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Effects of Fermented Oils on Alpha-Biodiversity and Relative Abundance of Cheek Resident Skin Microbiota

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Abstract: The skin microbiome is in a very close mutualistic relationship with skin cells, influencing their physiology and immunology and participating in many dermatological conditions. Today, there is much interest in cosmetic ingredients that may promote a healthy microbiome, especially postbiotics, mainly derived from fermented products. In the present work, we studied the effects on skin microbiota of new patented natural oils obtained by unique fermentation technology in vivo. Three fermented oils were evaluated: F-Shiunko (FS), F-Artemisia® (FA) and F-Glycyrrhiza® (FG). The active components were included as single active component or in combination (FSAG) in an emulsion system. A total of 20 healthy women were recruited, and skin microbiota from cheek were analyzed by mean of swab sampling at T0 and T1 (after 4 weeks of a one-day treatment). 16S sequencing revealed that the treatment with fermented oils improved microbiome composition and alpha-diversity. It was shown that higher biodiversity reflects in a healthier microbial ecosystem since microbial diversity decreases in the presence of a disease or due to aging. The treatment also resulted in a more "beneficial" and "younger" microbial community since a significant decrease in *Proteobacteria* and the increase in *Staphylococcus* were reported after the treatment with fermented oils.

Keywords: microbiota; skin; postbiotic; fermented oil; alpha-diversity

1. Introduction

The skin, as the largest organ is also the more complex and dynamic microbial ecosystem of the human body [1]. It is colonized by trillion of bacteria, archaea, fungi and viruses collectively referred to as the skin microbiome; they are in a very close mutualistic relationship with skin cells influencing their physiology [2] and immunology [3].

Due to its composition, especially as regards unusual and diversified lipids, skin results as a very distinct habitat both from a chemical and physical point of view compared to other human sites, especially the intestinal one [4,5]. Therefore, since the density and variety of glands vary considerably across different skin sites, different microbial populations characterize each skin site [1].

Cutibacterium (now *Propionibacterium*) and *Staphylococcus* are found as the most abundant genera in the sebaceous area (e.g., face, upper chest and back) [6] and *Corynebacterium*, *Staphylococcus* and *Proteobacterium* in moist areas (e.g., armpits, elbow, antecubital fossa) [7].

On the other side, as reported by recent scientific evidence [8], unexplored host and microbial factors can drive differences at species [8] and strain level [9].

Findings from the scientific community have brought to light the involvement of skin microbiota in human health as in dermatological conditions [10–14].

Many factors can influence the microbiota composition of the skin. For sure the presence of dermatological conditions leads to microbial community changing [1]. Other factors are genetic, environment, aging, diet, but also skin type (e.g., oiliness, hydration) and skin-care routine [15]. More recently, negative changes in the microbiota composition have been associated with the use of cosmetics [16,17]. Indeed, many skin reactions such as allergies have been associated with synthetic ingredients in cosmetics [18–23]. For these reasons, natural ingredients must be preferred and there is a continuous effort both by researchers and formulators to discover new active ingredients that may help in improving skin appearance and structure, but at the same time able to promote a healthy microbiome. In this view, the cosmetic market as the research is focused on offering solutions coming from nature such as especially, prebiotics and probiotics although inactivated probiotics are preferred to the latter because of the difficulty of maintaining them viable in a topical formulation [23,24]. A new frontier in the field is that represented by postbiotic, byproducts of probiotic bacteria and which are responsible for their main activities [23]. The main advantages deriving from the use of postbiotics are related to their higher specificity of action on resident microbiota as of interaction whit cells of the host compared to probiotics. Most importantly, they have no need for survival in the topical formulation [25]. For these reasons, they can be helpful in all the dermatological conditions in which the role of the microbiome has been hypothesized. Postbiotics are usually obtained as the results of microbial metabolism in a fermented matrix. Benefits deriving from fermentation are largely reported for gut microbiome modulation [25–28], but their effect also on skin microbiome has been hypothesized [29]. Indeed, fermentation is linked to the overproduction or ex-novo production of bioactive molecules whit health benefits ranging from antimicrobial to immunomodulatory, anti-inflammatory, anti-proliferative, antioxidants, anti-obesogenic and anti-hypertensive activity [24,25].

