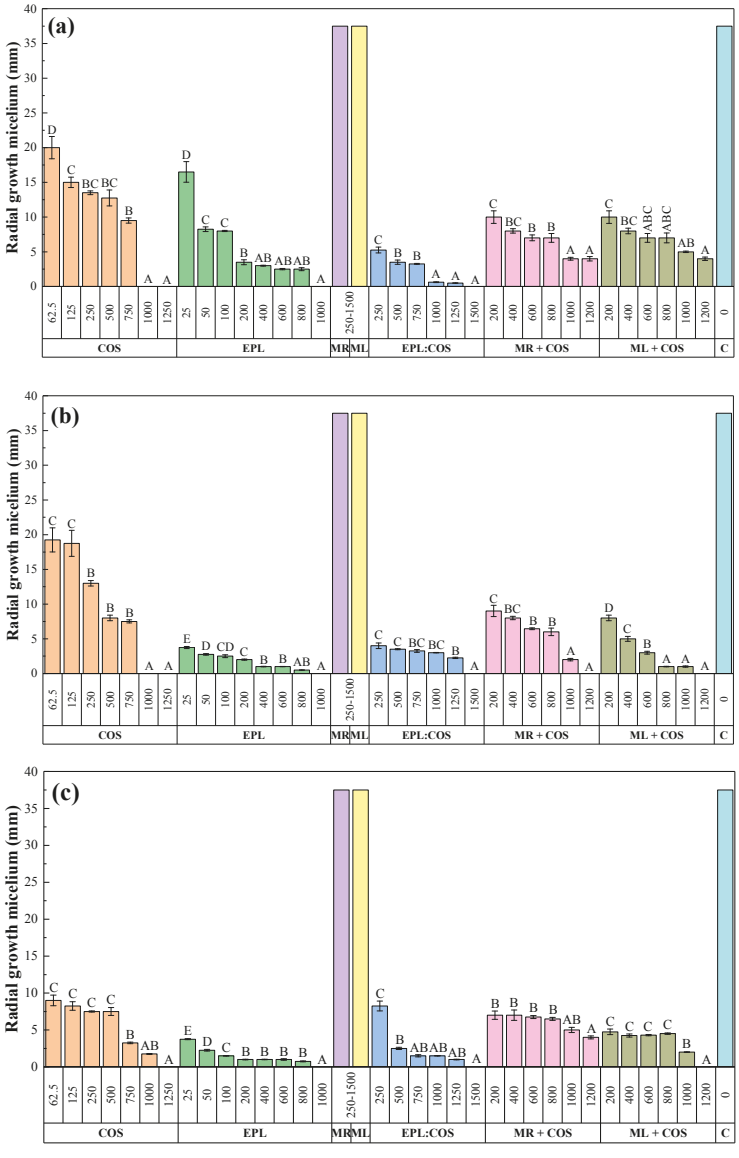

Figure 3. Radial growth values of (**a**) *N. parvum*; (**b**) *D. seriata*; and (**c**) *B. dothidea* in the presence of the different treatments under study at different concentrations (in μg·mL^{-1}). COS, EPL, MR, ML and C stand for chitosan oligomers, ε-polylysine, *S. rochei* secondary metabolites, *S. lavendofoliae* secondary metabolites and control, respectively. For MR and ML only one column is shown, since no inhibition was detected at any concentration in the 250–1500 μg·mL^{-1} range. Concentrations labelled with the same uppercase letters are not significantly different at $p < 0.05$ by Tukey's test. All values are presented as the average of three repetitions. Error bars represent the standard deviation across three replicates.

The increase in the treatment doses resulted in a reduction in the radial growth of the mycelium in all cases, with statistically significant differences amongst the various concentrations (Figure 3),

except for the *S. rochei* and *S. lavendofoliae* secondary metabolites-only based treatments (MR and ML, respectively), for which no inhibition was observed.

Doses in the 1000–1500 $\mu g \cdot mL^{-1}$ range were required to attain full inhibition of the three Botryosphaeriaceae species for the COS, EPL, and EPL:COS conjugate treatments. As regards the activity of MR+COS and ML+COS treatments, differences were observed as a function of the fungal pathogen species. Full inhibition of *D. seriata* was attained for both treatments at a dose of 1200 $\mu g \cdot mL^{-1}$, whereas it was only observed for ML+COS in the case of *B. dothidea*. MR+COS treatment led to 89% inhibition at the same dose for this latter pathogen. In the case of *N. parvum*, the highest doses of MR+COS and ML+COS led to 83% and 89% inhibition, respectively.

The sensitivity tests results may also be expressed in terms of effective concentrations EC_{50} and EC_{90}, that is, the concentrations that reduce mycelial growth by 50% and 90%, respectively (Table 1). Goodness-of-fit analyses revealed good r^2 and low sum of standard errors, showing that parameter fits of sigmoid curves to the dose-response data were significant. In view of the obtained theoretical values, the activity of the treatments—in general terms—would follow the sequence EPL > EPL:COS > ML+COS > COS > MR+COS.

Table 1. Effective concentrations that inhibited mycelial growth by 50% and 90% (EC_{50} and EC_{90}, respectively).

Pathogen	Concentration ($\mu g \cdot mL^{-1}$)	Treatment				
		COS	**EPL**	**EPL:COS**	**MR + COS**	**ML + COS**
N. parvum	EC_{50}	60.7	16.0	11.2	67.2	46.7
	EC_{90}	1270.0	227.0	507.5	2074.2	1101.7
D. seriata	EC_{50}	94.3	0.3	11.6	45.1	30.7
	EC_{90}	1120.7	26.9	580.2	906.9	498.2
B. dothidea	EC_{50}	1.8	0.4	4.2	15.8	10.7
	EC_{90}	689.5	22.5	497.4	1019.0	490.3

3. Discussion

3.1. Efficacy of the Treatments

In relation to the efficacy of the composites, although the review paper by Mondello et al. [2] provides a qualitative comparison of different treatment against GTDs, specific inhibition rates with their associated concentrations or effective concentrations were not provided. A survey of such values against the three Botryosphaeriaceae species under study is summarized in Table 2 for comparison purposes.

It may be observed that the EC_{50} values for the treatments presented herein (Table 1), in particular those of EPL and EPL:COS conjugate, were comparable to or better than those of popular synthetic organic compounds used to control GTDs, and only slightly lower than the excellent activities reported for AgNPs.

The results presented for COS were in excellent agreement with those reported by Nascimento et al. [3] and Cobos et al. [4]. However, with regard to this latter study, it should be noted that while the use of polyphenols, such as vanillin or those found in garlic extract, may be suitable against *D. seriata* and other Botryosphaeriaceae strains [17], it may not be advisable against *N. parvum*. Galarneau et al. [5] recently found that *N. parvum* was either uninhibited or promoted by phenolic compounds such as gallic acid, epicatechin, rutin, or piceid. In fact, the authors explained that the ability of *N. parvum* to tolerate these phenolics or utilize them as carbon sources would contribute to its greater virulence compared to *D. seriata*.

Table 2. Concentration values and associated inhibition rates, or EC_{50} values, reported in the literature for other active compounds against the three Botryosphaeriaceae species under study.

