



*fermentation*

Special Issue Reprint

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# 10th Anniversary of *Fermentation*

Feature Papers in the “Fermentation for  
Food and Beverages” Section

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Edited by  
Alice Vilela

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**10th Anniversary of Fermentation:  
Feature Papers in the “Fermentation for  
Food and Beverages” Section**



# **10th Anniversary of Fermentation: Feature Papers in the “Fermentation for Food and Beverages” Section**

Guest Editor

**Alice Vilela**



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Erlenmeyers with alginate beads

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# Contents

|                            |     |
|----------------------------|-----|
| About the Editor . . . . . | vii |
|----------------------------|-----|

|                   |    |
|-------------------|----|
| Preface . . . . . | ix |
|-------------------|----|

|                                                                                                                                                                                                                                                                                                                                                                                                   |   |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---|
| <b>Valeria Novikova, Anna Shiker, Anastasia Vostrikova, Egor Parkhomenko, Ilya Demyanenko, Olga Korneeva, et al.</b><br>Lactic Acid Bacteria Fermentation as a Tool for Creating Texture in Plant-Based “Clean-Label” Cheeses<br>Reprinted from: <i>Fermentation</i> 2026, 12, 111, <a href="https://doi.org/10.3390/fermentation12020111">https://doi.org/10.3390/fermentation12020111</a> . . . | 1 |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---|

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |    |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| <b>Adriana Barros de Cerqueira e Silva, Eric de Lima Silva Marques, Rachel Passos Rezende, Cristiano Santana, Angelina Moreira Freitas, Maria Clara Bessa Souza, et al.</b><br>Use of <i>Pichia manshurica</i> as a Starter Culture for Spontaneous Cocoa Fermentation in Southern Bahia, Brazil<br>Reprinted from: <i>Fermentation</i> 2025, 11, 694, <a href="https://doi.org/10.3390/fermentation11120694">https://doi.org/10.3390/fermentation11120694</a> . . . | 22 |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|

|                                                                                                                                                                                                                                                                                                                                                                |    |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| <b>Yi-Hsuan Chen, Yi-Wen Fang, Po-An Tu, Ching-Yi Chen and Han-Tsung Wang</b><br>Fermentation-Based Preservation of Okara and In Vitro Evaluation of Its Application in Dairy Cattle Diets<br>Reprinted from: <i>Fermentation</i> 2025, 11, 559, <a href="https://doi.org/10.3390/fermentation11100559">https://doi.org/10.3390/fermentation11100559</a> . . . | 36 |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|

|                                                                                                                                                                                                                                                                                                                                                                                 |    |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| <b>Penghui Zhang, Kelyn Seow, Leo Wein, Rachel Steven, Rebecca J. Case, Yulan Wang and Patricia L. Conway</b><br>Production of Nutritional Protein Hydrolysates by Fermentation of Black Soldier Fly Larvae<br>Reprinted from: <i>Fermentation</i> 2025, 11, 524, <a href="https://doi.org/10.3390/fermentation11090524">https://doi.org/10.3390/fermentation11090524</a> . . . | 55 |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|

|                                                                                                                                                                                                                                                                                                               |    |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| <b>Yanzhuo Kong, Suhasna Palihakkara, Leo Vanhanen and Venkata Chelikani</b><br>Fermentation Performance of Epigenetically Modified Yeast<br>Reprinted from: <i>Fermentation</i> 2025, 11, 515, <a href="https://doi.org/10.3390/fermentation11090515">https://doi.org/10.3390/fermentation11090515</a> . . . | 71 |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |    |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| <b>Natali Hernández-Parada, Hugo Gabriel Gutiérrez-Ríos, Patricia Rayas-Duarte, Oscar González-Ríos, Mirna Leonor Suárez-Quiroz, Zorba Josué Hernández-Estrada, et al.</b><br>Screening Sourdough Starter Cultures from Yeast and Lactic Acid Bacteria Isolated from Mexican Cocoa Mucilage and Coffee Pulp for Bread Quality Improvement<br>Reprinted from: <i>Fermentation</i> 2025, 11, 498, <a href="https://doi.org/10.3390/fermentation11090498">https://doi.org/10.3390/fermentation11090498</a> . . . | 84 |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|

|                                                                                                                                                                                                                                                                                                                                                                                               |     |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| <b>Muhammet Ercan, Mehmet Akbulut, Hacer Çoklar and Talha Demirci</b><br>Impacts of Sonication on Fermentation Process and Physicochemical, Microbiological and Sensorial Characteristics of Fermented Black Carrot Juice<br>Reprinted from: <i>Fermentation</i> 2025, 11, 475, <a href="https://doi.org/10.3390/fermentation11080475">https://doi.org/10.3390/fermentation11080475</a> . . . | 110 |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|

|                                                                                                                                                                                                                                                                                                                                                                      |     |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| <b>Inés M. Ramos, Samuel Rodríguez García and Justa M. Poveda</b><br>Development and Characterization of Pistachio Yogurt Analog: A Healthy, Sustainable, and Innovative Plant-Based Alternative<br>Reprinted from: <i>Fermentation</i> 2025, 11, 467, <a href="https://doi.org/10.3390/fermentation11080467">https://doi.org/10.3390/fermentation11080467</a> . . . | 125 |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|

|                                                                                                                                                                                                                                                                                                                                                                                                              |     |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| <b>Yanping Lu, Yalan Li, Bing Wan, Yuzhu Li, Jian Su, Jia Zheng, et al.</b><br>Correlation Analysis Between Volatile Flavor Compounds and Microbial Communities During the Fermentation from Different Ages Mud Pits of Wuliangye Baijiu<br>Reprinted from: <i>Fermentation</i> 2025, 11, 388, <a href="https://doi.org/10.3390/fermentation11070388">https://doi.org/10.3390/fermentation11070388</a> . . . | 140 |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|

- Sandra Cermeño, José Manuel Veiga-del-Baño, Miguel Ángel Cámara, Pedro Andreo-Martínez and José Oliva**  
Processing Factors and Risk Assessment of Pesticide Residues in Wine  
Reprinted from: *Fermentation* **2025**, *11*, 318, <https://doi.org/10.3390/fermentation11060318> . . . **155**
- Alejandro Coloma, Maria Mamani-Mamani, Cristina Valencia-Sullca, Javier Mamani Paredes, Herbert Callo, Nancy Curasi Rafael, et al.**  
Ultrasound-Assisted Kinetics of Alcoholic Fermentation at Varying Power Levels for the Production of Isaño Wine (*Tropaeolum tuberosum*)  
Reprinted from: *Fermentation* **2025**, *11*, 268, <https://doi.org/10.3390/fermentation11050268> . . . **170**
- Samira Arroum, Amel Sboui, Imen Fguiri, Mohamed Dbara, Naziha Ayeb, Mohamed Hammadi and Touhami Khorchani**  
Influence of Kefir Grain Concentration on the Nutritional, Microbiological, and Sensory Properties of Camel Milk Kefir and Characterization of Some Technological Properties  
Reprinted from: *Fermentation* **2025**, *11*, 170, <https://doi.org/10.3390/fermentation11040170> . . . **187**

# About the Editor

## Alice Vilela

Alice Vilela has a degree in enology, a master's degree in genetics and species improvement, and a PhD in microbiology from UTAD. She began her teaching career at UTAD in 1999 and is currently an Assistant Professor in the Department of Agronomy at the School of Agricultural and Veterinary Sciences (ECAV). Her teaching experience encompasses undergraduate, master's, and doctoral courses, and she serves as the head of several curricular units, advising students across three teaching cycles. She is involved in university management activities, holding leadership positions as director of the master's degree in enology and viticulture and vice-director of the bachelor's degree in enology. Moreover, she is the director of the UTAD Tasters panel, actively disseminating knowledge, organizing, and participating in international and national events, as well as university extension activities within and outside Portugal. She has participated in more than 20 strategic projects with competitive financing and national and global partnerships. Her research focuses on the bioreduction of volatile acidity in wines, biodealcoholization of wines, sensory evaluation of food and wines, and development of sensory tests. She has published more than 110 articles in international scientific journals, 14 technical articles, 25 book chapters, and over 120 conference proceedings publications, with her h-index currently being 32. She is an active reviewer for several SCI journals, including *Food Research International*, *Journal of the Science of Food and Agriculture*, *Food Chemistry*, *Journal of Gastronomy and Food Science*, and several MDPI journals (*Foods*, *Fermentation*, *Beverages*, and *Molecules*). Additionally, she serves as an Academic Editor and is a member of the Editorial Board of several SCI journals. From 2021 to 2025, Stanford University, USA, recognized her as among the world's 2% most cited scientists. On March 20, 2026, she received the '2025 Researcher of the Year Award' from UTAD.



# Preface

The reprint *10th Anniversary of Fermentation: Feature Papers in the “Fermentation for Food and Beverages” Section* celebrates recent advances in one of the oldest and most versatile biotechnological processes. Fermentation has long played a fundamental role in the production of safe, nutritious, and high-quality foods and beverages. Today, it is also a key technology for developing sustainable, clean-label, functional, and plant-based products that address evolving consumer expectations and global food challenges. This volume brings together outstanding contributions that illustrate the breadth and impact of contemporary research in food and beverage fermentation.

The scope of this reprint encompasses microbial fermentation, novel starter cultures, plant-based fermented products, sustainable valorization of food by-products, innovative processing technologies, and the quality and safety assessment of fermented foods and beverages. Its aim is to provide an up-to-date overview of emerging scientific developments while highlighting innovative approaches that contribute to healthier, more sustainable, and consumer-oriented food production.

The motivation for compiling this collection arises from the rapid growth of fermentation research and the increasing need to consolidate interdisciplinary advances in a single reference. By presenting high-quality original research and review articles published in the *Fermentation* journal, this reprint seeks to promote scientific exchange, stimulate future research, and facilitate the translation of innovative fermentation strategies into industrial and commercial applications.

This volume is intended for researchers, food scientists, microbiologists, biotechnologists, nutritionists, graduate students, and professionals in the food industry, as well as all readers interested in the latest advances in food and beverage fermentation and their contribution to sustainable and functional food production.

**Alice Vilela**  
*Guest Editor*



## Article

# Lactic Acid Bacteria Fermentation as a Tool for Creating Texture in Plant-Based “Clean-Label” Cheeses

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## Abstract

The growing demand for clean-label plant-based cheese alternatives underscores the need for products with desirable properties. A key technological challenge is replicating the firmness of traditional cheese without synthetic additives. This study explores lactic acid bacteria (LAB) fermentation as a natural texturizing method for clean-label plant-based cheeses. We investigated the link between LAB metabolic traits and the firmness of three substrates: cashew, soybean, and sunflower seed. A strong correlation ( $r \approx -0.88$ ) was found between final pH and firmness in cashew paste, where strains achieving a pH of ~4.0–4.5 (near the protein isoelectric point) produced the firmest gels (up to 3.35 N). *Lactiplantibacillus plantarum* 729/23 and *Lacticaseibacillus helveticus* NK-1 were most effective. Soybean paste firmness increased moderately (to 1.93 N), while sunflower seed paste showed no significant improvement ( $\leq 1.14$  N) despite active acidification, indicating substrate-specific limitations. This study, supported by a comprehensive analysis of the literature on firmness measurement in plant-based cheeses, demonstrates the potential of targeted selection of natural starter cultures to create clean-label products with minimal ingredients.

**Keywords:** plant-based cheese; lactic acid bacteria (LAB); clean label; fermentation; firmness

## 1. Introduction

A growing body of research highlights the critical scientific and practical interest in plant-based cheese alternatives, driven by their environmental benefits and the rising demand for clean-label, health-oriented products [1–4].

According to the comprehensive study by Poore and Nemecek [5], which encompassed data from 38,700 farms across 119 countries and 40 food products accounting for 90% of global caloric and protein intake, animal-based foods—while providing only 37% of global protein and 18% of calories—are responsible for 83% of all agricultural land use, 58% of



food-related greenhouse gas emissions, and the majority of impacts on eutrophication and acidification. A shift to a vegan diet, according to the authors' estimates, could potentially reduce land use by 76%, lower food-related greenhouse gas emissions by 49% (in CO<sub>2</sub>-equivalents per year), decrease freshwater use by 19%, cut waterway pollution from eutrophication by 56%, and reduce environmental acidification by 50%.

Traditional cheese production significantly contributes to greenhouse gas emissions, land degradation, and excessive water consumption. The production of raw milk alone accounts for 79–95% of the cheese's global warming potential, mainly due to methane emissions, manure management, and feed production [6,7]. Cheese manufacturing further intensifies environmental burdens, requiring up to 5000 L of water per kilogram of product and often lacking efficient wastewater treatment systems [8,9]. Thus, despite its high nutritional value and health benefits, cheese remains one of the most resource-intensive and environmentally taxing animal-derived food products. This underscores the need to develop plant-based alternatives capable of delivering comparable sensory and technological properties while dramatically reducing ecological impact.

While plant-based cheeses offer reduced ecological impact and suit populations with dietary restrictions, their textural properties often fall short of consumer expectations, presenting significant technological challenges for formulation and process design [1,10–12].

A major bottleneck in the development of plant-based cheeses is the ability to mimic the firmness, meltability, and sensory profile of dairy cheese, given the fundamentally different protein and carbohydrate matrices of plant substrates [1,11–16]. Traditional texturizers and stabilizers can improve these properties but undermine the “clean label” appeal for many consumers [1]. Recent research has focused on the potential for lactic acid bacteria (LAB) and other fermentation-active strains to serve as natural structuring agents, leveraging their ability to synthesize exopolysaccharides (EPSs), drive acidification, and modulate substrate rheology, thereby enhancing texture and reducing reliance on additives [3,17].

The global market for plant-based cheese is growing, yet products often rely on additives to compensate for poor texture, conflicting with clean-label demand [18,19]. Fermentation with lactic acid bacteria is a promising, natural alternative. However, industrial application is hindered by the lack of systematic data linking specific microbial traits to textural outcomes across different plant substrates [3,9]. Traditional starter cultures used in cheese production have been selected and refined over long periods of time, whereas the plant-based cheese industry is still in its early stages. Given the distinct compositional differences between plant biomass and dairy milk, there is no guarantee that cultures proven effective in traditional cheesemaking will perform optimally in plant-based matrices. This highlights the need to develop targeted screening methods for identifying strains compatible with each specific type of plant substrate. The novelty of this study lies in its direct, comparative approach: we correlate key metabolic activities of five LAB strains with the firmness they generate in three distinct, commercially relevant substrates (cashew, soy, sunflower). This provides a rational framework for selecting starters based on substrate composition, moving beyond empirical testing for cleaner-label product development.

Several studies have demonstrated that inoculation with selected bacterial combinations can improve both texture and flavor in plant-based cheese matrices, especially when exopolysaccharide-producing LAB are employed [2,13,17]. EPS improves rheological properties, increasing yield and texture similarity to dairy cheese by creating a more continuous protein matrix [2,17,20,21]. Acidification by bacterial metabolism is central for reaching the isoelectric point and facilitating curd formation. This step is critical not only for dairy, but also for protein-rich plant bases [3,22]. A third key parameter is amylolytic activity, relevant particularly in legume and starch-rich systems. Starch hydrolysis increases the amount

of fermentable sugars, which enhances acid production but may lower viscosity if not controlled [23,24]. While the significance of proteolytic activity has been well documented in dairy cheese maturation, its role in plant-based systems remains less clear. Proteolysis can release bioactive peptides and modulate texture but, in the context of plant matrices, excessive proteolysis might lead to softer, less cohesive textures [23,25,26]. Evidence suggests that managing the balance between hydrolysis and structure maintenance is critical for optimal texture in plant-based cheeses [23].

Despite these advances, many current studies are hampered by a diversity of plant substrates (nuts, legumes, oilseeds) and the need for tailored microbial solutions for each matrix [3,10]. There is also a lack of systematic exploration linking microbial metabolic activity to firmness of plant-based cheeses. The goal of this study is to elucidate the relationship between the firmness properties of plant-based cheeses and the key metabolic traits of bacterial strains used as starters, including acidification, viscosity, proteolytic and amylolytic activities. By systematically comparing these parameters using three distinct plant-protein substrates—cashew (nut), soy (legume), and sunflower (oilseed)—this study seeks to identify optimal microbial strategies for structuring clean-label, functional, plant-based cheeses with superior mechanical qualities and minimal additive use.

## 2. Materials and Methods

### 2.1. Bacterial Strains

Five lactic acid bacterial strains were used in this study. Three strains *Lactobacillus helveticus* NK-1, *Lactobacillus helveticus* AC-V and *Lactiplantibacillus plantarum* 729/23 were obtained from the Collection of Microorganisms and Bacteriophages, All-Russian Research Institute of Butter- and Cheesemaking (VNIIMS, Uglich, Russia), and one strain *Lactiplantibacillus plantarum* B-5466 was acquired from the All-Russian Collection of Industrial Microorganisms (VKPM, Moscow, Russia). In addition, one strain *Leuconostoc mesenteroides* H32-02 Ksu was isolated in our laboratory from naturally fermented milk obtained in the Novosibirsk region and identified by 16S rRNA gene sequencing.

All strains were maintained as frozen stocks at  $-80\text{ }^{\circ}\text{C}$  in MRS broth supplemented with 20% (*v/v*) glycerol. Prior to experiments, cultures were subcultured twice in MRS broth at  $30\text{ }^{\circ}\text{C}$  under anaerobic conditions and standardized to approximately  $10^9$  CFU/mL for inoculation of the substrates.

The most probable number (MPN) of microorganisms in plant substrates was determined [27]. The initial sample (paste from cashews, soy or sunflower seeds) was homogenized in sterile LB in a ratio of 1:10, successive tenfold dilutions were prepared ( $10^{-1}$ – $10^{-9}$ ), and 1 mL of each dilution was added to 3 parallel tubes with LB nutrient medium. After incubation at  $30\text{ }^{\circ}\text{C}$  for 3 days, the number of positively grown tubes was recorded for each dilution, forming a code combination, and the most probable number of viable microorganisms in 1 g (mL) of the sample was calculated using the NPVH tables with confidence limits.

### 2.2. Preparation of Fermented Plant Substrates and Mechanical Property Analysis

#### 2.2.1. Preparation of Fermented Plant Substrates for Mechanical Property Analysis and Estimation of Gas Formation Activity

Dried cashew kernels, hulled sunflower seeds, and soybeans were soaked in an excess of water for 12 h. The residual water was then decanted, and the hydrated seeds were blended to a homogeneous paste consistency. Individual portions of the paste were aseptically inoculated with bacterial suspensions. The bacterial suspensions were pre-cultured in MRS broth to an optical density  $\text{OD}_{600} = 1.5$  and added to wet substrate at a concentration of 1% (*w/w*). The mixtures were thoroughly homogenized, and the initial

pH of the substrates was measured. The inoculated paste was subsequently transferred into sterile syringes with 20 mm internal diameter and incubated at 30 °C for 24 h. The pH of the final fermented product was also determined using pH meter (Milwaukee MW102-FOOD PRO+, Milwaukee Instruments, Inc., Rocky Mount, NC, USA). All experiments were carried out in triplicate. As a control, non-sterile mass of the corresponding plant substrate without the addition of lactic acid bacteria culture was used.

Gas formation was evaluated by determining the increase in fermented substrate volume within a syringe. The cultures were classified as gas-forming if volume expansion exceeded 10%.

#### 2.2.2. Analysis of the Mechanical Properties of Fermented Plant Substrates

The mechanical properties of the samples were analyzed using a compression test performed on an RSA-G2 mechanical analyzer (TA Instruments – Waters LLC, New Castle, DE, USA) [13]. Each sample was cut into a cylindrical shape (20 mm in height and 20 mm in diameter) and compressed by 5 mm at a constant speed of 1 mm/s using 25 mm diameter parallel plates. The force required to achieve 5 mm compression was recorded, representing the mechanical parameter used for comparison between different samples. The mean value from three replicate measurements per sample was recorded. Firmness was defined as the peak force recorded during this single compression cycle, corresponding to the first-bite force typically reported in standard TPA protocols.

#### 2.3. Milk Fermentation and Final pH Determination

The acidifying activity of lactic acid bacteria (LAB) strains was assessed in ultra-pasteurized milk with an initial pH of 6.70. Bacterial cultures were inoculated into sterile 50 mL plastic tubes (Accumax Lab Devices Pvt. Ltd., Gandhinagar, India) at a concentration of  $10^9$  CFU/mL, representing a 1% (*v/v*) inoculum. The inoculated samples were incubated statically at 37 °C for 24 h. After fermentation, the final pH of the fermented milk was measured at room temperature ( $23 \pm 2$  °C) using a calibrated digital pH meter with a glass electrode, following the procedure outlined in the Russian State Standard GOST R 53359-2009 [28].

#### 2.4. Determination of Dynamic Viscosity of Fermented Milk

Viscosity was measured on an AND SV-10 vibrational viscometer (A&D Company, Limited, Tokyo, Japan) at  $23 \pm 2$  °C. Samples were homogenized through 3–5 gentle inversions before transferring a minimum of 40.00 cm<sup>3</sup> into the measurement vessel, avoiding air bubbles. With the probe immersed to the specified depth, measurements were taken over 60 s until stabilization.

#### 2.5. Determination of Proteolytic Activity

Proteolytic activity was evaluated by measuring zones of protein hydrolysis using a modified milk agar assay. The MRS agar medium was supplemented with 5% (*w/v*) of skimmed milk powder (fat content < 0.5%) and sterilized by autoclaving at 115 °C for 10 min to prevent protein denaturation. Wells were aseptically created in the solidified agar using the reverse end of sterile pipette tips, and a 100 µL aliquot of the bacterial culture ( $OD_{600} = 0.80 \pm 0.05$ ) was dispensed into each well.

The plates were incubated at 37 °C for 24 h. Proteolytic activity was indicated by the formation of clear zones around the bacterial colonies resulting from casein hydrolysis. The diameter of the zones was measured, with three replicate measurements taken per sample. The hydrolysis zone width was calculated using the following formula:

$$HP = (D - D_0)/2 \quad (1)$$

where  $HP$  is the casein hydrolysis zone width,  $D$  is the total diameter of the clear zone, and  $D_0$  is the well diameter.

The results are expressed as the mean diameter of the proteolytic zones  $\pm$  standard deviation.

## 2.6. Determination of Amylolytic Activity

Amylolytic activity of the bacterial cultures was assessed by measuring the zones of starch hydrolysis [29,30]. An agarized MRS medium supplemented with 2 g/L of soluble starch was prepared and sterilized by autoclaving at 121 °C for 15 min. Wells were aseptically punched into the solidified agar using the reverse end of sterile pipette tips. A 100  $\mu$ L aliquot of the bacterial culture ( $OD_{600} = 0.80 \pm 0.05$ ) was dispensed into each well. The plates were incubated at 37 °C for 24 h under aerobic conditions.

Following incubation, the agar surface was flooded with Lugol's solution (1%  $I_2$  in 2% KI,  $w/v$ ) for 2 min to visualize starch hydrolysis. Amylolytic activity was indicated by the formation of clear zones around the bacterial growth against a dark blue background. The width of the hydrolysis zone was calculated using the following formula:

$$HA = (D - D_0)/2 \quad (2)$$

where  $HA$  is the hydrolysis zone width,  $D$  is the total diameter of the clear zone, and  $D_0$  is the well diameter.

The mean value from three independent measurements was recorded.

## 2.7. Correlation Analysis

To assess the potential relationship between final pH values and gel firmness on different plant-based substrates, a correlation analysis was performed. For the calculations, mean values obtained for each microbial strain were used. The Pearson correlation coefficient ( $r$ ) was calculated according to the standard formula as follows:

$$r = \frac{\sum (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{(\sum (x_i - \bar{x})^2)(\sum (y_i - \bar{y})^2)}} \quad (3)$$

where  $x_i$  represents pH values and  $y_i$  the corresponding firmness values.

All calculations were carried out in Python 3.11 using the NumPy and Pandas libraries. Scatter plots with linear regression trend lines were generated for visualization of the observed relationships (library matplotlib).

Given the limited number of observations (one mean value per strain), the correlation analysis was considered descriptive and was used to illustrate general trends rather than to provide strict statistical inference.

## 2.8. Statistical Analysis

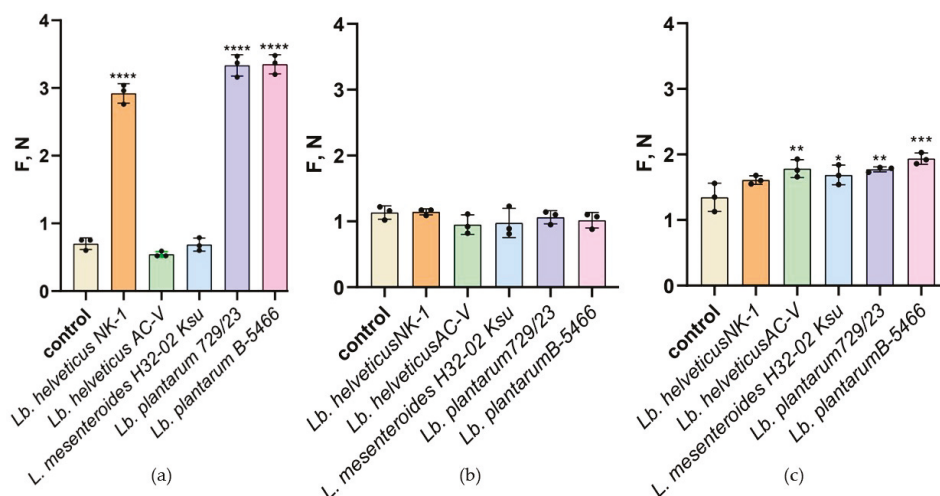
Statistical data analysis was performed using GraphPad Prism version 8.4 (GraphPad Software, Boston, MA, USA). To evaluate the significance of differences between the control group and experimental samples, a one-way analysis of variance (ANOVA) was employed, followed by Dunnett's post hoc multiple comparisons test, which is specifically designed for comparing all groups against a single control. Differences were considered statistically significant at a threshold of  $p < 0.05$ . The results are presented as the mean value  $\pm$  confidence interval (or  $\pm$  standard deviation), and on the graphs, statistical significance is denoted by symbols (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ ).

### 3. Results

To evaluate the potential of three plant-based substrates for the production of cheese-like products through fermentation with different bacterial strains and to assess the influence of strain-specific properties on curd firmness, a series of analyses was performed. The summarized results are presented in Table 1.

#### 3.1. Analysis of the Mechanical Properties and Gas Formation of Fermented Plant Substrates

The evaluation of the rheological properties of fermented cashew paste revealed statistically significant differences in firmness depending on the bacterial strain used (Figure 1). Firmness was assessed by compression force, expressed in Newtons (N). The results demonstrate a clear separation of the tested strains into two groups according to their ability to modify the structural characteristics of the product.



**Figure 1.** Comparative evaluation of the firmness of fermented cashew paste (a), sunflower seed paste (b), and soy paste (c) obtained using different strains of lactic acid bacteria. Data are presented as mean  $\pm$  SD. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , and \*\*\*\*  $p < 0.0001$  by Dunnett's post hoc test for significant one-way ANOVA data.

In fermented cashew paste (a), the highest firmness values were observed in samples inoculated with *Lb. plantarum* B-5466 ( $3.35 \pm 0.141$  N,  $p < 0.0001$ ), *Lb. plantarum* 729/23 ( $3.33 \pm 0.158$  N,  $p < 0.0001$ ), and *Lb. helveticus* NK-1 ( $2.92 \pm 0.144$  N,  $p < 0.0001$ ). These values were significantly greater than those of the control group ( $0.7 \pm 0.086$  N), underscoring the pronounced capacity of these strains to foster a dense protein matrix. In contrast, *Lb. helveticus* AC-V ( $0.54 \pm 0.04$  N,  $p = 0.3916$ ) and *L. mesenteroides* H32-02 Ksu ( $0.68 \pm 0.096$  N,  $p = 0.9998$ ) exhibited no substantial hardening effect, with firmness levels statistically indistinguishable from the control.

These findings affirm that the selection of starter strain constitutes a pivotal factor in dictating the textural attributes of fermented cashew paste. Notably, *Lb. plantarum* B-5466 and *Lb. plantarum* 729/23 emerge as the most promising candidates for the deliberate engineering of products with tailored rheological profiles.

The evaluation of the rheological properties of fermented sunflower seed paste (b) showed that none of the tested strains resulted in a significant increase in firmness compared to the control sample ( $1.13 \pm 0.102$  N).

**Table 1.** The functional profiles of the bacterial strains and characteristics of the resulting fermented plant-based gels. “+” indicates visible gas formation during fermentation; “–” indicates no visible gas formation.

|                                       | E, N<br>(Cashews) | E, N (Sun-<br>flower<br>Seeds) | E, N (Soy)      | pH<br>Cashews | pH<br>Sunflower<br>Seeds | pH Soy      | Gas<br>Formation<br>in Soy | Gas<br>Formation<br>in<br>Sunflower<br>Seeds | Gas For-<br>mation<br>in<br>Cashews | Final<br>pH in<br>Milk | Milk<br>Viscos-<br>ity,<br>mPa·s | Proteolytic<br>Activity,<br>mm | Amylolytic<br>Activity,<br>mm |
|---------------------------------------|-------------------|--------------------------------|-----------------|---------------|--------------------------|-------------|----------------------------|----------------------------------------------|-------------------------------------|------------------------|----------------------------------|--------------------------------|-------------------------------|
| <i>Lb. helveticus</i> NK-1            | 2.92 ±<br>0.144   | 1.14 ±<br>0.046                | 1.61 ±<br>0.065 | 4.02 ± 0.02   | 4.28 ± 0.01              | 4.17 ± 0.03 | +                          | +                                            | –                                   | 3.48                   | 102                              | 2.25 ± 0.25                    | 5.00 ± 0.33                   |
| <i>Lb. helveticus</i> AC-V            | 0.54 ± 0.04       | 0.95 ±<br>0.147                | 1.78 ±<br>0.136 | 5.58 ± 0.02   | 4.34 ± 0.01              | 4.33 ± 0.01 | +                          | +                                            | +                                   | 3.57                   | 37                               | –                              | –                             |
| <i>L. mesenteroides</i><br>H32-02 Ksu | 0.68 +<br>0.096   | 0.97 ±<br>0.223                | 1.68 ± 0.15     | 4.64 ± 0.04   | 4.66 ± 0.03              | 4.41 ± 0.02 | +                          | +                                            | +                                   | 3.86                   | 33.4                             | –                              | 2.00 ± 0.33                   |
| <i>Lb. plantarum</i><br>729 /23       | 3.33 ±<br>0.158   | 1.06 ± 0.1                     | 1.77 ±<br>0.037 | 3.98 ± 0.04   | 4.27 ± 0.03              | 4.05 ± 0.03 | +                          | +                                            | –                                   | 4.61                   | 107                              | 2.25 ± 0.25                    | –                             |
| <i>Lb. plantarum</i><br>B-5466        | 3.35 ±<br>0.141   | 1.01 ±<br>0.119                | 1.93 ± 0.86     | 4.04 ± 0.02   | 4.35 ± 0.01              | 4.07 ± 0.07 | +                          | +                                            | –                                   | 4.82                   | 84                               | 3.50 ± 0                       | 7.0 ± 0.33                    |



In contrast, *Lb. plantarum* B-5466 ( $1.01 \pm 0.119$  N,  $p = 0.7352$ ), *L. mesenteroides* H32-02 Ksu ( $0.97 \pm 0.223$  N,  $p = 0.5015$ ), *Lb. plantarum* 729/23 ( $1.06 \pm 0.1$  N,  $p = 0.9482$ ), and *Lb. helveticus* AC-V ( $0.95 \pm 0.147$  N,  $p = 0.3657$ ) exhibited a tendency toward reduced firmness compared with the control group. The results obtained with *Lb. helveticus* NK-1 ( $1.14 \pm 0.046$  N,  $p = 0.9999$ ) were closest to the control. However, as evident from the data, the variability among replicates was considerable, and confidence intervals overlapped broadly. This indicates the absence of statistically significant differences among most of the tested samples.

Thus, unlike the pronounced effects observed in cashew paste, fermentation with the same strains did not lead to any significant modification of the structural characteristics of sunflower seed paste under the conditions of this experiment.

The evaluation of the rheological properties of fermented soybean paste (c) revealed a statistically significant influence of microbial strains on the firmness characteristics of the product. As shown by the results, tested strains significantly increased the firmness of soybean paste compared to the control ( $1.34 \pm 0.215$  N).

The most pronounced strengthening effect was observed for strains *Lb. helveticus* AC-V ( $1.78 \pm 0.136$  N,  $p = 0.0057$ ), *Lb. plantarum* B-5466 ( $1.93 \pm 0.86$  N,  $p = 0.0005$ ), and *Lb. plantarum* 729/23 ( $1.77 \pm 0.037$  N,  $p = 0.0067$ ). A significant, albeit less marked, increase in firmness was also recorded for *L. mesenteroides* H32-02 Ksu ( $1.68 \pm 0.15$  N,  $p = 0.0287$ ). Conversely, the firmness value for the strain *Lb. helveticus* NK-1 ( $1.61 \pm 0.065$  N,  $p = 0.1015$ ) did not exhibit a statistically significant difference from the control.

Thus, it was established that fermentation with lactic acid bacteria is an effective approach for modifying the structural characteristics of soybean paste.

Visible gas formation was observed in the majority of samples during fermentation, as indicated by partial biomass extrusion from cultivation molds. However, this effect was not universal: gas release was not detected in several cashew-based samples, specifically those fermented with *Lb. helveticus* NK-1, *Lb. plantarum* 729/23, and *Lb. plantarum* B-5466. Notably, these same samples, those in which no visible gas release was observed, exhibited the highest firmness values among all tested combinations ( $2.92 \pm 0.144$  to  $3.35 \pm 0.141$  N), whereas samples that showed gas extrusion demonstrated considerably lower firmness ( $0.54 \pm 0.04$  to  $1.93 \pm 0.86$  N). This indicates that gas formation during fermentation is associated with a looser and less structured matrix.

Although gas formation was assessed using a semi-quantitative criterion (more than 10% biomass displacement from the syringe mold), this approach allowed for consistent classification of samples into two distinct groups: with or without visible gas release. Importantly, samples categorized as “non-gassy” did not exhibit any noticeable extrusion, not even below the threshold, indicating a true absence of visible gas formation.

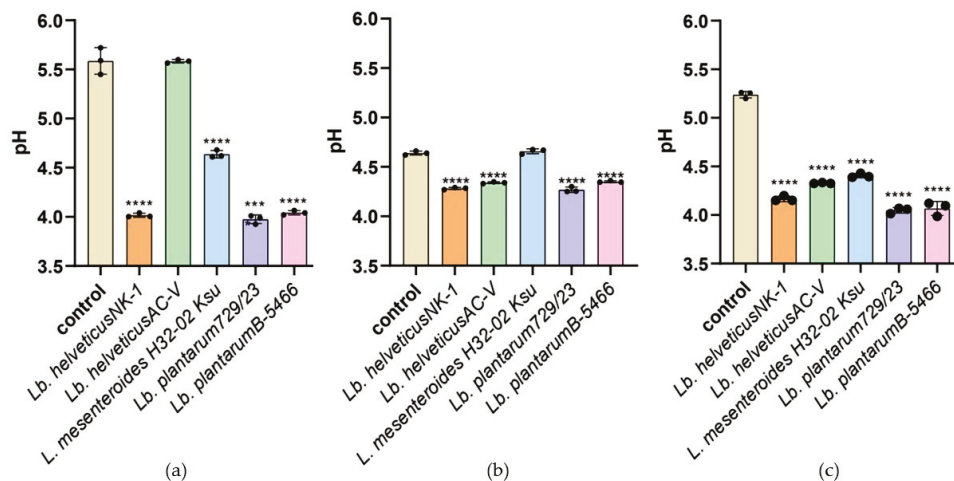
### 3.2. Acidifying Ability of Strains During Fermentation of Plant Substrates

In addition to rheological characteristics, changes in acidity (pH) during fermentation of cashew paste with different microbial strains were investigated (Figure 2).

Analysis of the final pH values during fermentation of cashew paste (a) with various microbial strains revealed substantial acidification of the substrate compared to the control sample ( $\text{pH } 5.59 \pm 0.14$ ).

The obtained data allowed for the investigated strains to be clearly divided into two groups according to their acidifying capacity on this substrate.

The first group, which demonstrated the most pronounced acidification, included *Lb. helveticus* NK-1, *Lb. plantarum* 729/23, and *Lb. plantarum* B-5466. The final pH values in these samples were  $4.02 \pm 0.02$  ( $p < 0.0001$ ),  $3.98 \pm 0.04$  ( $p < 0.0001$ ), and  $4.04 \pm 0.02$  ( $p < 0.0001$ ), respectively.



**Figure 2.** The effect of fermentation by different microbial strains on the final pH value of cashew (a), sunflower (b), and soybean plant pastes (c). Data are presented as the mean  $\pm$  SD. \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$  by Dunnett's post hoc test for significant one-way ANOVA data.

The second group, represented by *Lb. helveticus* AC-V and *L. mesenteroides* H32-02 Ksu, was characterized by much weaker acidification. The final pH for *Lb. helveticus* AC-V was  $5.58 \pm 0.02$  ( $p > 0.9999$ ), which was nearly identical to the initial level, whereas *L. mesenteroides* H32-02 Ksu reached  $4.64 \pm 0.04$  ( $p < 0.0001$ ).

Thus, it was established that *Lb. helveticus* NK-1, *Lb. plantarum* 729/23, and *Lb. plantarum* B-5466 exhibit high fermentative activity toward cashew paste, as reflected in the substantial decrease in pH, while *Lb. helveticus* AC-V and *L. mesenteroides* H32-02 Ksu displayed a low acidification capacity under the tested conditions.