The system used to deliver actives is also important. In this sense, emulsions possess many advantages for skincare applications [30]. Due to the special and unique lipo/hydrophilic balance, the use of a cream on the skin has many advantages compared to the use of a simple system such as a hydrophilic solution or an oil.

The lipophilic component of an emulsion is mainly constituted by oils. They act as nourishing agents for the skin and promote its natural defense, but they can also be metabolized by microorganisms and act as antimicrobial agents [31,32].

The earliest emollients in the history of cosmetics were naturally occurring fats and vegetable oils [33]. they consist of ester salts of glycerin with a large number of organic acids such as stearic acid, oleic acid and palmitic acid-forming stearin, olein and palmitin, respectively [34]. Stearin and palmitin prevail in the solid oils and fats, while olein is dominant in the liquid oils [35].

Natural oils are excellent emollients and have the advantage of being rich in several antioxidant compounds. Oils may also act as natural antimicrobial substances and help to control the growth of microorganisms also contributing to the maintenance of the skin structure which is essential for a healthy microbiome [36].

On the other hand, vegetable oils are often very greasy and unpleasant on the skin and hard to stabilize in formulations. For these reasons, many efforts in the last years have been done to study how to stabilize vegetable oils and improve texture, while maintaining their unique activity also on the skin microbiome [37]

In the present work, we studied in vivo the effects on skin microbiota of a new patented category of natural oils obtained by unique fermentation technology according to which the vegetable oil was fermented by *Pseudozyma* sp. SY-16 [38].

2. Materials and Methods

2.1. Materials

All materials used in this work are listed in Table 1, including identifying name, INCI name and manufacturer/distributor.

Table 1. Overview of all materials used in this work.

	Identifying Name	INCI * Name	Manufacturer/Distributor
Active	F-Shiunko	*Pseudozyma epicola*/ apricot kernel oil/olive fruit oil/sunflower seed oil/sweet almond oil/(*Angelica gigas*/*Lithospermum erythrorhizon*) root extract ferment filtrate	LABIO Co., Ltd., Korea
	F-Artemisia	*Pseudozyma epicola*/ apricot kernel oil/olive fruit oil/sweet almond oil/sunflower seed oil/*Artemisia princeps* extract ferment extract filtrate	LABIO Co., Ltd., Korea
	F-Glycyrrhiza	*Pseudozyma epicola*/ apricot kernel oil/olive fruit oil/sweet almond oil/sunflower seed oil/licorice root extract ferment extract filtrate	LABIO Co., Ltd., Korea
Preservative	Euxyl K712®	Aqua, sodium benzoate, potassium sorbate	Schuelke & Meyr, Italy
Emulsifier	Pemulen TR-1®	Acrylates c10-30 alkyl acrylates crosspolymer	Lubrizol, USA
	Carbopol Ultrez 21®	Acrylates c10-30 alkyl acrylates crosspolymer	Lubrizol, USA
Others	Glicerina	Glycerin	Acef, Italy
	EDTA bisodico	Disodium EDTA	Acef, Italy
	Emultop Velvet IP®	Lecithin	Labio LAB, Italy

* International Nomenclature of Cosmetic Ingredients

Three fermented actives were used, named, respectively: F-Shiunko (FS), F-Artemisia® (FA) and F-Glycyrrhiza® (FG). The actives were included as single active or in combination (FSAG) in an emulsion system.

The emulsions were designed with the basic concept to get a pleasant and stabilized system, able to deliver the oil properly. The emulsifying system consists of an acrylic hydrophilic polymer. Lecithin is used to stabilize the emulsion and a very simple and microbiota-safe preservative system (potassium sorbate and sodium benzoate) [39] is added to ensure a low microbiological risk.

2.2. Study Design

The study was in the form of a spontaneous, open, clinical trial, conducted by one center and under dermatological control. Twenty healthy volunteers (female, median aged 45 ± 12.5 years) were enrolled after signing informed consent.