Fungicide	Fungal Species	Concentration ($\mu g \cdot mL^{-1}$)	Inhibition rate (%)	EC_{50} ($\mu g \cdot mL^{-1}$)	Ref.
Tebuconazole	*N. parvum* *D. seriata*			90 150	[18]
Pyraclostrobin	*N. parvum* *D. seriata*			100 250	
Carbendazim, tebuconazole, iprodione, fludioxonil, fluazinam, flusilazole, penconazole, procymidone, myclobutanil, pyraclostrobin	*N. parvum* *D. seriata* *B. dothidea*			360–440 * 530–620 * 450 *	[19]
Carbendazim Tebuconazole Iprodione	*N. parvum*			40 130 750	[20]
Tecobunazole	*D. seriata*			300	[21]
Fe NPs (FeNPs + neem leaf extract)	*D. seriata* *B. dothidea*	100 (FeNPs / FeNPs+neem 1:1)	79/80.3 83/82.5		[22]
AgNPs AgNPs	*N. parvum*	40 30	84 81		[23] [24]
Lemon essential oil (limonene, neral, β-pinene, and γ-terpinene) in DMSO	*B. dothidea*	2500	48.1		[25]
Chitosan oligosaccharin (mol. wt. <3 kDa)	*Botryosphaeria* sp.			1.56	[3]
Chitosan oligosaccharides Vanillin Garlic extract	*D. seriata*	1000 1000 40000	100 89.8 75.3		[4]

* Data pooled across fungicides to provide mean EC_{50} values for isolate sensitivity in the original study.

The *Streptomyces* spp. secondary metabolites-based treatments showed an unexpected lack of activity when used alone. In fact, the percentage of inhibition of radial growth (PIRG) values, shown in Tables S1–S3, were negative, i.e., the growth of the pathogens was promoted. This was not a case of hormetic response, provided that increasing the concentration did not result in inhibition. The observed mycelial growth promotion may be tentatively ascribed to the presence of molasses and yeast extract in the culture filtrates, together with a poor absorption and bioavailability of the active ingredients in the water-based culture filtrates, resulting from their insolubility or very poor solubility in water.

In relation to one of the active compounds present in the culture filtrates under test, lankacidin, Harada et al. [26] stated that lankacidin-group antibiotics are scarcely soluble in water and that the parts that dissolved are rapidly decomposed to compounds with no antimicrobial activity. To overcome this problem, they prepared inclusion compounds with cyclodextrins. In this study, this solubility problem was solved by forming polyelectrolyte complexes (PECs) with a polycationic polymer, i.e., chitosan oligomers. These chitosan-based PECs have been reported to exhibit favorable physicochemical properties and to preserve chitosan's biocompatible characteristics [27], which has made this approach very popular in the drug delivery fields [28,29]. In fact, Zhang et al. [30] previously reported that chitosan behaves as an efficient carrier to deliver streptomycin.

3.2. Mechanism of Action

Concerning the mechanism of action (MOA) of the proposed treatments, although the antimicrobial activity of EPL is well documented, its MOA has only been vaguely described. Hyldgaard et al. [31] hypothesized that EPL destabilizes membranes in a carpet-like mechanism by interacting with negatively charged phospholipid head groups, which displace divalent cations and enforce a negative curvature folding on membranes that leads to formation of vesicles/micelles. According to Ye et al. [32], the antimicrobial mechanism of EPL may be attributed not only to disturbances on membrane integrity, but also to oxidative stress by ROS, and to its effects on various gene expressions.

It is worth noting that the fungicidal activity would likely benefit from the substitution of lysine with arginine residues, provided that previous works have demonstrated the superior cell permeability by

arginine polymers over lysine-containing ones [33,34]. Mechanistic evidences indicate that arginine can enhance the activity of both translocating and membrane permeabilizing peptides [35,36]. This would be a potential direction for future studies.

Regarding the inhibition mode of chitosan oligomers, Ing et al. [37] proposed several MOAs. The interaction of chitosan's positive charge with negatively charged phospholipid components would result in an increased permeability and in leakage of cellular contents. Its chelating action would deprive fungi of trace elements essential for their normal growth. Moreover, its binding to fungal DNA would inhibit mRNA synthesis and affect proteins and enzymes production.

Consequently, the activity of EPL:COS conjugates, as noted by Liang et al. [7], should be referred to an enhanced disruption of their cell membranes, leading to damages of structure, function, and permeability, leakage of intracellular components and the ultimate lysis of the cell.

3.3. Applicability to GTDs in vivo

As regards the applicability of the proposed treatments to GTDs in vivo, although it was not covered in this preliminary study, several systems may be envisaged [38]. To reduce symptoms in the field, once the wood is already infected, an approach to be explored would be to apply the products to the soil (injector pole) or to the trunk (trunk injections), mimicking the mechanism activated by winter spraying of sodium arsenite [39]. However, it would be expensive and time-consuming if applied on a large scale [40], and would only be cost-effective when applied in high-value vineyards [41,42].

The proposed antifungal agents may also be administrated by foliar pulverization with minor changes to the formulations (e.g., adding a surfactant as Tween-80). This would be the most practical approach considering the experience of winegrowers. According to Roblin et al. [43], the compounds sprayed on the leaf blades would be able to migrate to the fungal target in the trunk or to trigger the plant defense reaction in distal parts of the plant. In fact, successful use of foliar sprays of chitosan on grapevine plants artificially infested with *Phaeomoniella chlamydospora* or *Neonectria liriodendri* have been reported in the literature [3]. However, this application method has the major drawback that the treatments may be easily washed off by rainfall [44]. If this approach was to be chosen, sprays after the period of vintage should be useful since, at this period, the phloem sap begins to be directed in a descending flow towards the roots, assuring the transport of the compounds towards the fungi [43].

Alternatively, as a preventive measure, the active ingredients may also be used to protect pruning wounds to avoid grapevine infection and to limit fungal expansion in the plant, either as painted pastes or as liquid formulations. This application method was evaluated against *D. seriata* and *P. chlamydospora* in field trials by Cobos et al. [4], using chitosan oligosaccharides, vanillin, and garlic extract, and resulted in a significant decrease in plant mortality and in the infection rate. Nonetheless, to improve the adherence of the treatments, thickener agents would need to be added to the formulations: e.g., starches, vegetable gums, pectin, or clays such as halloysite.

3.4. Significance of the Reported Findings

Although follow-up studies involving in vivo assays and field tests would be necessary to draw firm conclusions on the effectiveness of the application of the proposed treatments, the fact that they reached higher mycelial growth inhibition than that of commercial fungicides makes them promising candidates for the effective control of botryosphaeriaceous diseases.

It is also worth noting that the three fungal species tested in the present study are not only pathogens of grapevine, but also of other commercially important woody plants. For instance, *D. seriata* and *B. dothidea* are phytopathogens of apple [22], *N. parvum* causes dieback in avocado [45], *B. dothidea* causes branch dieback of olive [46], and the three of them are associated with branch cankers on almond trees [47]. Consequently, the results of this study may also find application in other pathosystems, resulting in an even higher ecological and economic impact.

4. Materials and Methods

4.1. Reagents, Bacteria and Fungi

High molecular weight chitosan (CAS 9012-76-4; 310000-375000 Da) was purchased from Hangzhou Simit Chemical Technology Co., Ltd. (Hangzhou, China). ε-polylysine (CAS 25104-18-1), phosphate buffer (for microbiology, APHA, pH 7.2), ethyl acetate (CAS 141-78-6; ≥99.5%), and citric acid (CAS 77-92-9; ≥99.5%) were supplied by Sigma-Aldrich Química S.A. (Madrid, Spain). Neutrase® 0.8L enzyme was supplied by Novozymes (Bagsvaerd, Denmark). Potato dextrose agar (PDA), yeast extract, and Bacto™ Peptone were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Starch casein agar (SCA), Mueller Hinton agar, and malt extract agar (MEA) came from Oxoid Ltd. (Hampshire, UK). Molasses were supplied by ACOR, Sociedad Cooperativa General Agropecuaria (Castilla y León, España).