For sunflower seed paste (b), changes in pH during fermentation were investigated in parallel with the assessment of rheological characteristics. Analysis of the data revealed statistically significant acidification of the substrate in experimental samples compared to the control (pH  $4.64 \pm 0.02$ ).

The strongest acidification capacity was observed for *Lb. plantarum* 729/23 and *Lb. helveticus* NK-1, with final pH values of  $4.27 \pm 0.03$  ( $p < 0.0001$ ) and  $4.28 \pm 0.01$  ( $p < 0.0001$ ), respectively. Slightly less pronounced, but still statistically significant, acidification was detected in samples fermented with *Lb. plantarum* B-5466 ( $4.35 \pm 0.01$ ,  $p < 0.0001$ ) and *Lb. helveticus* AC-V ( $4.34 \pm 0.01$ ,  $p < 0.0001$ ). Conversely, the final pH value for the sample fermented with *L. mesenteroides* H32-02 Ksu ( $4.66 \pm 0.03$ ,  $p = 0.6369$ ) showed no statistically significant difference from the control.

In soybean paste, changes in acidity (pH) during fermentation were also investigated (c). The results demonstrated statistically significant acidification in all experimental samples compared to the control (pH  $5.24 \pm 0.03$ ).

Analysis of the final pH values revealed a clear differentiation of the tested strains according to their acidification capacity in the soybean substrate. The most pronounced effect was observed for *Lb. plantarum* 729/23, which reached a pH of  $4.05 \pm 0.03$  ( $p < 0.0001$ ). *Lb. plantarum* B-5466 ( $4.07 \pm 0.07$ ,  $p < 0.0001$ ) and *Lb. helveticus* NK-1 ( $4.17 \pm 0.03$ ,  $p < 0.0001$ ) also demonstrated strong acidification activity. Moderate acidification capacity was recorded for *Lb. helveticus* AC-V and *L. mesenteroides* H32-02 Ksu, with final pH values of  $4.33 \pm 0.01$  ( $p < 0.0001$ ) and  $4.41 \pm 0.02$  ( $p < 0.0001$ ), respectively.

### 3.3. Analysis of Pearson Correlation Coefficients

In the analysis of the relationship between final pH and the strength of fermented samples across different substrates, a general trend was observed: lower pH values were



associated with higher gel strength. This effect was most pronounced in the cashew matrix, with a strong negative correlation ( $r \approx -0.88$ ), while soy and sunflower seed substrates showed weaker associations ( $r \approx -0.43$  and  $-0.55$ , respectively).

It should be noted that the correlation analysis has inherent limitations, as each strain was represented by a single averaged value, and the dataset is therefore discrete and small in size. These results should thus be interpreted cautiously. Nonetheless, the observed trend aligns with established concepts regarding the role of acidity in protein gelation and supports the hypothesis that pH reduction represents a critical determinant of the textural properties of plant-based substrates.

#### 3.4. Total Microbial Load of Raw Plant-Based Substrates

The MPN in the studied plant substrates varied in the range from  $2.5 \times 10^2$  to  $3.1 \times 10^4$  CFU/g depending on substrate. In the cashew paste, the MPN was  $2.5 \times 10^2$  CFU/g, indicating a low initial level of microbial contamination. In the sunflower seed paste, this value was an order of magnitude higher, at  $2.5 \times 10^3$  CFU/g, while the soybean paste had the highest level of contamination ( $3.1 \times 10^4$  CFU/g).

#### 3.5. Milk Fermentation and Final pH

Fermentation of ultra-pasteurized milk with the tested LAB strains for 24 h at 37 °C resulted in a substantial decrease in pH.

The initial pH of milk (6.70) decreased to a range of 3.48–4.82 depending on the strain used, corresponding to pH changes ( $\Delta$ pH) of 1.88 to 3.22 units. The highest acidifying activity was demonstrated by *Lb. helveticus* NK-1 (final pH 3.48;  $\Delta$ pH 3.22) and *Lb. helveticus* AC-V (final pH 3.57;  $\Delta$ pH 3.13). *L. mesenteroides* H32-02 Ksu showed intermediate activity (final pH 3.86;  $\Delta$ pH 2.84), whereas the *Lactiplantibacillus* strains exhibited more moderate acidification: *Lb. plantarum* 729/23 ( $\Delta$ pH 2.09) and *Lb. plantarum* B-5466 ( $\Delta$ pH 1.88).

#### 3.6. Dynamic Viscosity of Fermented Milk

The dynamic viscosity of fermented milk varied widely from 33.4 to 107.0 mPa·s, demonstrating substantial strain-specific differences in structuring capacity.

The highest viscosity was achieved by *Lb. plantarum* 729/23 (107.0 mPa·s), followed by *Lb. helveticus* NK-1 (102.0 mPa·s). Moderate viscosity values were observed for *Lb. plantarum* B-5466 (84.0 mPa·s), whereas *Lb. helveticus* AC-V (37.1 mPa·s) and *Leuconostoc mesenteroides* H32-02 Ksu (33.4 mPa·s) produced samples with the lowest viscosity.

Analysis of the relationship between acidification and viscosity revealed no direct correlation between these parameters. Strains exhibiting strong acidification ( $\Delta$ pH > 2.5) showed varying abilities to induce textural changes, with only *Lb. helveticus* NK-1 combining intensive acidification with high product viscosity.

#### 3.7. Proteolytic and Amylolytic Activities of the Strains

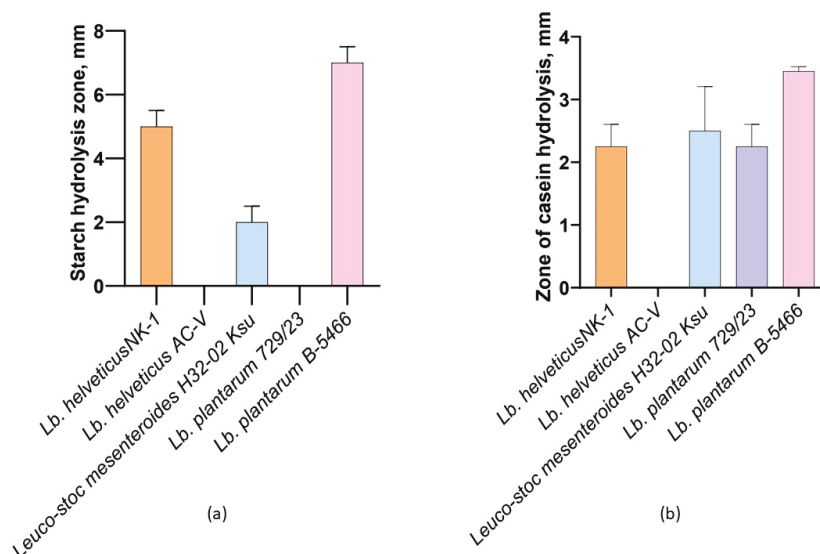
Screening for extracellular enzymatic activities revealed substantial differences in functional profiles among the tested strains (Figure 3). Proteolytic activity was detected in four out of five strains, with clearing zone diameters ranging from 2.25 to 3.50 mm.

The highest proteolytic activity was demonstrated by *Lb. plantarum* B-5466 ( $3.50 \pm 0$  mm), followed by *L. mesenteroides* H32-02 Ksu ( $2.50 \pm 0.50$  mm), *Lb. helveticus* NK-1, and *Lb. plantarum* 729/23 (both  $2.25 \pm 0.25$  mm). *Lb. helveticus* AC-V exhibited no proteolytic activity under the experimental conditions.

Amylolytic activity was detected in three out of the five tested strains.

The highest starch-hydrolyzing capacity was demonstrated by *Lb. plantarum* B-5466, with an average clearing zone of  $7.0 \pm 0.33$  mm, followed by *Lb. helveticus* NK-1 with a hydrolysis zone of  $5.00 \pm 0.33$  mm. Moderate amylolytic activity was observed in

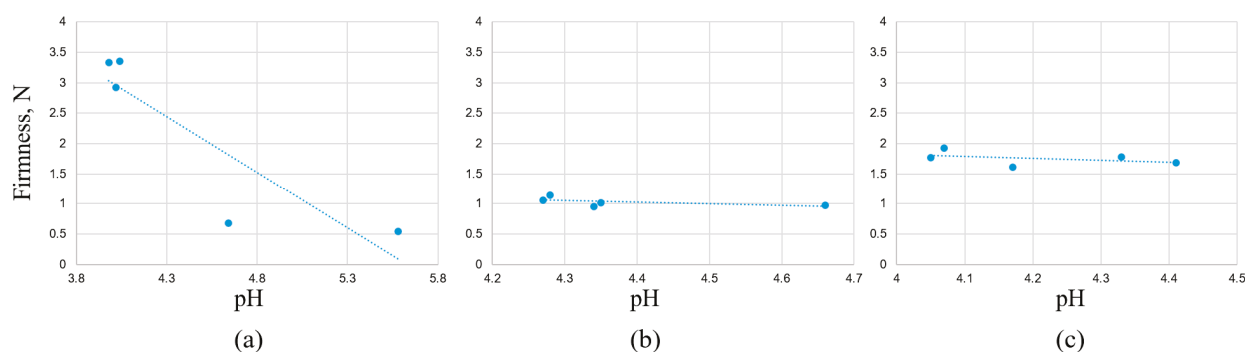
*L. mesenteroides* H32-02 Ksu ( $2.00 \pm 0.33$  mm). *Lb. helveticus* AC-V and *Lb. plantarum* 729/23 showed no detectable amylolytic activity under the tested conditions.



**Figure 3.** Widths of the enlightenment zones when assessing amylolytic (a) and proteolytic activities (b).

#### 4. Discussion

The obtained results demonstrate a clear relationship between the acidifying capacity of the strains and the rheological properties of the fermented cashew paste (Figure 4). The highest firmness values were recorded for strains that provided the most significant pH reduction (*Lb. plantarum* 729/23, *Lb. plantarum* B-5466, and *Lb. helveticus* NK-1). The Pearson correlation coefficient, calculated with the corresponding assumptions, was  $-0.88$ . This suggests that the key mechanism of texture formation in this case is the acid-induced denaturation of cashew proteins, leading to the formation of a dense coagulated network. An exception was the strain *Lb. helveticus* AC-V, which, despite moderate acidification, did not induce gel strengthening. Therefore, the development of cashew-based products with a defined dense texture requires strains possessing high and consistent acidifying activity.



**Figure 4.** The dependence of the firmness of the gel obtained through fermentation of vegetable mass on the final pH: cashew (a), sunflower (b), and soybean plant pastes (c).

Fermentation of the sunflower seed paste revealed a fundamentally different pattern compared to cashew. Despite statistically significant acidification by all tested strains, none resulted in a substantial increase in gel strength compared to the control. This substrate-specific limitation can be attributed to several intrinsic factors of sunflower seeds. Firstly, the inability of sunflower protein to form a strong gel under the conditions of our fermentation protocol can be attributed to its poor intrinsic gelling properties, which are

critically dependent on high temperature denaturation. As comprehensively reviewed by González-Pérez and Vereijken, gel formation by globular proteins like helianthinin, the main sunflower storage protein, is a complex process requiring denaturation, aggregation, and network formation, with denaturation typically achieved by heating [31]. Sunflower protein isolates are known for their weak gelling capacity, forming irregular, foamy structures with low mechanical strength even in concentrated dispersions. A direct comparison highlights this limitation: a 10% (*w/v*) sunflower protein isolate dispersion forms a very weak gel with a storage modulus ( $G'$ ) of only about 500 Pa after heating and cooling, whereas a similar soy glycinin dispersion achieves a  $G'$  above 5000 Pa under identical conditions [31,32]. A key reason is that the denaturation temperature of sunflower protein isolate is approximately 100 °C, meaning that significant denaturation does not occur below this threshold [33]. Notably, some studies report that gelation may not occur even when heating to 110 °C [34]. This explains the observed lack of firmness increase despite active acidification in our experiment. The pH shift alone was insufficient to overcome the innate poor gelling functionality of non-thermally denatured sunflower proteins. Secondly, the major storage proteins in sunflower, helianthinin and 2S albumins, exhibit isoelectric points in a relatively broad and alkaline range ( $pI \sim 5.0\text{--}8.8$ ), which is notably higher than the pH achieved during lactic fermentation (4.27–4.66) [31,35]. Thirdly, sunflower seeds contain significant amounts of phenolic compounds, primarily chlorogenic acid, which can constitute 1–4% of the kernel weight [36]. Polyphenols are known to reduce the gel strength of sunflower seed protein isolates [37]. Therefore, for modifying the texture of sunflower pastes, a strategy based solely on biological acidification is insufficient and necessitates the search for additional technological approaches (e.g., protein isolation, use of texture-modifying enzymes, or blending with other plant proteins) or strains with alternative modes of action.

The results of soybean paste fermentation confirmed the efficacy of biological acidification for modifying its rheological properties, yet revealed a more complex dependency. All strains significantly increased the paste's firmness, with the most pronounced effect demonstrated by *Lb. plantarum* B-5466 and *Lb. helveticus* AC-V. However, a direct correlation with the pH level was not absolute: the strain *Lb. plantarum* 729/23, which showed the greatest pH reduction, did not form the firmest texture. This indicates that in the case of soy protein, alongside acid denaturation, other factors also contribute significantly to texture formation.

Lactic fermentation reduces substrate pH to approach protein isoelectric points ( $pI$ ), facilitating protein precipitation and enhancing substrate firmness. Cashew proteins exhibit  $pI$  values at pH 3.0 and 5.0 [38,39], with optimal precipitation at pH 4.0–4.5 [39]. Soybean proteins demonstrate optimal isoelectric precipitation at pH 4.5, with acid precipitation at this pH resulting in distinct effects on protein composition and functional properties compared to precipitation at pH 3.5 or 5.5 [40]. Sunflower seed proteins display  $pI$  ranges from pH 4.1 to 5.6, varying by protein fraction [41]. Lactic acid bacteria used in our study effectively target these critical pH ranges, with the majority of strains achieving pH values below 4.5 necessary for protein coagulation. This pH reduction enables formation of protein networks that enhance firmness while maintaining functional properties in fermented plant-based products.

Texture profile analysis (TPA) is a standard method used to characterize the mechanical behavior of food gels by simulating the action of chewing. It typically provides multiple parameters, including firmness, cohesiveness, springiness, gumminess, and chewiness, which together describe the texture and mouthfeel of the product. In the present study, we focused exclusively on the firmness parameter, defined as the peak force during the first compression cycle, as a primary indicator of gel strength.

This decision was based on the exploratory nature of the current work, which aimed to identify promising combinations of plant-based substrates and LAB strains capable of forming dense, self-supporting gels suitable for further development as plant-based cheese analogs. At this screening stage, firmness alone was sufficient to differentiate between successful and unsuccessful fermentations. In subsequent stages, once optimal substrate-strain pairs are selected, full TPA profiling will be performed to evaluate additional textural properties relevant to sensory quality and product acceptability.

To account for the potential influence of structural heterogeneity introduced by gas formation, we adapted our firmness assessment protocol. Prior to measurement, the fermented material was gently extruded from the syringe mold to allow for gas release and permit collection of a cohesive portion of the matrix. From this portion, a representative sample was excised for texture analysis. While this approach enabled standardized sampling across replicates, we acknowledge that gas-induced porosity and fragmentation could introduce some variability, and firmness values obtained from such samples should be considered indicative rather than absolute.

It is important to emphasize, however, that gas-forming samples were not considered viable candidates for further development as plant-based cheese products. Gas production in these cases was likely the result of native microbiota activity not sufficiently suppressed by the added LAB strains. Given the importance of structural uniformity and visual quality in cheese analogs, strains that failed to prevent excessive gas formation were categorically excluded from consideration at this stage. Accordingly, although we reported firmness values for such samples, the primary reason for their exclusion was gas production itself. Texture measurements in this context primarily served to reinforce the functional inadequacy of these fermentations, highlighting the lack of gel formation in addition to their structural instability.

Comprehensive sensory evaluation, including the assessment of texture, mouthfeel, and flavor acceptability, will be addressed in the next stage of formulation development, where selected strains will be evaluated under refined processing conditions.

Direct comparison of published data, however, is complicated by considerable methodological heterogeneity. Tests have been conducted at different temperatures, on samples of varying dimensions, and using a wide range of deformation rates and compression levels (20–80% of the original sample height) [12,42,43]. Moreover, even within the same research groups, distinct protocols have been applied in separate publications [13,44]. As a result, reported values are not always directly comparable and must be interpreted with caution.

Nevertheless, ranges can be delineated from the literature. For dairy cheeses, firmness values span from 4–5.8 N in soft varieties such as Camembert and Mozzarella to ~69 N in Emmental and 74.9–126.8 N in Cheddar [12,43]. Plant-based analogs exhibit an even broader spectrum, ranging from 4.4–5.7 N in cashew- and soy-based products (tofu) to ~90 N in formulations incorporating refined coconut oil and starch [43]. Cheddar-type analogs have been reported with values of 56.7–101.9 N [12], high-protein cheeses based on fava bean protein with 16.1–76.4 N [12], and soy-starch formulations with 89.8–116.6 N [45]. Collectively, these data show that plant-based products range from very soft matrices to textures approaching those of semi-hard dairy cheeses.

In contrast, the values obtained in the present study were markedly lower, ranging from 0.54 to 3.35 N. These values are an order of magnitude below those reported for most plant-based analogs. The discrepancy is likely attributable to differences in formulation and processing. Whereas the majority of studies describe multi-component formulations containing starch, hydrocolloids, and vegetable fats prepared without microbial fermentation, our approach employed raw substrates fermented by lactic acid bacteria. The absence

of structuring additives and the reliance on microbial activity alone may account for the lower gel firmness observed.

The work most comparable in methodology is that of Masiá et al. (2023) [13], in which pea protein isolate gels fermented with different bacterial blends exhibited firmness values of 3.5–7 N. In our study, the maximum firmness, achieved with cashew fermentation, was 3.33–3.35 N. Differences can be attributed to the composition of the substrate (pea protein isolate supplemented with sunflower oil, glucose, and sucrose in Masiá's study), the bacterial consortia employed, and longer ripening of the analogs.

It is well established that the firmness of various cheeses, both dairy-based and plant-based, increases during the maturation process [46–48].

For instance, studies by Łopusiewicz et al. demonstrated that the firmness of a plant-based Camembert alternative made from lupin seeds increased from ~2.02 N to ~15.8 N after 7 days of ripening [49], while a similar product made from flaxseed increased from ~2.1 N to ~3.66 or ~5.37 N, depending on the fungal component used [50].

Accordingly, the firmness values we obtained for cashew-based samples were comparable to the initial values observed in lupin- or flaxseed-based Camembert alternatives without additional structuring ingredients, suggesting potential for further improvement during the ripening stage of the final product.

In our study, we focused exclusively on the initial 24 h incubation phase, as the goal of this study was to elucidate the relationship between the firmness properties of plant-based cheeses and the key metabolic traits of bacterial strains used as starters, including acidification, viscosity, and proteolytic and amylolytic activities.

Closer examination of individual substrates highlights the decisive influence of raw material composition and processing parameters on gel firmness.

For cashew, our results (3.33–3.35 N) were lower than those reported by Campos et al. (2024), who observed 6.0–9.7 N in cashew-based analogs compressed to 20% of sample height [42]. The higher values may be explained by the inclusion of red seaweed, differences in microbial cultures, and alternative fermentation conditions (temperature and duration), all of which can promote a denser structure. Our cashew-based gels, in contrast, represent a softer variant, which is consistent with a formulation lacking external stabilizers.

A similar trend was observed for soy. In the classic study of Yang & Taranto (1982) [51], mozzarella-type analogs prepared from soy protein isolate with gelatin and other additives exhibited firmness values of 6.8–12.8 N, whereas our soy-based gels yielded only 1.61–1.93 N. The absence of texturizing agents and the higher moisture content of our samples likely account for this difference. The negative correlation between water content and firmness is well established for both dairy and plant-based cheeses [42,52] and the higher hydration of the soy substrate in our experiments is expected to have markedly reduced firmness.

For sunflower seeds, virtually no data on cheese-like products are available. However, it is well known that polyunsaturated lipid content negatively correlates with the firmness of both cheese analogs and other food matrices [42]. This relationship has also been demonstrated in dough systems, where sunflower oil produced the softest textures [53]. Our results for fermented sunflower seeds (0.95–1.14 N) are consistent with these expectations, supporting the interpretation that the low firmness of sunflower-based products is determined by their lipid composition.

Overall, the comparative analysis suggests that the low firmness of our samples results from both the absence of starch-based stabilizers and the intrinsic properties of the raw substrates: cashew and soy are characterized by high water content and the presence of unsaturated fats, whereas sunflower additionally contains elevated levels of polyunsaturated lipids, which hinder the formation of a dense protein matrix.



Cashew showed particularly encouraging performance in our experiments. Even without any additional structuring agents, and immediately after fermentation, the simple combination of cashew paste and bacterial cultures reached firmness values of ~3.3 N. This demonstrates that a clean-label substrate can already develop measurable firmness within a short time frame. Considering that previous studies reported values of 6–10 N only after supplementing the matrix with hydrocolloids or subjecting it to extended ripening, our results indicate substantial potential for further improvement. With appropriate strain selection and/or a short maturation period, cashew-based products potentially could achieve textural properties comparable to soft dairy cheeses while retaining a minimal-ingredient formulation.

In traditional cheese and dairy production, mild pasteurization is commonly applied to ensure the suitability of milk for coagulation and to reduce the microbial load of the raw material. Studies on the production of plant-based dairy alternatives also often employ pasteurization or autoclaving, although this is mostly the case for products made from protein isolates and involving emulsification steps [54–57]. Dense plant-based pastes derived from nuts, seeds, or legumes—as opposed to liquid plant “milks” or emulsified protein systems—are difficult to pasteurize or sterilize evenly under laboratory conditions without substantially altering their structure and nutritional properties. Moreover, methods ensuring complete sterility (e.g., autoclaving) are generally unsuitable for industrial applications due to high energy consumption and potential deterioration of product quality. This is particularly relevant in the context of “clean-label” product development, where minimal processing and the absence of artificial additives are prioritized.

In studies using homemade nut pastes as the starting material, hot water blanching is commonly applied after soaking [42,58]. According to the Fermented Foods Guideline (Plant-based Cheese) [59], blanching nuts in boiling water ( $\geq 90$  °C for 2 min) is considered sufficient to achieve a 5-log reduction in *Salmonella* prior to soaking, blending, and fermentation.

It is well established that plant raw materials are often contaminated with spore-forming bacteria such as *Bacillus* spp. and *Clostridium* spp., which are resistant to mild thermal treatments [60] and may germinate during storage, leading to uncontrolled fermentation and product spoilage.

Lactic acid bacteria are widely used as biopreservation agents that inhibit the growth of undesirable microbiota. The addition of LAB strains such as *Latilactobacillus sakei*, *Lacti-caseibacillus casei*, *Lactiplantibacillus plantarum*, and *Leuconostoc mesenteroides*—all of which possess QPS (Qualified Presumption of Safety) status [61]—has been shown to inhibit pathogens including *Listeria monocytogenes*, *Salmonella* sp., and *E. coli* O157:H7, as well as spoilage organisms such as *Pseudomonas* sp. and *Enterobacteriaceae* [62]. Incorporating LAB into plant-based products enables the suppression of undesirable microbiota through substrate competition and production of antimicrobial metabolites. Recent studies have demonstrated the effectiveness of LAB in extending the shelf life and enhancing the microbiological safety of meat analogs [63–66].

In strictly controlled laboratory experiments, substrate sterilization is often standard practice for isolating the effects of the inoculated strains. However, the aim of our study was to simulate conditions that closely resemble real-world industrial fermentation of plant substrates, where complete sterilization is economically and technologically impractical. This approach allows for an evaluation of strain performance in a competitive environment that includes natural microbiota, which is more representative of actual production settings. To account for the possible influence of native microbiota on the fermentation process, we included parallel control samples without LAB inoculation, incubated under identical conditions (i.e., the same temperature and time). This setup was designed to assess

whether inoculation performs better than spontaneous fermentation in terms of acidity and texture development.

The results of acidity and texture measurements in the control samples were analyzed and compared to those of the inoculated variants (Figure 4). We observed that in the absence of the added strains, acidification and gel formation occurred to a much lesser extent or were not observed at all. Specifically, the strength of control samples was  $0.7 \pm 0.086$  N (cashew, pH =  $5.59 \pm 0.14$ ),  $1.13 \pm 0.102$  N (sunflower, pH =  $4.64 \pm 0.02$ ), and  $1.34 \pm 0.215$  N (soy, pH =  $5.24 \pm 0.03$ ). The limited texturization and weak or absent acidification in the control samples confirm that the observed gel structure and pH reduction in the tested substrates were primarily due to the metabolic activity of the inoculated LAB, not the native microbiota. These findings support the dominant role of the added cultures in shaping the final properties of the product. Therefore, the use of non-sterile but controlled substrates combined with parallel controls allowed us to reliably assess the contribution of inoculated LAB strains while maintaining the relevance of the experiments for potential industrial application.

We also evaluated the total microbial load of the initial plant pastes. The total microbial number of LAB inoculated into the substrates was  $\sim 1 \times 10^7$  CFU/g, which exceeded the native microbiota levels by 3–5 orders of magnitude. Of course, when developing the final product—a cashew-based cheese analog—it will be essential to monitor the presence of both pathogenic and spoilage spore-forming microorganisms in the raw material and final product, and to select an appropriate treatment method for the nut base. We propose that mild thermal processing combined with LAB fermentation could eliminate the need for additional preservatives to control undesirable microbiota, which is especially important for clean-label formulations.

One of the goals of this study was to assess whether conventional technological characteristics of lactic acid bacteria, routinely used in dairy fermentation, could be repurposed to predict their functional performance in plant-based substrates. Such an approach is highly relevant for the development of clean-label plant-based cheese alternatives, where rapid and cost-effective screening of large LAB strain libraries is often necessary.

To this end, we evaluated the strains using two complementary strategies: (1) fermentation performance in milk, a well-established model matrix for assessing acidification and viscosity development, and (2) enzymatic activity on agar-based model systems (proteolysis and amylolysis), commonly used to characterize LAB functional potential in standardized conditions.

Fermentation of milk allowed us to measure final pH and dynamic viscosity—two parameters directly associated with acidifying ability and texture-forming potential in dairy contexts. Our results showed that the final pH in milk did not strongly correlate with acidification or structural outcomes in cashew-based matrices. For instance, *Lb. plantarum* 729/23 and B-5466 displayed relatively mild acidification in milk but achieved the lowest pH values and highest firmness in cashew paste. Conversely, *Lb. helveticus* NK-1, which showed the most pronounced acidification in milk (pH 3.48), also performed well in cashew, suggesting that pH in milk alone may not be a reliable predictor of performance across diverse plant substrates.

In contrast, milk viscosity demonstrated a clearer relationship with cashew matrix structuring. The two strains with the highest viscosity in fermented milk—*Lb. plantarum* 729/23 and *Lb. helveticus* NK-1—were also the ones that produced the firmest cashew gels (over 3 N). Strains with low milk viscosities, such as *L. mesenteroides* H32-02 Ksu and *Lb. helveticus* AC-V, failed to generate significant gelation in cashew. This suggests that milk viscosity may serve as a practical screening criterion for selecting strains with texture-forming potential in nut-based substrates.

To further explore functional differentiation, we also assessed the enzymatic activity profiles of the strains using agar-based model systems. While we acknowledge the limitations of these assays—their inability to replicate the complex macromolecular environment of real food matrices—the goal was not to draw direct mechanistic conclusions, but to test whether enzymatic capacity assessed under standardized conditions could reflect substrate-specific performance.

Strikingly, all three strains that demonstrated proteolytic activity on milk agar (*Lb. plantarum* 729/23, *Lb. plantarum* B-5466, and *Lb. helveticus* NK-1) were also the only ones to successfully structure the cashew matrix into firm gels. This consistency suggests that proteolytic potential, even when measured in simplified conditions, may be a relevant marker for predicting structuring behavior in nut-based substrates. No such relationship was observed for amylolytic activity, indicating that starch degradation capacity did not influence textural outcomes in the tested matrices.

Together, these findings suggest that traditional dairy-based screening parameters—particularly milk viscosity and proteolytic activity—may offer preliminary predictive value when selecting LAB strains for plant-based cheese applications. While the correlations were identified within a limited strain panel, they offer a foundation for future high-throughput studies. Such studies could validate the utility of these markers and streamline the selection of promising strains for fermentation trials in complex plant matrices.

Ultimately, integrating predictive screening tools with targeted plant–substrate evaluations may help reduce the reliance on full factorial fermentation trials and accelerate the development of high-quality, clean-label plant-based dairy alternatives.

## 5. Conclusions

In the evaluation of the mechanical properties of fermented plant substrates, significant strain-dependent modulation of firmness was observed. Cashew paste fermented with *Lactiplantibacillus plantarum* 729/23 and *Lacticaseibacillus helveticus* B-5466 and NK-1 strains exhibited markedly higher firmness (2.92–3.35 N) than the control ( $0.70 \pm 0.09$  N), indicating robust protein network formation, whereas *Lacticaseibacillus helveticus* AC-V ( $0.54 \pm 0.04$  N) and *Leuconostoc mesenteroides* H32-02 Ksu ( $0.68 \pm 0.10$  N) showed no strengthening effect. Sunflower seed paste displayed no significant firmness enhancement across all strains, with values comparable to control ( $1.13 \pm 0.10$  N) and with wide confidence intervals. Conversely, soybean paste firmness moderately increased for all strains (1.77–1.93 N) versus control ( $1.34 \pm 0.22$  N). These results underscore the critical influence of strain selection on the texturizing capacity of fermented plant substrates.

This study demonstrates that the formation of a firm plant-based curd can be achieved using only cashew paste and starter cultures, without the need for stabilizers or structuring additives. Within just 24 h of fermentation, firmness values comparable to soft cheese analogs were obtained, highlighting the potential of this approach for the development of clean-label products. These findings open opportunities for further optimization: the selection of strains with high texturing capacity, the incorporation of additional natural ingredients, and variations in processing and ripening conditions could further enhance firmness and improve sensory characteristics. The results also indicate that a similar strategy may be applicable to soybean substrates, provided that moisture content is carefully controlled.

Sunflower seeds did not form a dense curd; microbial fermentation nevertheless occurred, as evidenced by pH reduction by all tested strains. This observation suggests that combining different plant substrates may help balance the nutritional composition and confer textural properties closer to those of traditional dairy cheeses.



It is important to emphasize that the approach presented here relies specifically on microbial fermentation. In contrast to most current plant-based cheese analogs, which are typically blends of starch, fats, and hydrocolloids, the product obtained in this study is formed through the natural activity of microorganisms. This makes it closer to the classical principles of cheesemaking and opens new avenues for the development of fermented plant-based products.

Future work should focus on (1) optimizing sterilization conditions, fermentation parameters (time, temperature, inoculum size) and post-fermentation ripening for each successful substrate–strain pair; and (2) investigating blends of plant substrates (e.g., cashew with soy) to improve nutritional balance and texture.

Key challenges for industrial implementation include (1) Scalability and cost-effectiveness: transitioning from laboratory-scale fermentation in syringes to large vats while maintaining texture and preventing phase separation; (2) Process control: developing reliable biomarkers (e.g., specific pH endpoints, metabolite profiles, gas formation) for process monitoring to guarantee product consistency; (3) Regulatory and consumer perception: navigating food safety regulations for novel microbial starters and clearly communicating the benefits of “fermented” versus “formulated” plant-based cheese to consumers.

Addressing these challenges through further research and industry collaboration is essential to harness the full potential of LAB fermentation for the next generation of sustainable, clean-label, and sensorily appealing plant-based cheeses.

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## Abbreviations

The following abbreviations are used in this manuscript:

|     |                          |
|-----|--------------------------|
| LAB | Lactic acid bacteria     |
| EPS | Exopolysaccharides       |
| TPA | Texture profile analysis |

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## Article

# Use of *Pichia manshurica* as a Starter Culture for Spontaneous Cocoa Fermentation in Southern Bahia, Brazil

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## Abstract

To improve cocoa fermentation and the quality of its final products, microbial cultures with potential as starters were investigated. Yeasts were considered a promising option due to their adaptability to biotechnological processes and ease of laboratory manipulation. From 185 strains previously isolated from spontaneous cocoa fermentation, those producing protease, amylase, and cellulase were identified. Strain CII87b (*Pichia manshurica*) exhibited the most favorable results and was evaluated for cytotoxicity using the MTT assay, showing no adverse effects. This culture was subsequently inoculated into freshly harvested cocoa almonds during the secondary (winter) harvest. The inoculum accelerated and increased the average fermentation temperature from 25 to 50 °C, reduced internal mold incidence, decreased defect rates, increased total fermentation, and resulted in a more desirable pH compared to the control. These findings demonstrate that the use of *P. manshurica* CII87b as a starter culture in winter harvests can improve fermentation efficiency and product quality, offering a biotechnological tool with potential benefits for cocoa producers and the chocolate industry.

**Keywords:** biotechnology; *Theobroma cacao* L.; yeast

## 1. Introduction

Yeasts play a fundamental role as starter organisms in spontaneous cocoa fermentation. They are primarily responsible for the degradation of citric acid in the pulp, increasing the pH from approximately 3.5 to 4.2 and creating favorable conditions for the growth



of bacterial communities. Under low-oxygen and high-sugar conditions, yeasts produce ethanol, organic acids (such as oxalic, phosphoric, succinic, malic, and acetic acids), and volatile organic compounds that serve as precursors of chocolate flavor compounds [1]. Additionally, some yeast species contribute to the degradation of pectin in the pulp, improving seed aeration and promoting the growth of acetic acid bacteria [1]. Variations in the concentration of reducing sugars and shifts in the metabolic profile of the microbial community during fermentation have also been reported [2].

*Pichia manshurica* is a yeast commonly found in fermented products, including Daqu [3], wines [4], animal feed, silage, Ishizuchi-kurocha tea, and decomposing plant matter. Its genome is closely linked to species such as *P. membranifaciens* and *P. kudriavzevii* [5]. *P. manshurica* has been used in cocoa fermentation processes across several cocoa-producing regions worldwide, including Cuba, Ghana, Ivory Coast, Colombia, Malaysia, and the Brazilian Amazon [6–10]. More recently, this species has been successfully used as an inoculum in cocoa fermentations conducted in the Brazilian Amazon [11].

Cocoa fermentation can be performed using different methods, including stacked methods using wooden boxes (square or circular), boxes composed of alternative materials (Styrofoam or plastic), and even in trays made from different materials. In southern Bahia, the traditional method involves the use of square wooden boxes covered with banana leaves [12].

Since the first reports on the use of microbial inoculants in cocoa fermentation, significant efforts have been made to develop starter cultures aimed at improving process control and final product quality. Several studies have demonstrated the effectiveness of this strategy [13–16]. Criteria commonly used for the selection of potential starter strains include their prevalence in spontaneous fermentations [17,18], their ability to accelerate the fermentation process [19], microbial diversity, and enzymatic production capacity [20,21]. Additional considerations include the microorganisms' resistance to high temperatures and elevated ethanol and acid concentrations, as well as their ability to degrade the pectin layer surrounding pulp sugars [22].

Inoculating microorganisms in cocoa fermentation can lead to several consequences, including possible acceleration of fermentation [19,23], increased levels of volatile compounds [23,24], changes in the levels of phenolic compounds [19,24], control of the growth of filamentous fungi [18,25], improved quality of fermented almonds [24], products with high antioxidant potential [20], and improved flavor precursors in the fermented product [21]. These outcomes aim to improve the sensory characteristics of chocolate and/or achieve greater control of the fermentation process. Thus, the present study aimed to evaluate the biotechnological potential of inoculating native starter yeasts in the cocoa fermentation process in southern Bahia during the secondary harvest.

## 2. Materials and Methods

### 2.1. Yeast Reactivation

The yeasts used in this study were obtained from the Cacao Fermentation Yeast Bank of the Microbial Biotechnology Laboratory at the State University of Santa Cruz. Dextrose Sabouraud broth with chloramphenicol (0.05 g/L) was used to reactivate the yeasts. The flasks (Erlenmeyer 125 mL) were incubated at 28 °C with constant agitation at 200 rpm for up to 120 h. Yeasts that showed growth were cultivated on Petri dishes containing Sabouraud agar with chloramphenicol at the same concentration and incubated at 28 °C for the same period to observe the morphological characteristics of the colonies. The yeasts were then subcultured again to ensure the purity of the culture.

## 2.2. Enzyme Production Evaluation

A previous study by the research group evaluated the enzymatic activities (protease, lipase, and amylase) of these yeasts [26]. Cellulase was evaluated in yeasts that tested positive for protease and amylase in the previous study [26]. The evaluation was performed in YNB Difco™ medium containing sucrose (0.5%) as a carbon source and carboxymethylcellulose (0.5%) as a cellulose source and incubated in an oven at 28 °C for 5, 10, and 15 days. Plates were washed with 1M NaCl and developed with 0.1% Congo red. Positive halos were then measured.

Invertase was quantitatively evaluated using the DNS method. In this method, 1 unit of enzymatic activity was defined as the amount of enzyme capable of releasing 1 µmol of reducing sugars per minute using glucose as a standard [27]. The positive control used was a commercial *Saccharomyces cerevisiae*.