Exclusion criteria included: i) cutaneous or systemic infection; ii) any aesthetic facial procedure including laser therapy and tissue/dermal injectables within the last 6 months; iii) light-based therapy in the last 4 months; iv) facial dermatological conditions that may interfere with the present study; v) antibiotics use in the last 30 days; vi) any therapy with immunosuppressant and/or cortisone in the last 4 months; vii) probiotics in the last 15 days; viii) pregnancy or breastfeeding.

All subjects were also asked to do the last washing with soap the evening before sampling and not to use make-up in the morning of the sampling procedure.

Products were assigned randomly to each subject and two products were assigned for a half-face treatment. Subjects were instructed to apply the products once a day in the morning and use the products assigned as the only facial cream for the entire duration of the study and to not use the product on the day of sampling until swabbing had taken place.

The study was performed following the Declaration of Helsinki and the International Conference of Harmonization (ICH) Guidelines for Clinical Practice. Study approval was given by the Ethical Independent Committee for Clinical, not pharmacological investigation in Genoa (Italy).

2.3. Swab Sample Collection

Enrolled subjects were sampled before to start the treatment (T0) and after 1 month of treatment (T1). Cheek swabs were obtained with the eNAT™ kit consisting of 1 mL eNAT™ transport and preservation medium in 12 × 80 mm screw cap tubes and a regular FLOQSwab™ (Copan, Brescia, Italy). Immediately after sampling, swabs were immersed in the eNAT™ medium and stored at 4 °C, until DNA extraction.

2.4. DNA Extraction and 16S Amplicon Generation, Sequencing and Analysis

Bacterial DNA from cheek swabs was extracted by mean of QIAamp Dneasy Tissue kit (Qiagen, Milan, Italy) according to manufacturer protocol, with minor modifications [40] and quantified by the QIAexpert system (Qiagen, Milan, Italy) before sequencing.

DNA samples were amplified for the variable region V3-V4 using the universal prokaryotic primers: 341 F CTGNCAGCMGCCGCGGTAA [41,42] and 806bR GGACTACNVGGGTWTCTAAT [43–45] at Personal Genomics (Verona, Italy) following the method of Caporaso et al. [46] and Kozich et al., [47] with minor modifications. Libraries were generated using 300PE instrument (Illumina, San Diego, CA, USA) and bioinformatic analysis performed as previously reported [11].

Amplicon reads were also analyzed as regards alpha diversity by mean of Shannon index, using QIIME v1.9.

2.5. Statistical Analysis

Statistically significant differences in alpha diversity and bacterial communities were obtained by t-tests for independent samples corrected by using Welch's t-test. All datasets were normally distributed. Analyses were performed with GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA, USA). Differences between groups were considered significant at a p-value < 0.05.

3. Results

Semi-Quantitative Bacterial Composition: Alpha-Biodiversity and Relative Abundance

A total of 20 healthy women were recruited and skin microbiota from cheek were analyzed by mean of swab sampling at T0 (before the first application) and T1 (after 4 weeks of treatment).

The bacterial 16S rRNA gene V3-V4 region (16S) was sequenced using the 300PE Illumina platform. We first analyzed the alpha diversity (species richness) at baseline, and after 4 weeks of treatment in the analyzed skin site.

After one month of treatment with fermented oils, all groups displayed a significant ($p < 0.001$) increase in the α-diversity based on the observed and Chao1-estimated OTU number and the Shannon index (Figure 1).

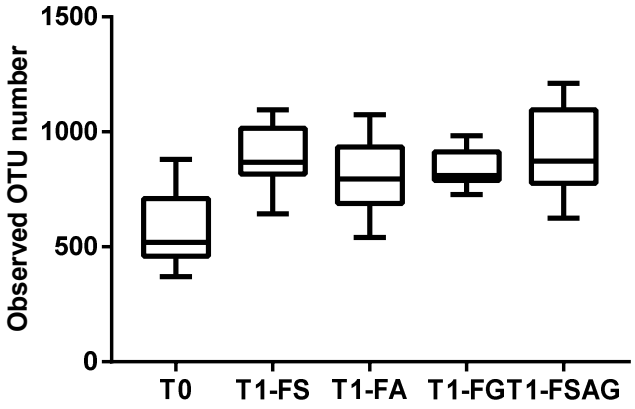
In particular, the analysis of the observed OTU numbers showed a significant increase of the species richness in the treated groups (FS: $p = 0.0007$; FA: P = 0.0217; FG: $p < 0.0001$; FSAG: $p = 0.0027$) compared to baseline (TO) (Figure 1a).