The three fungal isolates under study, viz. *Diplodia seriata* (ITACYL_F079), *Neofusicoccum parvum* (ITACYL_F111), and *Botryosphaeria dothidea* (ITACYL_F141), were supplied by ITACYL, Instituto Tecnológico Agrario de Castilla y León (Castilla y León, España).

The two *Streptomyces* spp. strains from which secondary metabolites were produced, *Streptomyces lavendofoliae* (DSM 40217) and *Streptomyces rochei* (DSM 41729) were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen; Braunschweig, Germany).

4.2. Equipment

A probe-type UIP1000hdT ultrasonicator (Hielscher, Teltow, Germany; 1000 W, 20 kHz) was used for solutions sonication.

To incubate the flasks, controlling the temperature and the stirring speed, an ECOLAN 60 (Labolan; Esparza de Galar, Navarra, Spain) orbital stirrer incubator was used.

Functional groups were identified by Fourier-Transform Infrared spectroscopy with a Nicolet iS50 (Thermo Scientific, Waltham, MA, USA) apparatus equipped with a diamond attenuated total reflection (ATR) module. The spectra were collected in the 400–4000 cm^{-1} region with a 1 cm^{-1} spectral resolution; 64 scans were co-added and the resulting interferogram was averaged. The ATR-FTIR spectra were corrected using the advanced ATR correction algorithm [47] available in OMNIC™ software suite.

4.3. Preparation of Chitosan Oligomers

Chitosan oligomers were obtained according to the enzymatic procedure described by Santos-Moriano et al. [48], with slight modifications. 20 g of high molecular weight chitosan were dissolved in 1000 mL of Milli-Q water by adding citric acid under constant stirring at 60 °C. Once dissolved, Neutrase® 0.8 L (1.67 $g{\cdot}L^{-1}$) was added in order to degrade the polymer chains. The mixture was sonicated for 3 min in cycles of 1 min with sonication and 1 min without sonication to keep the temperature in the 30–60 °C range [14]. At the end of the process, a solution with a pH in the four to six interval with oligomers of molecular weight < 2000 Da was obtained.

4.4. ε-polylysine Treatment

For the preparation of the ε-polylysine treatment, 2 g of EPL were dissolved in 1000 mL of Milli-Q water. The mixture was sonicated for 3 min in cycles of 1 min with sonication and 1 min without sonication so that the temperature remained in the 30–60 °C range.

4.5. Synthesis of ε-polylysine: Chitosan Oligomers Conjugates

Conjugated complexes of ε-polylysine and chitosan oligomers were prepared at different mass ratios, namely 1:1, 1:3, 1:5 1:8, 1:10, and 1:12.5 *w*/*w*, respectively. The appropriate amounts of each component were dissolved in Milli-Q water using sonication (5 cycles of 5 min/cycle, taking care

not to exceed 60 °C). The resulting solutions were lyophilized, and then heated at 60 °C under 60% relative humidity for 24 h. This synthesis procedure was analogous to other procedures described in the literature for the preparation of EPL:COS conjugates through Maillard reaction [7,49,50]. Only the conjugate with the highest expected activity was assayed in the mycelial growth inhibition tests.

4.6. Secondary Metabolites Production from Streptomyces spp. Strains

Two strains of the genus *Streptomyces*, viz. *Streptomyces lavendofoliae* DSM 40217 and *Streptomyces rochei* DSM 41729 were seeded on starch casein agar medium plates at 28 °C for 10 days. The plates were stored at 4 °C. For long-term storage, lyophilizates from both strains were used.

In order to obtain the secondary metabolites, the method described by Sadigh-Eteghad et al. [51] was followed. Once the fermentation was complete, each final solution of the cultures of both strains was treated with 50 mL of phosphate buffer (pH 6.4) and was sonicated for 5 min. The solutions were then filtered through sterile muslin cloth twice. These solutions (culture filtrates) were used for the mycelial growth inhibition tests.

In order to determine the concentration of bioactive compounds in aforementioned solutions (and the doses used in the inhibition tests), the filtrates were centrifuged, and the supernatant was extracted with 100 mL of ethyl acetate. The solvent with the crude bioactive compounds was concentrated under reduced pressure and then lyophilized. The culture filtrates had a concentration of approx. 2000 $\mu g \cdot mL^{-1}$ (1958 $\mu g \cdot mL^{-1}$ for *S. lavendofoliae* secondary metabolites and 1877 $\mu g \cdot mL^{-1}$ for *S. rochei* secondary metabolites), in agreement with Pazhanimurugan et al. [52]. The bioactive compounds in the secondary metabolites of *S. lavendofoliae* and *S. rochei* are summarized in Table S4.

4.7. Synthesis of Chitosan Oligomers-secondary Metabolites Inclusion Compounds

Secondary metabolites, either from *S. lavendofoliae* or from *S. rochei*, and chitosan oligomers mixtures were prepared by mixing in 1:1 (*w*/*w*) ratio of their respective solutions (2000 $\mu g \cdot mL^{-1}$ of bioactive compounds + 2000 $\mu g \cdot mL^{-1}$ COS), followed by sonication. The resulting solutions (ML+COS and MR+COS) were assayed at different concentrations in the inhibition tests.

4.8. In vitro Mycelial Growth Inhibition Tests

The biological activity of the treatments under study was determined by the agar dilution method: aliquots of the original solutions of the various treatments, obtained by dilution of the respective "mother" solutions, were incorporated into the PDA medium to obtain the final concentrations indicated in Table 3. It should be clarified that the tested concentrations were not the same all treatments due to difficulties associated with the estimation of the molecular weights of the polymeric reagents from their viscosities. Petri dishes containing only PDA culture medium (20 mL) were used as the control.

The mycelial discs of pathogen (5 mm in diameter) were removed from the margins of 7-day-old PDA cultures and transferred to the petri dishes (in triplicate). Plates were incubated at 25 °C. The measurements of fungal growth for *D. seriata* and *N. parvum* were taken two, four and five days after inoculation. In contrast, for *B. dothidea*, measurements were carried out two, four and six days after inoculation, provided that mycelial growth was slower for this later fungus in the control plates.

The inhibition of mycelial growth, or the efficacy of the compound analyzed, for each treatment and concentration, was calculated by the formula:

$$\text{Percentage inhibition of radial mycelium growth (\%)} = ((R_1 - R_2)/R_1) \times 100 \quad (1)$$

where R_1 and R_2 correspond to the average radial growth of the fungal mycelium in the control medium (pure PDA) and in the fungicide-amended medium, respectively.

The results were also expressed as the effective concentrations that reduced mycelial growth by 50% and 90% (EC_{50} and EC_{90}, respectively), which were determined by the regression of the radial growth inhibition values (%) against the $\log_{10}$ values of the concentrations of antifungal compounds

using PROBIT in IBM SPSS Statistics v.25 software. This regression procedure fits the dose-response curve to a sigmoid and calculates the values, with 95% CI, of the dose variable that correspond to a series of probabilities.

Table 3. Concentrations assayed for each of the treatments in the mycelial growth inhibition tests. COS, PL, MR and ML stand for chitosan oligomers, ε-polylysine, *S. rochei* secondary metabolites, and *S. lavendofoliae* secondary metabolites, respectively.

Treatment	Concentrations Assayed in the Mycelial Growth Inhibition Tests ($\mu g \cdot mL^{-1}$)
COS	62.5, 125, 250, 500, 750, 1000, 1250, 1500
EPL	25, 50, 100, 200, 400, 600, 800, 1000
MR	250, 500, 750, 1000, 1250, 1500
ML	250, 500, 750, 1000, 1250, 1500
EPL:COS	250, 500, 750, 1000, 1250, 1500
MR+COS	200, 400, 600, 800, 1000, 1200
ML+COS	200, 400, 600, 800, 1000, 1200

4.9. Statistical Analyses

Data were subjected to analysis of variance (ANOVA) in IBM SPSS Statistics v.25 software. Tukey's HSD test at 0.05 probability level ($p < 0.05$) was used for the *post hoc* comparison of means.