## 2.3. Assessment of Resistance to Stress Conditions

The yeasts that showed the best enzymatic results were submitted to a qualitative evaluation of growth on Sabouraud agar under different conditions of pH (2.5, 3.5, and 4.5), glucose concentrations (5%, 15%, and 30%), and ethanol concentrations (8%, 10%, and 12%), simulating possible environmental conditions present in the fermentation box. The presence of growth was evaluated qualitatively at 24 and 48 h.

## 2.4. Evaluation of the Cytotoxicity of *Pichia manshurica*

To evaluate whether the yeast chosen for inoculum was cytotoxic to a HmVII cell line (BCRJ 0316) and to assess the maintenance of their viability, the MTT cell proliferation test was performed. This test measures the reduction of MTT to formazan crystals. The yeasts were first cultivated in Sabouraud broth for 48 h at 28 °C. After this period, 10 mL of the broth was transferred to a 15 mL sterile tube and centrifuged for 15 min at 5000 × g. The supernatant was discarded, and the yeast extract was resuspended with a 0.9% saline solution. The suspension was centrifuged again for 15 min at 5000 × g to remove any possible residues from the culture medium. The contents were then resuspended once more with 0.9% saline, and the optical density was adjusted at 600 nm.

Next, the yeast extract was inoculated in RPMI medium supplemented with 5% fetal bovine serum and placed in contact with human cells in 96-well plates. The plates were then incubated in an incubator at 37 °C with 5% CO<sub>2</sub> for 24 h to allow for confluence and cell adhesion at the bottom of the plate. After this period, 50 µL of MTT reagent at 5 mg/mL diluted in RPMI was added to the medium, and the plate was again incubated in the incubator at 37 °C for 4 h and protected from light. MTT crystals that formed were dissolved with the addition of DMSO, and the plate was incubated again at 37 °C for another 15 min before being read in a spectrophotometer at 540 nm. The percentage of cell viability was calculated using the following equation: % viability = [(O.D. tested yeast – O.D. blank)/(O.D. negative control – O.D. blank)] × 100.

## 2.5. Yeast Inoculum

### 2.5.1. Preparation of the Inoculum

The cultivation process was performed as described by Santos et al., 2020 [21] with modifications. Yeasts were cultivated in 250 Erlenmeyer flasks with YEPD medium (peptone 2%; yeast extract 1%; dextrose 2%; chloramphenicol 0.05 g/L) for 48 h at 200 rpm at room temperature. This culture was then used as a pre-inoculum for 3 L of YEPD medium under the same conditions. The mixture was subjected to centrifugation (3000 × g at 28 °C for 10 min), and the pellet was resuspended in 2 L of 1% peptone water. The inoculum



reached to  $10^8$  cells  $\text{mL}^{-1}$  (OD approximately 0.5 at 600 nm), and 1 L of this suspension mixture was inoculated in each experimental fermentation box.

#### 2.5.2. Fermentation Box Inoculation

The experiment was conducted at Fazenda Luz do Vale ( $14^{\circ}42'51.17''$  S— $39^{\circ}16'28.76''$  W), Agrícola Conduru, which belongs to the municipality of Ilhéus, BA, in July 2019 (secondary harvest). The study began with selective harvesting of only fully ripe, undamaged cocoa pods free from disease. A standardized fermentation blend (18°Brix) was created using Parazinho (Brazilian Forastero amelonado) and PS1319 (hybrid) beans, which were then allocated to the fermentation boxes.

Three square fermentation boxes measuring  $50 \times 50 \times 50$  cm and containing 80 kg of wet cocoa mass from a varietal mix were used for each box, including one control and two experimental replicates. Yeast was added at time 0 h, and fermentation was performed according to the protocol described by Ferreira, 2017 [12]. Temperature measurements were taken at three different points of the boxes (lower, central, and upper part) three times a day using a long-stem thermometer Gulton® model GULterm 180 (Gulton, São Paulo, Brazil). Measurements of soluble solids were also taken in the first 36 h using an Instrutherm® portable refractometer model RT-30ATC (Instrutherm, São Paulo, Brazil). In addition, 300 g of samples was collected every 12 h throughout the fermentation for further analysis. At the end of the process, the almonds were dried in a transparent oven with a UV filter given the rains that occurred during the period. A total of 6 kg of dried almonds was collected for further analysis.

#### 2.5.3. pH Analysis

The pH of the pulp and cotyledon were evaluated every 12 h during the fermentation using the potentiometry technique with a Tecnal® model TEC-51 pH meter (Tecnal, Piracicaba, Brazil). The pH of the pulp was measured directly. The methodology described by the Association of Official Analytical Chemists [28] was used to obtain the pH of the cotyledon. For this method, 10 g of the almonds, manually separated from the testa and pulp was crushed with 90 mL of water, and the pH of the resulting solution was measured.

#### 2.5.4. Assessment of Almond Cut Quality

The evaluation of the cut quality of the almonds and the pH of the dried beans were performed using the ISO 2451/2014 methods (ISO, 2014) [29] at the Cocoa Innovation Center (CIC) in Ilhéus, Bahia, Brazil.

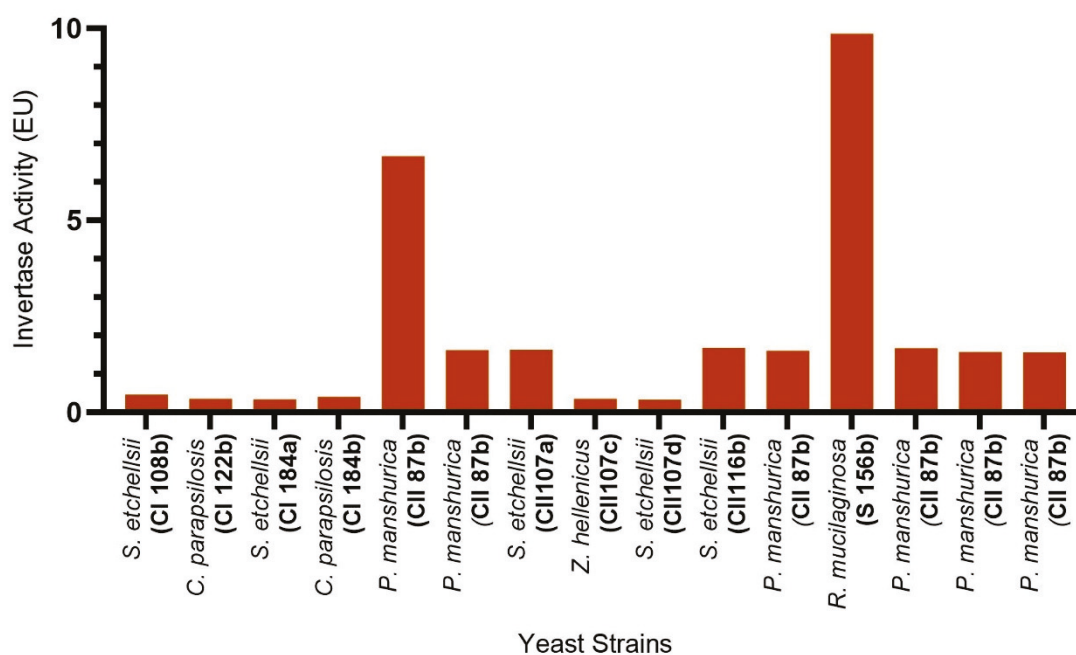
#### 2.5.5. Assessment of Microbiological Quality of Almonds

The evaluation of the microbiological quality of the beans was performed after the cocoa was dried by counting coliforms at  $45^{\circ}\text{C}$  and searching for *Salmonella* sp., as recommended for this type of food by Resolution RDC n°12/2001 of the National Health Surveillance Agency (ANVISA) [30]. Both protocols were performed as recommended by the American Public Health Association.

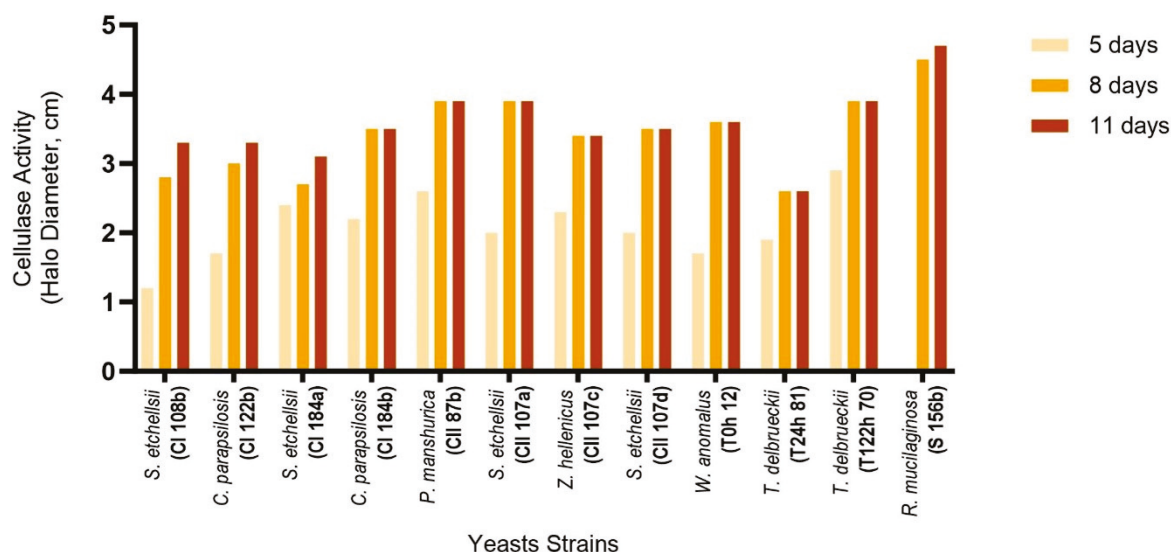
### 3. Results and Discussion

#### 3.1. Enzyme Production

To identify yeast strains with the highest biotechnological potential for cocoa fermentation, isolates that previously tested positive for protease activity [26] were subjected to quantitative assays for invertase production. Additionally, positive strains for both protease and amylase were assessed qualitatively for cellulase production, as shown in Figures 1 and 2.



**Figure 1.** Quantitative evaluation of invertase production by yeast strains that tested positive for protease. Invertase activity is expressed in enzyme units (EUs), defined as the amount of enzyme capable of releasing 1  $\mu$ mol of reducing sugars per minute using glucose as a standard.



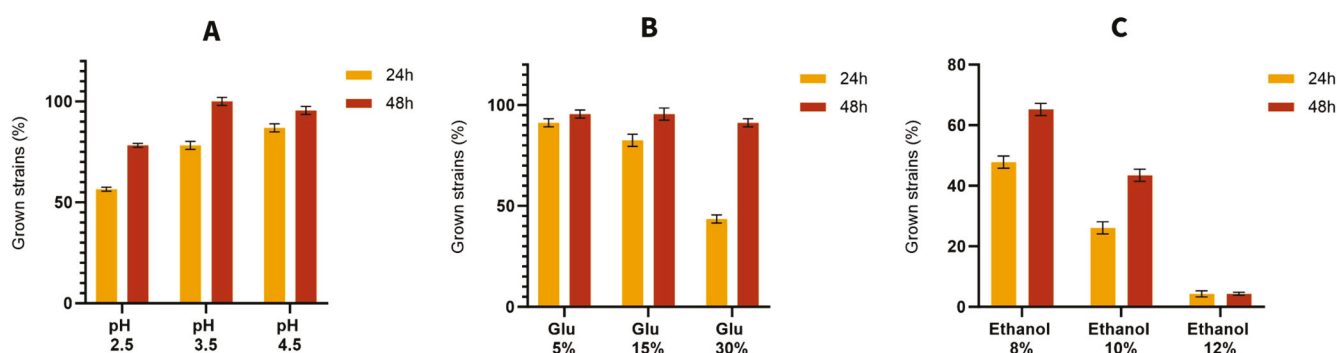
**Figure 2.** Qualitative evaluation of cellulase production by yeast strains that tested positive for protease and amylase. The evaluation was based on the halo diameter (cm) after 5, 8, and 11 days of incubation. The graph includes only strains that tested positive for cellulase.

Among all candidate inoculants, strains CII87b and S156B exhibited the highest levels of invertase production. The most prominent cellulolytic halos (greater than 3.5 cm in diameter after 8 days of incubation) were observed with strains CII87b, CII107a, T0h 12, T122h 70, and S156B. High invertase activity was expected, given the presence of microorganisms capable of producing this enzyme in the fermentation environment, which is rich in sucrose, its preferred substrate. Moreover, the production of invertase by fungi has been previously reported [31]. Cellulase production is particularly important in this context because it enables the hydrolysis of cellulose- and hemicellulose-rich biomass into

fermentable sugars [8], thereby enhancing ethanol production during the initial and critical stages of cocoa fermentation (within the first 48 h).

### 3.2. Resistance to Stressful Conditions

After identifying strains with desirable enzymatic profiles, we evaluated their resistance to conditions that mimic those encountered during the cocoa fermentation process. Yeasts previously identified as positive for protease and amylase activity [26] were tested for growth under varying pH levels (2.5, 3.5, and 4.5), glucose concentrations (5%, 15%, and 30%), and ethanol concentrations (8%, 10%, and 12%) at both 24 h and 48 h intervals, as illustrated in Figure 3.



**Figure 3.** Stress assays. The graphs show the percentage of yeast strains that showed growth on Sabouraud agar medium under conditions of stress. (A) The percentage of strains that grew at different pH values (2.5, 3.5, and 4.5) at 24 and 48 h. (B) The percentage of strains that grew under different glucose concentrations (5%, 15%, and 30%) at 24 and 48 h. (C) The percentage of strains that grew under different alcoholic concentrations (8%, 10%, and 12%) at 24 and 48 h. The presence of growth was evaluated to simulate environmental conditions within the fermentation box. Error bars indicate the standard deviation of the mean of the experimental replicates.

Regarding pH, over 78% of strains showed growth under all tested conditions (Figure 3A). However, at the most acidic pH (2.5), this level of growth was only achieved after 48 h. This delay is not necessarily problematic, as studies on cocoa fermentation in southern Bahia [32] have shown that the pH of cocoa pulp typically ranges from 4.0 to 5.0, a range similar to that observed in the present study (3.6 to 4.6).

Regarding glucose concentrations, more than 82% of the yeasts demonstrated growth under all tested conditions. At the highest glucose concentration (30%), this was only achieved after 48 h, likely associated with the Crabtree effect (Figure 3B) [33]. Such a delay may be attributed to metabolic adjustments that occur in response to stress caused by increased osmotic pressure from high sugar concentrations [34].

In contrast, ethanol concentrations had a more pronounced inhibitory effect (Figure 3C). In 8% ethanol, 65.2% growth was noted after 48 h. This value dropped to 43.5% in 10% ethanol and further to below 4.3% in 12% ethanol, with minimal variation between 24 and 48 h. The extent of stress-induced damage depends on various factors, including the yeast cell cycle stage at the time of exposure [35]. Alcoholic fermentation is itself a stressful process, involving elevated osmotic pressure and increasing ethanol concentrations [36]. Ethanol can alter membrane polarity and, at higher concentrations, reduce both the growth rate and cell viability [37]. This decline in growth at ethanol concentrations above 10% has been reported in other fermentation processes, such as those involving sugarcane juice and cocoa of various origins [38,39]. However, ethanol concentrations above 10% are rarely observed during cocoa fermentation [38,40].

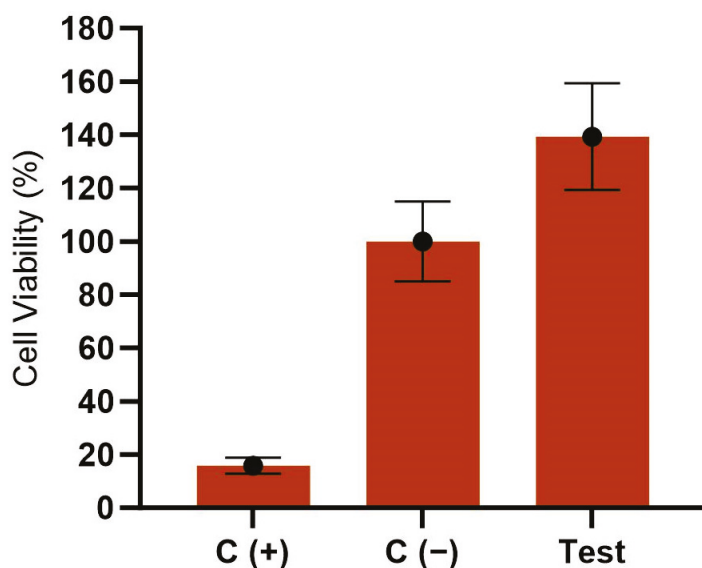
### 3.3. Inoculum Selection

Following the identification process [26] and based on the results described previously, the selected inoculum was CII87b (*Pichia manshurica*). This strain was the only strain to exhibit positive activity in all enzymatic assays. Moreover, it demonstrated one of the highest enzymatic indices for invertase. Notably, species of the genus *Pichia* are widely regarded as microorganisms with high potential for use in fermentation processes [41].

### 3.4. Cytotoxicity Evaluation of *Pichia manshurica*

To assess the cytotoxicity of the selected yeast inoculum, the MTT cell proliferation assay was performed to evaluate the viability of human HMVII cells exposed to *P. manshurica* extract. This assay measures mitochondrial dehydrogenase activity through the conversion of the yellow salt MTT into purple formazan crystals, serving as an indicator of cellular metabolic activity [42].

Figure 4 presents the cell viability results for the treatment group (cells + *P. manshurica*) compared to positive and negative controls. The results provide compelling evidence for the safety profile of *P. manshurica* CII 87b, a critical prerequisite for its application as a starter culture. Although the positive control (cells + DMSO) exhibited only 15.84% cell viability, cells exposed to the yeast extract not only maintained full viability relative to the negative control but demonstrated a remarkable increase to 139.3%. This finding indicates that the strain is not merely non-cytotoxic, but that it may possess biocompatible or even proliferative-stimulating properties.



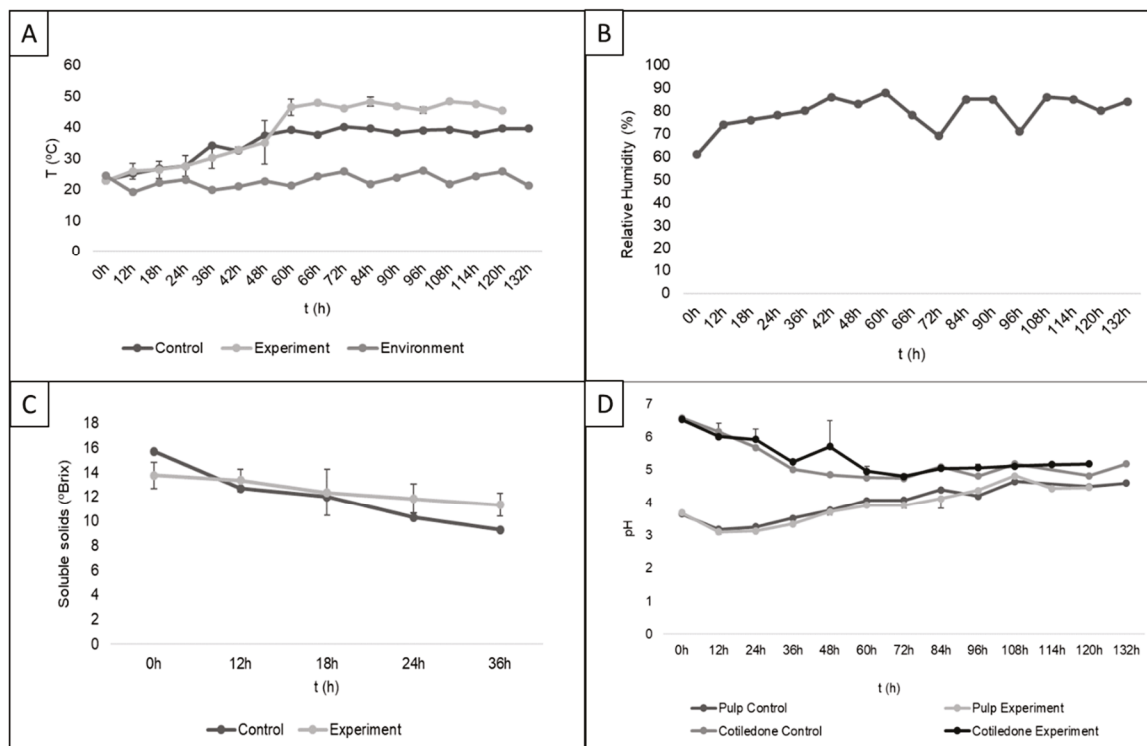
**Figure 4.** Percentage of HMVII line cell viability after exposure to the yeast *Pichia manshurica*. Cell viability was assessed using the MTT assay, which measures mitochondrial dehydrogenase activity through the conversion of the yellow MTT salt into purple formazan crystals. The graph compares the viability of the treatment group (HMVII cells + yeast extract), which showed a notable increase to 139.3%, with the positive C (+) and negative C (−) controls. The positive control C (+) showed only 15.84% cell viability. Error bars indicate the standard deviation of the mean of the experimental replicates.

Our results are consistent with findings from diverse ecosystems, as exemplified by the low cytotoxicity observed in mangrove yeasts (*Candida albicans* and *Saccharomyces cerevisiae*) cultured with VERO cells [43]. Such a robust safety profile is paramount for the development of commercial inoculants and aligns with previous findings where phylogenetically related yeasts, such as *Saccharomyces cerevisiae* strains isolated from wine

fermentation, also showed minimal effects on macrophage viability [44]. This positions *P. manshurica* CII 87b as a highly promising and safe candidate for further development in controlled cocoa fermentation and potentially other food applications.

### 3.5. Inoculum Application in Fermentation Boxes

Figure 5 presents the physicochemical parameters monitored during experimental fermentation. In Figure 5A,  $t = 0$  marks the start of the fermentation process. Here,  $t = 114$  and  $t = 120$  h indicate the endpoint for the experimental replicates, and  $t = 132$  h represents the endpoint for the control group (without inoculum).



**Figure 5.** Physicochemical parameters monitored during cocoa fermentation in the secondary harvest. (A) The average temperature in the fermentation boxes and the environment throughout the process, highlighting that the experimental boxes consistently showed higher internal temperatures after 48 h. (B) The percentage of relative air humidity during fermentation, which remained consistently above 60%. (C) The progressive decrease in soluble solids (°Brix) in the control and experimental boxes during the first 36 h. (D) The pH measurements of the pulp and cotyledon throughout the process. The pulp pH increased from 3.6 to 4.46 (experiment) and 4.6 (control), whereas the cotyledon pH decreased to 5.18.

During the initial 48 h, the temperature profiles of the experimental and control boxes were similar. However, beyond this period, the experimental boxes consistently exhibited higher internal temperatures than the control group. The ambient temperature remained significantly lower than the internal temperature of the fermentation mass, reflecting the climatic conditions of the region during the study period.

The control group maintained the lowest temperatures throughout fermentation, peaking at 40.2 °C at 72 h. In contrast, the experimental group reached a maximum temperature of 48.3 °C at 108 h, indicating a more robust thermogenic response. These results demonstrate that the introduction of the starter culture effectively elevated the fermentation temperature, even under challenging environmental conditions, specifically

the low ambient temperatures (19.1 °C minimum, 26.1 °C maximum), high rainfall, and elevated humidity typical of the winter season in the southern region of Bahia, Brazil.

The external environment has a direct influence on the cocoa mass in the fermentation box, and during summer, the mass temperature is always higher than during the colder seasons of the year [12]. Moreover, fermentation boxes with small dimensions, such as those used in this experiment, tend to lose temperature more easily than larger boxes due to the smaller volume of cocoa mass they store. However, the inoculated boxes still had temperatures comparable to those of fermentations conducted under more favorable climatic conditions. The addition of the inoculum accelerated the fermentation of the secondary harvest by 12 h compared to the control group, consistent with previous studies involving *Saccharomyces cerevisiae* in cocoa from southern Bahia [45]. Additionally, fermentation temperatures ranging from 25 °C to 50 °C during summer fermentations in the southern Bahia region have been reported [46], which closely match the temperature range observed in this study.

Figure 5B shows that relative humidity during fermentation remained consistently above 60%, as expected for the region and season. The soluble solids (°Brix) of the samples were measured during the first 36 h of fermentation (Figure 5C), a period in which the pulp was still present. A progressive decrease in the values was observed throughout the evaluated period for both the control and the experimental boxes. The fermentable sugars decrease during the fermentation process due to their transformation into alcohol and CO<sub>2</sub> by yeasts, as well as the production of pectinolytic enzymes that hydrolyze the polysaccharides present in the pulp [47].

Figure 5D shows the measurements of pulp and cotyledon pH values throughout the fermentation process. The pH of the pulps from both the control and the experiment exhibited similar behavior, starting at 3.6 and increasing during the process and ending at 4.6 (control) and 4.46 (experiment). The high pulp acidity at the beginning of the process is due to the presence of 2 to 2.5% citric acid, which degrades during the process and justifies the increase in pH to values close to 5.0 [48]. During the first 36 h, there was a slight decline in the pH of both pulp samples, followed by a progressive increase. This behavior has also been observed in previous works, which can be explained by the proliferation of microorganisms in the cocoa mass at low pH [46,49–51].

Similar behavior between the control and experiment samples was also observed in the cotyledon pH, with the samples starting at a pH of approximately 6.5 and decreasing during the process, ending at 5.18. This decay is expected and occurs due to the acidification of the almond after embryo death caused by the activity of microorganisms, which leads to the penetration of ethanol and acetic acid mainly through the testa into the cotyledon. This results in a decrease in the internal pH, which, together with the increase in temperature, leads to the development of flavor precursors and pigment degradation by endogenous enzymes, such as invertase, glycosidases, proteases, and polyphenoloxidase [52–54].

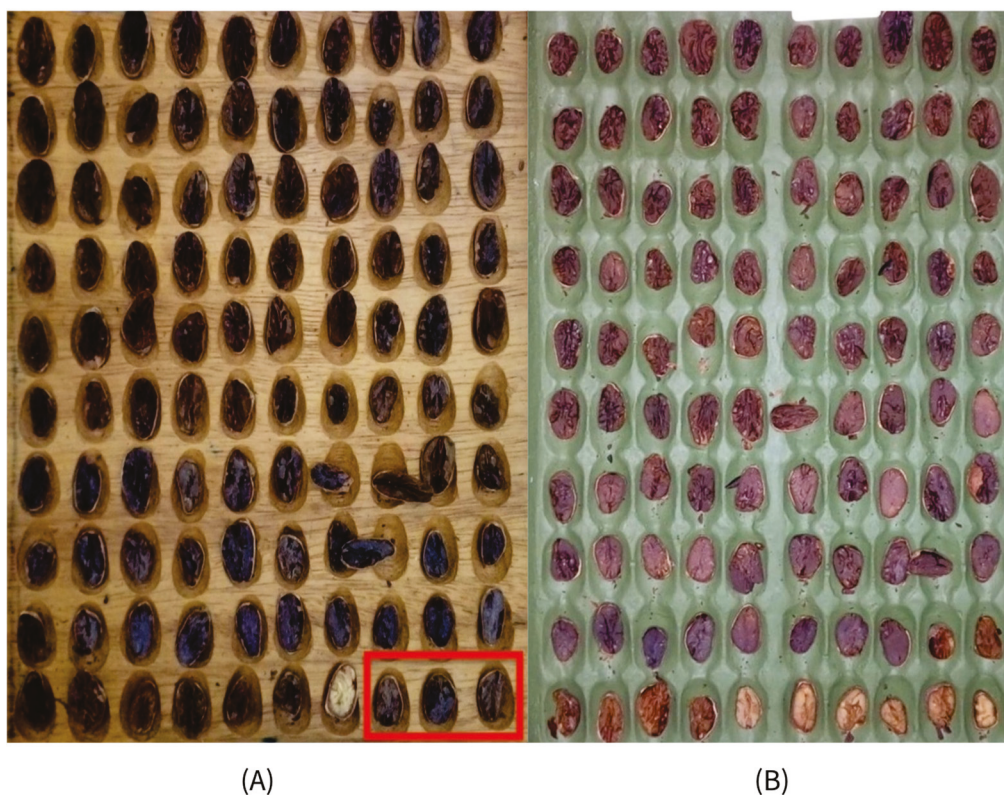
### 3.6. Impact of Starter Culture on the Final Quality and Safety of Cocoa Beans

All samples showed moisture values within the expected range, thus avoiding the loss of edible material and minimizing the risk of fungal and bacterial growth. Such growth compromises the food safety, flavor, and quality of the final product. However, the control sample showed a moisture content between 6.0% and 6.5%, which is also undesirable because it can make the beans brittle. According to ISO (2017) [55], the moisture content of almonds should be around 7%, with a maximum of 7.5%.

Furthermore, all samples displayed a typical external appearance of brown cocoa beans, without visible signs of contamination, and the aroma of both samples was deemed characteristic (Figure 6). The absence of external mold in both samples suggests that



drying was adequately performed during both harvests, despite the challenging environmental conditions in southern Bahia, such as high rainfall and humidity, which favor fungal growth.



**Figure 6.** Photograph of the cut test plate of 100 almonds showing the cut test for the control (A) and the experiment (B) samples according to the ISO 2014 standard [29] for robust assessment of cocoa quality. The image shows the typical external appearance of brown cocoa beans, with no visible defects. A rectangle highlights the presence of internal mold as a defect in the control (A), whereas the experiment with the starter yeast shows no such defect.

It should also be noted that the final percentage of brown beans, a key factor in the cut test's colorimetric evaluation, can be influenced by factors other than fermentation effectiveness, such as the cocoa's genetic variety and its initial phenolic compound content [55].

The subjectivity of the cut test [56] is particularly evident when assessing almonds in intermediate states of fermentation, which complicates consistent interpretation across different analysts. For this reason, complementing the visual cut test with objective analytical methods is crucial for a more robust assessment of cocoa quality [21,55].

According to Table 1, starter culture inoculation led to a marked improvement in the final quality and safety of fermented beans when compared to the control group. The most significant impact was observed in the reduction of critical defects, as the experimental samples showed a notably lower incidence of internal mold (Figure 6). Although the control sample had a slightly higher well-fermented bean index based on color (70.3% vs. 62.5%), this single metric should be interpreted with caution. The cut test is a subjective method with known limitations and does not consider key physicochemical properties that strongly influence flavor. In this context, the more favorable pH of the experimental beans (5.35 vs. 5.20 in the control) is a more objective and arguably more important indicator of a well-controlled fermentation, as excessive acidity is known to negatively impact chocolate quality. Therefore, when considering the combined benefits of significant

defect reduction and superior physicochemical balance, inoculated fermentation represents a clear improvement over the spontaneous process.

**Table 1.** Almond cut quality.

| Parameter            | Control Sample                                                                               | Experimental Sample                                                                 |
|----------------------|----------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|
| Defects              | Higher rate (internal mold, flattened, insect-damaged, germinated, and violet kernels > 50%) | Lower defect rate                                                                   |
| Fermentation-Related | Higher % of compartmentation (white/brown almonds) and well-fermented almonds (70.3%)        | Higher % of flat white, partially/sub-fermented almonds; fermentation index (85.1%) |
| pH                   | More acidic (5.20)                                                                           | Less acidic (5.35)—more desirable                                                   |
| External Appearance  | Brown almonds, no contamination                                                              | Brown almonds, no contamination                                                     |
| Aroma                | Characteristic                                                                               | Characteristic                                                                      |
| Mold Presence        | No external mold (drying well conducted)                                                     | No external mold (drying well conducted)                                            |
| Key Notes            | Higher % brown almonds                                                                       | Higher fermentation index                                                           |

### 3.7. Microbiological Safety Assessment of Cocoa Beans

Both the control and experimental samples were assessed for microbiological quality using coliform counts at 45 °C (<3.0 MPN/g) and testing for *Salmonella* spp. (absent). The results were within the standards set by ANVISA Resolution RDC No. 12/2001 [29], which stipulates that dried in natura cocoa beans must not exceed 10<sup>2</sup> MPN/g for coliforms at 45 °C, and *Salmonella* must be absent in 25 g of sample. Thus, both samples were deemed suitable for human consumption.

It is widely accepted that food microbiological quality is closely tied to the adoption of Good Manufacturing Practices. Satisfactory final product outcomes are typically the result of a well-managed production chain, encompassing harvesting, pod breaking, bean fermentation, drying, packaging, and transportation. Although fermentation in cocoa relies on a succession of microbial activity to develop desirable physicochemical and sensory properties, it is essential to prevent cross-contamination by pathogenic microorganisms to ensure product safety, as demonstrated by the results obtained in this study.

## 4. Conclusions

The use of *Pichia manshurica* as an inoculum was effective in accelerating fermentation and increasing the average temperature of cocoa mass during the secondary harvest, a period when achieving optimal fermentation conditions is more challenging due to cooler regional climates. The use of starter yeast leads to increased productivity, allowing producers to process more cocoa in a shorter period, thereby improving efficiency and quality while reducing costs. The inoculum also helped reduce internal mold formation, improved the total fermentation index, and resulted in a more desirable pH with lower acidity.

Future studies should jointly evaluate how different inoculum concentrations affect fermentation time, comparing the production of higher alcohols and acetate esters when using the inoculum. Additionally, research should focus on achieving more precise control over the final product quality. Experiments using single cocoa varieties or different inoculum concentrations could help optimize its effects.

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## Article

# Fermentation-Based Preservation of Okara and In Vitro Evaluation of Its Application in Dairy Cattle Diets

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## Abstract

Okara, a protein-rich byproduct of soymilk production, is highly perishable because of its high moisture content. This study evaluated the preservation and nutritional value of okara fermented by lactic acid bacteria for use in dairy cattle diets. Fermentation effectively reduced pH within 2 weeks and maintained quality for up to 6 weeks. However, aerobic exposure increased the concentration of ammonia, indicating a decline in stability. In vitro assessments revealed no significant differences in in vitro true dry matter digestibility, in vitro neutral detergent fiber digestibility, or gas production between fermented and fresh okara, although fermented okara had a higher concentration of ammonia nitrogen. In situ analysis revealed slightly lower dry matter effective degradability (ED) in fermented okara, but similar rumen-degradable and undegradable protein fractions. When fermented okara was used to replace soybean meal in total mixed rations, 25–50% inclusion-maintained digestibility and fermentation characteristics, with 25% replacement yielding the highest ED at a low ruminal passage rate (0.02 h<sup>-1</sup>). Taken together, these results suggest that fermented okara can be strategically incorporated into dairy rations as a sustainable protein alternative, supporting both rumen function and bypass protein supply.

**Keywords:** okara; fermentation; in vitro digestibility; in situ; soybean meal replacement

## 1. Introduction

Okara, a major byproduct of tofu and soymilk production, is generated in large quantities worldwide, particularly in Asia, where soybean processing is widespread. Globally, approximately 14 million metric tons of okara are produced annually, with China and Japan contributing approximately 2.8 and 0.8 million metric tons, respectively [1]. In terms of dry matter (DM), okara contains a relatively high crude protein (CP) content, ranging from 15.2% to 33.4% [2], indicating its major potential as a protein source for animal feed. However, the extremely high moisture content of okara, which typically ranges from 70% to 80% [3], poses major challenges for storage and transportation, often resulting in rapid microbial spoilage and nutrient degradation [2]. Rahman et al. [1] recommended using fresh okara within 3 days in cases of direct feeding to livestock. Notably, the presence of antinutritional compounds, such as trypsin inhibitors, saponins, phytic acid, hemagglutinins, stachyose, and raffinose, limits the direct inclusion of okara in animal diets [4,5]. Thus, manufacturers often dispose of okara through composting or incineration, which leads to the loss of a valuable protein-rich resource.



Global events such as regional conflicts have increased the prices of imported feed ingredients, including soybean meal. This economic pressure has increased interest in sustainable, locally sourced, and cost-effective feed alternatives [6]. Microbial fermentation offers a promising approach for enhancing the stability and nutritional quality of okara. Fermentation not only extends the shelf life of okara but also mitigates the effects of antinutritional compounds [2]. Lactic acid bacteria (LAB) fermentation is recognized as one of the most effective techniques for preservation. This technique lowers pH through lactic acid production, which inhibits the growth of spoilage microorganisms [7,8]. Additionally, LAB fermentation may enhance the nutritional value of feed by breaking down fibers and proteins, thereby increasing the concentration of free amino acids, and converting macronutrients into smaller, more digestible molecules [3,8–10].

To evaluate the nutritional value of okara in ruminant diets, the digestibility of okara should be examined. Although *in vivo* trials provide direct evidence, they are often expensive, time-consuming, and difficult to conduct for single ingredients [11]. *In vitro* and *in situ* techniques offer valuable alternatives for evaluating rumen digestibility with minimal animal use [12,13]. *In vitro* methods can be used to evaluate DM and neutral detergent fiber (NDF) digestibility, fermentation characteristics, and gas production patterns [14,15]. *In situ* methods can be used to estimate dynamic DM and CP degradation, including effective degradability (ED) [16,17]. The kinetics of feedstuff degradation can be evaluated through fermentative gas production. Gas measurements also provide valuable information on the digestion kinetics of both the soluble and insoluble fractions of feedstuffs [18]. A well-constructed model may link gas production data to animal performance by estimating the extent of ruminal degradation [19]. Combining the results of *in vitro* digestibility and fermentation products, such as volatile fatty acids (VFAs) and ammonia, with gas kinetics may offer a practical approach for examining how ruminant diets are utilized [20].

This introduces a two-phase approach addressing the challenges of okara preservation and protein source diversification in ruminant nutrition. In summary, this study aimed to develop an optimal fermentation process for okara preservation by evaluating its effects on chemical composition, fermentation quality, and aerobic stability. Concurrently, it sought to determine the feeding value of the optimally fermented okara, including its degradability and potential as a soybean meal replacement in dairy cattle total mixed rations (TMRs), through comprehensive *in vitro* and *in situ* digestibility trials.