Similarly, the treatment results in a higher Chao1-estimated OTUs (Figure 1b) than the non-treated group. No significant differences were found as regards the Shannon index (Figure 1c). Taking together these data indicated that the treatment induces a tendency toward a higher alpha diversity.

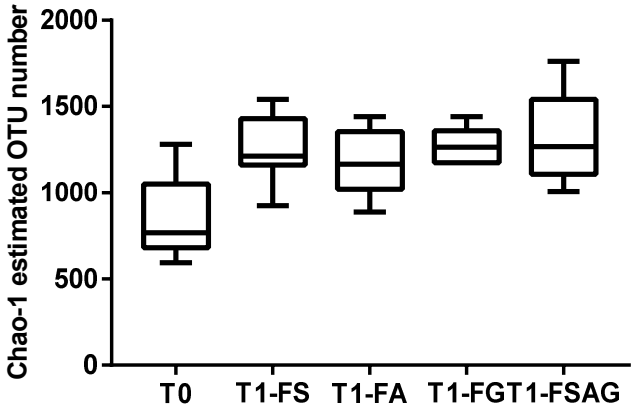
Statistical significance compared to baseline (T0) is reported in Table 2.

The measurements of semi-quantitative bacterial composition (relative abundance) were done through16S-rDNA metagenomic approach.

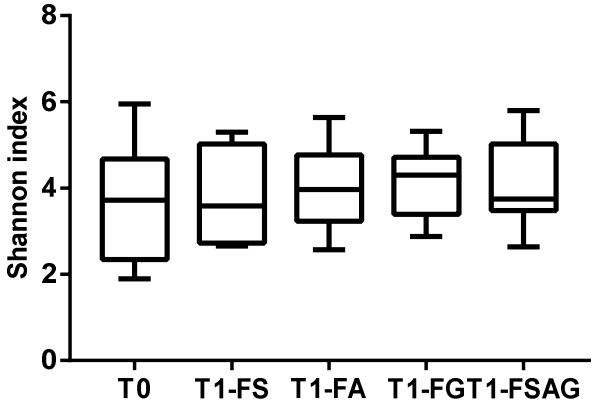
At the phylum-level, the four dominating phyla were Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria. Among these, Actinobacteria resulted as the most predominant in all groups (61%, 57%, 50%, 50% and 53%, respectively for T0, FS, FA, FG and FSAG) (Figure 2).



(**a**)



(**b**)



(**c**)

Figure 1. Alpha diversity of analyzed skin microbiome samples based on the (**a**) observed and (**b**) Chao1-estimated Observed Taxonomic Unit (OTU) numbers and the (**c**) Shannon index. T0: baseline; F-Shiunko (T1-FS); F-Artemisia (T1-FA); F-Glycyrrhiza (T1-FG); combination (T1-FSAG).

Table 2. Alpha diversity's statistical significance.

Metric	Samples	*p*-Value [1]
Observed OTU number	T1-FS	0.0007
	T1-FA	0.0217
	T1-FG	<0.0001
	T1-FSAG	0.0027
Chao-estimated OTU number	T1-FS	0.0012
	T1-FA	0.0116
	T1-FG	<0.0001
	T1-FSAG	0.0031
Shannon Index	T1-FS	0.7991
	T1-FA	0.5369
	T1-FG	0.2997
	T1-FSAG	0.4258

[1] Welch's *t*-test.