5. Conclusions

The efficacy of ε-polylysine, chitosan oligomers, ε-polylysine:chitosan oligomers conjugates, two *Streptomyces* spp. secondary metabolites, and the combinations of the latter two with chitosan oligomers were examined in vitro against *N. parvum*, *D. seriata* and *B. dothidea*. On the basis of vibrational spectroscopy data, a 1:1 *w/w* mass ratio was chosen for the EPL:COS conjugate, for which an optimum Schiff base was formed. From the mycelial growth inhibition tests it was found that, in spite of the remarkable contents in bioactive compounds in the culture filtrates, the secondary metabolites of *S. rochei* and *S. lavendofoliae* did not inhibit any of the GTD-related fungi, probably due to hydrophobicity reasons. In contrast, upon formation of polyelectrolyte complexes with chitosan oligomers, inhibitions above 80% were attained. In view of the calculated effective concentration values, the antifungal activity of the treatments would follow the sequence EPL > EPL:COS > ML+COS > COS > MR+COS. EC_{50} values below 100 $\mu g \cdot mL^{-1}$ were obtained for all the assayed treatments, suggesting that they could be a viable alternative to conventional synthetic fungicides. In particular, ε-polylysine and ε-polylysine:chitosan oligomers may be put forward as the most promising options, due to the high efficacy of the former and the trade-off between efficacy and cost associated with the latter. In the current context in which the use of synthetic chemical pesticides is more and more restricted, this work constitutes a necessary step for developing efficient treatments that take into account the importance of environmental protection within the scope of sustainable development.

Supplementary Materials: The following are available online at http://www.mdpi.com/2079-6382/8/3/99/s1, Figure S1: *D. seriata* mycelial growth inhibition assays, Figure S2: *B. dothidea* mycelial growth inhibition assays, Table S1: Radial growth of mycelium (RG) and percentage of inhibition of radial growth (PIRG) of the different treatments against *N. parvum* two, four and five days after inoculation, Table S2: Radial growth of mycelium (RG) and percentage of inhibition of radial growth (PIRG) of the different treatments against *D. seriata* two, four and five days after inoculation, Table S3: Radial growth of mycelium (RG) and percentage of inhibition of radial growth (PIRG) of the different treatments against *B. dothidea* two, four and six days after inoculation, Table S4: Bioactive secondary metabolites produced by *S. lavendofoliae* and *S. rochei*.

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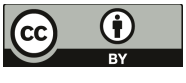

antibiotics

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Article

13-(2-Methylbenzyl) Berberine Is a More Potent Inhibitor of MexXY-Dependent Aminoglycoside Resistance than Berberine

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Abstract: We previously showed that berberine attenuates MexXY efflux-dependent aminoglycoside resistance in *Pseudomonas aeruginosa*. Here, we aimed to synthesize berberine derivatives with higher MexXY inhibitory activities. We synthesized 11 berberine derivatives, of which 13-(2-methylbenzyl) berberine (13-o-MBB) but not its regiomers showed the most promising MexXY inhibitory activity. 13-o-MBB reduced the minimum inhibitory concentrations (MICs) of various aminoglycosides 4- to 128 fold for a highly multidrug resistant *P. aeruginosa* strain. Moreover, 13-o-MBB significantly reduced the MICs of gentamicin and amikacin in *Achromobacter xylosoxidans* and *Burkholderia cepacia*. The fractional inhibitory concentration indices indicated that 13-o-MBB acted synergistically with aminoglycosides in only MexXY-positive *P. aeruginosa* strains. Time-kill curves showed that 13-o-MBB or higher concentrations of berberine increased the bactericidal activity of gentamicin by inhibiting MexXY in *P. aeruginosa*. Our findings indicate that 13-o-MBB inhibits MexXY-dependent aminoglycoside drug resistance more strongly than berberine and that 13-o-MBB is a useful inhibitor of aminoglycoside drug resistance due to MexXY.

Keywords: *Pseudomonas aeruginosa*; efflux; MexXY; aminoglycoside resistance; berberine

1. Introduction

Pseudomonas aeruginosa is a major cause of nosocomial infections. Treatment of *P. aeruginosa* infections with antimicrobial concentrations insufficient to inhibit *P. aeruginosa* growth results in the emergence of new multidrug resistant *P. aeruginosa* strains [1] that are difficult to eradicate and may increase mortality [2].

Drug efflux is a major mechanism leading to antimicrobial resistance in *P. aeruginosa* [3]. Four resistance-nodulation-division (RND)-type multidrug efflux pumps (MexAB-OprM [4], MexCD-OprJ [5], MexEF-OprN [6] and MexXY-OprM/OprA [7,8]) have been reported as drug efflux systems involved in the drug resistance of *P. aeruginosa*. Of these, only MexXY contributes to aminoglycoside drug resistance [8,9]. The MexXY-OprM system comprises a cytoplasmic membrane antibiotic-proton antiporter (MexY), an outer membrane porin (OprM), and a periplasmic membrane fusion protein (MexX) [10]. MexXY has multiple functions, including the expulsion of antibiotics. Wild-type *P. aeruginosa* expresses low MexXY levels but elevated MexXY has been detected in

aminoglycoside-resistant *P. aeruginosa* strains [11,12]. Therefore, the development of MexXY inhibitors would allow the use of lower concentrations of aminoglycoside drugs that can cause severe side effects such as kidney damage [13].

There have been various reports of inhibitors of RND-type multidrug efflux pumps, but no clinical applications have been published to date [14]. Phenyl-arginine-β-naphthylamide (PAβN, MC-207,110), a well-known efflux pump inhibitor, does not inhibit aminoglycoside resistance due to MexXY [15]. We previously reported that berberine attenuates MexXY-dependent aminoglycoside resistance in *P. aeruginosa* [15], consistent with a recent report that berberine has high affinity to a MexXY model protein in silico [16].

Berberine is an isoquinoline quaternary alkaloid isolated from many kinds of medicinal plants such as *Coptis chinensis*, *Coptis rhizome*, *Coptis japonica* and *Phellondendron amurense* [17] and has weak antibacterial activity against Gram-negative bacteria such as *P. aeruginosa* [18]. Various derivatives of berberine have been developed and studied for their anti-hyperglycemic, anti-cancer, anti-inflammatory, anti-Alzheimer's disease and anti-microbial activities [19]. Derivatives with multidrug resistance pump inhibitory activity against *Staphylococcus aureus* [20] and that reduce fluconazole resistance against *Candida albicans* [21] have been reported. In addition, quaternary ammonium compounds inhibit the biofilm formation in *P. aeruginosa* and *C. albicans* have been reported [22].

The optimum concentration of berberine to inhibit MexXY in *P. aeruginosa* cells is more than 512 μg/mL [15], which is too high for clinical application. In this study, we aimed to synthesize berberine derivatives with higher MexXY inhibitory activities.

2. Results

2.1. Antibacterial Activity of Berberine Derivatives toward P. aeruginosa

We first measured the minimum inhibitory concentrations (MICs) of 11 berberine derivatives (Figure 1) synthesized against *P. aeruginosa* mutants PAGU^{g}1927, which expresses MexXY, and PAGU^{g}1931, which does not express MexXY. A difference in the activity of a derivative toward the two strains indicates that the MexXY activity is not masked by the other four pumps (MexAB, MexCD, MexEF and MexVW) [15]. The MIC values of the berberine derivatives were lower in both strains compared to berberine (Table 1), suggesting that these berberine derivatives had higher anti-pseudomonas activity compared with berberine. These berberine derivatives showed similar MIC values that differed no more than, 4-fold. Their MIC values against PAGU^{g}1927 were 2-fold greater than against PAGU^{g}1931, indicating that the derivatives are MexXY substrates.