## 2. Materials and Methods

### 2.1. Okara Source

Fresh okara was obtained from Kuang Chuan Ranch in Taoyuan, Taiwan. It was immediately collected after soymilk processing, which involved soaking, grinding, and heating soybeans, followed by filtration. Upon sample arrival, the internal temperature of the okara samples ranged from 60 to 70 °C, with an average moisture content of approximately 80%. All samples were analyzed to determine their chemical composition, including DM, CP, ether extract (EE), NDF, acid detergent fiber (ADF), acid detergent lignin (ADL), ash, and water-soluble carbohydrates (WSCs).

### 2.2. Experimental Design

This study was conducted in two phases: (1) laboratory-scale fermentation of fresh okara and (2) large-scale evaluation of fermented okara as a replacement for soybean meal in dairy cattle diets through *in vitro* and *in situ* trials. The first phase focused on evaluating a small-scale fermentation process to examine its effects on the chemical composition, fermentation quality, and aerobic stability of okara. The second phase involved determining the feeding value of fermented okara through *in vitro* and *in situ* trials, particularly

focusing on its degradability and potential to replace soybean meal in dairy cattle total mixed rations (TMRs). These two complementary phases were designed to provide a comprehensive understanding of the storage stability and practical use of fermented okara in ruminant feeding.

### 2.2.1. Phase 1: Laboratory-Scale Fermentation Trial

In the first phase, a fermentation strategy was developed and evaluated to improve okara preservation by determining its effects on chemical composition, fermentation quality, and aerobic stability. Each replicate sample in a treatment group consisted of 200 g of fresh okara.

Fermentation conditions were tested using nine different combinations: moisture content at 60%, 70% or 80%, and inoculum concentration at  $10^6$ ,  $10^7$ , or  $10^8$  CFU/g. Changes in pH and ammonia concentration were monitored on days 3, 6, 12, and 24 after inoculation. These parameters were analyzed to identify the optimal conditions for future fermentation applications.

A commercial fermenting starter (Synferm Feed, Shenghe Biotech, Kaohsiung City, Taiwan) containing *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus paracasei*, *Lactobacillus rhamnosus*, and *Saccharomyces cerevisiae* was used. The concentrations of LAB and yeast were  $2.2 \times 10^{10}$  and  $5.2 \times 10^7$  CFU/g, respectively. The inoculum was diluted in sterile water and applied at 0.2% (*v/w*) okara. The mixture was then vacuum-sealed in plastic bags and fermented at room temperature. A control group without an inoculum or moisture adjustment was also included. After screening, fermentation was conducted at a moisture content (unadjusted) of 80% and an inoculation level of  $10^6$  CFU/g for 24 days for evaluation. Finally, the fermented samples were analyzed to determine their chemical composition, fermentation quality, and aerobic stability after 48 h of air exposure.

Fermentation quality was determined by opening the fermentation bags at 1, 2, 4, 6, and 8 weeks. Samples from the top, middle, and bottom layers were mixed and analyzed for pH, microbial count, VFAs, ammonia nitrogen ( $\text{NH}_3\text{-N}$ ), and chemical composition. Aerobic stability was evaluated by exposing 4 week fermented samples to the air for 48 h, with microbial counting conducted every 24 h [8].

### 2.2.2. Phase 2: Feed Utilization Trial

In this phase, the nutritive potential of fermented okara was evaluated by examining its digestibility, fermentation characteristics, and gas production patterns both in vitro and in situ. On the basis of the fermentation results, fermented okara was incorporated into TMRs at varying replacement levels for soybean meal, and the digestibility of each formulation was evaluated. These replacement levels were set at 0%, 25%, 50%, 75%, and 100%, resulting in five experimental groups. All diets were formulated in accordance with National Research Council [21] guidelines to maintain comparable levels of CP, rumen degradable protein (RDP), rumen undegradable protein (RUP), and net energy for lactation. These formulations were designed for a multiparous lactating dairy cow weighing 680 kg and producing 35 kg/day of milk containing 3.0% true milk protein and 3.5% milk fat. Differences in mineral and vitamin content between the experimental diets were adjusted using a premixture to meet standard dairy cattle requirements. Ingredient proportions and nutrient compositions are presented in Table 1. Each formulation was evaluated using in vitro and in situ methods.

**Table 1.** TMR formulas and nutrient compositions used in the okara replacement experiment.

| Ingredient %DM <sup>2</sup>             | Diets (Soybean Meal Replacement %) <sup>1</sup> |                 |      |      |      |
|-----------------------------------------|-------------------------------------------------|-----------------|------|------|------|
|                                         | Control                                         | Fermented Okara |      |      |      |
|                                         |                                                 | 25              | 50   | 75   | 100  |
| Alfalfa hay                             | 25.4                                            | 25.4            | 25.4 | 25.4 | 25.4 |
| Oat hay                                 | 25.4                                            | 25.4            | 25.4 | 25.4 | 25.4 |
| Soybean meal                            | 6.4                                             | 4.8             | 3.2  | 1.6  | 0.0  |
| Fermented okara                         | 0.0                                             | 1.6             | 3.2  | 4.8  | 6.4  |
| Corn meal                               | 23.7                                            | 19.7            | 15.8 | 11.5 | 7.6  |
| Wheat bran                              | 10.6                                            | 10.6            | 11.4 | 13.1 | 13.6 |
| Corn gluten meal                        | 4.7                                             | 3.4             | 2.5  | 1.7  | 0.6  |
| DDGS <sup>2</sup>                       | 2.1                                             | 7.4             | 11.2 | 14.7 | 19.3 |
| NaHCO <sub>3</sub>                      | 0.8                                             | 0.8             | 0.8  | 0.8  | 0.8  |
| Salt                                    | 0.4                                             | 0.4             | 0.4  | 0.4  | 0.4  |
| Vitamin premix                          | 0.5                                             | 0.5             | 0.5  | 0.5  | 0.5  |
| Nutrient Composition (%DM) <sup>3</sup> |                                                 |                 |      |      |      |
| CP                                      | 16.4                                            | 16.4            | 16.4 | 16.4 | 16.4 |
| RDP                                     | 10.4                                            | 10.4            | 10.4 | 10.4 | 10.4 |
| RUP                                     | 6.0                                             | 6.0             | 6.0  | 6.0  | 6.0  |
| NE <sub>1</sub>                         | 1.5                                             | 1.5             | 1.5  | 1.5  | 1.5  |
| NDF                                     | 36.8                                            | 38.8            | 40.5 | 42.5 | 44.4 |
| ADF                                     | 22.6                                            | 23.4            | 24.1 | 24.9 | 25.7 |
| Ca                                      | 0.7                                             | 0.7             | 0.7  | 0.7  | 0.7  |
| P                                       | 0.5                                             | 0.5             | 0.5  | 0.5  | 0.5  |

<sup>1</sup> Nutrient requirements recommended by the National Research Council [21] for dairy cows: CP = 16.4%, RDP = 10.4%, RUP = 6%, NE<sub>1</sub> = 1.52 Mcal/kg, Ca = 0.61%, and P = 0.35%. <sup>2</sup> DM = dry matter; DDGS = distilled dried grains with soluble components. <sup>3</sup> CP = crude protein; RDP = rumen degradable protein; RUP = rumen undegradable protein; NDF = neutral detergent fiber; ADF = acid detergent fiber.

### 2.3. Chemical Composition Analysis of Fresh Okara and Fermented Products

#### 2.3.1. Sample Preparation

10 g of fresh or fermented okara was homogenized with 90 mL of chilled sterile water (4 °C) by using a high-speed blender (Oster, Ontario, CA, USA) for 20 s and then filtered through four layers of cheesecloth. The filtrate was used for pH measurement and microbial counting. The remaining liquid was centrifuged twice at 12,000 × g at 4 °C for 15 min to remove impurities. The supernatants were then analyzed for microbial CP (MCP), VFA, and NH<sub>3</sub>-N.

Approximately 100 g of fresh and fermented okara was dried at 65 °C for 2 days, ground using a mill (DM-6, 25,000 rpm; XiangTai, Taishan District, New Taipei City), and sieved through a 20 mesh screen for use in a chemical composition analysis and in vitro and in situ digestion trials.

#### 2.3.2. pH, Microbial Count, Microbial Crude Protein, Volatile Fatty Acids, and NH<sub>3</sub>-N

After fermentation, the pH of the fluid was measured using a pH meter (F-71; Horiba Scientific, Kyoto, Japan). The serum bottle was then recapped and placed in an ultrasonicator containing cold water (approximately 4 °C) for 5 min and sonicated under cold water for 15 min to dislodge the microbes attached to the residual substrates. After the sample filtrate was diluted with phosphate-buffered saline, LAB and mold or yeast counting was conducted using a 3M Petrifilm Lactic Acid Bacteria Count Plate and a YM-Media Pad, respectively. The sonicated fermented fluid was then centrifuged at 400 × g at 4 °C for 5 min. At this point, half of each supernatant fraction was collected and lyophilized for MCP analysis by using the purine content method described by Zinn and Owens [22]. MCP values from the blank fermentation group (containing rumen inoculum but no substrate) were sub-

tracted during MCP synthesis calculations. The remaining half of the supernatant fraction was centrifuged at  $13,500 \times g$  at  $4\text{ }^{\circ}\text{C}$  for 15 min to analyze VFAs, lactic acid, and  $\text{NH}_3\text{-N}$ . The concentration of  $\text{NH}_3\text{-N}$  was immediately determined using a colorimetric method [23]. The samples used for VFA and lactic acid analysis were acidified with 25% metaphosphoric acid (4:1, *w/v*) and filtered through a  $0.22\text{ }\mu\text{m}$  membrane filter (Millipore, Bedford, MA, USA). All VFA samples were stored at  $-20\text{ }^{\circ}\text{C}$  for analysis. The VFA concentration was determined using high-performance liquid chromatography (LC-4000; Jasco, Tokyo, Japan) with a Rezex ROA-Organic Acid  $\text{H}^+$  (8%) column ( $300\text{ mm} \times 7.8\text{ mm}$ ; Phenomenex, Torrance, CA, USA) and a UV detector as an integrated part of the LC-4000 system [20].

### 2.3.3. Chemical Composition Analysis

The organic matter (AOAC 942.05), DM (AOAC 934.01), EE (AOAC 920.39), CP (AOAC 990.03), ADL (AOAC 973.18), and ash (AOAC 942.05) contents of fresh and fermented okara were determined using standard Association of Official Analytical Chemists (AOAC) [24] methods. NDF and ADF were analyzed in accordance with the procedure described by Van Soest et al. [25]. WSCs were determined using the methods reported by Dreywood [26] and Morris [27]. MCP synthesis efficiency is expressed as MCP (milligrams) per gram of digested organic matter.

### 2.4. *In Vitro* Digestibility and Gas Kinetics Assay

Rumen fluid was collected from two rumen-fistulated dry Holstein cows (approximately 800 kg body weight) housed in a livestock farm at the College of Agriculture, National Taiwan University. These cows were fed a TMR with a forage-to-concentrate ratio of 60:40, consisting of 180 g/kg Bermuda hay, 180 g/kg alfalfa hay, and 240 g/kg commercial concentrate (16% CP, DM basis). Each cow received 16 kg of food per day and had ad libitum access to fresh water. The two cows were housed in individual pens and fed the same diet for 14 days before rumen fluid collection. On the sampling day, rumen fluid was collected through a cannula from each cow 2 h after the morning feeding session. This fluid was then strained through four layers of cheesecloth into a prewarmed serum bottle ( $40\text{ }^{\circ}\text{C}$ ) flushed with carbon dioxide before use. Freeze-dried okara samples were used in the *in vitro* assay. A portion of the fermented okara was frozen at  $-80\text{ }^{\circ}\text{C}$  for 24 h and then freeze-dried at  $-50\text{ }^{\circ}\text{C}$  for 96 h. Freeze-dried samples were used for *in vitro* tests to minimize the impact of high-temperature drying on digestibility.

*In vitro* degradability was determined using an Ankom Daisy II Incubator (Ankom Technology, Macedon, NY, USA) over a 48 h incubation period. Digestion and sample collection were conducted as described by Spanghero et al. [28]. Briefly, F57 filter bags ( $25\text{ }\mu\text{m}$  pore size; Ankom Technology) were prewashed with acetone, soaked for 10 min, and dried overnight at  $65\text{ }^{\circ}\text{C}$ . After cooling, the bags were weighed to determine their initial weights. A total of 0.5 g of freeze-dried sample was placed in each filter bag, and all bags were sealed twice using a heat sealer (four replicates per treatment). Blank bags without samples were prepared in the same manner and served as controls. Weight loss was determined and used to calculate *in vitro* true DM digestibility (IVTDMD). Residual NDF was used to determine *in vitro* NDF digestibility (IVNDFD).

To conduct a gas production assay, 400 mg of each experimental diet was weighed and added to 100 mL serum bottles, with four replicates per treatment. Rumen fluid from each cow was then combined with artificial saliva solution, as described by Menke and Steingass [18], at a ratio of 1:4 (*v/v*). This mixture was maintained at  $39\text{ }^{\circ}\text{C}$  in the presence of continuous flushing with carbon dioxide. Subsequently, 80 mL of the rumen inoculum mixture was added to each serum bottle in the presence of carbon dioxide flushing. After inoculation, the bottles were connected to an Ankom pressure sensor module, and gas

production was monitored every 2 min by using a wireless Ankom RF gas production system (Ankom Technology) [29].

### 2.5. *In Situ Degradability Assay*

2 g of freeze-dried sample was sealed in a 5 cm × 10 cm nylon bag (50 µm pore size) and incubated in the rumen of cannulated cows, with three replicates per treatment. The cows were fed the same diet described in Section 2.4. The digestion bags were removed at 2, 4, 8, 12, 24, 48, and 72 h; washed; dried at 60 °C for 48 h; and analyzed for DM, CP, and NDF. Degradation parameters (a, b, c, and  $t_0$ ) were modeled following the method described by McDonald [30]. RDP, RUP, and ED were calculated using the models proposed by Cone et al. [15] and Ørskov and McDonald [16].

### 2.6. *Statistical Analysis*

The chemical composition of fresh and fermented okara, the fermentation characteristics of fermented okara, and the digestibility and fermentation parameters of the *in vitro* trials were analyzed using analysis of variance (ANOVA) in SAS software version 9.4 (SAS Institute, Cary, NC, USA). Significant differences between treatment means were evaluated using Tukey's honestly significant difference test. Gas production kinetics and *in situ* degradability data were modeled using the one-phase nonlinear association model in GraphPad Prism 8 version 2019 (GraphPad Software, San Diego, CA, USA). A nonlinear regression analysis was conducted using the NLIN procedure in SAS software, and gas production parameters were estimated using the Marquardt iterative method. The following model, based on McDonald [30], was used to calculate degradability values:

$$P1 = a \quad (t \leq t_0)$$

$$P2 = a + b (1 - e^{-ct}) \quad (t > t_0)$$

where P is degradation at time t a is the soluble fraction, b is the potentially degradable fraction, c is the degradation rate of the b fraction, and  $t_0$  is the lag time before degradation begins. A *p* value of <0.05 was considered statistically significant, and values between >0.05 and ≤0.10 were interpreted as indicative of a statistical trend.

## 3. Results

### 3.1. *Laboratory-Scale Fermentation*

#### 3.1.1. Fermentation Condition Selection

According to the results of the moisture content and inoculum concentration combination tests (Table 2), fermentation with a moisture content of 80% and an inoculum level of  $10^6$  CFU/g resulted in the lowest pH value (approximately 4.1) on day 12 after inoculation and maintained a relatively low ammonia concentration through day 24. These findings suggest that fresh okara can be directly fermented using a low inoculum concentration without requiring additional moisture adjustment. Based on these screening results (Table 2), the optimal condition, characterized by 80% moisture content and an inoculum concentration of  $10^6$  CFU/g, achieved the lowest pH on day 12 and maintained low ammonia through day 24, which effectively inhibited spoilage microorganisms and promoted a stable microbial population. Consequently, this condition was chosen for comprehensive evaluation of chemical composition, fermentation quality, and aerobic stability.



**Table 2.** Temporal changes in pH and ammonia nitrogen (NH<sub>3</sub>-N) content during fermentation at different inoculation levels and moisture conditions on days 3, 6, 12, and 24.

| Item                            | Days | Control           | Inoculation Level (CFU/g) |                   |                   |                   |                   |                    |                   |                   |                    | SEM <i>p</i> -Value |       |
|---------------------------------|------|-------------------|---------------------------|-------------------|-------------------|-------------------|-------------------|--------------------|-------------------|-------------------|--------------------|---------------------|-------|
|                                 |      |                   | 10 <sup>6</sup>           |                   |                   | 10 <sup>7</sup>   |                   |                    | 10 <sup>8</sup>   |                   |                    |                     |       |
|                                 |      |                   | Mositure %                |                   |                   | Mositure %        |                   |                    | Mositure %        |                   |                    |                     |       |
|                                 |      |                   | 80                        | 70                | 60                | 80                | 70                | 60                 | 80                | 70                | 60                 |                     |       |
| pH                              | 3    | 4.61 <sup>a</sup> | 4.32 <sup>b</sup>         | 4.26 <sup>c</sup> | 4.54 <sup>b</sup> | 4.25 <sup>c</sup> | 4.37 <sup>b</sup> | 4.61 <sup>a</sup>  | 4.39 <sup>b</sup> | 4.45 <sup>a</sup> | 4.54 <sup>a</sup>  | 0.03                | <0.01 |
|                                 | 6    | 4.47 <sup>a</sup> | 4.28 <sup>c</sup>         | 4.27 <sup>c</sup> | 4.38 <sup>b</sup> | 4.23 <sup>c</sup> | 4.34 <sup>c</sup> | 4.60 <sup>a</sup>  | 4.36 <sup>b</sup> | 4.43 <sup>b</sup> | 4.57 <sup>a</sup>  | 0.02                | <0.01 |
|                                 | 12   | 4.36 <sup>a</sup> | 4.15 <sup>c</sup>         | 4.26 <sup>b</sup> | 4.30 <sup>b</sup> | 4.28 <sup>b</sup> | 4.30 <sup>c</sup> | 4.42 <sup>a</sup>  | 4.32 <sup>b</sup> | 4.33 <sup>a</sup> | 4.41 <sup>a</sup>  | 0.02                | <0.01 |
|                                 | 24   | 4.37 <sup>b</sup> | 4.23 <sup>c</sup>         | 4.38 <sup>b</sup> | 4.34 <sup>b</sup> | 4.39 <sup>b</sup> | 4.41 <sup>b</sup> | 4.52 <sup>a</sup>  | 4.47 <sup>a</sup> | 4.50 <sup>a</sup> | 4.55 <sup>a</sup>  | 0.01                | <0.01 |
| NH <sub>3</sub> -N<br>(g/kg DM) | 3    | 7.90 <sup>a</sup> | 4.61 <sup>b</sup>         | 2.38 <sup>d</sup> | 1.15 <sup>e</sup> | 3.79 <sup>c</sup> | 2.77 <sup>d</sup> | 2.03 <sup>d</sup>  | 3.41 <sup>c</sup> | 2.66 <sup>d</sup> | 2.45 <sup>d</sup>  | 0.09                | <0.01 |
|                                 | 6    | 6.34 <sup>a</sup> | 4.68 <sup>b</sup>         | 1.56 <sup>d</sup> | 0.69 <sup>e</sup> | 3.41 <sup>c</sup> | 2.06 <sup>d</sup> | 1.38 <sup>de</sup> | 3.03 <sup>c</sup> | 2.40 <sup>d</sup> | 1.61 <sup>d</sup>  | 0.11                | <0.01 |
|                                 | 12   | 2.54 <sup>a</sup> | 1.58 <sup>b</sup>         | 1.21 <sup>b</sup> | 0.85 <sup>c</sup> | 1.70 <sup>b</sup> | 1.37 <sup>b</sup> | 0.96 <sup>c</sup>  | 1.73 <sup>b</sup> | 1.31 <sup>b</sup> | 1.06 <sup>bc</sup> | 0.07                | <0.01 |
|                                 | 24   | 2.23 <sup>a</sup> | 1.41 <sup>b</sup>         | 1.32 <sup>b</sup> | 0.93 <sup>b</sup> | 1.65 <sup>b</sup> | 1.27 <sup>b</sup> | 1.04 <sup>b</sup>  | 2.40 <sup>b</sup> | 1.53 <sup>b</sup> | 0.78 <sup>b</sup>  | 0.08                | <0.01 |

Different superscript letters indicate significant differences between treatments. CFU = colony-forming unit; DM = dry matter; SEM = standard error of the mean.

### 3.1.2. Chemical Composition of Fresh and Fermented Okara

Fresh okara contained 21.70% DM, 22.40% CP, and 7.72% EE on a DM basis (Table 3). Its fiber content included 34.29% NDF, 21.02% ADF, and 13.52% ADL. After 8 weeks of fermentation, DM ranged from 18.06% to 19.06% and CP ranged from 17.45% to 21.04% (Table 4). Fiber fractions fluctuated during fermentation, with NDF ranging from 34.31% to 39.84% and ADF ranging from 23.64% to 26.45%. Ash content ranged from 2.24% to 2.61%. All *p* values were >0.05, indicating that the chemical composition of okara remained stable throughout the fermentation process.

**Table 3.** Chemical composition of fresh okara.

| Item                                     | %DM Basis    |
|------------------------------------------|--------------|
| Dry matter (%)                           | 21.70 ± 0.09 |
| Crude protein (%DM)                      | 22.40 ± 0.13 |
| Ether extract (%DM)                      | 7.72 ± 0.07  |
| Neutral detergent fiber (%DM)            | 34.29 ± 0.75 |
| Acid detergent fiber (%DM)               | 21.02 ± 0.31 |
| Acid detergent lignin (%DM)              | 13.52 ± 1.79 |
| Ash (%DM)                                | 3.42 ± 0.46  |
| Water soluble carbohydrate extract (%DM) | 2.07 ± 0.03  |

Data are expressed as mean ± standard deviation. DM = dry matter.

**Table 4.** Chemical composition of fermented okara at constant moisture (80%) and inoculation concentration (10<sup>6</sup> CFU/g).

|                               | Week After Inoculation |       |       |       |       |       |      |                 |
|-------------------------------|------------------------|-------|-------|-------|-------|-------|------|-----------------|
| Item                          | 0                      | 1     | 2     | 4     | 6     | 8     | SEM  | <i>p</i> -value |
| Chemical composition (%DM)    |                        |       |       |       |       |       |      |                 |
| DM (%)                        | 18.72                  | 19.05 | 18.80 | 19.06 | 18.85 | 18.06 | 0.22 | 0.89            |
| Crude protein (%DM)           | 21.04                  | 20.41 | 20.28 | 20.27 | 20.06 | 17.45 | 0.52 | 0.33            |
| Neutral detergent fiber (%DM) | 37.31                  | 38.76 | 37.64 | 37.89 | 39.84 | 34.31 | 0.87 | 0.20            |
| Acid detergent fiber (%DM)    | 23.64                  | 25.92 | 25.08 | 25.42 | 26.45 | 25.28 | 0.53 | 0.47            |
| Ash (%DM)                     | 2.61                   | 2.33  | 2.39  | 2.34  | 2.24  | 0.23  | 0.04 | 0.87            |

DM = dry matter.



### 3.1.3. Quality of Fermented Okara

Table 5 presents the fermentation quality of fermented okara. Fermentation had a significant effect on pH, NH<sub>3</sub>-N, and lactate concentration ( $p < 0.01$ ). For example, pH dropped from 5.29 in week 1 to a minimum of 4.51 by week 4 and then increased to 5.58 by week 8. In week 4, the concentration of NH<sub>3</sub>-N significantly increased from 6.09 to 51.38 g/kg DM. In week 2, the concentration of lactate peaked at 44.89 g/kg DM and remained high through week 4 (44.74 g/kg DM) before substantially decreasing in subsequent weeks. LAB counts peaked in week 6 (10.16 log CFU/g), whereas yeast populations significantly declined after week 4.

**Table 5.** Fermentation quality of fermented okara at constant moisture (80%) and inoculation concentration (10<sup>6</sup> CFU/g).

| Item                                              | Week After Inoculation |                    |                    |                    |                    | SEM  | p-value |
|---------------------------------------------------|------------------------|--------------------|--------------------|--------------------|--------------------|------|---------|
|                                                   | 1                      | 2                  | 4                  | 6                  | 8                  |      |         |
| pH                                                | 5.29 <sup>a</sup>      | 4.65 <sup>b</sup>  | 4.51 <sup>b</sup>  | 5.18 <sup>a</sup>  | 5.58 <sup>a</sup>  | 0.15 | <0.01   |
| NH <sub>3</sub> -N <sup>1</sup> (g/kg DM)         | 6.09 <sup>b</sup>      | 13.20 <sup>b</sup> | 15.95 <sup>a</sup> | 42.81 <sup>a</sup> | 51.38 <sup>a</sup> | 5.59 | <0.01   |
| Lactate (g/kg DM)                                 | 27.54 <sup>b</sup>     | 44.89 <sup>a</sup> | 44.74 <sup>a</sup> | 6.96 <sup>c</sup>  | 4.21 <sup>c</sup>  | 5.58 | 0.01    |
| Acetate (g/kg DM)                                 | 7.47                   | 6.23               | 7.58               | 17.90              | 44.76              | 2.16 | 0.08    |
| Lactic acid bacteria (log CFU/g FM <sup>2</sup> ) | 9.39 <sup>bc</sup>     | 9.98 <sup>ab</sup> | 9.88 <sup>a</sup>  | 10.16 <sup>a</sup> | 8.73 <sup>c</sup>  | 0.19 | 0.02    |
| Yeast (log CFU/g FM)                              | 5.24 <sup>a</sup>      | 5.42 <sup>a</sup>  | -                  | 3.78 <sup>b</sup>  | -                  | 0.22 | 0.01    |

<sup>1</sup> NH<sub>3</sub>-N = ammonia nitrogen. <sup>2</sup> FM = fresh matter. Different superscript letters indicate significant differences between weekly treatments. - Indicates not analyzed. CFU = colony-forming unit; DM = dry matter; SEM = standard error of the mean.

### 3.1.4. Aerobic Stability of Fermented Okara

After 48 h of aerobic exposure, major changes were observed in the chemical and microbial composition of fermented okara, particularly in NH<sub>3</sub>-N and acetate concentrations and microbial proliferation (Table 6). The temperature of fermented okara remained stable and did not significantly differ over time ( $p = 0.32$ ), indicating minimal heat generation during the aerobic exposure period. The pH values ranged from 4.56 to 4.91 and did not significantly differ ( $p = 0.16$ ).

**Table 6.** Changes in the chemical composition and microbial count of fermented okara after 48 h of aerobic exposure.

| Item                                              | Time After Open (h) |                    |                    |                    | SEM  | p-value |
|---------------------------------------------------|---------------------|--------------------|--------------------|--------------------|------|---------|
|                                                   | 12                  | 24                 | 36                 | 48                 |      |         |
| Temperature above RT (°C)                         | 1.50                | 0.60               | 1.10               | 1.20               | 0.31 | 0.32    |
| pH                                                | 4.91                | 4.56               | 4.64               | 4.86               | 0.12 | 0.16    |
| NH <sub>3</sub> -N <sup>1</sup> (g/kg DM)         | 12.21 <sup>b</sup>  | 15.66 <sup>a</sup> | 14.79 <sup>a</sup> | 16.27 <sup>a</sup> | 0.63 | 0.01    |
| Lactate (g/kg DM)                                 | 32.92               | 27.20              | 23.24              | 22.88              | 6.28 | 0.23    |
| Acetate (g/kg DM)                                 | 8.81 <sup>b</sup>   | 12.65 <sup>a</sup> | 14.67 <sup>a</sup> | 14.53 <sup>a</sup> | 0.80 | <0.01   |
| Lactic acid bacteria (log CFU/g FM <sup>2</sup> ) | -                   | 9.24               | -                  | 9.27               | 0.05 | 0.72    |
| Yeast (log CFU/g FM)                              | -                   | 5.02 <sup>b</sup>  | -                  | 6.79 <sup>a</sup>  | 0.12 | 0.01    |
| Aerobic bacteria (log CFU/g FM)                   | -                   | 9.20 <sup>b</sup>  | -                  | 10.05 <sup>a</sup> | 0.29 | 0.03    |

<sup>1</sup> NH<sub>3</sub>-N = ammonia nitrogen. <sup>2</sup> FM = fresh matter. Different superscript letters indicate significant differences between weekly treatments. - Indicates not analyzed. CFU = colony-forming unit; DM = dry matter; SEM = standard error of the mean.

The concentration of NH<sub>3</sub>-N significantly increased over time ( $p = 0.01$ ), rising from 12.21 g/kg DM at 12 h to 16.27 g/kg DM at 48 h. The concentrations observed at 24, 36, and 48 h were significantly higher than that observed at 12 h. The concentration of acetate

also significantly increased from 8.81 g/kg DM at 12 h to approximately 14.5 g/kg DM at 48 h ( $p < 0.01$ ). By contrast, the concentration of lactate remained stable over time ( $p = 0.23$ ).

Microbial counts demonstrated signs of aerobic deterioration. Yeast populations significantly increased from 5.02 to 6.79 log CFU/g FM ( $p = 0.01$ ), and aerobic bacterial counts increased from 9.20 to 10.05 log CFU/g FM ( $p = 0.03$ ), indicating active microbial growth under aerobic conditions. By contrast, LAB counts remained relatively stable between 24 and 48 h (9.24 vs. 9.27 log CFU/g FM,  $p = 0.72$ ), suggesting the persistence of LAB during aerobic exposure.

### 3.1.5. In Vitro Fermentation and Digestibility of Fermented Okara

After 48 h of in vitro fermentation, differences in fermentation end-products and gas kinetics were observed among the three treatment groups: control, fresh okara (lyophilized), and fermented okara (Table 7). No significant between-group difference was observed in total gas production ( $p = 0.13$ ). The pH values remained constant across the three groups, averaging approximately 5.6, indicating stable fermentation conditions ( $p = 0.26$ ). The fermented okara group had a significantly higher  $\text{NH}_3\text{-N}$  concentration (6.71 mg/kg) than did the control (4.26 mg/kg) and fresh okara (4.06 mg/kg) groups ( $p < 0.01$ ), suggesting increased protein deamination during fermentation. No significant differences were observed in the profiles of VFAs (acetate, propionate, and butyrate) or MCP production ( $p > 0.05$ ).

**Table 7.** Fermentation products, gas kinetic parameters, and in vitro digestibility of fresh and fermented okara after 48 h of in vitro fermentation.

| Item <sup>1</sup>                           | Control           | Fresh             | FO                | SEM  | <i>p</i> -Value |
|---------------------------------------------|-------------------|-------------------|-------------------|------|-----------------|
| Fermentation Parameters                     |                   |                   |                   |      |                 |
| Gas production (mL/g DM)                    | 183.6             | 195.1             | 219.2             | 11.8 | 0.13            |
| pH                                          | 5.65              | 5.65              | 5.62              | 0.01 | 0.26            |
| $\text{NH}_3\text{-N}$ <sup>2</sup> (mg/kg) | 4.26 <sup>b</sup> | 4.06 <sup>b</sup> | 6.71 <sup>a</sup> | 0.37 | <0.01           |
| Acetate (mM)                                | 52.11             | 48.62             | 62.25             | 6.32 | 0.57            |
| Propionate (mM)                             | 24.54             | 24.04             | 27.18             | 2.45 | 0.37            |
| Butyrate (mM)                               | 1.21              | 1.10              | 1.78              | 0.15 | 0.84            |
| MCP <sup>3</sup> (mg/g DM)                  | 28.97             | 27.86             | 21.07             | 4.46 | 0.91            |
| Gas Kinetics Parameter                      |                   |                   |                   |      |                 |
| Soluble fraction a (%)                      | 1.61              | 0.92              | 1.75              | 0.62 | >0.05           |
| Degradable fraction b (%)                   | 98.40             | 99.08             | 98.25             | 0.62 | >0.05           |
| Degradable rate of b ( $\text{h}^{-1}$ )    | 0.10              | 0.10              | 0.12              | 0.01 | >0.05           |
| In Vitro Digestibility                      |                   |                   |                   |      |                 |
| IVTDMD <sup>4</sup> (%)                     | 85.76             | 88.42             | 86.83             | 2.44 | 0.70            |
| IVNDFD <sup>5</sup> (%)                     | 58.62             | 65.09             | 57.26             | 8.44 | 0.84            |

<sup>1</sup> Control = ensiled okara without incubation; Fresh = lyophilized fresh okara; FO = lyophilized fermented okara (80% moisture,  $10^6$  CFU/g inoculation concentration, 4 week fermentation). <sup>2</sup>  $\text{NH}_3\text{-N}$  = ammonia nitrogen. <sup>3</sup> MCP = microbial crude protein; DM = dry matter; SEM = standard error of the mean. <sup>4</sup> IVTDMD = in vitro true dry matter digestibility. <sup>5</sup> IVNDFD = in vitro neutral detergent fiber digestibility. Different superscript letters indicate significant differences between groups.

Regarding gas kinetics, no significant between-group differences were observed in the soluble fraction (a) or the degradable fraction (b) ( $p > 0.05$  for both). However, no significant between-group difference was observed in the values of in vitro digestibility. In addition, no significant between-group difference was observed in the degradation rate constant (c).

In terms of digestibility, IVTDMD and IVNDFD did not significantly differ between the groups ( $p > 0.05$ ). The IVTDMD values ranged from 85.8% to 88.4%, and the IVNDFD values ranged from 57.3% to 65.1%, indicating that fermentation did not negatively affect the DM or fiber digestibility of okara.

### 3.1.6. In Situ Degradation of Fresh and Fermented Okara

Table 8 presents the in situ rumen degradation kinetics of DM and CP for fresh and fermented okara. Significant between-group differences were observed in the DM degradation characteristics. The rapidly degradable DM fraction (a) was highest in the fresh okara group (30.69%), followed by the fermented okara (21.42%) and control (20.69%) groups ( $p < 0.01$ ). By contrast, the slowly degradable DM fraction (b) was significantly lower in the fresh okara group (65.73%) than in the fermented okara (78.52%) and control (79.22%) groups ( $p < 0.01$ ). No significant between-group differences were observed in the degradation rate constant ( $K_d$ ) ( $p = 0.12$ ). The ED of DM, calculated at a rumen passage rate of 0.08/h, was significantly higher in the fresh okara group (64.29%) than in the fermented okara group (58.78%,  $p < 0.01$ ), whereas the ED of DM in the control group (60.96%) did not significantly differ from that of the fresh okara group, but was significantly higher than that of the fermented okara group (58.78%,  $p < 0.01$ ).

**Table 8.** Gas kinetic parameters, and in vitro digestibility of fresh and fermented okara after 48 h of in vitro fermentation.

| Item <sup>1</sup>                        | Control            | Fresh              | FO                 | SEM  | <i>p</i> -Value |
|------------------------------------------|--------------------|--------------------|--------------------|------|-----------------|
| DM Degradation Parameters                |                    |                    |                    |      |                 |
| Rapidly degradable fraction a (%)        | 20.69 <sup>c</sup> | 30.69 <sup>a</sup> | 21.42 <sup>b</sup> | 0.96 | <0.01           |
| Slowly degradable fraction b (%)         | 79.22 <sup>b</sup> | 65.73 <sup>c</sup> | 78.52 <sup>b</sup> | 2.89 | <0.01           |
| Rate of degradation ( $K_d$ , %/h)       | 0.08               | 0.08               | 0.07               | 0.01 | 0.12            |
| Effective degradability <sup>2</sup> (%) | 60.96 <sup>a</sup> | 64.29 <sup>a</sup> | 58.78 <sup>b</sup> | 1.44 | <0.01           |
| Protein Degradation Parameters           |                    |                    |                    |      |                 |
| Rapidly degradable fraction a (%)        | 2.98               | 5.38               | 4.75               | 3.63 | 0.54            |
| Slowly degradable fraction b (%)         | 20.52              | 30.07              | 38.26              | 5.72 | 0.98            |
| Indigestible fraction c (%)              | 76.50              | 64.55              | 56.99              | 6.77 | 0.23            |
| Rate of degradation ( $K_d$ , %/h)       | 0.09               | 0.05               | 0.03               | 0.25 | 0.76            |
| RDP <sup>3</sup> (% CP)                  | 13.83              | 17.48              | 15.53              | 4.11 | 0.98            |
| RUP <sup>4</sup> (% CP)                  | 86.17              | 82.52              | 84.47              | 4.11 | 0.98            |

<sup>1</sup> Control = ensiled okara without incubation; Fresh = lyophilized fresh okara; FO = lyophilized fermented okara (80% moisture,  $10^6$  CFU/g inoculation concentration, 4 week fermentation). <sup>2</sup> Effective degradability =  $a + (b \times K_d) / (K_d + K_p)$ , where  $K_p$  is the assumed rumen particle passage rate (0.08/h).

<sup>3</sup> RDP = rumen degradable protein; CP = crude protein; DM = dry matter; SEM = standard error of the mean.

<sup>4</sup> RUP = rumen undegradable protein. Different superscript letters indicate significant differences between groups.

No significant between-group differences were observed in protein degradability. However, the fermented okara group had a numerically higher slowly degradable protein fraction ( $p > 0.05$ ) compared with the other groups. The RDP and RUP contents also did not significantly differ among the three groups.