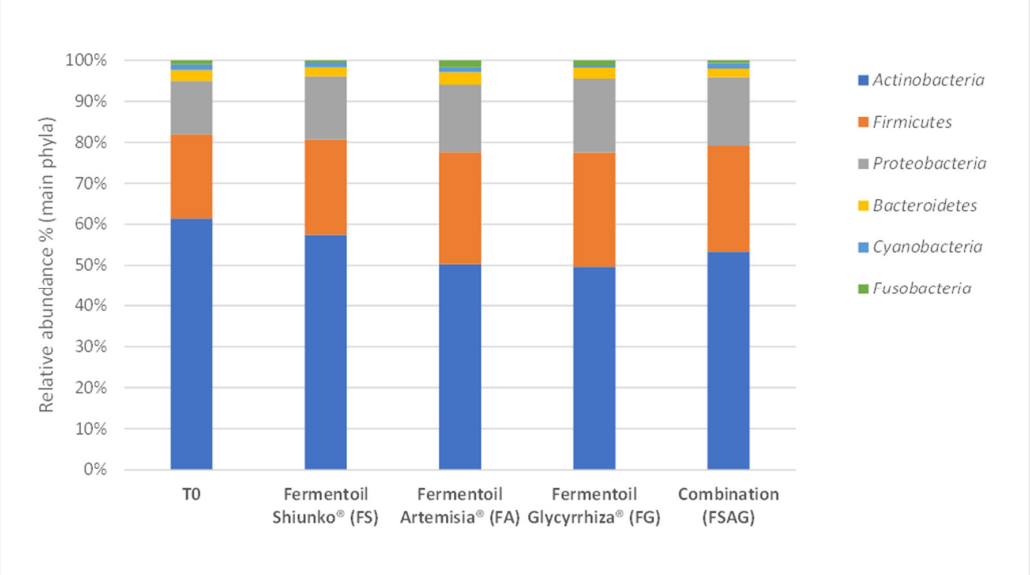


Figure 2. Semi-quantitative bacterial composition: relative abundance at phylum level.

Comparing the abundance of the major phyla between groups revealed that Actinobacteria showed a lower abundance after one month of treatment with fermented oils. The reduction of Actinobacteria was most striking in FA, FG and FSAG groups (Figure 3), but this reduction is not significant.

The treatment leads also to a significantly lower abundance in Proteobacteria than the baseline (T0) with a concurrent increase in Firmicutes (Figure 2).

Analysis at the genus level revealed *Propionibacterium*, *Corynebacterium*, *Streptococcus*, *Staphylococcus* and *Acinetobacter* as the five most abundant genera in all groups (Figure 3). The average abundance was ≥1%. Among them, *Propionibacterium* was the most abundant, accounting for 75%, 55%, 63%, 59% and 66% of the total reads in T0, FS, FA, FG and FSAG groups, respectively (Figure 3).

Comparative analysis of the abundance of five dominant genera between groups showed that the abundance of *Propionibacterium* was significantly ($p < 0.001$) lowered in the Fermentoil®-groups than baseline (T0). These data were consistent with the above reported marked reduction of *Actinobacteria* after treatment. In addition, the abundance of *Staphylococcus* was significantly ($p < 0.001$) increased in the Fermentoil®-groups, while *Streptococcus* was significantly decreased in FA and FG groups (Figure 3). No differences were found as regards the *Acinetobacter* genus except for the FG group in which a significant ($p < 0.001$) decrease was reported.