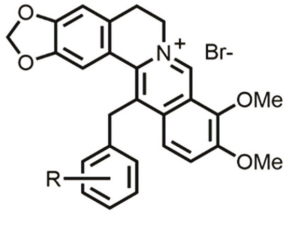


Figure 1. Structure of berberine derivatives.

Table 1. Antibacterial activities of berberine derivatives against PAGU^{g}1931 and PAGU^{g}1927.

Compound	-R	MIC of (μg/mL)	
		PAGU^{g}1927	PAGU^{g}1931
GM[2]	-	1024	8
Ber[3]	-	>512	>512
1	-H	256	128
2	*o*-Br	256	128
3	*p*-Br	128	64
4	*o*-F	512	256
5	*o*-Cl	256	128
6	*p*-Cl	256	128
7	*o*-CH_3	512	256
8	*m*-CH_3	256	128
9	*p*-CH_3	256	128
10	*o*-NO_2	512	256
11	2,6-Cl	128	64

Note: R, side chain of the benzyl group of 13-benzylberberine derivatives; GM, gentamicin; Ber, berberine.

2.2. Inhibition of Drug Resistance in *P. aeruginosa* Using Combined Berberine Derivatives

We investigated the MexXY inhibitory activities of the berberine derivatives by measuring the MICs of gentamicin in the presence of the derivatives against *P. aeruginosa* mutants PAGU^{g}1927 and PAGU^{g}1931 (Table 2). The concentrations of the berberine derivatives were 1/2, 1/4, or 1/8 that of the MICs for PAGU^{g}1931.

The MIC of gentamicin for PAGU^{g}1927 in the presence of 256 μg/mL berberine was 128 μg/mL (Table 2), which is one-eighth that of gentamicin alone (Table 1). Compounds 1–5 and 7 exhibited apparently increased MexXY inhibitory activity, with compound 7 reducing the MIC of gentamicin 64-fold. Compound 7 was named 13-o-MBB. Compounds 8 and 9, which are regioisomers of 13-o-MBB, increased sensitivity to gentamicin by up to 4-fold and were the weakest MexXY inhibitors.

Table 2. Increase in sensitivity to gentamicin by combination with berberine derivatives.

Concomitant Compound	-R	GM MIC with Berberine Derivative (μg/mL)											
		PAGU^{g}1927						PAGU^{g}1931					
		256 *	128	64	32	16	8	256	128	64	32	16	8
Ber	–	128	256	256	512	-	–	8	8	8	8	–	–
1	-H	–	–	32	64	128	–	–	–	8	8	8	–
2	*o*–Br	–	–	32	64	64	–	–	–	4	8	8	–
3	*p*–Br	–	–	–	128	256	512	–	–	–	4	8	8
4	*o*–F	–	32	64	128	–	–	–	4	8	8	–	–
5	*o*–Cl	–	–	32	64	128	–	–	–	4	8	8	–
6	*p*–Cl	–	–	256	256	256	–	–	–	4	8	8	–
7	*o*–CH_3	–	16	32	64	–	–	–	4	8	8	–	–
8	*m*–CH_3	–	–	256	256	512	–	–	–	4	8	8	–
9	*p*–CH_3	–	–	256	256	512	–	–	–	4	8	8	–
10	*o*–NO_2	–	128	128	256	–	–	–	8	8	8	–	–
11	2,6–Cl	–	–	–	128	256	512	–	–	–	4	8	8

Note: R, side chain of the benzyl group of 13–benzyl–berberine derivatives; GM, gentamicin; Ber, berberine, *; combined concentration (μg/mL).

We also examined changes in sensitivity to drugs other than gentamicin by combination with the berberine derivatives (Table 3). The combined use of 13-o-MBB 128 μg/mL reduced the MIC values of various substrate drugs (amikacin, tobramycin, kanamycin, gentamicin, spectinomycin, norfloxacin, ciprofloxacin, erythromycin, carbenicillin, ethidium bromide, tetracycline, chloramphenicol, azithromycin and cefepime) targeting MexXY by 2-fold to 16-fold (Table 3). The regiosomer 13-(3-methylbenzyl) berberine bromide (13-m-MBB) increased the spectinomycin sensitivity of PAGU^{g}1927 8-fold and that of cefepime 4-fold at 64 μg/mL, whereas the other derivatives

did not change the sensitivity to spectinomycin more than 2-fold. In addition, the combined use of 64 μg/mL of 13-(4-methyl-benzyl)-berberine bromide (13-p-MBB) increased sensitivity t cefepime 4-fold for PAGU^{g}1927. Moreover, the sensitizing action of 13-(3-methyl-benzyl)-berberine bromide and 13-(4-methyl-benzyl)-berberine bromide did not exceed that of 13-o-MBB for PAGU^{g}1927. Taken together, these results suggest that the *o*-methyl group of 13-o-MBB increases antimicrobial sensitivity in a MexXY-dependent manner.

Table 3. Increase in sensitivity to antibiotic resistance due to 13−o−MBB and its regioisomers.

Drug	MIC in the Presence of Berberine Derivative (μg/mL)									
	PAGU^{g}1927					PAGU^{g}1931				
	Ber	13−o−MBB	13−m−MBB	13−p−MBB	−	Ber	13−o−MBB	13−m−MBB	13−p−MBB	−
AMK	4	2	4	4	8	1	0.5	0.5	0.5	1
TOB	0.25	0.5	0.5	0.5	0.5	0.25	0.25	0.5	0.125	0.25
KM	128	64	256	256	512	64	32	32	64	64
SPCM	256	64	128	128	1024	64	8	8	32	64
NLFX	0.25	0.0625	1	1	1	0.015625	0.015625	0.015625	0.015625	0.015625
EM	128	64	512	256	512	16	16	16	8	16
CBPC	1	1	1	1	1	1	1	1	1	1
EtBr	512	128	256	256	256	64	8	8	16	64
Tc	2	1	8	8	16	0.125	0.125	0.125	0.125	0.25
Cp	4	2	8	8	8	2	2	2	1	2
AZM	64	64	256	256	512	8	8	8	4	8
CEF	1	0.25	0.125	2	8	0.0625	0.125	0.125	0.125	0.125

Note: Ber, combined berberine 256 μg/mL; 13−o−MBB, combined 13−o−MBB 128 μg/mL; 13−m−MBB, combined 13−m−MBB 64 μg/mL; 13−p−MBB, combined 13−p−MBB 64 μg/mL; AMK, amikacin; TOB, tobramycin; KM, kanamycin; SPCM, spectinomycin; NLFX, norfloxacin; EM, erythromycin; CBPC, carbenicillin; EtBr, ethidium bromide; Tc, tetracycline; Cp, chloramphenicol; AZM, azithromycin; CEF, cefepime.

We investigated whether the inhibitory action of 13-o-MBB against MexXY-dependent drug resistance can be observed in PAGU 1606, a multidrug resistant *P. aeruginosa* clinical strain, and its MexXY-deficient strain PAGU^{g}1659 (Table 4). The MIC of amikacin alone against PAGU 1606 was 256 μg/mL and 64 μg/mL when combined with berberine. In contrast, the combined use of 13-o-MBB and amikacin decreased the MIC to 16 μg/mL. Thus, 13-o-MBB inhibits amikacin resistance 4-fold more effectively than berberine in a MexXY-dependent drug resistant strain. Another aminoglycoside drug, 13-o-MBB, inhibited drug resistance two to four times stronger than berberine but had no greater effect on the drug resistance of PAGU 1606 than the other aminoglycosides. However, the MICs of norfloxacin, erythromycin and azithromycin were increased towards PAGU^{g}1659, a pump-deficient strain.