## 3.2. Feed Utilization Trial

### 3.2.1. Gas Production and Fermentation Characteristics of Total Mixed Rations with Okara Replacement

The inclusion of fermented okara at various replacement levels (0–100%) in TMRs significantly affected several in vitro fermentation parameters at both 24 and 48 h incubation periods (Table 9). At 24 h, gas production significantly increased with 25–75% okara replacement, reaching 175.5 mL/g DM at 25% replacement ( $p < 0.01$ ). The concentration of  $\text{NH}_3\text{-N}$  was significantly higher in all fermented okara replacement groups than in the control group ( $p < 0.01$ ), indicating enhanced protein degradation or nitrogen release. The concentrations of acetate and propionate were lowest at the 25% replacement level ( $p < 0.05$ ), whereas acetate peaked at 75% (49.78 mM) and propionate peaked at 100%

(18.84 mM). No significant differences were observed in pH, butyrate concentration, or MCP production across treatments at a 25% replacement level.

**Table 9.** Gas production and fermentation products of TMR groups with different fermented okara replacement ratios.

| Item                                      | Replacement (%)    |                    |                    |                    |                    | SEM  | p-value |
|-------------------------------------------|--------------------|--------------------|--------------------|--------------------|--------------------|------|---------|
|                                           | 0                  | 25                 | 50                 | 75                 | 100                |      |         |
| 24 h <sup>1</sup>                         |                    |                    |                    |                    |                    |      |         |
| Gas production (mL/g DM)                  | 126.3 <sup>b</sup> | 175.5 <sup>a</sup> | 159.0 <sup>a</sup> | 173.2 <sup>a</sup> | 163.0 <sup>b</sup> | 6.1  | <0.01   |
| pH                                        | 5.85               | 5.81               | 5.80               | 5.87               | 5.80               | 0.02 | 0.12    |
| NH <sub>3</sub> -N <sup>2</sup> (g/kg DM) | 0.53 <sup>b</sup>  | 1.08 <sup>a</sup>  | 1.09 <sup>a</sup>  | 1.17 <sup>a</sup>  | 1.06 <sup>a</sup>  | 0.07 | <0.01   |
| Acetate (mM)                              | 32.34 <sup>a</sup> | 24.92 <sup>b</sup> | 44.86 <sup>a</sup> | 49.78 <sup>a</sup> | 46.80 <sup>a</sup> | 2.49 | 0.02    |
| Propionate (mM)                           | 12.15 <sup>b</sup> | 9.74 <sup>c</sup>  | 15.72 <sup>b</sup> | 18.09 <sup>a</sup> | 18.84 <sup>a</sup> | 0.96 | <0.01   |
| Butyrate (mM)                             | 3.03               | 2.61               | 3.24               | 4.02               | 4.14               | 0.36 | 0.72    |
| MCP <sup>2</sup> (mg/g DM)                | 19.40              | 20.13              | 23.36              | 19.60              | 13.91              | 2.50 | 0.50    |
| MCP (mg/g OM digestibility)               | 26.79              | 28.31              | 33.42              | 38.66              | 20.82              | 4.28 | 0.41    |
| 48 h <sup>1</sup>                         |                    |                    |                    |                    |                    |      |         |
| Gas production (mL/g DM)                  | 220.5 <sup>a</sup> | 228.7 <sup>a</sup> | 224.0 <sup>a</sup> | 183.5 <sup>c</sup> | 206.1 <sup>a</sup> | 5.9  | <0.01   |
| pH                                        | 5.61 <sup>c</sup>  | 5.64 <sup>c</sup>  | 5.66 <sup>c</sup>  | 5.70 <sup>b</sup>  | 5.79 <sup>a</sup>  | 0.01 | <0.01   |
| NH <sub>3</sub> -N (g/kg DM)              | 2.24 <sup>b</sup>  | 2.57 <sup>a</sup>  | 2.56 <sup>a</sup>  | 1.83 <sup>b</sup>  | 2.06 <sup>b</sup>  | 0.08 | <0.01   |
| Acetate (mM)                              | 56.14              | 58.93              | 56.37              | 44.49              | 52.55              | 2.75 | 0.08    |
| Propionate (mM)                           | 18.91              | 19.47              | 18.55              | 15.55              | 17.48              | 1.60 | 0.17    |
| Butyrate (mM)                             | 4.98 <sup>a</sup>  | 5.46 <sup>a</sup>  | 4.94 <sup>a</sup>  | 3.91 <sup>b</sup>  | 4.20 <sup>b</sup>  | 0.25 | 0.02    |
| MCP (mg/g DM)                             | 43.78              | 49.22              | 56.40              | 46.56              | 44.38              | 2.96 | 0.79    |
| MCP (mg/g OM digestibility) <sup>3</sup>  | 55.49              | 63.78              | 73.73              | 61.06              | 58.98              | 3.85 | 0.26    |

<sup>1</sup> 24 h = incubated for 24 h; 48 h = incubated for 48 h. <sup>2</sup> NH<sub>3</sub>-N = ammonia nitrogen; MCP = microbial crude protein; DM = dry matter; OM = organic matter; SEM = standard error of the mean. Different superscript letters indicate significant differences between groups.

At 48 h, total gas production remained high in the 0–50% replacement groups but significantly declined at 75% replacement ( $p < 0.01$ ), suggesting a threshold for optimal fermentability. The pH values linearly increased with higher replacement levels ( $p < 0.01$ ), with the highest pH observed at 100% replacement (5.79), indicating decreased acid production or buffering effects. The concentration of NH<sub>3</sub>-N was significantly higher in the 25% and 50% replacement groups than in both the control and the 75% replacement group ( $p < 0.01$ ). The concentration of butyrate was significantly lower in the 75% and 100% replacement groups than in the other groups ( $p = 0.02$ ). No significant differences were observed in acetate, propionate, or MCP production among treatments at 48 h ( $p > 0.05$ ).

### 3.2.2. In Vitro Gas Kinetics and Digestibility of Total Mixed Rations

Replacing soybean meal with fermented okara in TMRs significantly affected gas production kinetics (see Section 2.6), particularly the soluble and degradable fractions (Table 10). The soluble gas fraction (a) significantly differed among treatments ( $p = 0.03$ ), with the highest value observed at 50% replacement (5.91%), indicating the increased availability of rapidly fermentable components. The degradable fraction (b) significantly decreased from 82.95% in the control group to 68.32% with increasing replacement levels of okara (75%,  $p < 0.01$ ). The degradation rate of the degradable fraction (c) was significantly lower at 75% replacement in the fermented okara group ( $0.116 \text{ h}^{-1}$ ) than in the other groups ( $0.122\text{--}0.124 \text{ h}^{-1}$ ,  $p = 0.03$ ), suggesting a slower fermentation process at higher replacement levels. Consistently, the half-time for gas production was significantly longer at 75% replacement for fermented okara (6.09 h,  $p = 0.02$ ), further indicating delayed

substrate utilization. No significant differences were observed in IVTDMD or IVNDFD among the treatments.

**Table 10.** In vitro gas production kinetics of TMR groups with different soybean meal replacement ratios.

| Item                                  | Replacement (%)    |                    |                    |                    |                    | SEM   | p-value |
|---------------------------------------|--------------------|--------------------|--------------------|--------------------|--------------------|-------|---------|
|                                       | 0                  | 25                 | 50                 | 75                 | 100                |       |         |
| Soluble fraction a (%)                | 3.90 <sup>bc</sup> | 5.17 <sup>ab</sup> | 5.91 <sup>a</sup>  | 2.94 <sup>c</sup>  | 4.59 <sup>ab</sup> | 0.52  | 0.03    |
| Degradable fraction b (%)             | 82.95 <sup>a</sup> | 81.81 <sup>a</sup> | 79.80 <sup>a</sup> | 68.32 <sup>c</sup> | 72.88 <sup>b</sup> | 1.32  | <0.01   |
| Degradable rate (c, h <sup>−1</sup> ) | 0.124 <sup>a</sup> | 0.122 <sup>a</sup> | 0.122 <sup>a</sup> | 0.116 <sup>b</sup> | 0.123 <sup>a</sup> | 0.019 | 0.03    |
| Half time (h)                         | 5.57 <sup>b</sup>  | 5.52 <sup>b</sup>  | 5.68 <sup>b</sup>  | 6.09 <sup>a</sup>  | 5.66 <sup>b</sup>  | 0.11  | 0.02    |
| IVTDMD <sup>1</sup> (%)               | 78.90              | 77.17              | 76.52              | 76.40              | 75.11              | 1.28  | 0.15    |
| IVNDFD <sup>2</sup> (%)               | 42.60              | 41.11              | 42.11              | 44.53              | 43.99              | 3.09  | 0.69    |

<sup>1</sup> IVTDMD = in vitro true dry matter digestibility. <sup>2</sup> IVNDFD = in vitro neutral detergent fiber digestibility. SEM = standard error of the mean. Different superscript letters indicate significant differences between groups.

### 3.2.3. In Situ Degradation of Total Mixed Rations with Okara Replacement

Table 11 presents the in situ rumen degradation characteristics of TMRs containing varying levels of fermented okara as a replacement for soybean meal. DM degradability exhibited minimal changes at 25% and 75% replacement levels. ED, calculated at a rumen passage rate of 0.02/h, significantly differed among the treatments ( $p = 0.04$ ). The 25% replacement group had the highest ED value (81.47%), followed by the 0% (80.92%) and 75% (80.19%) groups. However, no significant differences were observed at higher assumed passage rates of 0.06/h and 0.08/h ( $p = 0.17$  and  $0.19$ , respectively), where ED values ranged from 66.66% to 71.38%.

**Table 11.** In situ rumen DM degradation kinetics of TMR groups with different fermented okara replacement ratios.

|                                                      | Replacement (%)     |                    |                    | SEM   | p-Value |
|------------------------------------------------------|---------------------|--------------------|--------------------|-------|---------|
|                                                      | 0                   | 25                 | 75                 |       |         |
| Rapidly degradable fraction a (%)                    | 43.31               | 44.43              | 44.85              | 0.60  | 0.09    |
| Slowly degradable fraction b (%)                     | 47.06 <sup>a</sup>  | 45.59 <sup>b</sup> | 44.61 <sup>b</sup> | 0.34  | 0.15    |
| Rate of degradation ( $K_d$ , %/h)                   | 0.083               | 0.087              | 0.077              | 0.007 | 0.59    |
| Effective degradability <sub>0.02</sub> <sup>1</sup> | 80.92 <sup>ab</sup> | 81.47 <sup>a</sup> | 80.19 <sup>b</sup> | 0.24  | 0.04    |
| Effective degradability <sub>0.06</sub>              | 70.23               | 71.38              | 69.84              | 0.59  | 0.17    |
| Effective degradability <sub>0.08</sub>              | 66.93               | 68.15              | 66.66              | 0.57  | 0.19    |

<sup>1</sup> Effective degradability =  $a + (b \times K_d) / (K_d + K_p)$ , where  $K_p$  is the assumed rumen passage rate (0.02, 0.06, or 0.08/h). SEM = standard error of the mean. Different superscript letters indicate significant differences between groups.

## 4. Discussion

### 4.1. Fermentation Efficacy and Preservation

#### 4.1.1. Fermentation Characteristics

In the laboratory-scale fermentation trials, the pH of all treatments decreased below 4.5 by day 12, reaching a minimum at that time point (Table 2). The group treated with  $10^6$  CFU/g at 80% moisture had the lowest pH, which dropped to 4.15 ( $p < 0.01$ ) and remained low through day 24 (4.23,  $p < 0.01$ ). This acidification process was primarily attributable to lactic acid production by *Lactobacillus* spp. [31], which creates unfavorable conditions (pH < 4.5) for undesirable microorganisms such as *Escherichia coli* and *Staphylococcus aureus* [32]. Excessive moisture can reduce porosity and increase viscosity, whereas insufficient moisture can limit substrate solubility [33]. These findings are consistent with



those of a previous study on solid-state fermentation [34].  $\text{NH}_3\text{-N}$  reflects protein and amino acid degradation [35], any reduction in its concentration over time indicates active nitrogen utilization. Unlike LAB, which rarely assimilate inorganic nitrogen for amino acid synthesis [36], *S. cerevisiae* likely contributes to  $\text{NH}_3\text{-N}$  reduction by utilizing yeast-assimilable nitrogen compounds, such as glutamine and asparagine, during alcoholic fermentation [37]. In the present study, by day 24, treatment with  $10^6$  CFU/g at 80% moisture resulted in both the lowest pH and a relatively high concentration of  $\text{NH}_3\text{-N}$ , making this the most favorable condition for scaling up okara fermentation.

During storage, the chemical composition (DM, CP, NDF, ADF) remained stable over the 8-week period (Table 4), highlighting the role of optimal fermentation in maintaining nutritional quality. However, preservation stability declined after week 4, as evidenced by rising pH, declining lactate, and increasing  $\text{NH}_3\text{-N}$  concentrations (Table 5). The increased lactic acid level at this point reflects the active proliferation of LAB and rapid acidification, which are both essential for suppressing spoilage microorganisms and maintaining silage quality [7]. Lactic acid is a primary catabolic product of pyruvate metabolism by *Lactobacillus* spp. during fermentation [31]. Its accumulation reduces environmental pH, which in turn inhibits the growth and reproduction of undesirable microbes such as *E. coli* and *S. aureus* [32]. This study confirmed that LAB fermentation effectively reduced the pH of okara, thereby enhancing its preservation potential. The pronounced pH decline observed after 4 weeks of fermentation was primarily attributable to lactic acid accumulation, consistent with findings reported by Takenaka and Echo [38]. These results support previous studies indicating that LAB fermentation can stabilize high-moisture byproducts, such as okara, when the fermentation conditions are optimized [2,8].

Overall, LAB fermentation effectively stabilized okara during the early weeks of storage, but preservation beyond 4 weeks showed signs of instability, suggesting a practical feeding window of 2–4 weeks for maintaining quality. These changes may be attributable to the depletion of available carbon sources, decreased LAB activity, or microbial contamination [39]. By weeks 6 and 8, the concentration of  $\text{NH}_3\text{-N}$  exceeded 40 g/kg DM, a threshold commonly associated with low silage quality and decreased palatability for ruminants [40]. Simultaneously, a marked increase was observed in acetate concentration and LAB counts, followed by a resurgence of yeast populations during the early stages and a subsequent decline by week 6, further supporting the occurrence of microbial succession and aerobic instability.

#### 4.1.2. Aerobic Stability

During the aerobic stability test (week 4),  $\text{NH}_3\text{-N}$  concentrations increased significantly with prolonged air exposure, peaking at 48 h, while acetate also rose markedly from 12 h onward (Table 6). Yeast and aerobic bacterial counts increased after 48 h, confirming a progressive decline in aerobic stability. Aerobic stability of silage is defined as the period after aerobic exposure during which the internal temperature of silage remains within 2 °C of the ambient temperature. Once aerobic microorganisms begin oxidizing organic acids and WSCs, carbon dioxide and water are produced, typically leading to an increase in internal silage temperature [35]. In this study, although no significant increase was observed in temperature, likely because of the high ambient temperature, the concentrations of  $\text{NH}_3\text{-N}$  and acetate significantly increased after 24 h of aerobic exposure. Yeast and aerobic bacterial counts also markedly increased after 48 h, indicating a gradual decline in silage quality during aerobic exposure. This pattern is characteristic of aerobic deterioration, in which microbial activity accelerates nutrient breakdown. The increase observed in the concentration of acetate suggests a shift from anaerobic lactic acid fermentation to aerobic metabolism, likely driven by spoilage yeast, such as *S. cerevisiae* [37]. From a practical



perspective, fermented okara can be stored anaerobically for up to 4 weeks with acceptable quality, but once opened, its aerobic stability declines rapidly within 24–48 h. To maintain nutritional value and palatability, fermented okara should be fed within 12–24 h of opening, emphasizing the need for airtight storage and timely incorporation into TMRs.

#### 4.2. Enhanced Nutritional Value of Fermented Okara

##### 4.2.1. Fermented Okara Characteristics and Digestibility for Feed Use

The comparison among control, fermented okara, and fresh okara treatments showed no significant differences in gas production, *in vitro* kinetics, IVTDMD, or IVNDFD, indicating that LAB fermentation did not negatively affect the digestibility of okara. However, the fermented okara group exhibited significantly higher  $\text{NH}_3\text{-N}$  concentrations, consistent with reports that fermentation increases protein solubility and non-protein nitrogen release [2,9]. Despite these positive findings, high  $\text{NH}_3\text{-N}$  concentrations may also reflect suboptimal microbial protein synthesis, particularly when not accompanied by adequate fermentable carbohydrate availability [13]. This inefficiency may be linked to the acetate-to-propionate (A/P) ratio, which serves as an indicator of energy utilization efficiency in the rumen. A lower A/P ratio generally corresponds to higher energetic efficiency [41]. A decrease in this ratio is associated with a reduction in  $\text{NH}_3\text{-N}$  accumulation as a result of enhanced microbial nitrogen capture [42]. Chen et al. [20] reported a negative correlation between the A/P ratio and MCP synthesis efficiency. In the present study, the A/P ratios were 2.1, 2.0, and 2.29 for the control, fresh, and fermented okara groups, respectively. The high A/P ratio observed in the fermented okara group suggests low-efficiency fermentation and nitrogen utilization. According to the literature,  $\text{NH}_3\text{-N}$  accumulation may result from an imbalance between protein degradation and carbohydrate availability, thereby limiting microbial protein synthesis [43].

Gas production is strongly associated with microbial activity and fermentation dynamics in the rumen [44]. Yang [45] reported a gas yield of 207 mL/g DM after 48 h of ensiled okara fermentation in the rumen. This value is comparable to those observed in the present study, along with similar acetate and propionate concentrations. VFAs, including acetate, propionate, and butyrate, are key end-products of the microbial fermentation of organic matter in the rumen. They serve as essential energy sources and substrates for gluconeogenesis [46]. Therefore, VFA profiles not only reflect fermentation characteristics but also have direct implications for ruminant growth and milk production. Overall, although the fermented okara group exhibited a high  $\text{NH}_3\text{-N}$  concentration, gas production and MCP synthesis did not significantly differ among treatments, suggesting stable overall fermentation efficiency and microbial growth potential. This supported the feasibility of fermented okara inclusion in dairy cow diets. Provided that adequate fermentable carbohydrates are supplied, fermented okara can serve as a stable protein source in TMRs without impairing digestibility.

##### 4.2.2. In Situ Rumen Degradability in Feed Applications

*In situ* rumen degradation kinetics revealed that fermentation significantly affected the DM degradability of okara, although its CP degradation dynamics remained unchanged (Table 8). Specifically, fermented okara exhibited a lower ED of DM compared with fresh okara (58.78% vs. 64.29%,  $p < 0.01$ ), primarily because of a reduction in the rapidly degradable fraction (a). By contrast, the slowly degradable fraction (b) was significantly higher in both the control and fermented okara groups than in the fresh okara group, suggesting that fermentation induces structural modifications into the okara matrix. Given that dietary fibers constitute approximately 40–60% of okara's dry weight, which is predominantly composed of insoluble components such as cellulose and hemicellulose [2], this increase in

fraction b can be attributed to enzymatic action, particularly  $\beta$ -glucanase production by *S. cerevisiae*, which can degrade glucans and alter fiber structure [9,31]. In addition, the sum of a + b remained high in both the control (99.9%) and fermented okara (99.6%) groups, exceeding that of fresh okara (96.4%), indicating an overall increase in the proportion of rumen-accessible substrates after fermentation. Although a shift from rapid to slowly degradable fractions may reduce the immediate availability of fermentable substrates, it may also promote more sustained nutrient release, thereby enhancing rumen stability, particularly in diets rich in rapidly fermentable carbohydrates. In the present study, the ED of fermented okara (58.78%) was comparable to that of commonly used forage ingredients, such as oat hay (59.97%) and alfalfa hay (58.89%) [47], indicating its potential as a functional fiber and protein source in dairy cow diets formulated for gradual nutrient delivery and enhanced rumen function.

In terms of protein utilization, no significant between-group difference was observed in the concentrations of RDP and RUP. This finding indicated that LAB-based fermentation preserved the protein fraction without inducing excessive proteolysis or altering the ruminal degradability profile. In situ test results revealed that both fresh and fermented okara had a higher proportion of RUP (approximately 82–86%) compared with the higher proportion of RDP (approximately 65%) found in soybean meal [48]. Notably, the high degradability of soybean meal may not be suitable for high-producing dairy cows because it increases urinary nitrogen loss [49]. This high RUP content of okara positions it as a promising source of bypass proteins, which are capable of delivering a constant amino acid supply to the small intestine of ruminants. This characteristic may help reduce feed cost and mitigate reliance on expensive animal-derived protein supplements in ruminant diets.

#### 4.3. Application of Okara in Ruminant Diets

##### Effects of Fermented Okara Replacement Levels on TMRs

Analysis of the in vitro fermentation trial results revealed that partial replacement of soybean meal with fermented okara in TMR formulations can positively affect ruminal fermentation dynamics and nutrient utilization (Table 9). Moderate inclusion of fermented okara (25–50% replacement of soybean meal) exhibited significantly increased gas production at 24 h, indicating enhanced fermentability and microbial activity [44]. This finding suggests that moderate fermented okara inclusion provides a fermentable substrate profile favorable for ruminal microbial metabolism, potentially because of the presence of lactic acid, pre-fermented carbohydrates, and reduced fiber complexity [2]. This interpretation is further supported by the VFA profiles. Although the concentrations of acetate and propionate increased with fermented okara inclusion, the highest values were observed at 50–100% replacement levels, indicating a shift toward more efficient fermentation pathways. Despite these findings, the decline observed in total gas production and VFA yield at 75% replacement at 48 h indicates that excessive replacement may impair fermentation efficiency, likely as a result of fiber recalcitrance, an imbalanced energy-to-protein ratio, or a decline in ruminal buffering capacity. In addition, the increased  $\text{NH}_3\text{-N}$  concentration observed at 24 h in all fermented-okara-containing diets indicates increased protein solubility and deamination. Although this phenomenon may increase nitrogen availability for microbial use, it also underscores the importance of balancing fermented okara inclusion with adequate fermentable carbohydrate sources to optimize microbial protein synthesis [13].

An increase in the soluble-gas-producing fraction (a) at 25–50% fermented okara replacement levels indicates that moderate inclusion enhances the availability of rapidly fermentable substrates (Table 10), likely because of the increased levels of WSCs and small peptides generated during fermentation [7]. These components may contribute to rapid initial microbial fermentation and improved energy availability for ruminal microbes

during early digestion. By contrast, the reduction in the degradable fraction (b) and degradation rate observed at 75% fermented okara replacement suggests that excessive inclusion impairs fermentability. This reduction may be attributable to an increase in structural fiber content or the accumulation of residual fermentation products that inhibit microbial access to fermentable substrates [2,9]. This interpretation is further supported by the prolonged half-time for gas production at high fermented okara levels, indicating delayed microbial activity and decreased substrate utilization efficiency [15]. Although IVTDMD and IVNDFD remained unaffected, consistent with earlier findings that okara inclusion does not impair digestibility when properly balanced [4,50]. Taken together, these results suggest that moderate fermented okara inclusion supports or enhances fermentation dynamics, whereas excessively high replacement levels ( $\geq 75\%$ ) may reduce gas production efficiency without compromising final digestibility outcomes.

In this study, the in situ DM degradation kinetics of TMRs formulated with varying levels of fermented okara (Table 11) revealed that neither the rapidly degradable fraction (a) nor the slowly degradable fraction (b) was significantly affected by fermented okara replacement. This finding indicates that fermented okara inclusion does not alter the inherent degradability characteristics of basal TMR ingredients. Research has indicated that ruminal passage rates and digestive metabolism significantly differ between high-producing dairy cows and animals with lower productivity or intake, such as dry cows or those exposed to heat stress [13]. In this study, ED at a low ruminal passage rate (0.02/h), a condition representative of slow rumen turnover, was found to be significantly higher at 25% fermented okara inclusion than at 75% fermented okara inclusion. This finding indicates that moderate fermented okara inclusion (i.e., 25%) may improve nutrient availability under conditions of decreased DM intake, supporting a degradability profile comparable to that of soybean meal-based rations.

## 5. Conclusions

LAB fermentation stabilizes okara by enhancing its aerobic stability and reducing pH, particularly under conditions of approximately 80% moisture and an inoculation level of  $10^6$  CFU/g. Although fermentation improves certain nutritional and preservation characteristics, its integration into ruminant diets must be carefully balanced with fermentable energy and nitrogen sources to prevent excessive nitrogen excretion, as indicated by increased  $\text{NH}_3\text{-N}$  concentrations. Notably, the relatively high RUP content of fermented okara highlights its potential as a valuable bypass protein source, capable of delivering a constant amino acid supply to the small intestine. From a ration formulation perspective, fermented okara can be strategically incorporated at a 25–50% replacement level for soybean meal (on a CP basis). Overall, fermented okara is a feasible alternative protein source for on-farm feeding and stably contributes to the bypass protein fraction in TMRs.

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## Article

# Production of Nutritional Protein Hydrolysates by Fermentation of Black Soldier Fly Larvae

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## Abstract

The black soldier fly (*Hermetia illucens*) has become one of the most promising alternative protein sources in the feed and food industry. The aim of this work was to utilize microbial fermentation to enhance the nutritional properties of black soldier fly larvae (BSFL) as a food ingredient for human consumption by optimizing the amino acid profile and small peptide content. Free amino acids (FAA) have a critical role in human nutrition and bioavailability. Unlike whole proteins that require enzymatic breakdown in the digestive tract, FAA are directly absorbable by the small intestine, allowing for rapid utilization in protein synthesis and metabolic functions. BSFL pastes were fermented using *Lacticaseibacillus paracasei* (PCB 030) or a mixed starter culture preparation, and results were compared to pea protein and BSFL pastes that were enzymatically hydrolyzed. The resultant hydrolyzed BSFL pastes were analyzed for free amino acids and small peptides. The *L. paracasei* PCB 030 fermented BSFL pastes yielded significantly higher amounts of free amino acids than the control or pastes fermented using a commercial starter culture (named F-LC). The increased FAA availability in fermented BSFL makes it a more efficient protein source for human consumption. The *L. paracasei* PCB 030 fermented pastes showed an increase in small peptides after three days fermentation; nearly 80% of normalized abundances of small peptides increased by over 100 times compared to day zero (before the fermentation started). Over 90% of these small peptides consisted of more than 50% hydrophobic amino acids, which may contribute to their antioxidant and antibacterial properties. This study provides a promising and industrially practical process for hydrolyzing BSFL protein to yield a functional protein hydrolysate with an enhanced nutritional profile.

**Keywords:** protein hydrolysate; black soldier fly larvae; fermentation; *Lacticaseibacillus paracasei* (PCB 030); free amino acids; small peptides

## 1. Introduction

The growth of the human population and increased global demand for food protein drives the development of sustainable and efficient protein production methods. The black soldier fly (BSF, *Hermetia illucens*), which has a higher protein content than conventional animal livestock, is a promising candidate for industrial rearing to supplement food production as it can bioconvert organic waste to protein with a high feed conversion efficiency, but a lower carbon footprint [1,2]. BSF contains a diversity of micro- and macro-nutrients, with high protein and fat content, as well as being rich in zinc, iron, and calcium among other micronutrients [3,4]. Because of this valuable nutritional profile, BSF is utilized as a feed ingredient, particularly in rearing fish, shrimp, chicken, swine and even pet animals [1]. This impressive nutritional profile, combined with the observed high feed conversion efficiency, fast growth rate, and ability to recycle organic matter led to the BSF being identified as a possible alternative food source for human consumption [5–8].

Despite the advantages from both sustainable and nutritional standpoints, negative perceptions associated with eating insects are a major barrier to its widespread adoption as food [9,10]. One way to overcome consumer acceptance challenges is to develop refined insect-based protein ingredients, rather than include them in their original form, so they can be readily included in familiar foods [11,12]. A strategy to refine insect-derived ingredients and improve their nutrient profile is to take advantage of liquid-solid-state fermentation [1,13] as a processing method. For example, fermentation of BSFL by lactic acid bacteria (LAB) was recently described by Luparelli et al. [14], with the aim to enhance its nutritional value. The extensive utilization of *Lactocaseibacillus paracasei* as a starter culture for food fermentation means it is generally recognized as safe (GRAS) by the US Food and Drug Authority (FDA) [15–17]. In addition, the proteolytic enzyme system of *Lactocaseibacillus paracasei* is well documented, including cell-envelope proteinases and multiple peptidases that hydrolyze proteins into free amino acids and peptides [18–20]. Enzymatic hydrolysis of BSFL has been carried out in order to produce nutritional ingredients such as amino acids and small peptides [21–23]. To meet the global demand for food protein, efficient utilization of high nutritional value insects, such as BSF, is required; however, most BSFL research has been focused on rearing strategies and characterization of its nutritional composition, including proteins, lipids, amino acids, and minerals. Only a few studies have explored BSFL refinement via fermentation. For example, Meng et al., fermented the BSFL with *Lactobacillus crispatus* and *Pichia kudriavzevii* in combination with neutral protease for enzymolysis [24], which mainly focused on evaluating the quality and safety of the fermented BSFL paste. Saadoun et al., fermented black soldier fly prepupae using *Lactocaseibacillus rhamnosus* and *Lactiplantibacillus plantarum* as starter cultures, and reported the in vitro antimicrobial activity of the fermented prepupae [25].

This study aimed to produce nutritional ingredients from BSF larvae (BSFL), potentially for human consumption, by hydrolyzing BSFL using a fermentation process and *Lactocaseibacillus paracasei* (PCB 030) as the starter culture. The strain *L. paracasei* PCB 030 was chosen as it was previously isolated and had been shown to cause a decrease in pH and a fermented-paste aroma, as well as proteolytic activity in preliminary screening assays (unpublished data). The *L. paracasei* PCB 030 showed a more favorable fermentation profile than the other tested candidates, including *Lactobacillus rhamnosus*, *Limosilactobacillus fermentum*, *Lactiplantibacillus plantarum*, *Saccharomyces cerevisiae*, and *Lactobacillus acidophilus*. Initially the fermentation conditions were optimized in terms of numerous parameters including the initial glucose and salt concentrations, pH, pre-treatment processes to create the BSFL paste, and the elimination of contaminating bacteria. The final product of the fermented material was assessed by monitoring the degree of hydrolysis as well as analyzing the free amino acids and small peptides in the hydrolysate. In addition, from the perspec-

tive of industrial application, we monitored dynamic changes during fermentation and established optimum fermentation conditions. This systematic investigation of procedures for hydrolyzing BSFL using *L. paracasei* PCB 030 provides a protocol for screening other bacteria with proteolytic properties and has established a foundation for functional black soldier fly-based ingredients for human consumption.

## 2. Materials and Methods

### 2.1. Chemicals and Materials

Black soldier fly larvae (BSFL; *Hermetia illucens*) were provided as frozen packages by Protenga Sdn Bhd (Senai, Malaysia), which were reared on plant-based, pre-consumer, traceable agricultural by-products that comply with EU feedstock regulations. Phosphate-buffered saline (PBS, 10 mM, pH = 7.4), sodium chloride (NaCl), sodium nitrite (NaNO<sub>2</sub>), D-(+)-Glucose, and glacial acetic acid were purchased from Sigma Aldrich (Singapore). Deionized (DI) water used in this study had a resistivity of >15 MΩ·cm. The PBS, NaCl, NaNO<sub>2</sub>, glucose, and deionized water were autoclaved before use.

Liquid Chromatography Mass Spectrometry (LC-MS)-grade methanol, acetonitrile (ACN), formic acid, water, and ammonium formate reagent as well as amino acid standards including Alanine (Ala), Arginine (Arg), Asparagine (Asn), Aspartic acid, (Asp), Glutamic acid (Glu), Glutamine (Gln), Histidine (His), Isoleucine (Ile), Leucine (Leu), Lysine (Lys), Methionine (Met), Phenylalanine (Phe), Proline (Pro), Serine (Ser), Threonine (Thr), Tryptophan (Trp), Tyrosine (Tyr), and Valine (Val) were all purchased from Sigma-Aldrich (Singapore).

*Lactocaseibacillus casei* PCB 030 was obtained from PC Biome Pte. Ltd. (Singapore). *L. paracasei* PCB 030 was routinely grown using de Man, Rogosa and Sharpe (MRS) broth and agar, which were obtained from Sigma-Aldrich (Singapore) and prepared according to the manufacturer's instructions. F-LC starter culture contained *Pediococcus acidilactici*, *Staphylococcus xylosus*, *Latilactobacillus curvatus* (Chr Hansen, Hørsholm, Denmark) was used according to the manufacturer's instructions, i.e., adding 25 g of the starter culture powder per 100 kg BSFL paste.

### 2.2. BSFL Paste Preparation

Frozen BSFL were thawed and then washed with tap water three times and steamed for 60 min prior to being pressed to separate the exoskeleton and endoskeleton that were then mixed homogenously to form the BSFL paste. The water content of the samples was analyzed by weighing the mass before and after freezing and drying.

Overnight growth of *L. paracasei* PCB 030 in MRS broth was harvested by centrifugation (2000 rpm, 5 min) and washed twice with sterile PBS (pH = 7.4) solution and resuspended in the PBS prior to use. Aliquots (30 g) of BSFL paste were mixed with NaCl/NaNO<sub>2</sub> and glucose to yield the final concentrations, 2.8 wt%/0.015 wt% and 2.5 wt%, respectively. Acetic acid (300 µL) was added to adjust the pH of the mixture, which was then homogenized by vortexing, before the addition of *L. paracasei* PCB 030 (5 mL; final concentration was 7.5 log CFU/g) washed suspension (the total volume of *c.a.* 41 mL). The BSFL paste with the same amount of PBS but no *L. paracasei* PCB 030 was used as the control. The mixtures were aliquoted into 15 mL falcon tubes. The lids on the tubes were tightly closed prior to incubation at 37 °C. Samples were collected at 0, 1, 2, 3, and 7 d of fermentation and all the fermentation experiments were carried out in triplicate. The F-LC fermented BSFL paste was prepared with the same conditions, except that it was dosed at 25 g per 100 kg BSFL paste; final concentration was over 11 log CFU/g.

### 2.3. Online pH Measurement

Online pH measurements were carried out using pH sensors (Vernier, Beaverton, OR, USA) inserted into the BSFL paste mixture. The pH sensors and 15 mL falcon tubes were sealed with parafilm and kept at 37 °C using metallic thermal beads in an Elite Dry Bath Incubator (Major Science, Saratoga, CA, USA). The pH was recorded automatically every 5 min by connecting with the data-collection interface LabQuest® 3 (Vernier, USA) during the fermentation process.

### 2.4. Viable Counts

Viable counts of bacteria in the fermented BSFL paste mixture were determined using the standard plate count method. Briefly, after the sample collection at different time points, 0.5 mL of samples were added to 4.5 mL sterile PBS and then vortexed for 30 s prior to 10-fold serial dilutions in PBS. Aliquots of 5 µL of appropriate dilutions were plated in triplicate on MRS agar plates and incubated at 37 °C for 48 h after drying. Colonies were counted and analyzed on a log plot of colony forming units (CFU) per mL.

### 2.5. *L. paracasei* PCB 030 Fermented BSFL Paste Morphology

FESEM (Hitachi High-Tech, Singapore) was used to study the morphology of *L. paracasei* PCB 030 fermented BSFL paste at 3 d. The sample was fixed in 2.5% glutaraldehyde at 4 °C overnight followed by dehydration in a graded concentration ethanol series (25–100%) and dried at room temperature for 24 h. The dried samples were then coated with platinum before imaging.

### 2.6. Degree of Hydrolysis

The degree of hydrolysis (DH) was determined as the ratio of 10% trichloroacetic acid (TCA) soluble nitrogen to total nitrogen in the sample before fermentation according to the method of Hoyle and Merritt (1994) with some modification [26]. Briefly, 0.9 mL samples were added to 0.9 mL of 20% TCA and the mixture was vortexed before being stored in a fridge at 4 °C for 30 min. The mixture was then centrifuged at 10,000 rpm at 4 °C for 10 min before filtering. Aliquots (0.5 mL) of the filtrate were added into 19.5 mL Deionized H<sub>2</sub>O for nitrogen measurement using a total organic carbon analyzer (TNM-L, Shimadzu, Kyoto, Japan). To measure the total nitrogen content, 100 mg un-fermented and dried BSFL samples were completely hydrolyzed in 5 mL of 6 N HCl at 110 °C for 24 h [27]. After the hydrolysis with HCl, the suspension was filtrated before nitrogen analysis.

The DH was calculated as:

$$\text{DH} = (\text{TCA soluble N}) / (\text{Total N}) \times 100\%$$

### 2.7. Quantification of Free Amino Acids

After fermentation, collected samples (1.0 mL) were mixed with 4.0 mL methanol to extract the free amino acids by vortexing vigorously prior to being centrifuged at 10,000 rpm for 10 min. The supernatant was collected (250 µL), completely dried by a vacuum concentrator system with a −84 °C cold trap then reconstituted in 1000 µL of 80%/20% acetonitrile/H<sub>2</sub>O. Aliquots (150 µL) of the solution were syringe filtered (0.22 µm) before liquid chromatography-mass spectrometry (LC-MS) analysis. The calculation of the free amino acids (in mg/kg) was based on the dry mass of the BSFL paste.