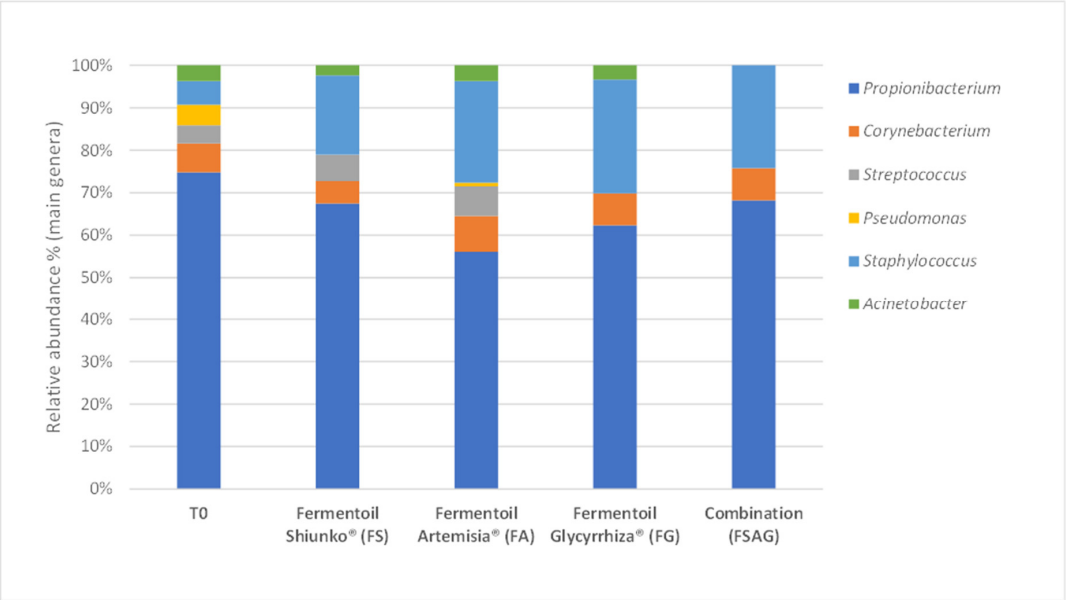


Figure 3. Semi-quantitative bacterial composition: relative abundance at genus level.

Pseudomonas also showed higher abundance at baseline (Figure 3).

Altogether, these data indicated global taxonomic differences in the analyzed groups.

4. Discussion

In the present work, we focused on the study microbiota of three different actives obtained by a patented fermentative process using natural oils as fermentation matrix [38].

In cosmetic products, oils and lipid substances in general play an essential role due to their high eudermic potential.

The ideal system of delivery of oils in cosmetic products is nanoliposomes and nanostructured lipid carriers are [48]. Emulsions too are good systems [30]. They can get the right hydro-lipophilic balance to achieve a good bioavailability both hydrophilic and lipophilic active ingredients [49]. At the same time, they improve the sensorial characteristics of the single components.

Indeed, a simple oil would be too much occlusive and tacky on the skin, while a hydrophilic compound would be too dry and not able to interact with the complex skin structure properly. Emulsion's texture and pleasantness are key points in terms of "adherence to therapy" by the users.

In a complex system such as an emulsion, the action of hydrophilic and lipophilic components is synergic [50]. Both hydrophilic and lipophilic components are released on the skin surface in a performing manner: first hydrophilic and then lipophilic components [50]. The oil phase in an oil in water (o/w) emulsion becomes more available due to the action of the high oil/water interface that allows bioavailability of active ingredients through the skin layers.

In this review, we focused on the study of the lipophilic component of this system, which is often related to the most active part of the formulation; molecule as polyphenols, isoflavones, terpenes are, among others, some of the most important molecules related to antioxidant [51–53] and anti-inflammatory [52,53] activity on the skin.

Oils can also act as anti-bacterial substances of natural origin, helping to control the growth of microorganisms [54–56]. Some examples are represented by Jojoba oil [56] and lavender essential oils [55]. Oil components are reported to act by disturbing the lipid structure of cell membranes of bacteria and, after penetration, they are also able to block their metabolism [57]. The chemical composition of oils can have an influence on their microbiological properties [58].

Natural oil fermentation [38] enhances emulsion stability, texture, moisturizing and penetration capacity, but also changes the chemical composition of fermented natural oils. The fermentation

of oils is made by yeast, *Pseudozyma* sp. SY-16 [KCTC 8950P] [39]. As a result of fermentation, a higher content of free fatty acid (4–6% vs. <1% in natural plant oil) and α-tocopherol is obtained. In addition, active polyphenols were produced. This increases the antioxidant potential of this type of oil. Finally, due to the anabolic activity of the yeast also mannosylerythritol lipid (MEL) is produced, which is responsible for the enhanced emulsifying potential of fermented oils. Therefore, both MEL and free fatty acids decrease the surface tension of oil. Fermented oils are obtained from shiunko oil, artemisia and licorice, respectively.

Shiunko oil is a Japanese traditional remedy used in moxibustion therapy [58]. *Lithospermi radix* (LR), *Angelicae gigantis Radix*, sesame seed oil, beeswax and swine oil are the main components of Shiunko oil [59]. It is widely used for treating eczema, psoriasis, hair loss, burns, topical wounds and atopic dermatitis [60].