Table 4. Inhibited resistance to aminoglycoside-based drugs by 13-o-MBB in PAGU 1606

Drug	MIC (μg/mL)													
	PAGU 1606							PAGU^{g}1659						
	-	Ber (256) [1]	Ber (128)	Ber (64)	13-o-MBB (256)	13-o-MBB (128)	13-o-MBB (64)	-	Ber (256)	Ber (128)	Ber (64)	13-o-MBB (256)	13-o-MBB (128)	13-o-MBB (64)
AMK	256	64	128	128	16	32	32	16	8	8	8	8	8	8
TOB	256	64	128	128	16	32	32	8	8	8	8	8	8	8
KM	>2048	1024	1024	2048	256	512	1024	256	256	256	256	256	256	256
GM	64	4	8	16	2	4	4	0.5	0.25	0.25	0.5	0.25	0.25	0.5
SPCM	>2048	>2048	>2048	>2048	2048	>2048	>2048	2048	2048	2048	2048	1024	2048	2048
NLFX	256	256	256	256	256	256	256	64	256	256	256	128	128	128
CPFX	64	64	64	64	32	32	32	64	64	64	64	32	32	32
EM	256	128	256	256	128	256	256	128	256	256	256	256	256	256
CBPC	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512
EtBr	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512
Tc	32	16	16	16	16	16	16	16	16	16	16	16	16	16
Cp	128	64	128	128	64	64	64	128	128	128	128	128	128	128
AZM	256	64	64	128	64	64	128	32	256	256	256	128	128	128
CEF	512	512	512	512	512	512	512	512	512	512	512	512	512	512

Note: [1], values in parentheses are combined concentrations (μg/mL); Ber, berberine; AMK, amikacin; TOB, tobramycin; KM, kanamycin; GM, gentamicin; SPCM, spectinomycin; NLFX, norfloxacin; CPFX, ciprofloxacin; EM, erythromycin; CBPC, carbenicillin; EtBr, ethidium bromide; Tc, tetracycline; Cp, chloramphenicol; AZM, azithromycin; CEF, cefepime.

The sensitizing action of 13-o-MBB for various aminoglycosides was compared with that of berberine at the same concentrations as tested against *P. aeruginosa* clinical strains but using *Burkholderia cepacia* PAGU 0013 and *Achromobacter xylosoxidans* PAGU 0002 (Table 5). The two non-*P. aeruginosa* strains are naturally resistant to aminoglycosides due to the presence of MexXY orthologs [7,23]. 13-o-MBB at the same concentration as berberine increased the sensitivity to the aminoglycosides more than 4-fold over that of berberine. In addition, comparison of the MICs of the aminoglycosides in combination with 13-o-MBB towards a clinical strain of *P. aeruginosa* and its *mexXY*-deficient strain provided similar MIC values. 13-o-MBB greatly increased the sensitivity to aminoglycoside drugs for *P. aeruginosa*, *B. cepacia*, and *A. xylosoxidans*, increasing the sensitivity to amikacin more than 128-fold and to gentamycin more than 512-fold for *A. xylosoxidans*.

Table 5. Inhibition by 13−o−MBB of aminoglycoside resistance in *P. aeruginosa* clinical strains.

Strain	MIC of Aminoglycoside (μg/mL)														
	AMK			GM			TOB			KM			SPEC		
	–	Ber	13-o-MBB	–	Ber	13-o-MBB	–	Ber	13-o-MBB	–	Ber	13-o-MBB	–	Ber	13-o-MBB
PAGU 0974	4	1	0.5	4	0.5	0.25	0.5	0.125	0.125	128	32	32	512	128	32
PAGU^{g} 0975	1	0.5	0.5	0.25	0.125	0.25	0.25	0.25	0.125	64	32	32	32	32	32
PAGU 1498	32	8	1	1024	128	8	256	32	8	>2048	512	256	512	128	32
PAGU^{g}1565	2	1	1	8	8	8	8	8	8	512	256	256	32	32	32
PAGU 1569	256	64	32	256	32	8	16	8	4	>2048	>2048	1024	512	256	128
PAGU^{g}1627	32	32	32	8	8	8	8	8	4	1024	512	1024	128	128	128
*PAGU 0013	128	32	4	128	32	4	64	8	1	64	8	2	1024	128	16
PAGU 0002	>2048	256	16	>2048	32	4	512	16	4	>2048	2048	256	>2048	512	64

2.3. Interaction between 13-o-MBB and Aminoglycoside Drugs

The fractional inhibitory concentration (FIC) values were determined using 13-o-MBB or berberine and gentamicin or amikacin in combination with *P. aeruginosa* strains PAGU 1606 and PAGU^{g}1927 and their MexXY-defective mutants PAGU^{g}1659 and PAGU^{g} 1931 (Table 6). The combination of 13-o-MBB and amikacin or gentamicin showed a synergistic effect in the MexXY-expressing strain, showing that the MexXY-dependent aminoglycoside resistance inhibitory action of 13-o-MBB is synergistic. In addition, the MICs of 13-o-MBB and berberine were reduced only in combination with amikacin or gentamicin and only in the MexXY-expressing strain, showing that the combination of amikacin or gentamicin in the presence of MexXY increases the accumulation of 13-o-MBB and berberine in the cell.

Table 6. Antibacterial activities of berberine derivatives against *P. aeruginosa.*

Strain	MIC (μg/mL) for AMK in the Presence of:		MIC (μg/mL) for 13-o-MBB in the Presence of:		FIC	Mode of Interaction
	–	13-o-MBB	–	AMK		
PAGU^{g}1931	1	1	256	256	2.0	Indifferent
PAGU^{g}1927	8	2	512	128	0.5	Synergy
PAGU^{g}1659	16	8	>512	>512	>1.5	Indifferent
PAGU 1606	256	16	>512	64	<0.5	Synergy