LC-MS analysis was performed following the protocol by Andrea et al. [28], with some modification; a Waters UPLC BEH Amide column (Waters Corporation, Milford, MA, USA) (1.7 µm, 2.1 × 100 mm) was used in the LC-MS. The mobile phase was composed of 30% acetonitrile + 70% H<sub>2</sub>O + 0.1% formic acid + 10 mM ammonium formate (eluent A) and 95% acetonitrile + 5% H<sub>2</sub>O + 0.1% formic acid + 10 mM ammonium formate (eluent B). The

flow rate was set at 0.5 mL/min, with an injection volume of 5  $\mu$ L, column temperature 45 °C and sample temperature 4 °C. Detection was performed using a Waters Xevo TQ-S (Milford, MA, USA) mass spectrometer and the parameters settings were as follows: the ESI source was in positive ionization mode, capillary voltage 3.0 kV, source temperature 150 °C, desolvation temperature 500 °C, cone gas flow 150 L/h, and desolvation gas flow 1000 L/h. Calibration was performed with standard amino acid solutions in the range of 10 nM to 10,000 nM.

## 2.8. Small Peptides Analysis by LC-MS/MS

Fermented BSFL samples (1.0 mL) were mixed with 4.0 mL methanol to extract the small peptides by vortexing for 30 s and centrifugation at 10,000 rpm for 10 min. Supernatant was collected (250  $\mu$ L) and completely dried before being reconstituted with 1000  $\mu$ L of 0.1% formic acid. A 150  $\mu$ L sample was filtered before injection into LC-MS/MS.

LC-MS/MS was performed by Singapore Phenome Centre (SPC, Singapore) at NTU using an ACQUITY ultra-performance liquid chromatography and quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF MS) system. Furthermore, 20  $\mu$ L samples were injected into a Phenomenex bioZen XB-C18 column (Phenomenex Inc., Torrance, CA, USA) (1.7  $\mu$ m, 2.1  $\times$  150 mm) which was operated at 60 °C and the flow rate was 0.2 mL/min. The total run time was 100 min with mobile phase A (water + 0.1% formic acid) and mobile phase B (acetonitrile + 0.1% formic acid). The gradient during the run time window was 98% A + 2% B from 0 to 55 min, 60% A + 40% B from 55 to 85 min, 5% A + 95% B from 85 to 96 min, 98% A + 2% B from 96 to 100 min. The separated small peptides were injected into the Xevo G2-XS QTOF (Waters, MA, USA) with the standard ESI source in positive mode, with 3.0 kV capillary voltage, 150 °C source temperature, 350 °C desolvation temperature, 25 L/h cone gas flow, and 800 L/h desolvation gas flow. MS spectra had a *m/z* range of 50–2000 Da with a scan time of 0.2 s. After acquisition, the MS spectra were uploaded into Progenesis QI v3.0 software for data pre-processing with a *Hermetia illucens* database (UniProt). Since no calibration with peptide standards was conducted, this method provides relative abundance data based on ion intensity rather than absolute quantification for the small peptide concentrations.

## 2.9. Fat Content Measurement

Fat content was measured with a HCl Hydrolysis System (ANKOM) and Extractor (ANKOM). Briefly, samples were completely dried before being added into filter bags containing diatomaceous earth. The filter bags were then sealed and put into a hydrolysis vessel with 500 mL of 3N HCl at 90 °C for 60 min. The filter bags were rinsed with sufficient water and dried at 110 °C for 3 h to completely remove the HCl residue. Hexane was used to extract the fat content at 90 °C for 60 min. The fat content was calculated based on the mass change after the whole process and drying.

## 2.10. Ash Content Measurement

The freeze-dried samples were put into crucible and then placed in a muffle furnace and burned at 550 °C for 0.5 h until ash was formed. The ash content was calculated by weighing the mass before and after the burning process.

## 2.11. Statistical Analysis

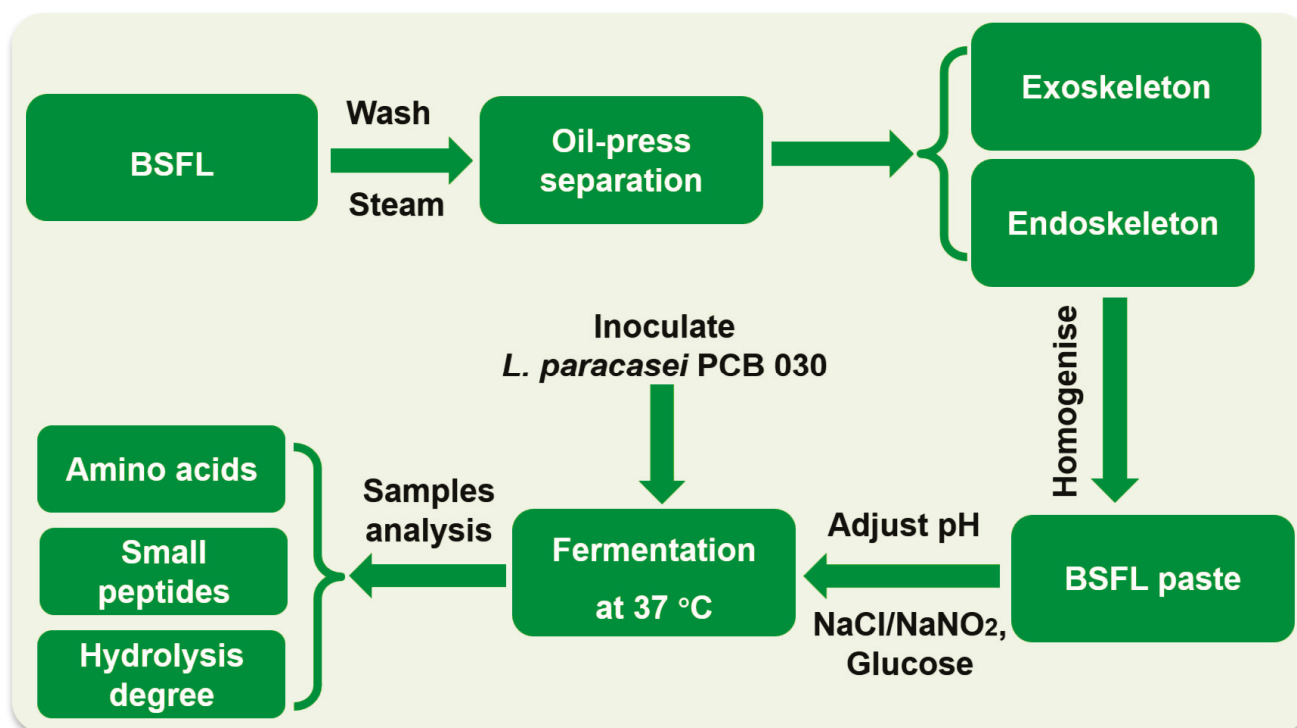
All the results are presented as the mean  $\pm$  standard error from triplicate samples. Data was analyzed using a one-way ANOVA test, with *p* < 0.05 considered statistically significant.



### 3. Results and Discussion

#### 3.1. BSFL Pre-Processing and Fermentation Condition

The pre-treatment and fermentation of BSFL paste is illustrated in Figure 1. The washing and steaming of BSFL was performed sequentially to remove solid residues (such as soil, stone, or wood, etc.) and contaminating bacteria. For better efficiency of fermentation and industrial application, the BSFL were pressed using a mechanical oil press and the endoskeleton and exoskeleton parts of BSFL were separated. Figure S1 shows the fat and ash contents of BSFL of the endoskeleton, exoskeleton and the mixtures. The ash content of the endoskeleton and exoskeleton, 11.6 wt% and 8.8 wt%, respectively, were similar ( $p = 0.008$ , two-sample  $t$ -test). However, the fat content of the endoskeleton (32.9 wt%) was much higher than that of the exoskeleton (7.2 wt%) ( $p = 0.0004$ , two-sample  $t$ -test), resulting in an overall fat content in larval paste of 26.4 wt%. These results are consistent with previously reported values [29,30]. The mixed endoskeleton and exoskeleton BSFL paste was then subjected to fermentation. Glucose was added to accelerate the fermentation process as the BSFL paste contains limited fermentable sugar [31,32]. After glucose and salt were added, the pH was adjusted, and the mixture was inoculated with *L. paracasei* PCB 030 (initial cell density was  $\sim 7.5$  Log CFU/mL), the BSFL paste was fermented, and samples were collected on specific days. During the fermentation process, vigorous bubbling was observed (Figure S2b), presumably  $\text{CO}_2$  produced during the fermentation, which resulted in a fermented BSFL protein hydrolysate with a porous powder form after freeze drying (Figure S2c).



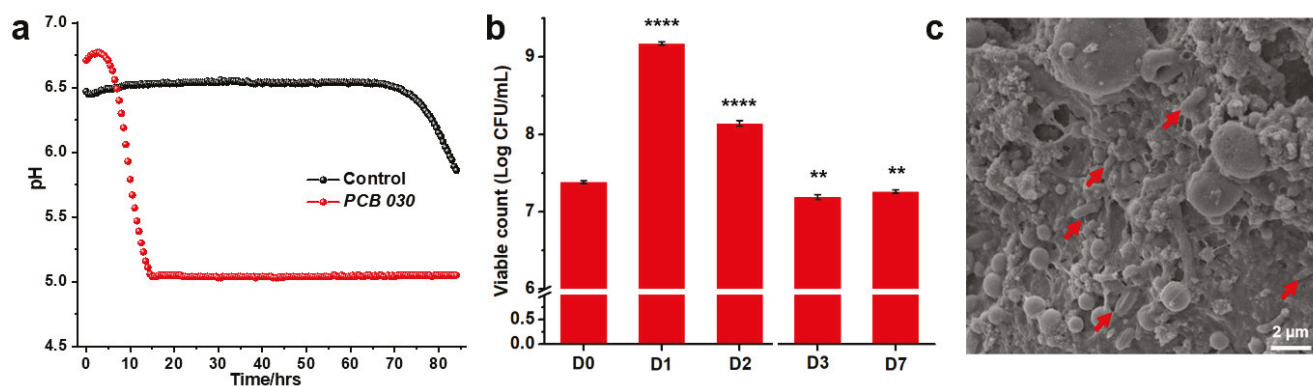
**Figure 1.** Schematic diagram of the pre-treatment and fermentation of the black soldier fly larvae (BSFL) and subsequent analyses. The BSFL was washed and steamed before the exoskeleton and endoskeleton parts were separated with an oil-press machine. After mixing to obtain a homogenous BSFL paste, the *L. paracasei* PCB 030 was inoculated as the starter culture.

#### 3.2. Phenotypic Characterizations of the Fermentation Process

It is essential for *L. paracasei* PCB 030 (or other fermenting organisms) to produce fast acidification to inhibit growth of pathogens and spoilage microbes [33–35]. The pH



changes in the BSFL paste during the fermentation period is shown in Figure 2a. Upon inoculation of *L. paracasei* PCB 030, the pH declined from *c.a.* 6.7 to *c.a.* 5.0 within 16 h and stayed constant at pH5 for the remainder of the experiment. However, the pH of the control sample was *c.a.* 6.5 over 65 h which indicates that there was no fermentation and hence no contamination by fermenting bacteria after the pre-treatment of the BSFL. The rapid decrease in pH after inoculated with the *L. paracasei* PCB 030 would benefit the fermentation process. In addition, the pH value dropped to 5.0 at 1 d, this is regarded as an essential threshold from a food safety perspective [32]. Lactobacilli selective MRS agar was used to enumerate the viable *L. paracasei* PCB 030 during fermentation. The initial *L. paracasei* PCB 030 loading was 7.4 Log CFU/mL (Figure 2b) while the initial viable count was not detectable (below 2.6 Log CFU/mL) for the control (Figure S3a,b), which implies again that the steaming pre-treatment was effective to eliminate the contaminating bacteria. The *L. paracasei* PCB 030 concentration rapidly increased to 9.2 from 7.4 Log CFU/mL after one day of incubation, the doubling time was calculated to be 4.04 h. The viable count dropped down to 8.1, 7.2, and 7.3 Log CFU/mL at D2, D3, and D7, respectively. The maximum viable count of 9.2 Log CFU/mL during the fermentation was very close to the work reported by Borremans et al. [36]. The volume of the BSFL paste expanded and formed a sponge-like paste during the fermentation process (Figure S3c), which may be explained by the production of carbon dioxide as a by-product of the carbohydrate metabolism of *L. paracasei* PCB 030 [37]. This observation is consistent with the pH drop as shown in Figure 2a. During fermentation, rod-shaped *L. paracasei* PCB 030 cells were also observed on the surface of the BSFL paste using a field emission scanning electron microscope, which suggested an interaction between the inoculum and BSFL protein (Figure 2c), resulting in the production of BSFL protein hydrolysate.



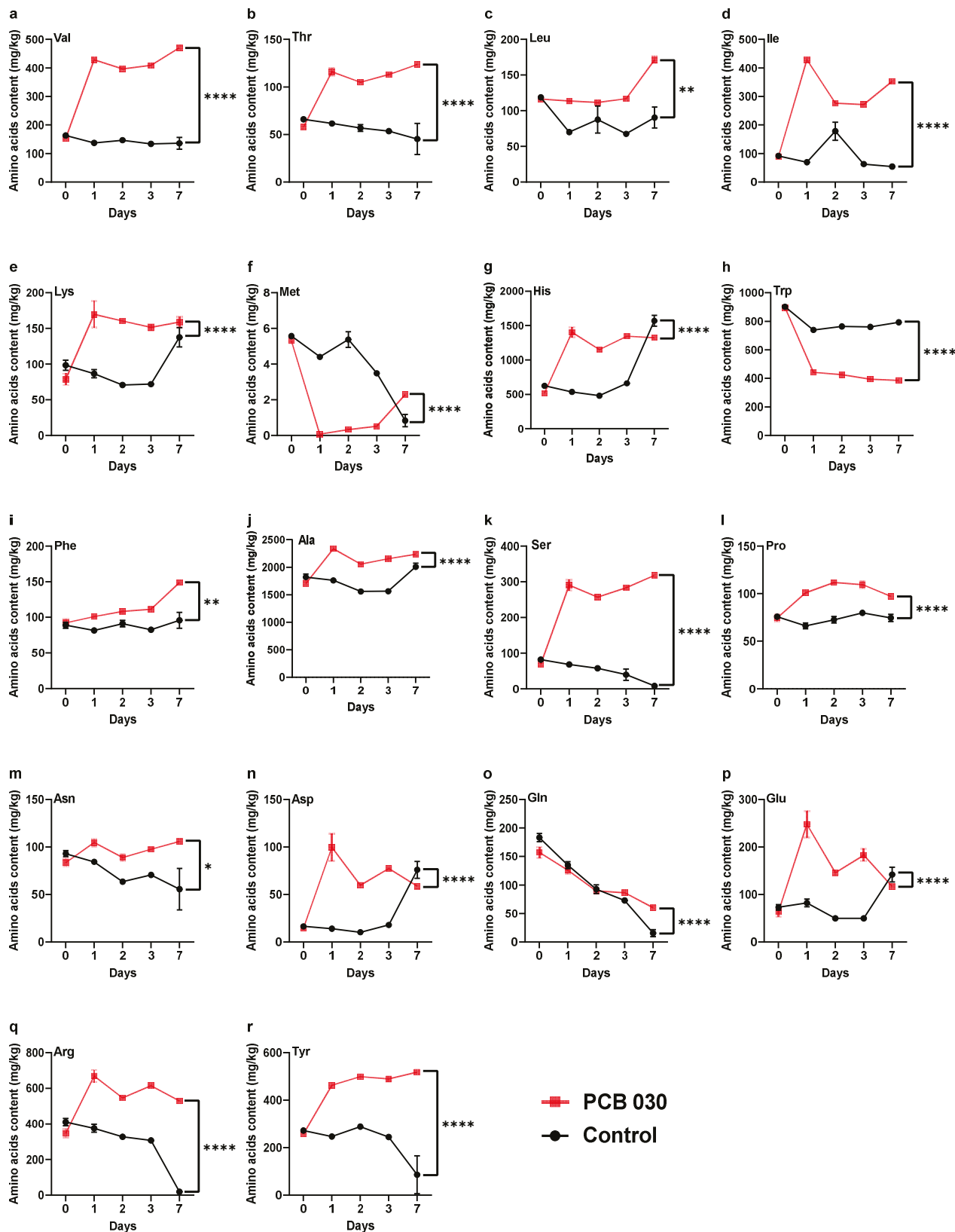
**Figure 2.** Characterizations of the fermentation process of black soldier fly larvae (BSFL). (a) pH changes of the BSFL paste after inoculating with *L. paracasei* PCB 030 (Log CFU/mL is around 7.5). (b) Viable counts of the BSFL paste at the start of the experiment (0 d) and at 1, 2, 3, and 7 d of fermentation. Each data point is expressed as the mean and standard error of triplicate samples. Significant differences were determined using unpaired t-tests, with each time point compared to day 0 (D0). Results are reported as \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ . (c) Field Emission Scanning Electron Microscope image of the *L. paracasei* PCB 030 fermented BSFL paste, the red arrows indicate rod-shaped cells.

### 3.3. Hydrolysate Assessment

The profile of the free amino acids in the BSFL protein hydrolysate and the control from 0 to 7 d was examined with Ultra-Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS) and the results are presented in Figure 3. The free amino acids (AA), including essential amino acids (EAA) and non-essential amino acids (non-EAA), increased during the first day of fermentation, except leucine (Leu), methionine (Met), tryptophan (Trp), and glutamine (Gln). The rapid increase in the AAs is an indication of the high activity of the

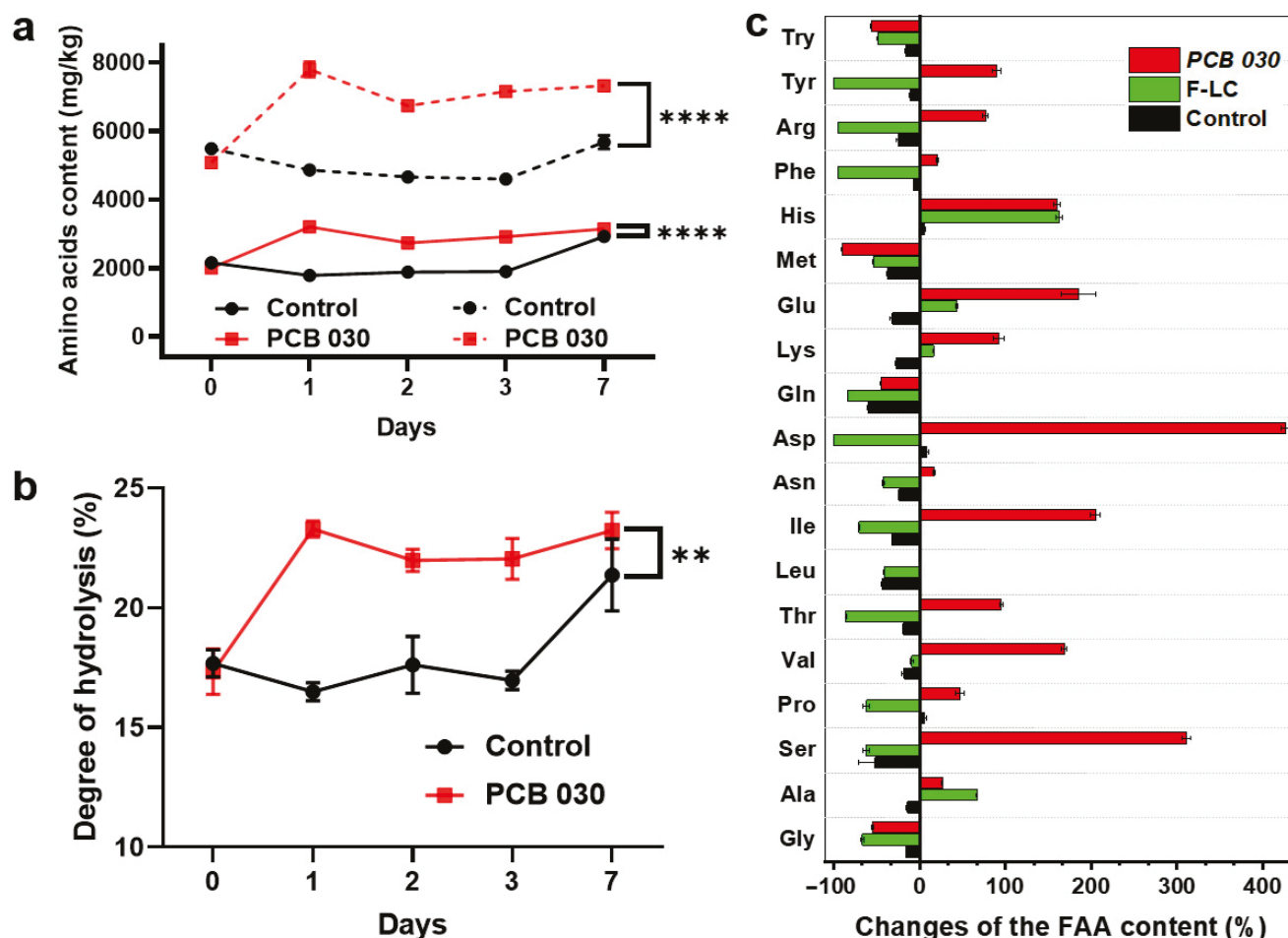
*L. paracasei* PCB 030 metabolism, which is consistent with the observation of pH decrease and viable count increase within one day (Figure 2a,b). Some of the EAA, e.g., valine (Val), isoleucine (Ile), lysine (Lys), and histidine (His) significantly increased 223.2%, 381.0%, 161.1%, and 171.0%, respectively. For the non-EAA, serine (Ser), aspartic acid (Asp), and glutamic acid (Glu) increased by 322.0%, 578.4%, and 287.1%, respectively, after one day of fermentation with *L. paracasei* PCB 030. Most of the free amino acid contents of the control remained relatively unchanged over 0–3 d of fermentation, some of the free amino acids content decreased after fermentation, such as Lys, His, Asn, Gln, etc. In addition, the amino acids contents of the control had not significantly changed in the first three days, compared to the *L. paracasei* PCB 030 fermented samples. It has been shown that the free amino acids of Asp and Glu contribute to the umami taste in fermented meat products [33,38]. The increase in these FAA contents after fermentation would benefit their acceptance and consumption as human food. The total free essential amino acids (TFEAA) and total free amino acids of the *L. paracasei* PCB 030 fermented BSFL pastes showed the same trend of change as a function of fermentation time (Figure 4a).

The degree of hydrolysis (DH) is considered as the percentage of peptide bonds of the cleaved protein [26]. As shown in Figure 4b, the DH increased from 17.3% to 23.3% after one day of fermentation with *L. paracasei* PCB 030 with no further increase detected after three days of fermentation, while the control retained its DH at 16.5–17.7% during the first three days of fermentation. This observation indicates that there was limited BSFL protein hydrolysis in the control and the increased DH in the presence of *L. paracasei* PCB 030 is due to bacteria hydrolysis. In addition, the change of the degree of hydrolysis and total free amino acids content is consistent (Figure 4a,b). A notably increased DH at 7 d in the control was observed, which likely results from (a) the residual endogenous protease activity that hydrolyzes proteins during incubation [39]; (b) prolonged incubation at 37 °C would induce protein denaturation and structural breakdown, and hence unfolded proteins would be more susceptible to hydrolysis. A limited change of the DH and TFAA at 3 d until 7 d fermentation was observed. Therefore, 3 d was considered the optimal fermentation in this study. Three days fermentation of BSFL paste using F-LC as a starter culture was also investigated as a benchmark. It was used at the dosage recommended in sausage production. The changes of free amino acids before and after fermentation were compared and are shown in Figure 4c. At 3 d fermentation with F-LC as the starter culture, 11 of the 19 free amino acids decreased by more than 50%. This decrease is likely due to the mixed culture rapidly assimilating and catabolizing extracellular amino acids to support their growth and aroma formation, while providing limited proteolysis of BSFL proteins. *Staphylococcus xylosus*, for example, is known to actively catabolize free amino acids, particularly branched-chain amino acids (Val, Leu, Ile), into aroma precursors through catabolism pathways [40]. *Pediococcus acidilactici* and *Lactobacillus curvatus* also contribute to amino acid uptake for growth [41,42]. In contrast, when *L. paracasei* PCB 030 was used as the starter culture, only three of the nineteen free amino acids decreased by more than 50%, while six out of nineteen free amino acids increased over 100%, including Ser, Val, Ile, Asp, Glu, and His. Most of the free amino acids in the control have no significant change in their contents after three days of fermentation with the F-LC starter culture. The DH of the F-LC fermented BSFL protein hydrolysate showed a decrease from 22.7% to 20.3% (Figure S4). When comparing the results for *L. paracasei* PCB 030 with those obtained when F-LC was used, the FAA and DH results provided evidence that the *L. paracasei* PCB 030 plays an important role in hydrolyzing the BSFL protein during the fermentation process.



**Figure 3.** Free amino acids profile of black soldier fly larvae (BSFL) paste during seven days of fermentation. Essential free amino acids and non-essential amino acids (mg/kg, based on the dry BSFL paste) in the BSFL paste inoculated with *L. paracasei* PCB 030 (red curves) and the control (black

curves). Essential amino acids include (a) valine, (b) threonine, (c) leucine, (d) isoleucine, (e) lysine, (f) methionine, (g) histidine, (h) tryptophan, and (i) phenylalanine; non-essential amino acids include (j) alanine, (k) serine, (l) proline, (m) asparagine, (n) aspartic acid, (o) glutamine, (p) glutamic acid, (q) arginine, and (r) tyrosine. Samples were collected at the start of the experiment (0 d) and at 1, 2, 3, and 7 d of fermentation. Each data point is presented as the mean and standard error of triplicate parallel fermentations. Significant differences relative to the control are reported as \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ . Amino acids are represented as a three-letter code according to the IUPAC-IUB. Detailed data can be found in Table S1.



**Figure 4.** Total free amino acids content and degree of hydrolysis of black soldier fly larvae (BSFL) protein hydrolysate at different fermentation days when fermented with either *L. paracasei* PCB 030 or F-LC commercial starter culture. (a) Total essential free amino acids (TEFAA) and total free amino acids (TFAA) content (mg/kg, based on the dry BSFL paste) in the *L. paracasei* PCB 030 fermented BSFL paste and the control (no added bacteria) at the start of the experiment (0 d) and at 1, 2, 3, and 7 d of fermentation. (b) The degree of hydrolysis of the *L. paracasei* PCB 030 fermented BSFL paste and the control at the start of the experiment (0 d) and at 1, 2, 3, and 7 d of fermentation. (c) Relative change of free amino acids content after 3 d fermentation by *L. paracasei* PCB 030 and F-LC. Each data point is expressed as the mean and standard error of triplicate parallel fermentations. Significant differences relative to the control are reported as \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .

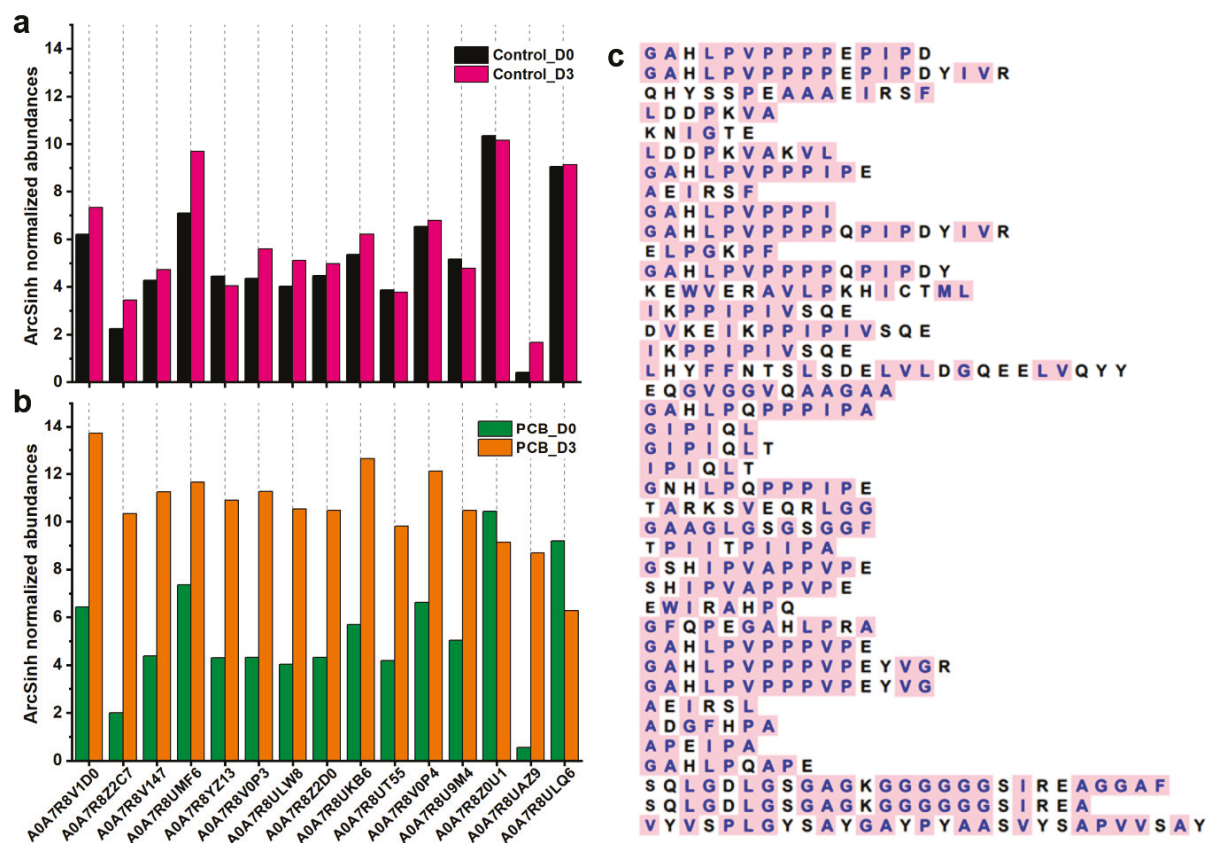
### 3.4. Small Peptides Analysis

*L. paracasei* PCB 030 fermented samples and control samples at 0 and 3 d were selected for the small peptides analysis using a UPLC/Q-TOF-MS system. Peptides were identified using Progenesis QI v3.0 data analysis software and *Hermetia illucens* database in UniProt. The relative abundance of small peptides identified is shown in Figure 5 and their detailed information is listed in Table S2. These peptides have between 6 and 29 amino acids,





three days. Most of the small peptide ions in the *L. paracasei* PCB 030 3 d fermented BSFL paste show a significantly higher abundance compared to those of the control. (a) Control fermentation at day zero (D0) and day three (D3); (b) *L. paracasei* PCB 030 at zero and three days fermentation. \* represents where there were no related peptides for the samples. Data is presented as the mean of ArcSinh normalized abundances of average normalized abundances of triplicated fermented samples with  $p < 0.01$ . Detailed information, including score, ANOVA (p), fold,  $m/z$ , and retention time, etc., can be found in Table S2.



**Figure 6.** The hypothetical protein accession number and the amino acids sequence of the detected peptide ions. The relative average normalized abundances of hypothetical proteins used in quantitation of the small peptides for the control (a) and *L. paracasei* PCB 030 fermented BSFL (b) paste at the start of the experiment (D0) and after three days (D3) of fermentation. Data is presented as the mean of ArcSinh normalized abundances of average normalized abundances of triplicated samples. Detailed information, including score, Anova (p), fold, and protein source description, can be found in Table S3. Database: OS = *Hermetia illucens* and OX = 343,691 in UniProt. (c) The amino acid sequence of the small peptides, the hydrophobic amino acids are highlighted with pink boxes and blue letters.

#### 4. Conclusions

This work demonstrates an effective method for hydrolyzing BSFL protein by fermentation with *L. paracasei* PCB 030. Inoculation with around 7.5 log CFU/mL *L. paracasei* PCB 030, with the addition of glucose and salts, and a suitable pH (6.2–6.8), results in fermentation with rapid acidification and production of gas bubbles resulting in BSFL protein hydrolysate with a porous powder form after freeze drying. After one day of fermentation, the essential amino acids, such as Val, Lys, and His, significantly increased from  $152.23 \pm 2.68$  to  $429.03 \pm 3.68$ ,  $89.22 \pm 1.49$  to  $429.15 \pm 2.69$ ,  $78.54 \pm 7.89$  to  $169.74 \pm 18.60$ , and  $517.60 \pm 25.80$  to  $1402.45 \pm 68.76$  mg/kg (based on the dry BSFL paste), respectively, while the control samples had no significant change in the content of amino acids. The *L. paracasei* PCB 030 fermented BSFL protein hydrolysate shows a better performance than



that of commercial starter cultures (F-LC) used at recommended dosage rates when compared in terms of amino acid production. Six of nineteen free amino acids increased their concentration more than 100% after fermentation with *L. paracasei* PCB 030, but none of the free amino acids increased in the F-LC fermented BSFL. In addition, over 79.3% of small peptides at 3 d notably increased in their relative abundances by over 10,000% for the *L. paracasei* PCB 030 fermented BSFL protein hydrolysate, compared to those in the extracts of samples without fermentation. Furthermore, most of these small peptides comprised over 50% of hydrophobic amino acids in their sequencing, the function of these peptides requires further investigation. Our study provides a method for efficient fermentative BSFL hydrolysis and a steppingstone for the development of sustainable, functional and nutritionally enhanced BSFL protein hydrolysate ingredients.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fermentation11090524/s1>, Figure S1: The chemical content of the BSF larvae samples. The paste refers to the homogenous mixture of the exoskeleton and endoskeleton. Results expressed as percentage (%) of total dry weight. Figure S2: Digital photos of (a) raw BSFL before steaming, (b) BSFL paste in 50 mL tubes during fermentation, and (c) freeze-dried BSFL powder after fermentation; Figure S3. Digital photos show the viable counts on MRS agar plates of (a) control and (b) PCB 030 fermented BSFL paste at the start of experiment (0 D). (c) Digital photo of the BSFL paste (*c.a.* 7 mL) fermented with PCB 030 (left two tubes) where the BSFL paste is sponge-like at 3 D, while the control (right two tubes) is not; Figure S4: The degree of hydrolysis of the PCB 030 and F-LC fermented BSFL paste and the control at the start of the experiment (D0) and after 3 days of fermentation. Each data point was expressed as the mean and standard error of triplicate parallel fermentations; Figure S5: (a) Kinetic OD@600 nm change of the PCB 030 in MRS broth at 37 °C for 24 h using Tecan Infinite M200 Plate Reader. (b) Microscope (Zeiss Primostar) image of PCB 030 after gram staining. Figure S6. The 2D montage of *m/z* vs. retention time of different peptide ions used in quantitation of normalized abundances for the samples of the control and PCB 030 fermented BSFL paste at the start of the experiment (D0) and after 3 days of fermentation; Table S1: Free essential amino acids content (mg/kg, based on the dry BSFL paste) of larvae paste inoculated with PCB 030 as the starter culture and incubated at 37 °C. Samples were collected at the start of the experiment (D0) and after 1, 2, 3, and 7 days of fermentation; Table S2: The average normalized abundances of selected peptide ions (with their corresponding amino acids sequence) used in quantitation; Table S3: The average normalized abundances of small peptides accession to their corresponding proteins. The relative quantitation using Hi-3, Hypothetical protein OS = *Hermetia illucens* OX = 343,691. Database: UniProt.

**Author Contributions:** Conceptualization: Y.W., P.L.C. and L.W.; methodology: P.Z., K.S. and R.S.; supervision: Y.W., P.L.C., R.J.C. and L.W.; writing—original draft: P.Z.; writing—review and editing: P.Z., Y.W., P.L.C., R.J.C., L.W., K.S. and R.S.; project Administration, Y.W. and P.L.C.; funding acquisition, P.L.C. and Y.W. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** Author Leo Wein was employed by the company Protenga Pte Ltd. Author Rachel Steven was employed by the company Protenga Sdn Bhd. Author Patricia L. Conway was employed by the company PC Biome Pte Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Article

# Fermentation Performance of Epigenetically Modified Yeast

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## Abstract

This study investigates the impact of epigenetic modification on *Saccharomyces cerevisiae* using sodium butyrate (SB), a histone deacetylase inhibitor (HDACi), to enhance sensory characteristics in beer fermentation. Epigenetics offers a non-GMO approach to modifying gene expression, with potential for cost-effective strain development in brewing. A commercial ale yeast was cultured under different SB exposure regimes and used to ferment wort. Sensory evaluation was conducted with untrained participants, alongside GC-MS and enzymatic assays for ethanol, glycerol, and residual sugars. While no significant differences were found in ethanol production or smoothness and creaminess—likely due to uniform wort composition—flavor and taste scores varied between treatments. Notably, yeast pre-treated with SB but fermented without additional SB (1G W/O) received the highest flavor acceptability. Treatments involving SB during fermentation showed reduced sensory scores, likely due to butyric off-notes. Higher alcohol levels remained within acceptable thresholds and were more likely influenced by wort amino acid content than epigenetic modification. Though SB had a limited impact on metabolic pathways, this study highlights the feasibility of using dietary epigenetic modifiers to develop novel yeast strains with improved sensory profiles in beer or other fermented beverages and warrants further investigation with alternative compounds.

**Keywords:** fermentation; microbial epigenetics; yeast; beer brewing

## 1. Introduction

Epigenetics is the study of phenotypic changes that occur through the alteration in gene expression in organisms. The heritable changes in gene expression predominantly occur through changes in the chromatin structure, more specifically altering the nucleotides and histones present in the chromatin structure. These gene expression mechanisms do not alter the DNA sequence in the organism; hence, epigenetics is considered as a non-GMO approach [1]. DNA methylation, a well-researched mechanism, mainly occurs by the transferring of a methyl group in at the 5th position of the pyrimidine ring of the cytosine residue [2]. Histone modification usually occurs at the amino acids of the N-terminal tails of histone proteins by the addition or removal of an acetyl group to the lysine residue. The addition of the acetyl group is known as acetylation while the removal of the acetyl group is called deacetylation. The post-translation modifications of histone proteins are controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs) [1].