Artemisia, also known as Korean Tea tree, is a traditional herbal medicine [61]. Cosmetics containing *Artemisia annua* extract are reported effective to repair sensitive skin, inhibit inflammation, repair skin barrier, improve damaged skin and reduce redness and other sensitive skin symptoms [62]. These effects are due to the high content in sesquiterpenes and polyphenolic antioxidants.

Licorice is the root of *Glycyrrhiza glabra* its extracts have been used in herbalism and traditional medicine for years for its anti-inflammatory properties [63]. Glycyrrhiza glabra Linn. belongs to the Fabaceae family [63]. It is an herbaceous perennial legume native to the Middle East, southern Europe and parts of Asia and was recognized since ancient times for its ethnopharmacological values. This plant contains different phytocompounds, such as glycyrrhizin, 18β-glycyrrhetinic acid, glabridin and isoflavones, that have demonstrated various pharmacological activities [64].

Either shiunko, artemisia and licorice were fermented by *Pseudozyma* sp. SY-16.

In the present study, we revealed that the treatment with fermented oils impacts on microbiota composition and biodiversity.

It was shown that higher biodiversity reflects in a healthier microbial ecosystem [65,66]. In the presence of a disease [65] or due to aging [67] microbial diversity decreases. Therefore, comparison with skin microbial composition of Western and uncontaminated rural Papua New Guinea and Paraguay populations confirmed that exposure to synthetic compounds leads to the lowering of microbial diversity [68]. Data on the present work are in line with these previous findings. Treatment of cheek with natural fermented oils significantly increases microbial diversity as showed by alpha-diversity analysis with all three metrics used.

High similarity in the species diversity and bacterial composition was found between treated groups. The treatment also resulted in a more "beneficial" and "younger" microbial community since a significant decrease in *Proteobacteria* and the increase in *Staphylococcus* were reported after the treatment with fermented oils and this was in line with previous studies from Shibagaki and collaborators [67] and Wilantho and collaborators [68]. Since a decrease of *Propionibacterium* was reported in treated groups it could be hypothesized a sebum-regulatory activity of fermented oils.

Lecithin has been used as a stabilizing ingredient. Recently, Nejrup and collaborator [69] reported the effect of phospholipid fractions, including lecithin from soybean on gut microbiome composition when used in infant formulas. According to this finding, the role of lecithin on microbial diversity reported in the present study could be hypothesized. As was suggested for the gut, phospholipid could influence lipid degradation and, as a consequence could affect the growth of specific bacterial groups and the overall metabolic activity of the bacterial community.

Future works will aim at also investigating the role of fermented oils on bacterial virulence in the hypothesis of their action as putative quorum sensing inhibitors of pathogens (e.g., *S. aureus*, *P. aeruginosa*) [70]. Indeed, the study of microbial biodiversity and bacterial virulence represent two schools of thought: the first focused on the basic mechanisms of the pathogen host interaction and the second in which virulence is one of several parameters affecting pathogen spread in the host population [71,72].

In summary, this study highlighted the effect of fermented oils on biodiversity and microbial composition on the cheek of healthy women. It can be speculated that microbial change reflects changes in skin physiology and appearance. This could be the rationale of new dermatologic and aesthetic treatments.

5. Conclusions

The involvement of the microbiota for skin health is becoming increasingly popular and there is a growing effort from the scientific community aimed at the discovery of new cosmetic active ingredients that can positively interact with skin microbial community.

Prebiotics, probiotics—and, more recently, postbiotics—represent a valid option for this purpose. The main source of postbiotics is represented by fermentation—the benefits of which for gut and skin microbiota have been reported.

We highlighted the positive effect of different oils obtained by a special patented fermentative process on the alpha-biodiversity and relative abundance of the microbiota of the cheek of 20 healthy volunteers after one month of treatments.

Limitations of the present work include the small sample size of enrolled subjects.

Future work will aim at deeply studying the effect also on skin structure, as well as aspects of fermented oils. Second, it will be conducted over a larger population and for a longer period to give a deep understand of these ingredients and skin microbiome ecosystems.

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