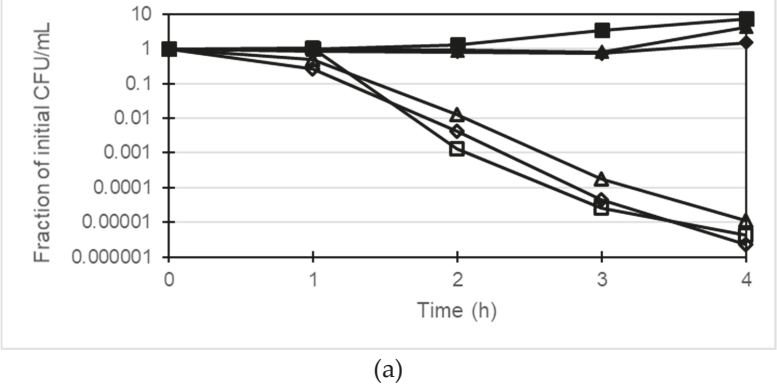
Table 6. *Cont.*

Strain	MIC (μg/mL) for AMK in the Presence of:		MIC (μg/mL) for Berberne in the Presence of:		FIC	Mode of Interaction
	–	Berberine	–	AMK		
PAGU^{g}1931	1	1	>512	>512	>2.0	Indifferent
PAGU^{g}1927	8	4	>512	512	<1.0	Synergy or Addition
PAGU^{g}1659	16	8	>512	>512	1.5	Indifferent
PAGU 1606	256	64	>512	512	<0.75	Synergy or Addition
Strain	**MIC (μg/mL) for GM in the Presence of:**		**MIC (μg/mL) for 13-o-MBB in the Presence of:**		**FIC**	**Mode of Interaction**
	–	13-o-MBB	–	GM		
PAGU^{g}1931	8	8	256	256	2.0	Indifferent
PAGU^{g}1927	1024	32	512	4	0.04	Synergy
PAGU^{g}1659	0.5	0.5	>512	>512	>1.0	Indifferent
PAGU 1606	64	2	>512	8	<0.5	Synergy
Strain	**MIC (μg/mL) for GM in the Presence of:**		**MIC(μg/mL) for Berberine in the Presence of:**		**FIC**	**Mode of Interaction**
	–	Berberine	–	GM		
PAGU^{g}1931	8	8	>512	>512	>1.0	Indifferent
PAGU^{g}1927	1024	128	>512	8	<0.5	Synergy
PAGU^{g}1659	0.5	0.5	>512	>512	>1.0	Indifferent
PAGU 1606	64	8	>512	256	<0.5	Synergy

Note: GM, gentamicin; AMK, amikacin; FIC, fractional inhibitory concentration index.

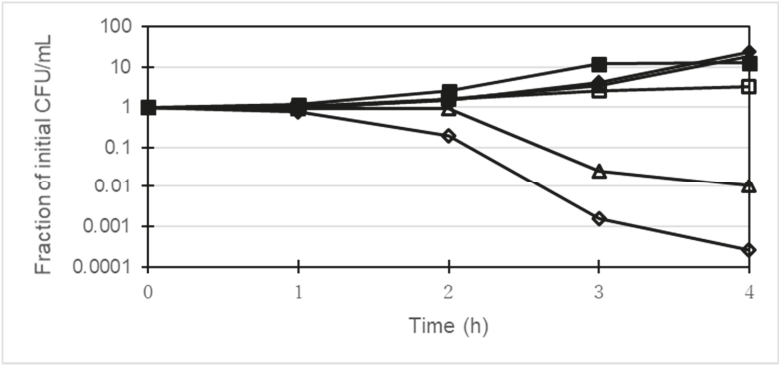
2.4. Time-Killing Assay

The bactericidal activity of gentamicin together with berberine and 13-o-MBB against *P. aeruginosa* was investigated using PAGU^{g}1933 and PAGU^{g}1929. PAGU^{g}1933 was killed after 4 h treatment with gentamicin at 2 μg/mL whereas the growth of PAGU^{g}1929 was suppressed but no bactericidal action was observed (Figure 2). Treatment of PAGU^{g}1929 for 4 h with 2 μg/mL gentamicin in combination with 256 μg/mL berberine reduced the number of colonies about 100-fold. In addition, treatment of PAGU^{g}1929 with a combination of 2 μg/mL gentamicin and 64 μg/mL 13-o-MBB enhanced the bactericidal action of gentamicin more than 10-fold over that of 256 μg/mL berberine.



(a)

Figure 2. *Cont.*



(b)

Figure 2. (**a**) Time-kill curves by the combination of gentamicin with berberine or 13-o-MBB against PAGU^{g}1933, closed squares, control; closed triangles, berberine 256 μg/mL; closed diamonds, 13-o-MBB 64 μg/mL; open squares, gentamicin 2 μg/mL; open triangles, gentamicin 2 μg/mL with berberine 256 μg/mL; open diamonds, gentamicin 2 μg/mL with 13-o-MBB 64 μg/mL; (**b**) Time-kill curves by the combination of gentamicin with berberine or 13-o-MBB against PAGU^{g}1929, closed squares, control; closed triangles, berberine 256 μg/mL; closed diamonds, 13-o-MBB 64 μg/mL; open squares, gentamicin 2 μg/mL; open triangles, gentamicin 2 μg/mL with berberine 256 μg/mL; open diamonds, gentamicin 2 μg/mL with 13-o-MBB 64 μg/mL.

3. Discussion

The addition of 128 μg/mL 13-o-MBB increased the sensitivity to aminoglycosides by 2-fold to 8-fold in comparison with 256 μg/mL berberine in the MexXY-positive *P. aeruginosa* strain PAGU^{g}1927 (Tables 2 and 3). The antimicrobial activity of 13-o-MBB was not significantly different from that of the 13-o-MBB regioisomers 13-(3-methylbenzyl) berberine bromide and 13-(4-methylbenzyl) berberine bromide, although the drug resistance inhibitory action of 13-o-MBB on the MexXY system is greater than that of these two regioisomers. This indicates that 13-o-MBB has greater inhibitory action against MexXY-dependent drug resistance than berberine and the other berberine derivatives we synthesized.

The deletion of *mexXY* from PAGU 1606 strain generated the PAGU^{g}1659 strain. The addition of 13-o-MBB increased PAGU^{g}1659 resistance towards norfloxacin, erythromycin and azithromycin 2-fold to 4-fold. Norfloxacin, erythromycin and azithromycin are substrates for MexCD-OprM and increased resistance towards norfloxacin, erythromycin and azithromycin may be due to the induction of MexCD-OprJ [24].

The addition of 13-o-MBB 256 μg/mL increased the efficacies of azithromycin and gentamicin to a Clinical and Laboratory Standards Institute (CLSI) breakpoint (amikacin is 64 μg/mL, gentamycin is 16 μg/mL) in a clinical strain of *P. aeruginosa* highly resistant to aminoglycosides. Amino acid residue Y613 within the loop of the drug binding pocket of MexY is directly involved in the recognition of aminoglycoside drugs, based on a decrease in sensitivity to aminoglycoside drugs upon mutation of Y613 have been reported [25]. Tobramycin and berberine have been reported to compete for Y613 on the docking simulations of tobramycin or berberine on MexY [16]. Furthermore, they claimed that the results of a combined berberine/tobramycin assay on different clinical isolates of *P. aeruginosa* were consistent with the in silico findings [16]. The results of our combination assay using berberine and 13-o-MBB with aminoglycosides are consistent with this report [16] and substantiate that the main mechanism of action of berberine and 13-o-MBB is competition for MexY inhibition. Another possible mechanism is suppression of MexY expression. However, Berberine decreased MexY mRNA only 0.8 to 0.9-fold have been reported [26]. Another reported that the MIC of amikacin and gentamicin was increased only up to 4-fold even in a strain *P. aeruginosa* that expresses 10–21 times more MexY mRNA than the PAO1 strain [12]. Our study of inhibited resistance by berberine showed that the gentamicin

MIC for PAGU1606 was reduced 4-fold to 16-fold by berberine (Table 4), suggesting that the inhibition of MexY expression is not the main mechanism of action of berberine and 13-o-MBB.

13-o-MBB showed cytotoxicity against Caco-2 cells, a human epithelial colorectal adenocarcinoma cell line, at 30 μg/mL (data not shown). Thus, a concentration of 256 μg/mL 13-o-MBB could be toxic to human cells. There is thus a need to synthesize a compound that exhibits inhibitory action against MexXY system-dependent drug resistance at a lower concentration than 13-o-MBB and that is non-toxic to human cells.

4. Materials and Methods

4.1. Bacterial Strains and Growth Conditions

The bacterial strains used in this study are described in Table 7. Bacterial cells were grown in Luria (L) broth and on L agar (1.5%) under aerobic conditions at 37 °C, as previously described [27].

Table 7. Bacterial strains and gene properties.