*Saccharomyces cerevisiae* is a eukaryotic organism and is an ideal model for genetic analysis due to its small genome, both haploid and diploid genome, and its rapid cell cycle [3]. DNA methylation is extensively present in both plants and animals, and its absent



presence in *S. cerevisiae* has led to histone modification, which is the epigenetic regulatory mechanism in this organism [4].

Epigenetic modifiers can modify genetic material to bring forth or suppress specific phenotypes in target organisms. Epigenetic modifiers are abundant and available as dietary nutrients in many foods. Many of these compounds are available as methyl donors, amino acids, vitamins, minerals, phytochemicals, and fatty acids [5]. This study focuses on sodium butyrate (SB), a fatty acid produced in the intestinal lumen by microbial fermentation. Sodium butyrate has been linked to many clinical studies [6] and has been shown to act as an HDAC inhibitor [7]. It hyperacetylates histones and loosens the chromatin structure, increasing accessibility of DNA and activating gene expression [8].

Consistent with previous work [9], sodium butyrate is a potent HDAC inhibitor in *S. cerevisiae*, capable of inducing histone modifications and altering phenotypes without DNA sequence changes. Kong et al. (2022) [9] investigated the HDAC inhibition capability of different HDAC modifiers. The study revealed that sodium butyrate had the highest HDAC inhibition of 17.6%, following curcumin. Sodium butyrate was chosen in this project due to its solubility and its cost-effectiveness [10]. Curcumin, in comparison, has poor solubility [11] and is prone to precipitate. Hence, sodium butyrate was chosen as the epigenetic modifier in this study.

This study aims to investigate and possibly develop a new *S. cerevisiae* strain with desirable sensory characteristics for brewing beer by treating the yeast with an epigenetic modifier, sodium butyrate. Sodium butyrate is a known HDACi and, therefore, was selected in addition to its low cost and solubility in aqueous solutions. This study looks at how *S. cerevisiae*, under the influence of sodium butyrate, affects the flavor and aroma profile of beer.

Beer is one of the most popular alcoholic drinks available on the market, with it being valued at USD 680.9 Billion in 2021 and, therefore, is a competitive industry [12]. The epigenetic modification of brewer's yeast can be a cost-effective method for producing new strains, compared to the isolation of wild yeast, use of non-conventional yeast strains, and creating new malted grains or hops. This study attempts to demonstrate the possibility of using epigenetic modifiers like sodium butyrate to develop a non-GMO yeast strain with desirable sensory characteristics.

## 2. Materials and Methods

### 2.1. Culture Preparation and Pitching of Wort

A commercially available freeze-dried *S. cerevisiae* yeast, "London", an English-style ale yeast (Lallemand Brewing, Montreal, QC, Canada), was used as the fermentation starter. Cultures were grown in YPD broth adjusted to pH~7, and sodium butyrate was added to a final concentration of 5 mM for pretreatment. Two types of broths were prepared with 300 mL of regular YPD and 200 mL of YPD with 5 mM sodium butyrate. The media was autoclaved at 121 °C for 15 min prior to inoculation. The yeast was dissolved in 7.5 mL of regular YPD and vortexed, followed by the transfer of 1 mL of the preparation into 25 mL of regular and 5 mM sodium butyrate YPD each. Five different treatments were produced using the regular YPD and 5 mM sodium butyrate YPD broth. These can be seen in Table 1.

**Table 1.** Yeast treatment conditions.

| Code * | Strain                         | Pretreatment                                                                                 | Fermentation Treatment |
|--------|--------------------------------|----------------------------------------------------------------------------------------------|------------------------|
| WT W/O | wild type <i>S. cerevisiae</i> | None                                                                                         | No sodium butyrate     |
| WT SB  | wild type <i>S. cerevisiae</i> | None                                                                                         | 500 µM sodium butyrate |
| SS W/O | <i>S. cerevisiae</i>           | 5 mM sodium butyrate for 100 h/<br>subculture every 20 h                                     | No sodium butyrate     |
| SS SB  | <i>S. cerevisiae</i>           | 5 mM sodium butyrate for 100 h/<br>subculture every 20 h                                     | 500 µM sodium butyrate |
| 1G W/O | <i>S. cerevisiae</i>           | 5 mM sodium butyrate for 100 h/<br>subculture every 20 h and 20 h with<br>no sodium butyrate | No sodium butyrate     |

\* WT = wild type, no subculturing; W/O = no sodium butyrate treatment; SB = sodium butyrate treatment; SS = wild type, with subculturing; 1G = wild type with alternative subculturing.

## 2.2. Fermentation and Monitoring

The treatments were performed in triplicate. The wort provided by Three Boys Brewery (Woolston, Christchurch, New Zealand) were filled into 2 L Schott bottles, and each was pitched with the above-prepared yeast at  $1 \times 10^6$  CFU/mL. The fermentation was monitored for 13 days, and the vessels were kept at 20 °C in a water bath. The temperatures and specific gravity readings were taken throughout the 13-day fermentation process with a refractometer and digital thermometer respectively.

## 2.3. Sensory Analysis

A total number of nine untrained participants (n = 9) was recruited from Three Boys Brewery (Woolston, Christchurch, New Zealand). According to Lincoln University's policies and procedures, they were asked to complete the consent form regarding human ethics before they started tasting the beer samples. A brief introduction was given to the participants at the beginning of the sensory session, and they were instructed to complete a questionnaire while tasting the beer samples. In the questionnaire, the participants were asked to rate the acceptability of six attributes of each beer sample, including taste, flavor, aroma, smoothness, creaminess, and overall liking. The acceptability test was based on a 9-point hedonic scale, which represents 9 hedonic responses using number 1 (dislike extremely) to 9 (like extremely), with a mutual response as 5 (neither like nor dislike). In the second part of the questionnaire, sweetness, sourness, dairy flavor, smoothness, and creaminess will be evaluated using the just-about-right-scale (JAR) in terms of both intensity and acceptability (1 = too little, 2 = just about right, 3 = too much). The last section of the questionnaire was the evaluation of emotional responses.

## 2.4. Ethanol and Higher Alcohol Analysis

### 2.4.1. Sample Preparation

A total of 0.9 mL of the sample was pipetted into a 20 mL amber SPME vial and diluted with 8.0 mL of 5 g/L tartaric acid buffer pH 3.5, and 40 µL of composite internal standard was added, followed by 4.5 g of sodium chloride before the vial was immediately capped.

### 2.4.2. Preparation of Working Standards

The composite standard was diluted in a suitable sample matrix (rotary evaporated to remove volatile background and reconstituted to give the sample ethanol content and pH, to prepare the top standard of the calibration curve). This was then serially diluted in the provided matrix to ensure each vial had a maximum volume of 0.9 mL of matrix present. Each vial was then diluted further with 8.0 mL of tartaric acid buffer, as in the samples, with

40 µL of composite internal standard being added followed by 4.5 g of sodium chloride before the vials were immediately capped.

#### 2.4.3. GC-MS Instrumentation

GC-MS analysis was carried out according to Tomasino (2015) [13]. In brief, the samples were run on a Shimadzu QP2010 GC-MS (Shimadzu Scientific Instruments, Columbia, MD, USA). The columns used were two columns in a series, an Rtx-wax column (30 m × 0.25 mm ID × 0.5 µm film thickness, polyethylene glycol, Restek, Bellefonte, PA, USA) and an Rxi-1MS column (15 m × 0.25 mm ID × 0.5 µm film thickness, 100% dimethyl polysiloxane, Restek, Bellefonte, PA, USA). The carrier gas was helium, at a linear velocity of 33.5 cm/s. The GC-MS oven temperature was held at 35 °C for 3 min, then ramped to 250 °C at 4 °C/min and then held for 10 min. The interface and MS source temperature was set at 250 °C and 200 °C, respectively, with the MS source operated in electron impact (EI) mode at an ionisation energy of 70 eV.

#### 2.5. Determination of Glycerol Concentration

The glycerol concentration of the five treatments was determined using a glycerol assay kit (K-GCROL 04/20) by Megazyme® (Megazyme, Wicklow, Ireland). The assay was conducted according to the manufacturer's protocols with a few alterations to the sample volumes. All samples were prepared in 2.5 mL visible plastic cuvettes, with 1950 µL distilled water, 50 µL sample/standard and 100 µL buffer solution (solution 1), 50 µL solution 2 (NADH/ATP/PEP), and 10 µL of suspension 3 (PK/L-LDH), except for the blanks, where distilled water was 2000 µL. The samples/standards were incubated, and the absorbance was measured using UV-VIS spectrophotometer (Shimadzu UV-1800, Kyoto, Japan), followed by the calculation of the glycerol concentration according to the manufacturer's instructions.

#### 2.6. Determination of Residual Sugar Concentration

The residual sugar concentrations were determined using the enzymatic kit Vintessential Laboratories, Tasmania, Australia) for the determination of D-glucose and D-fructose in grape juice and wine. Samples did not require dilution, as the readings were within 1 g/L. The reagents were mixed with the sample according to manufacturer's instructions, followed by seven times inversion of the cuvettes and an incubation time of 3 min. The absorbance readings were taken with a UV-VIS spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). The calculations were conducted according to the manufacturer's instructions.

#### 2.7. Statistical Analysis

Data was analyzed using analysis of variance (ANOVA) with a generalized linear model, followed by post-hoc Tukey's mean comparison test by using Minitab 20 (Minitab, LLC, State College, PA, USA). PCA and AHC were analyzed using XLSTAT Statistical Software 2016 (Addinsoft, Paris, France). A confidence level of 95% was applied to the statistical analysis, and the data were presented as mean ± SD.

### 3. Results and Discussion

#### 3.1. Sensory

Table 2 shows the mean and standard error of the liking score of different sensory attributes in five different beer treatments. There were no significant differences in the overall liking score. Similarly, there was no significant difference in aroma, smoothness, and creaminess across the five treatments. There was no significant difference in taste between the control and sodium butyrate-treated wild-type strain, although there was

a significant difference between SS W/O and SS SB. Furthermore, there was significant difference between the taste scores between SS SB and 1G W/O treatment. In terms of flavor, the 1G W/O treatment had the highest liking score. There was a significant difference between the acceptability score for flavor between treatments SS SB and 1G W/O.

**Table 2.** Acceptability scores \* of the five different treatments.

| Treatments ** | Sensory Attributes        |                          |                           |                          |                          |                          |
|---------------|---------------------------|--------------------------|---------------------------|--------------------------|--------------------------|--------------------------|
|               | Taste                     | Aroma                    | Flavour                   | Smoothness               | Creaminess               | Overall Liking           |
| WT W/O        | 4.44 ± 0.48 <sup>ab</sup> | 5.00 ± 0.69 <sup>a</sup> | 4.56 ± 0.50 <sup>ab</sup> | 5.56 ± 0.63 <sup>a</sup> | 5.56 ± 0.53 <sup>a</sup> | 4.89 ± 0.63 <sup>a</sup> |
| WT SB         | 5.11 ± 0.56 <sup>ab</sup> | 4.67 ± 0.60 <sup>a</sup> | 5.22 ± 0.49 <sup>ab</sup> | 5.56 ± 0.34 <sup>a</sup> | 5.22 ± 0.32 <sup>a</sup> | 5.00 ± 0.58 <sup>a</sup> |
| SS W/O        | 5.67 ± 0.29 <sup>a</sup>  | 5.78 ± 0.40 <sup>a</sup> | 5.56 ± 0.24 <sup>ab</sup> | 5.67 ± 0.29 <sup>a</sup> | 5.44 ± 0.34 <sup>a</sup> | 5.89 ± 0.31 <sup>a</sup> |
| SS SB         | 3.56 ± 0.44 <sup>b</sup>  | 3.67 ± 0.58 <sup>a</sup> | 4.11 ± 0.56 <sup>b</sup>  | 4.67 ± 0.44 <sup>a</sup> | 4.56 ± 0.41 <sup>a</sup> | 3.89 ± 0.59 <sup>a</sup> |
| 1G W/O        | 5.78 ± 0.57 <sup>a</sup>  | 5.89 ± 0.63 <sup>a</sup> | 6.11 ± 0.59 <sup>a</sup>  | 6.33 ± 0.44 <sup>a</sup> | 5.89 ± 0.42 <sup>a</sup> | 5.67 ± 0.47 <sup>a</sup> |

\* Acceptability scores were based on the 9-point hedonic scale (1 = dislike extremely, 5 = neither like nor dislike, 9 = like extremely); results were expressed as mean ± SEM (standard error of the mean, n = 67). Results with different superscripts in each column within each attribute indicate significant differences (Tukey's HSD test,  $p < 0.05$ ). \*\* WT = wild type, no subculturing; W/O = no sodium butyrate treatment; SB = sodium butyrate treatment; SS = wild type, with subculturing; 1G = wild type with alternative subculturing.

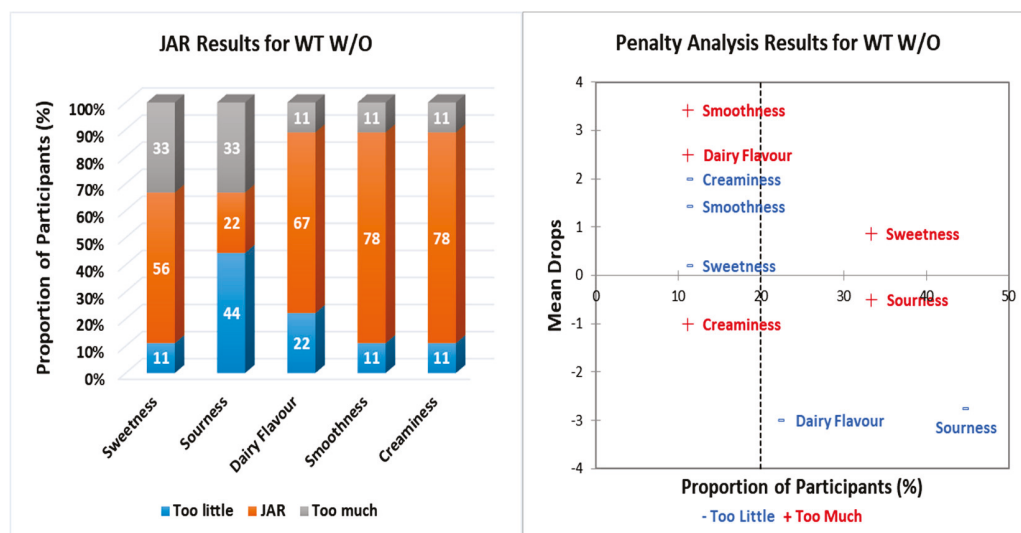
The epigenetically modified yeast-treated beer had different acceptability scores with regards to taste, while the treatment that did not contain sodium butyrate in the wort (SS W/O) had a higher acceptability score compared to the treated beer with sodium butyrate in it. Sodium butyrate (SB), which is a short-chain fatty acid, has shown functions as a histone modification inhibitor that has been used in clinical studies [14]. Although SB has shown promising results in the clinical field, it has its drawbacks due to its butyric smell and taste [15]. This would explain the results in terms of different acceptability scores for taste between the two epigenetically treated beers, SS W/O and SS SB. Yeast metabolism during fermentation mainly contributes to the sensory attributes such as flavor and aroma [16]. Attributes such as creaminess and smoothness are highly dependent on the wort. Hence, we do not see a significant difference between the treatments with regards to smoothness and creaminess, as the same wort was used for all five treatments. Overall, the acceptability scores across all sensory attributes were not high and could improve. As mentioned above, such low scores could be due to the negative sensory effects of sodium butyrate and other secondary metabolites present in the beer that will be discussed in later sections.

JAR sensory analysis was used in this experiment, as it is a tool used to link hedonic scales to the five specific attributes in the product being evaluated: smoothness, creaminess, sweetness, sourness, and dairy flavor.

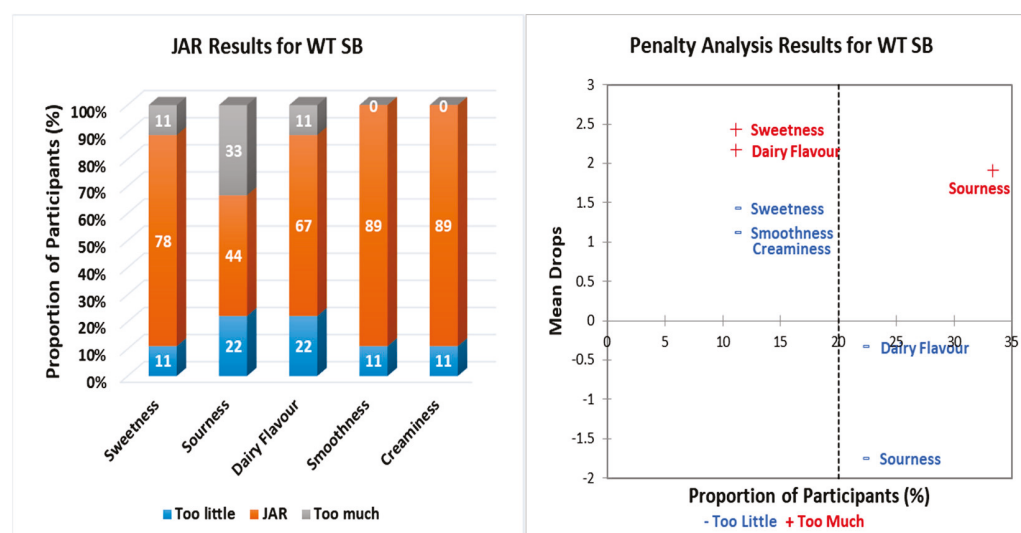
This type of diagnostic tool helps determine which product attribute is liked or disliked, is efficient in time and resources, and is useful in screening experiments and product development.

Figures 1–5 show the JAR frequencies (%) and mean drops based on the penalty analysis for the five different treatments regarding their sweetness, sourness, dairy flavor, smoothness, and creaminess. As shown in Figure 1, WT W/O had the highest selection of JAR for smoothness (78%) and creaminess (78%). The selection of “too much” was for both sweetness (33%) and sourness (33%), whereas the highest selection for “too little” was for sourness (44%). In Figure 2, WT SB had the highest selection of JAR for the same attributes for WT W/O with 89%, just about right. WT SB had the highest selection of “too little” for both sourness (22%) and dairy flavor (22%) in equal proportion, and “too much” for sourness (33%). With regards to epigenetically modified yeast without sodium

butyrate (SS W/O) (Figure 3), all participants showed JAR selection for sweetness (100%) and smoothness (100%), while the highest selection of “too little” was for both sourness (22%) and dairy flavor (22%) and “too much” for sourness, dairy flavor, and creaminess with equal frequencies (11%). In Figure 4, SS SB had the highest portion of participants that selected JAR for smoothness (78%), while the highest selection of “too little” and “too much” was for sourness (33%). For 1G W/O (Figure 5), all participants selected JAR for smoothness (100%), while the highest selection of “too little” was for dairy flavor (33%), and no participants selected “too much” for any of the attributes.

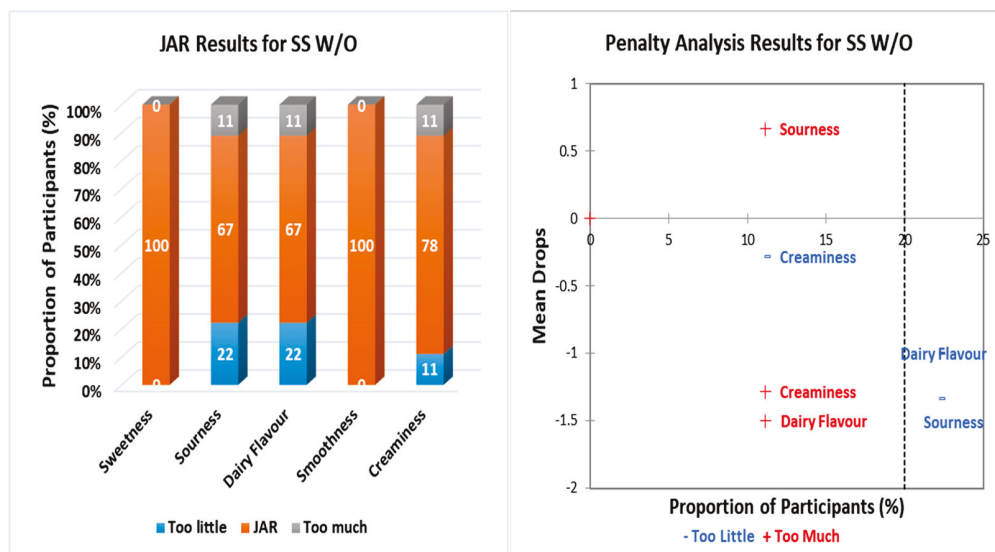


**Figure 1.** Just-about-right (JAR) frequencies and penalty analysis results regarding beer attributes for WT/WO. Penalty analysis was associated with overall liking scores (9-point hedonic scale). WT W/O: wild type *S. cerevisiae* continuously grown in YPD broth for 100 h/subculture every 20 h, without the addition of sodium butyrate.

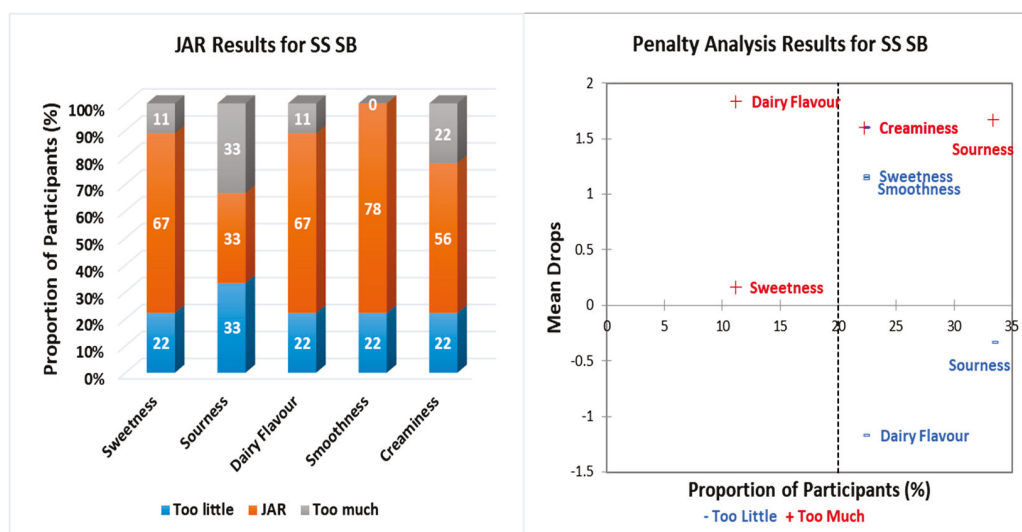


**Figure 2.** Just-about-right (JAR) frequencies and penalty analysis results regarding beer attributes for WT SB. Penalty analysis was associated with overall liking scores (9-point hedonic scale). WT SB: wild type *S. cerevisiae* continuously grown in YPD broth for 100 h/subculture every 20 h, with 500  $\mu$ M sodium butyrate addition during fermentation.





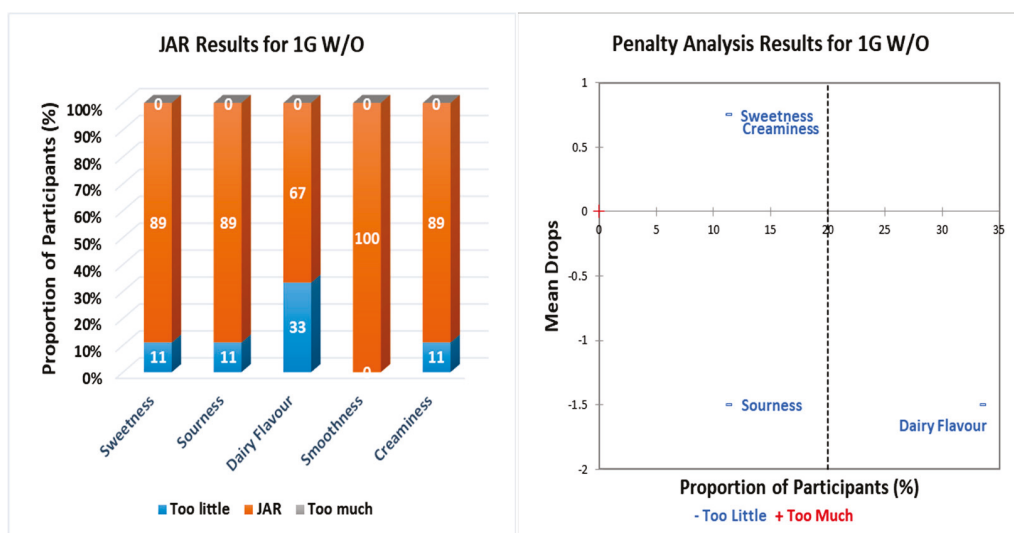
**Figure 3.** Just-about-right (JAR) frequencies and penalty analysis results regarding beer attributes for SS W/O. Penalty analysis was associated with overall liking scores (9-point hedonic scale). SS W/O: *S. cerevisiae* continuously treated with 5 mM sodium butyrate for 100 h/subculture every 20 h, without the addition of sodium butyrate during fermentation.



**Figure 4.** Just-about-right (JAR) frequencies and penalty analysis results regarding beer attributes for SS SB. Penalty analysis was associated with overall liking scores (9-point hedonic scale). SS SB: *S. cerevisiae* continuously treated with 5 mM sodium butyrate for 100 h/subculture every 20 h, with 500  $\mu$ M sodium butyrate addition during fermentation.

Penalty analysis was conducted based on both JAR frequencies and the overall liking scores of the 9-point hedonic scale. The threshold for population size was set at 20%, as shown in Figures 1–5. Therefore, values below 20% were considered of no consequence, as the number of respondents was small. Figure 1 shows that the WT W/O had a positive mean drop for sweetness, meaning less people found the sweetness of the beer “too much”. The negative mean drop for sourness shows that more people found the WT W/O-treated beer to be “too sour”, although a higher respondent percentage found the sourness being “too little”. More than 20% of respondents found that the dairy flavor was “too little”. The penalty analysis, as shown in Figure 2, shows that less people found that dairy flavor JAR. Over 30% of respondents found the sourness just about right, while less than 25% of respondents thought the sourness was “too little”. The penalty analysis for

SS W/O (Figure 3) shows that less people found the dairy flavor just about right, and a higher number of respondents found the sourness of the beer “too little”. Figure 4 shows that more people found that the creaminess is just about right. A similar percentage of respondents found that the sourness of the beer was “too little” and “too much”. Less people found the sweetness/smoothness “too little”, while less people found dairy flavor just about right. Figure 5 shows that, in the 1G W/O treatment, less people found the dairy flavor just about right.



**Figure 5.** Just-about-right (JAR) frequencies and penalty analysis results regarding beer attributes for 1G W/O. Penalty analysis was associated with overall liking scores (9-point hedonic scale). 1G W/O: *S. cerevisiae* continuously treated with 5 mM sodium butyrate for 100 h/subculture every 20 h, followed by 20 h in YPD broth without sodium butyrate and without the addition of sodium butyrate during fermentation.

In general, all treatments had a high JAR selection for smoothness (WT W/O: 78%; WT SB: 89%; SS W/O: 100%; SS SB: 78%; 1G W/O: 100%). These results aligned with the hedonic scale results. Likewise, creaminess JAR scores were of high percentage across all treatments and aligned with the results of the hedonic scale. As mentioned in Section 2.4.1, the creaminess and smoothness of the beer mainly depend on the type of wort and other fermentation parameters more than the yeast itself [15]. Hence, all treatments have high JAR scores. Attributes such as smoothness and creaminess can be deemed as positive attributes in the beer, although the JAR percentages did not have drastic differences from each other. The dairy flavor attribute is not normally considered as positive in context of beer. The JAR results and penalty analysis represent this, since the JAR scores were higher compared to the “too little” and “too much”. Moreover, the penalty analysis shows that few respondents found the beer to be JAR. The JAR and penalty analysis results, with regards to dairy flavor, could be attributed to the decision making of the respondents, where some respondents might find it difficult to compare the perceived intensity and ideal point of the attribute, estimate the ideal point of the attribute, and perceive the intensity of the attribute [17].

### 3.2. Ethanol and Higher Alcohol Analysis

As shown in Table 3, there is no difference in average ethanol content (%) among the five different treatments. Even though Kong (2022) [9] showed that sodium butyrate has high HDAC inhibition, it is not a potent inhibitor and is not specific in its upregulation or downregulation [4].

**Table 3.** Ethanol content \* of different treatments after fermentation.

| Treatment ** | Ethanol Content (%) |
|--------------|---------------------|
| WT W/O       | 6.15 ± 0.43         |
| WT SB        | 5.93 ± 0.35         |
| SS W/O       | 6.03 ± 0.20         |
| SS SB        | 6.16 ± 0.40         |
| 1G W/O       | 5.98 ± 0.25         |

\* Means are not significantly different ( $p < 0.05$ ). \*\* WT = wild type, no subculturing; W/O = no sodium butyrate treatment; SB = sodium butyrate treatment; SS = wild type, with subculturing; 1G = wild type with alternative subculturing.

Though sodium butyrate has been shown to have some amount of gene expression, it may have been sufficient to change core metabolic pathways such as ethanol production. Hence, we do not see any changes in ethanol concentration between the five treatments.

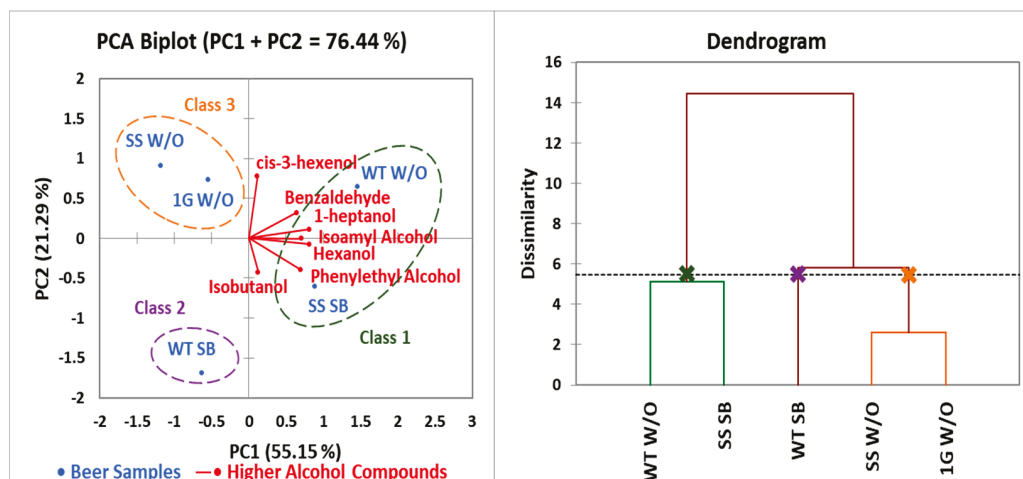
As shown in Table 4, isoamyl alcohol had the highest concentration out of the higher alcohols detected by GC-MS analysis with a range between 44,959–55,924 µg/L. Benzaldehyde, cis-3-hexenol, and 1-heptanol were detected in trace amounts compared to the other fusel alcohols. The fusel alcohol concentrations were not significantly different between treatments; the exception is benzaldehyde, an aldehyde. The benzaldehyde results for the WT W/O and 1G W/O treatments had higher results compared to other treatments but also a larger standard deviation, which could explain the significant test difference.

**Table 4.** Higher alcohol and aldehyde compounds (µg/L) \* detected using GC-MS analysis in the five different treatments.

| Compounds           | Treatments **              |                            |                           |                               |                            |
|---------------------|----------------------------|----------------------------|---------------------------|-------------------------------|----------------------------|
|                     | WT W/O                     | WT SB                      | SS W/O                    | SS SB                         | 1G W/O                     |
| Isobutanol          | 9638 ± 516.36 <sup>a</sup> | 7583 ± 262.88 <sup>a</sup> | 9210 ± 61.36 <sup>a</sup> | 10,387 ± 2352.95 <sup>a</sup> | 9497 ± 122.34 <sup>a</sup> |
| Isoamyl alcohol     | 55,924 ± 2757 <sup>a</sup> | 44,959 ± 1188 <sup>a</sup> | 51,840 ± 61 <sup>a</sup>  | 54,649 ± 5325 <sup>a</sup>    | 53,641 ± 111 <sup>a</sup>  |
| Hexanol             | 57.7 ± 2.69 <sup>a</sup>   | 44.2 ± 0.60 <sup>a</sup>   | 44.9 ± 0.98 <sup>a</sup>  | 57.0 ± 3.22 <sup>a</sup>      | 45.9 ± 3.41 <sup>a</sup>   |
| cis-3-Hexenol       | 4.73 ± 0.22 <sup>a</sup>   | 4.57 ± 0.34 <sup>a</sup>   | 5.11 ± 0.00 <sup>a</sup>  | 4.68 ± 0.30 <sup>a</sup>      | 5.22 ± 0.02 <sup>a</sup>   |
| 1-Heptanol          | 6.42 ± 0.57 <sup>a</sup>   | 4.76 ± 0.52 <sup>a</sup>   | 4.35 ± 0.05 <sup>a</sup>  | 5.92 ± 1.21 <sup>a</sup>      | 4.50 ± 0.32 <sup>a</sup>   |
| Benzaldehyde        | 11.02 ± 1.07 <sup>a</sup>  | 6.81 ± 0.06 <sup>ab</sup>  | 5.57 ± 0.33 <sup>ab</sup> | 8.90 ± 0.98 <sup>ab</sup>     | 10.09 ± 1.19 <sup>b</sup>  |
| Phenylethyl alcohol | 16,598 ± 368 <sup>a</sup>  | 13,116 ± 8 <sup>a</sup>    | 13,451 ± 181 <sup>a</sup> | 18,653 ± 3169 <sup>a</sup>    | 13,233 ± 392 <sup>a</sup>  |

\* Means that do not share a letter are significantly different ( $p < 0.05$ ). \*\* WT = wild type, no subculturing; W/O = no sodium butyrate treatment; SB = sodium butyrate treatment; SS = wild type, with subculturing; 1G = wild type with alternative subculturing.

The principal component analysis (PCA) and agglomerative hierarchical clustering (AHC) results are shown in Figure 6. The PCA biplot visualizes the relationship between the five different beer treatments and variance of their higher alcohol compounds detected using GC-MS analysis. Isoamyl alcohol, 1-heptanol, and hexanol were closely aligned with each other along the PC1 axis. Isoamyl alcohol and hexanol showed positive correlation due to the small angle formed between the compounds. Isoamyl alcohol and 1-heptanol also showed positive correlation between the two higher alcohols. Additionally, cis-3-hexanol was not well correlated with isoamyl alcohol, as the two are almost perpendicular to each other, while cis-3-hexanol are not correlated to each other due to being perpendicular to each other. The five treatments could be categorized into three classes: class 1, WT W/O and SS SB; class 2, WT SB; and class 3, SS W/O and 1G W/O.



**Figure 6.** Principal component analysis (PCA) biplot illustrating the relationship between beer samples fermented under different conditions and variance on their higher alcohol compounds (**left**); Beer samples grouped using agglomerative hierarchical clustering (HCA) according to their dissimilarity levels of GC-MS analysis (**right**).

The dendrogram based on AHC according to the dissimilarity levels of GC-MS analysis (Figure 6; right) shows that two main clusters were formed. The WT W/O and SS SB were similar, which was one cluster, and the second cluster consisted of SS W/O and 1G W/O treatment.

Higher alcohols are secondary metabolites that can have either a positive or negative effect on the flavor and aroma of the beer. The higher alcohols that were identified using GC-MS analysis are commonly found in beer and other alcoholic beverages. Isoamyl alcohol, which is the most abundantly found secondary metabolite in beer, showed the highest concentration throughout all five treatments. As isoamyl alcohol concentrations increase in beer, sensory analysis has shown that beer seems to have a “heavier” characteristic and influences drinkability of the beer [18], although there was no significant difference amongst the treatments for isoamyl alcohol. The isoamyl alcohol, which is detected above the threshold level in fruits such as banana, oranges, mango, and pineapple, imparts a banana characteristic in beer [19]. Isobutanol, which has similar sensory characteristics to amyl alcohols, further intensifies the sensory profile of isoamyl alcohol significantly [19]. All treatments were well below the threshold level (100 mg/L) with regards to isobutanol, while isoamyl alcohol fell within the threshold range (50–65 mg/L) [20] for all treatments except WT SB. The biosynthesis of higher alcohols is either through glycolysis, or the metabolism of amino acids present in the wort, also known as the Ehrlich pathway [21]. Therefore, a higher amount of amino acids present in the wort could have led to a higher isoamyl alcohol content throughout all treatments. There was no significant difference in isoamyl alcohol concentration between treatments; therefore, genes that encode nitrogen-sourced metabolic enzymes such as GAT1 GLN3 DAL80 and GZF3 [21] were either upregulated or downregulated. The AHC (Figure 6; right) shows that the WT W/O and SS SB were of the same cluster, which further proves that sodium butyrate did not have a substantial effect on the fusel alcohol profile of the beer.

### 3.3. Glycerol

Table 5 shows the glycerol content of the five different fermentation treatments. The epigenetically modified yeast did not produce higher or lower amounts of glycerol compared to the wild-type strains. Glycerol is one of the core metabolic pathways in yeast fermentation. Similar to the ethanol results, it is proposed that the sodium butyrate did not have sufficient capacity to make a significant enough regulation in gene expression that

would change the core metabolic pathways. Hence, the glycerol concentration between the five treatments was not significantly different from each other.