Strain Name	Relevant Characteristics	Reference
	Pseudomonas aeruginosa	
PAGU 0974	PAO1 (K. Poole Lab), wild type	[28]
PAGU^{g}0975	PAGU 0974Δ*mexXY*	[29]
PAGU 1498	PA7 Non-respiratory clinical isolate	[8]
PAGU^{g}1565	PA7Δ*mexXY-oprA*	[8]
PAGU 1569	K2162 Pan-aminoglycoside-resistant clinical isolate	[30]
PAGU 1606	NCGM2. S1 Multidrug-resistant clinical isolate	[31]
PAGU^{g}1627	K2162Δ*mexXY*	[30]
PAGU^{g}1659	PAGU 1606Δ*mexXY*	[8]
PAGU^{g}1927	YM34 Δ*mexZ*, *mexVW:: gfp-aacC1*	[15]
PAGU^{g}1929	YM34 Δ*mexZ*, *mexVW*	[15]
PAGU^{g}1931	PAGU^{g}1927::Δ*mexXY*	[15]
PAGU^{g}1933	PAGU^{g}1929::Δ*mexXY*	[15]
	Others	
PAGU 0002	ATCC 27061 *Achromobacter xylosoxidans* subsp. *xylosoxidans*	[32]
PAGU 0013	ATCC 25416 *Burkholderia cepacia*	[33]

4.2. Antibiotic Susceptibility Assay

MICs were assessed in cation-adjusted Mueller–Hinton (MH) broth after about 18–22 h of incubation at 37 °C (for *P. aeruginosa*) or after about 20–24 h of incubation at 35 °C (for *A. xylosoxidans* and *B. cepacia*) using the two-fold serial micro-titer broth dilution method described previously [15]. The categorization as susceptible, intermediate, and resistant was performed according to the interpretive standards of the CLSI.

The FIC index was calculated as described previously [15]. The effects of the drugs were interpreted to be indicative of synergy when the index was ≤0.5.

4.3. Time-Killing Assay

We examined the bactericidal activity of gentamicin monotherapy or combination therapy with berberine or berberine derivatives towards PAGU^{g}1929 and PAGU^{g}1933. Each measurement was started by inoculating between 5×10^6 to 2×10^7 CFU/mL in cation-adjusted MH broth and incubating at 150 rpm at 37 °C on a shaker. Samples were withdrawn to measure the survival counts on MH agar plates at 0, 1, 2, 3 and 4 h. The MH agar plates were incubated at 37 °C for 16–18 h. The concentrations of drugs tested were gentamicin 2 μg/mL, berberine 256 μg/mL, and 13-o-MBB 64 μg/mL. The fraction surviving vs. the control for each sample was determined by taking the average CFU/mL values of the

treated samples and dividing by the value for the same sample at 0 h. Each experiment was repeated at least three times, and a representative experiment is shown.

4.4. Synthesis

4.4.1. General Synthesis Information

Melting points were measured on a Yanagimoto micro melting point hot-stage apparatus (MP-S3) and are reported as uncorrected values. ^{1}H-NMR (TMS: δ: 0.00 ppm as an internal standard) and ^{13}C-NMR ($CDCl_3$: δ: 77.00 or DMSO-d_6: 39.52 ppm as an internal standard) spectra were recorded on JEOL JNM-AL400 (400 MHz and 100 MHz) spectrometers in $CDCl_3$ or DMSO-d_6. Mass spectra were obtained on a JEOL JMP-DX300 instrument (70 eV, 300 mA). Chromatographic separations were accomplished using silica gel 60N (Kanto Chemical Co., Inc., Tokyo, Japan) or aluminum oxide 90 standardized (Merck KGaA., Inc., Darmstadt, Germany). Thin-layer chromatography (TLC) was performed using silica gel 60F254 and aluminum oxide 60F254 neutral (Merck KGaA, Inc., Darmstadt, Germany). All reagents were purchased from Wako Pure Chemical Industry, Osaka, Japan. Kanto Chemical Co., Inc., Tokyo, Japan. Tokyo Chemical Industry Co., Ltd., Tokyo, Japan. Kishida Chemical Co., Ltd., Osaka Japan and Sigma-Aldrich Co., LLC. St. Louis, MO, USA. Dihydroberberine was synthesized by the reduction of berberine according to the reported procedure [21].

4.4.2. 13-Benzylberberine Derivatives; General Procedure

Each benzyl bromide (1.0 mmol) was added in a dropwise manner to a stirred solution of KI (310 mg, 1.86 mmol, 1.86 equiv) and dihydroberberine (337 mg, 1.0 mmol, 1 equiv) in CH_3CN (40 mL), and the resulting mixture was held at reflux for 4 h. The reaction mixture was then filtered, and the filtrate was collected and evaporated to dryness in vacuo to give the crude residue. The residue was purified by column chromatography over neutral alumina using $CHCl_3/CH_3OH$ (50:1 to 20:1) as eluent and recrystallization to give the final compounds 1–11. Compounds 1–10 were known compounds and their characterisation data were identical to those given in the literature. Their melting points (m.p.) were as follows: Compound 1; m.p. 198–200 °C [21], Compound 2; m.p. 179–180 °C [21], Compound 3; m.p. 235–240 °C [21], Compound 4; m.p. 214–216 °C [21], Compound 5; m.p. 210–211 °C [34], Compound 6; m.p. 218–220 °C [35], Compound 7; m.p. 216–220 °C [36], Compound 8; m.p. 222–225 °C [21], Compound 9; m.p. 204–207 °C [36] and Compound 10; m.p. 22–230 °C [21].

4.4.3. Characterisation Data of 13-(2,6-Dichlorobenzyl)berberine Bromide (11)

Compound 11 is a yellow solid. Yield: 41%. ^{1}H-NMR (DMSO-d_6) δ: 9.95 (1H, s), 8.11 (1H, d, J = 9.3 Hz), 7.84 (1H, d, J = 9.3 Hz), 7.56 (1H, s), 7.37 (2H, d, J = 7.8 Hz), 7.23 (1H, t, J = 8.3 Hz), 7.15 (1H, s), 6.18 (2H, s), 5.16 (2H, s), 4.84 (2H, br), 4.09 (3H, s), 4.01 (3H, s), 3.08 (2H, br). ^{13}C-NMR (DMSO-d_6) δ: 150.0 (s), 149.3 (s), 146.5 (s), 144.2 (d), 144.1 (s), 138.2 (s), 134.9 (s), 134.7 (s), 133.6 (s), 131.9 (s), 131.3 (s), 129.5 (d), 129.2 (d), 125.8 (d), 121.0 (s), 120.5 (d), 120.4 (s), 110.9 (d), 108.1 (d), 102.0 (t), 62.0 (q), 56.9 (q), 56.7 (t), 32.9 (t), 27.4 (t). MS m/z: 494 $(M–Br)^+$, 119, 85. m.p. 228–231 °C.

5. Conclusions

Eleven berberine derivatives were synthesized and tested for MexXY-dependent inhibition of gentamicin resistance using a *Pseudomonas aeruginosa* positive-MexXY strain and a negative-MexXY strain. 13-o-MBB showed the greatest inhibitory effect on MexXY-dependent gentamicin resistance. Regioisomers of 13-o-MBB exhibited no greater MexXY-dependent inhibition of gentamicin resistance than berberine. 13-o-MBB inhibited resistance to aminoglycosides 4-fold to 16-fold compared with berberine against the four tested *P. aeruginosa* clinical strains, and *Achromobacter xylosoxidans* and *Burkholderia cepacia*. These results indicate that 13-o-MBB inhibits the resistance to aminoglycosides

in a MexXY-dependent manner more strongly than berberine. 13-o-MBB is thus a useful inhibitor of aminoglycoside drug resistance due to MexXY.

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