**Table 5.** Mean glycerol content of fermented wort after treatments \*.

| Treatment ** | Glycerol (g/L) |
|--------------|----------------|
| WT W/O       | 1.74 ± 0.11    |
| WT SB        | 1.81 ± 0.14    |
| SS W/O       | 1.70 ± 0.09    |
| SS SB        | 1.83 ± 0.15    |
| 1G W/O       | 1.74 ± 0.14    |

\* Means are not significantly different ( $p < 0.05$ ). \*\* WT = wild type, no subculturing; W/O = no sodium butyrate treatment; SB = sodium butyrate treatment; SS = wild type, with subculturing; 1G = wild type with alternative subculturing.

### 3.4. Residual Sugars

There was no significant difference in average D-glucose and D-fructose concentrations (g/L) throughout the five treatments (Table 6).

**Table 6.** Mean D-glucose, D-fructose and total residual sugar concentrations (g/L) of the five different treatments \*.

| Treatment ** | D-Glucose      | D-Fructose     | Total Residual Sugar |
|--------------|----------------|----------------|----------------------|
| WT W/O       | 0.027 ± 0.0031 | 0.059 ± 0.0160 | 0.086 ± 0.005        |
| WT SB        | 0.028 ± 0.0013 | 0.164 ± 0.0141 | 0.192 ± 0.015        |
| SS W/O       | 0.025 ± 0.0024 | 0.140 ± 0.0332 | 0.165 ± 0.015        |
| SS SB        | 0.035 ± 0.0006 | 0.041 ± 0.0012 | 0.076 ± 0.005        |
| 1G W/O       | 0.026 ± 0.0030 | 0.141 ± 0.0436 | 0.167 ± 0.015        |

\* Means are not significantly different ( $p < 0.05$ ). \*\* WT = wild type, no subculturing; W/O = no sodium butyrate treatment; SB = sodium butyrate treatment; SS = wild type, with subculturing; 1G = wild type with alternative subculturing.

The WT W/O and SS SB showed similar total residual concentrations and were not significantly different from each with values of 0.086 and 0.076 g/L, respectively. Therefore, SB does not seem to have an effect on the metabolic pathway with regards to the metabolism of sugars present in the wort. Furthermore, WT SB, SS W/O and 1G W/O total residual sugar concentrations were not significantly different from each other.

## 4. Conclusions

This project investigated the potential effects of sodium butyrate on beer and how it affects certain sensory and biochemical characteristics of beer. Sensory results show that there was not much difference between treatments except for certain sensory attributes. SB showed a negative impact in terms of taste due to its butyric taste and was observed evidently between SS W/O and SS SB.

The ethanol concentration between different treatments did not show significant difference amongst the five treatments. Even though previous studies have shown that sodium butyrate has a high HDAC inhibition capacity, recent studies on prokaryotes have shown that, even though sodium butyrate has a high HDAC inhibition capacity compared to other epigenetic modifiers such as curcumin, genistein, and benzoic acid, sodium butyrate can regulate only a limited amount of genes compared to modifiers such as genistein [22]. The limited regulation of genes could have led to key metabolic pathways such as ethanol and glycerol production not being altered. Higher alcohols such as isobutanol and isoamyl alcohol, which are abundantly found in beer, were well detected across all treatments. The isoamyl alcohol content, which was within the threshold range,



could be due to the higher amount of amino acids present in the wort more so than due to the epigenetic effect of sodium butyrate, since all treatments were within the range of the threshold.

Our pretreatment regime of 5 mM sodium butyrate in YPD broth for 100 h was selected based on studies demonstrating HDAC inhibition and histone modifications in *S. cerevisiae*. Kong et al. (2022) [9] reported that sodium butyrate is among the most potent dietary HDAC inhibitors, capable of altering yeast phenotypes epigenetically without DNA sequence changes, supporting our interpretation that the fermentation outcomes observed here may be mediated through epigenetic mechanisms.

Although the culture pH was ~7, above the pKa of butyric acid (~4.8) and, thus, most butyrate was in its dissociated form, sodium butyrate retained HDAC inhibitory activity under these conditions. This indicates that epigenetic modulation can still occur even when passive uptake is reduced.

The commercial ale yeast strain used is likely polyploid or aneuploid, as is common among brewing strains. Such genetic variation can buffer stress and alter transcriptional responses, which may have influenced the magnitude of sodium butyrate's effects.

Even though sodium butyrate was not able to have a significant effect on the metabolic pathways of the yeast, other compounds can be explored to be utilized in creating epigenetically modified non-GMO yeast.

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**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** Not applicable.

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## Article

# Screening Sourdough Starter Cultures from Yeast and Lactic Acid Bacteria Isolated from Mexican Cocoa Mucilage and Coffee Pulp for Bread Quality Improvement

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## Abstract

This study aimed to identify and evaluate yeasts and lactic acid bacteria (LAB) isolated from Mexican cocoa mucilage (*Theobroma cacao*) and coffee pulp (*Coffea arabica*) for their potential use as sourdough starter co-cultures to improve bread quality. Functional screens included assessments of amylolytic, proteolytic, and phytase activities, CO<sub>2</sub> production, acidification capacity, and exopolysaccharide (EPS) synthesis. *Saccharomyces cerevisiae* YCTA13 exhibited the highest fermentative performance, surpassing commercial baker's yeast by 52.24%. *Leuconostoc mesenteroides* LABCTA3 showed a high acidification capacity and EPS production, while *Lactiplantibacillus plantarum* 20B3HB had the highest phytase activity. Six yeast–LAB combinations were formulated as mixed starter co-cultures and evaluated in sourdough breadmaking. The B3Y14 co-culture (LABCTA3 + YCTA14) significantly improved the bread volume and height by 35.61% and 17.18%, respectively, compared to the commercial sourdough starter, and reduced crumb firmness by 59.66%. Image analysis of the bread crumb revealed that B3Y14 enhanced the crumb structure, resulting in greater alveolar uniformity and a balanced gas cell geometry. Specifically, B3Y14 showed low alveolar regularity ( $1.16 \pm 0.03$ ) and circularity ( $0.40 \pm 0.01$ ), indicating a fine and homogeneous crumb structure. These findings highlight the synergistic potential of selected allochthonous yeast and LAB strains in optimizing sourdough performance, positively impacting bread texture, structure, and quality.

**Keywords:** sourdough; bread; yeast; lactic acid bacteria; coffee pulp; cocoa mucilage

## 1. Introduction

Sourdough is a leavening agent used in the production of bakery products. It is obtained through the spontaneous fermentation of flour and water. Lactic acid bacteria (LAB) and yeasts are the main microorganisms involved in this fermentation process. Through their metabolic activity, these microorganisms acidify and leaven the dough while producing antifungal and aromatic compounds. The latter compounds contribute to the

characteristic flavor of sourdough. During spontaneous fermentation, LAB and yeasts, autochthonous microorganisms from flour, dough ingredients, and the environment, add a unique flavor profile to the sourdough [1–3].

The metabolic activity of microorganisms during fermentation triggers biochemical changes in the main components of the dough—carbohydrates, proteins, and lipids—through enzymatic activities and metabolite production. These transformations impact bread quality, improving crumb firmness, shelf life, and aromatic and sensory profiles. Moreover, using starter co-cultures in sourdough fermentation with allochthonous microorganisms enables the development of specific bread attributes that enhance quality. Therefore, starter co-culture microorganisms must be selected based on criteria such as adaptation to the fermentation environment and metabolite production, enabling better fermentation control and desired product characteristics [2–5].

Yeast selection criteria include high CO<sub>2</sub> production, tolerance to low pH, and the ability to grow in the presence of lactic and acetic acids. For LAB, the criteria include acidification capacity and exopolysaccharide (EPS) production. Additionally, enzymatic activities such as amylolytic, proteolytic, and phytasic activities are advantageous over the other selection criteria for yeast and LAB. Ogunsakin et al. [6] evaluated and screened LAB and yeasts from sorghum as starter cultures for sourdough fermentation, selecting strains with functional attributes. For LAB, the selection was based on EPS production and acidification properties, while for yeast, the selection criteria included gas production and low pH and acetic acid tolerance. Similarly, Shen et al. [7] isolated yeast and LAB strains from Chinese sourdoughs and screened them for their ability to enhance the sensorial quality of whole wheat steamed bread. Their results demonstrated that *Saccharomyces cerevisiae* and *Lactobacillus johnsonii* exhibited high CO<sub>2</sub> and acid production compared to the other sourdough strains used. These microorganisms were combined with the acetic acid bacteria *Acetobacter pasteurianum* to formulate a mixed starter, leading to increased bread volume, enhanced volatile organic compound production, and improved breadcrumb softness.

Additionally, several studies have demonstrated that microorganisms present in different food sources, including kefir, yogurt, and plant-derived fermented foods rich in sugars, such as coffee, cocoa, pears, oranges, and grapes, can be used to start sourdough fermentation [8–15]. In addition, fermented coffee pulp and cocoa mucilage can be used as sources of diverse genera of LAB (*Lactobacillus* and *Leuconostoc*) and yeasts (*Saccharomyces*, *Pichia*, *Rhodotorula*, and *Kazachstania*), which have also been identified in sourdough fermentations [16–29]. Therefore, using the microbiota in fermented coffee pulp and cocoa mucilage is of interest for developing sourdough starter cultures. This study aims to select LAB and yeast strains from fermented coffee pulp and cocoa mucilage for their potential application as starter cultures in sourdough, contributing to bread production enhancement.

## 2. Materials and Methods

### 2.1. Chemicals, Enzymes, and Broths

Culture media and the main components were obtained from the following suppliers: yeast extract, MRS broth, and nutrient agar were purchased from DIBICO (Mexico City, Mexico); agar (CAS 9002-18-0), casein peptone (CAS 91079-40-2), dextrose (D-glucose, CAS 50-99-7), and potato dextrose agar (PDA) were supplied by MCD LAB (Mexico City, Mexico); and wheat flour was provided by Fábrica de Harina Elizondo (Mexico City, Mexico). Sugars and salts included D-glucose (CAS 50-99-7), sucrose (CAS 57-50-1) (CTR Scientific, Monterrey, Mexico), calcium chloride (CaCl<sub>2</sub>, CAS 10043-52-4), potassium chloride (KCl, CAS 7447-40-7) (Reasol, Mexico City, Mexico), magnesium sulfate heptahydrate (MgSO<sub>4</sub>·7H<sub>2</sub>O, CAS 10034-99-8), manganese sulfate monohydrate (MnSO<sub>4</sub>·H<sub>2</sub>O, CAS 10034-96-5), and iron sulfate heptahydrate (FeSO<sub>4</sub>·7H<sub>2</sub>O, CAS 7782-63-0) (Golden Bell,

Mexico City, Mexico). Additional reagents included starch (CAS 9005-25-8) and Lugol's iodine solution (iodine, CAS 7553-56-2; potassium iodide, CAS 7681-11-0) (HYCEL), as well as cobalt chloride hexahydrate ( $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , CAS 7791-13-1), ammonium nitrate ( $\text{NH}_4\text{NO}_3$ , CAS 6484-52-2), and hydrochloric acid (HCl, CAS 7647-01-0), all purchased from J.T. Baker through El Crisol S.A. de C.V. (San Luis Potosí, Mexico). Sodium phytate (Na-phytate, CAS 14306-25-3) was obtained from Makesy (Haltom City, TX, USA), and sodium hydroxide (NaOH, CAS 1310-73-2) was acquired from Química Suastes (Mexico City, Mexico). The commercial cultures used were the San Francisco Style Sourdough Starter Culture (Cultures for Health, Wake Forest, NC, USA) and *Saccharomyces cerevisiae* Saf-instant (Safmex, Toluca, Mexico). Sigma-Aldrich reagents, including  $\alpha$ -amylase from porcine pancreas (EC 3.2.1.1; CAS 9000-90-2), ammonium vanadate ( $\text{NH}_4\text{VO}_3$ , CAS 7803-55-6), iron (III) chloride hexahydrate ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , CAS 10025-77-1), lactic acid (CAS 50-21-5), phytase from wheat (*Triticum aestivum*, EC 3.1.3.26; CAS 9001-89-2), *Aspergillus melleus* proteinase (EC 3.4.21.63; CAS 9001-92-7), and skim milk powder (CAS 999999-99-4) were obtained from Tesla (Mexico City, Mexico). DNA extraction reagents were supplied by Qiagen Mexico (Mexico City, Mexico) in the Blood and Cell Culture DNA Kit.

## 2.2. Microorganisms and Growth Conditions

The strains used in this study were obtained from the microorganism collection of the Grupo de Tecnología de Alimentos at the Unidad de Investigación y Desarrollo de Alimentos (UNIDA), Instituto Tecnológico de Veracruz, Veracruz, Mexico. These strains were previously isolated from cocoa mucilage [30,31], and in the present work, they were identified and evaluated for their selection as sourdough starter cultures. The collection included four LAB strains identified as *Lactiplantibacillus pentosus* LABCTA1, *Leuconostoc mesenteroides* LABCTA3, *Lactiplantibacillus plantarum* LABCTA6 and LABCTA8, along with eight yeast strains identified as *Saccharomyces cerevisiae*: YCTA5, YCTA9, YCTA10, YCTA12, YCTA13, YCTA14, YCTA15, and YCTA16. Additionally, from coffee pulp, one LAB strain, *Lpb. plantarum* 20B3H, and two yeast strains, *Wickerhamomyces anomalus* YX1SR15 and *W. anomalus* YX1SR23, were previously identified and selected as candidate strains for sourdough starter cultures [32,33]. Yeast strains *W. anomalus* YX1SR15 and YX1SR23 isolated from coffee pulp were selected for their tolerance to a pH between 3.0 and 5.0 and for their high amylolytic, proteolytic and phytasic activities; furthermore, these strains were negative for hemolytic activity [32]. Lactic acid bacterial strain *Lpb. plantarum* 20B3H from coffee pulp was selected for its high amylolytic activity, which would allow the strain to grow in sourdough fermentation, as it also presents proteolytic and phytasic activities; furthermore, this strain was identified as EPS-producer, which is a key screening criterion [33]. *Lpb. plantarum* 20B3H was negative for hemolytic activity. Yeast strains were grown in YPD broth at 30 °C for 24 h, and LAB strains were cultured in MRS broth at 37 °C for 24 h. Commercial *Saccharomyces cerevisiae* was obtained from Saf-instant® brand (Toluca, Mexico). San Francisco Style Sourdough Starter Culture (Cultures for Health) was obtained from Cultures for Health (Wake Forest, NC, USA).

## 2.3. Molecular Identification of Yeast and LAB Strains from Cocoa Fermentation

Isolated yeast and LAB strains were incubated on YPD and MRS agar at  $30 \pm 1$  °C for 24 h for identification. DNA extraction was performed using the Blood and Cell Culture DNA kit. PCR amplification and sequencing of the extracted DNA were performed by MacroGen (Seoul, Republic of Korea). According to MacroGen's standard protocol, yeast identification was performed by amplifying the internal transcribed spacer (ITS) regions ITS1-5.8S-ITS2 using the ITS4/ITS5 primers and LAB identification targeted the V1–V3 region of the 16S rRNA gene using the 785F/907R primers. PCR-specific primer bands



and reagents were determined and provided by Macrogen. The sequences were analyzed using BLASTN version 2.13.0+ (GenBank, NCBI, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>, accessed on 5 May 2023), performed by Macrogen (Seoul, Republic of Korea).

## 2.4. Enzymatic Activities of Yeast and LAB Isolates

### 2.4.1. Phytase Activity

A phytate-selective medium (PSM) was used following the methodology of Jorquera et al. [34] PSM medium contained 10 g/L D glucose, 4 g/L  $C_6H_6O_{24}P_{6\bullet}12Na$  (sodium phytate), 2 g/L  $CaCl_2$ , 5 g/L  $NH_4NO_3$ , 0.5 g/L KCl, 0.5 g/L  $MgSO_4 \cdot 7H_2O$ , 0.01 g/L  $FeSO_4 (7H_2O)$ , 0.01 g/L  $MnSO_4 \cdot H_2O$ , and 15 g/L agar. A colony of each yeast and LAB strain was cultured in 10 mL of YPD and MRS broth, respectively, at 30 °C for 16 h. After this, a spot of 10 µL of yeast and BAL culture broth was inoculated into Petri dish. Degradation halos around colonies after eight days of incubation indicate phytase activity. To eliminate false positives, Petri dishes were flooded with an aqueous solution of 2% (*w/v*) cobalt chloride. After 5 min at room temperature, plates were then flooded with an equal-volume solution of ammonium molybdate (6.25% *w/v*) and ammonium vanadate (0.42% *w/v*) that was freshly prepared. After an additional 5 min of incubation, the solution was removed, and clear zones were examined. As a positive control, 10 µL of a 1% (*w/v*) phytase solution from wheat was used, while 10 µL of sterile water functioned as the negative control.

### 2.4.2. Proteolytic Activity

For yeasts, the screening medium consisted of PDA supplemented with 20 g/L of skim milk, while for LAB, the screening medium was composed of agar with 20 g/L of skim milk [6]. Two colonies of each yeast and LAB strain were cultured in 20 mL of YPD and MRS broth, respectively, and incubated at 30 °C for 16 h. Then, 10 µL of culture broth was inoculated onto Petri dishes. After 5 days of incubation, a 1 N HCl solution was added to cover the agar surface and incubated for 30 min. The acid precipitated non-hydrolyzed proteins, leaving clear zones where protease activity had degraded casein. Finally, the HCl solution was removed, and plates were rinsed with distilled water. The presence of degradation halos around colonies indicated proteolytic activity. As a positive control, 10 µL of a 1% (*w/v*) *Aspergillus melleus* proteinase solution was inoculated into the Petri dishes, while 10 µL of sterile water was used as the negative control.

### 2.4.3. Amylolytic Activity

For yeast and LAB, amylolytic activity screening was performed using nutrient agar supplemented with 0.2% (*w/v*) starch [35,36]. Two colonies of yeast and LAB strains were cultured in 20 mL of YPD and MRS broth, respectively, and incubated at 30 °C for 16 h. After 5 days of incubation, the plates were flooded with 1% Lugol solution. The presence of degradation halos around colonies indicates amylolytic activity. As a positive control, starch hydrolysis was tested using 10 µL of a 1% (*w/v*)  $\alpha$ -amylase solution from porcine pancreas, while 10 µL of sterile water was used as the negative control.

## 2.5. Gas Production by Yeast

CO<sub>2</sub> production by yeast, which determines its fermentation capacity in bread dough, was measured using the drainage method [37,38]. A mixture of 20 g of flour, 15 mL of distilled water, and 0.5 g of yeast pellet (obtained by centrifugation of an overnight cell culture at 2795 g for 15 min at 4 °C) was placed in an Erlenmeyer flask sealed with a perforated rubber stopper, coupled to a drainage glass tube leading to a 100 mL water-filled graduated burette. The displacement of water in the burette was recorded every hour for 4 h. Commercial *S. cerevisiae* was cultured and used as a control.

## 2.6. Lactic Acid Production Capacity

LAB strains were screened based on their acid production capacity. Two colonies of each LAB strain were inoculated into 20 mL of MRS broth and incubated at 30 °C for 16 h. The activated LAB was inoculated into 50 mL of MRS broth with 1% (*v/v*) inoculum and incubated at 30 °C. After 16 h of incubation, culture broth samples were collected to determine pH, titratable acidity (TTA), and the lactic acid concentration. TTA was measured using 0.1 N NaOH until pH 8.5, while the lactic acid concentration was determined by a spectrophotometric method.

### Determination of the Lactic Acid Concentration

The lactic acid concentration was determined using the method described by Borshchevskaya et al. [39], with modifications. The method is based on the spectrophotometric quantification of the colored product formed by the reaction between lactate ions with iron (III) chloride at 390 nm. A calibration curve was constructed within a range of 0–120 mM of lactic acid, with a correlation coefficient of 0.978. A 0.2% (*w/v*) iron (III) chloride solution was prepared. Then, 50 µL of lactic acid at the corresponding concentration was added to 2 mL of the iron (III) chloride solution. The reference solution contained 2 mL of the iron (III) chloride solution. For the determination of the lactic acid concentration in the culture supernatant, two colonies of each LAB strain were inoculated into 20 mL of MRS broth and incubated at 30 °C for 16 h. After the incubation, the supernatant was obtained by centrifugation (2795 g for 15 min) and diluted 20-fold with deionized water. Finally, the lactic acid concentration was determined using the calibration curve.

## 2.7. Screening LAB Exopolysaccharide (EPS) Production

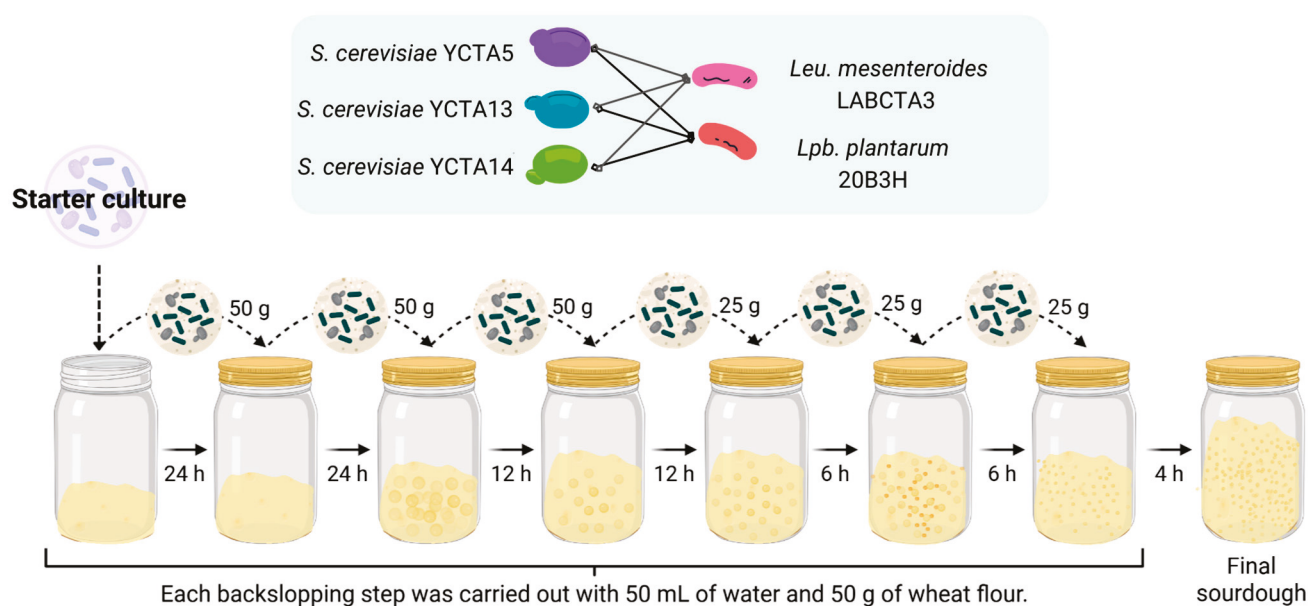
EPS production was tested with the method described by Menezes et al. [40] using MRS agar supplemented with 4% (*w/v*) sucrose. A single colony of activated bacteria was streaked onto the Petri dish, and the plates were incubated at 30 °C for 48 h. The presence of slimy or ropy colonies indicated EPS production.

## 2.8. Sourdough Elaboration and Breadmaking

To produce de novo sourdough with the selected microorganisms, six compound starter co-cultures with three yeast strains of *S. cerevisiae* (YCTA5, YCTA13, and YCTA14) and two LAB strains of *Lpb. plantarum* 20B3H and *Leu. mesenteroides* LABCTA3 were formulated to observe the individual effect of each yeast strain with LAB. The multistage sourdough feeding protocol (Figure 1) was adapted from an online sourdough course by Ramon-Simon [41]. To formulate the six starter co-cultures, a targeted combinatorial strategy was applied based on the functional screening results. Yeast and LAB strains were pre-selected due to their performance in functional assays, including CO<sub>2</sub> production, acidification rate, proteolytic and phytase activities, and EPS-producing ability. These strains were paired to reflect different technological profiles and potential synergistic interactions as combinations of high EPS-producing LAB with high CO<sub>2</sub>-producing yeasts. The resulting six co-cultures were thus not exhaustive of all possible combinations but were rationally selected to represent functional diversity and to evaluate the contributions of the selected microorganisms to sourdough bread quality.

To prepare the starter culture, cells from an overnight culture were harvested and washed twice with sterile water. Then, 1 mL of the starter culture inoculum, previously adjusted to 10<sup>7</sup> CFUs/g for yeast and 10<sup>8</sup> CFUs/g for LAB, was added to a mix of flour (50 g) and water (50 mL) to start sourdough production. Sourdough fermentation was performed at 30 °C with seven backslipping steps using different flour, water and dough proportions. The first three backslipping steps followed a flour/water/sourdough ratio (*w/w/w*) of

1:1:1 (24 h, 24 h, 12 h), while the last three were carried out using a 2:2:1 proportion (12 h, 6 h and 6 h) followed by a 4 h fermentation period to obtain the final sourdough (Figure 1). A commercial sourdough starter, San Francisco Style Sourdough Starter Culture, was used as control (Cultures for Health, Akron, OH, USA). To ensure experimental comparability, the entire content of the starter packet (~7 g) was directly inoculated into 50 g of wheat flour and 50 mL of sterile water, following the same backslopping conditions applied to mixed starters (Figure 1).



**Figure 1.** Sourdough preparation with the formulated mixed starter cultures.

Sourdough bread formulation was carried out using the AACC 10-10.03 method, with modifications. A formulation with 100 g of flour, 70 mL of water, 20 g of active sourdough, 1.5 g of salt, and 6 g of sugar was mixed using a KitchenAid mixer (KitchenAid, model KP26M9XCCU, Benton Harbor, MI, USA). The bread dough was placed in plastic bowls and covered with disposable food storage stretch lids and proofed at 30 °C for 6 h in an incubator (Gallenkamp, Harry Mazal S.A., Mexico City, Mexico). Then, the bread dough was manually shaped and molded in rectangular mini-loaf mold made of heavy-gauge steel with a non-stick coating with dimensions of 6.3 cm (W) × 16.0 cm (L) × 4.5 cm (H) (Cuisinart, Stamford, CT, USA); the mold was covered with the stretch lids and proofed for an additional 60 min at 30 °C. Finally, the bread dough was fermented overnight at 4 °C and the next day left, it rested at room temperature for 60 min before being baked in a convection oven (Gourmia, model GTF7660, Brooklyn, NY, USA) at 200 °C for 35 min with steam by adding water in the oven in a baking mold. The loaves were unmolded 15 min after baking, cooled for 120 min on a metallic rack, and then packed in polyethylene bags for further analysis.

### 2.9. Bread Quality

To determine which starter co-culture produced the best overall bread characteristics, bread quality was analyzed based on loaf volume (AACC Method 10-05.01), height (measured using digital Vernier calipers adapted as a height gauge), weight, crumb firmness (AACC Method 74-10.02), and crumb structure by image analysis. Loaf volume, height, and weight were evaluated 120 min after baking. For crumb firmness and structure, bread samples were kept in polyethylene hermetic bags until analyses were performed 24 h after baking.

### 2.9.1. Crumb Firmness Analysis

For crumb firmness, a compression test was carried out using a Texture Analyzer TA.XT Plus (Stable Micro Systems, Godalming, UK). From each loaf, 15-mm thick slices were obtained using a food slicer (Gourmia, model GFS-700, Brooklyn, NY, USA), discarding the central and ends slices. Two slices were compressed with a cylindrical P/25 probe (25 mm diameter) until a deformation of 3.0 mm was reached. The force required for compression was recorded as the firmness value.

### 2.9.2. Crumb Structure by Image Analysis

Crumb structure was assessed using digital image analysis in ImageJ software, version 1.54 (National Institutes of Health, Bethesda, MD, USA; <https://imagej.nih.gov/ij/>, accessed on 15 April 2025), based on standardized image processing steps. The crumb image analysis was conducted using high-resolution photographs captured with a SONY DSC-HX400V camera (zoom 2.5×, ISO 1000, F8.0, 1/30 s) (SONY, Tokyo, Japan). Images were saved as bitmap files, with a 300 DPI resolution and in real-color format (RGB, 256 million colors). All bread photos were scaled and standardized using a ruler. Images were cropped (ImageJ v1.54) to a field of view of 40 mm × 40 mm region from the crumb center to ensure comparability. The cropped color images were duplicated, and one was converted into an 8-bit grayscale image. Image contrast was enhanced by functions, normalized, and equalized. The grayscale images were automatically binarized/thresholded with the Otsu algorithm without manual adjustment [42–44]. This standardization ensures the objective, reproducible quantification of alveoli size distribution. Alveoli characteristics, including the area, circularity of pores (shape factor), and Feret’s diameter, were extracted from the binary images using the “Analyze Particles” function, excluding objects touching the edges. The circularity index parameter is defined as  $CI = \frac{4\pi A}{P^2}$ , where CI is the circularity index, A is the cell/alveoli area, and P is the cell perimeter; a perfect circle has a shape factor of 1.0, whereas a line has a shape factor approaching zero [45]. Alveoli were classified into five size categories: class 1 (0.05–0.49 mm<sup>2</sup>), class 2 (0.50–0.99 mm<sup>2</sup>), class 3 (1.00–4.99 mm<sup>2</sup>), class 4 (5.00–49.99 mm<sup>2</sup>), and class 5 (>50 mm<sup>2</sup>) [46]. Porosity was calculated as the ratio of the total alveolar area to the crumb area analyzed. Uniformity was assessed by calculating the coefficient of variation (CV) of Feret’s diameter, and circularity was reported as the average shape factor of all alveoli retained for analysis.

### 2.10. Statistical Analysis

All the experiments were performed in triplicate, and results are expressed as means with standard deviations. Statistical analysis was performed using the JMP Pro software (version 14.0, SAS Institute Inc., Cary, NC, USA) with one-way ANOVA followed by Tukey’s HSD test ( $p < 0.05$ ). Non-parametric data (e.g., alveolar distribution classes) were analyzed using the Kruskal–Wallis test.

## 3. Results and Discussion

### 3.1. Microorganisms Identified in Cocoa Mucilage

In this study, eight yeast and four LAB strains from cocoa mucilage were identified to screen their potential in formulating starter co-cultures (Table 1). Among yeasts, all strains were identified as *S. cerevisiae*, which was the dominant species of isolates from cocoa mucilage. *S. cerevisiae* has been widely reported as predominant in sourdough fermentation, mainly due to the favorable environmental conditions [47–52]. This yeast species is widely found in sourdough fermentation ecosystems due to its essential role as a leavening agent, its high fermentation efficiency, production of volatile organic compounds, and synergistic interactions with LAB, such as cross-feeding of metabolites, gas retention, and

mutual tolerance of inhibitory metabolites, which allows us to enhance the overall quality functional properties of bread. Together, these contributions are critical for developing the characteristic sensory properties of sourdough-based baked products [28,47,53–57].

**Table 1.** Identification of microorganisms in cocoa mucilage based on ITS and 16S rRNA.

| Identification Method | Microorganism                                | Length (bp) | Match/Total (bp) | Identity (%) |
|-----------------------|----------------------------------------------|-------------|------------------|--------------|
| ITS region            | <i>Saccharomyces cerevisiae</i> YCTA5        | 850         | 841/842          | 99.9         |
| ITS region            | <i>Saccharomyces cerevisiae</i> YCTA9        | 832         | 832/832          | 100          |
| ITS region            | <i>Saccharomyces cerevisiae</i> YCTA10       | 833         | 832/832          | 100          |
| ITS region            | <i>Saccharomyces cerevisiae</i> YCTA12       | 828         | 823/823          | 100          |
| ITS region            | <i>Saccharomyces cerevisiae</i> YCTA13       | 834         | 833/833          | 100          |
| ITS region            | <i>Saccharomyces cerevisiae</i> YCTA14       | 849         | 833/835          | 99.8         |
| ITS region            | <i>Saccharomyces cerevisiae</i> YCTA15       | 848         | 834/834          | 100          |
| ITS region            | <i>Saccharomyces cerevisiae</i> YCTA16       | 844         | 827/827          | 100          |
| 16S rDNA              | <i>Lactiplantibacillus pentosus</i> LABCTA1  | 1246        | 691/695          | 99.4         |
| 16S rDNA              | <i>Leuconostoc mesenteroides</i> LABCTA3     | 912         | 886/891          | 99.4         |
| 16S rDNA              | <i>Lactiplantibacillus plantarum</i> LABCTA6 | 1427        | 680/683          | 99.6         |
| 16S rDNA              | <i>Lactiplantibacillus plantarum</i> LABCTA8 | 1593        | 673/679          | 99.1         |

Regarding LAB species, one strain of *Lpb. pentosus* (LABCTA1), one strain of *Leu. mesenteroides* (LABCTA3), and two strain of *Lpb. plantarum* (LABCTA6 and LABCTA8) were identified in cocoa mucilage (Table 1). *Lpb. pentosus* is not commonly a dominant LAB species in spontaneous sourdoughs but is often used as a starter in laboratory sourdough. Nevertheless, it has also been isolated from artisan sourdoughs in Italy and cereal-based substrates [48,58]. *Lpb. plantarum* has been widely reported to be isolated in sourdough fermentation as it is predominantly found in sourdough elaborated in commercial bakeries rather than in the laboratory [59–63]. *Leu. mesenteroides* species are not common in sourdough fermentation; however, similar to *Lpb. pentosus*, it has been used as a starter culture in sourdough [64,65]. Despite their varying prevalence in sourdough, all three strains—*Lpb. pentosus*, *Lpb. plantarum*, and *Leu. Mesenteroides*—have been used as starter cultures in sourdough fermentation. Adding these LAB species to sourdough has been associated with improved bread sensory profiles, enhanced loaf volume, and reduced staling rates [27,66,67].

### 3.2. Screening and Selection of Yeast and LAB Strains

#### 3.2.1. Quantitative Halo-Based Enzymatic Screening of Strains from Cocoa Mucilage

The isolated strains were assessed for enzyme production using qualitative halo-based assays on agar plates to evaluate amylase, protease, and phytase activities, and the diameter of the hydrolysis zones (in mm) was used as indicator of enzymatic potential. The results of the degradation halos for these enzymatic activities are presented in Table 2. Notably, all strains exhibited low levels of amylase activity, showing halo diameter values between 0.20 and 1.43 mm. During sourdough fermentation, yeasts and LAB with amylolytic activity interact synergistically to metabolize starch. Acidic conditions during sourdough fermentation can induce structural and chemical modifications in both native and damaged starch granules, depending on pH, exposure time, and the type and concentration of organic acids present [68]. Prolonged exposure to the fermentative environment leads to starch surface damage (roughening, fissures, and localized erosion) and partial hydrolysis of  $\alpha$ -1,4-glycosidic linkages [69–71]. These changes occur mostly in the amorphous regions, enhancing the accessibility of starch to amylolytic enzymes and enabling yeast to efficiently metabolize starch [71,72]. This reduces the fermentation time by increasing fermentable sugar contents in the dough, which enhance gas formation during proofing and baking,



leading to an improved bread volume [73–75]. Furthermore, amylase reduces retrogradation by breaking down starch into smaller molecules, i.e., glucose, maltose, and dextrins. During bread staling, starch recrystallizes, which firms up the crumb. However, amylase disrupts this process by inhibiting the formation of double helices in amylose and in the linear regions of amylopectin, thereby reducing crystallinity, slowing retrogradation, and retarding the staling rate [76–78]. The screen of amylolytic activity showed that yeast strains had moderate amylolytic halos ( $1\text{ mm} < \text{halo} \leq 4\text{ mm}$ ). Although commercial *S. cerevisiae* typically lacks significant amylolytic activity and requires exogenous enzymes during industrial breadmaking, wild *S. cerevisiae* strains isolated from spontaneous fermentations have been reported to have amylolytic activity, possibly due to environment adaptation or horizontal gene transfer [79–81]. *S. cerevisiae* YCTA9 presented the largest amylolytic halo diameter, and *S. cerevisiae* YCTA10 showed a significantly smaller halo diameter compared to the former. In contrast, there were no significant differences in halo diameter among YCTA5, YCTA9, and YCTA12–YCTA16, as they all belonged to the same statistical group (Table 2). Amylolytic activity in yeasts was significantly higher than in LAB, as reported by Palla et al. [81] and Damayanti et al. [82]. Among LAB, amylolytic activity was generally low ( $<1\text{ mm}$ ), with *Lpb. plantarum* LABCTA6 showing the smallest halo diameter, and *Leu. mesenteroides* LABCTA3 the largest. However, the differences observed between LABCTA1, LABCTA6, and LABCTA8, and between LABCTA3 and LABCTA8 were not significant (Table 2). The amylolytic activity of LABCTA3 was significantly higher than LABCTA6, with halo diameters of, respectively, 0.90 mm and 0.81 mm. These findings align with previous research studies, such as Palla et al. [79], who analyzed 98 yeast strains isolated from PDO Tuscan sourdough and identified three *S. cerevisiae* strains as positive for amylase activity, showing hydrolysis halos of variable size up to 4 mm in diameter. Similarly, Akamine et al. [80] reported that one strain of *S. cerevisiae* isolated from laboratory-made sourdough showed a large amylase hydrolysis halo diameter ( $\geq 2\text{ cm}$ ). Regarding LAB, strains of *Lpb. plantarum* and *Leu. mesenteroides* have shown variable amylolytic activities, with halo diameters ranging from non-detectable (i.e., absence of a visible halo on starch-iodine agar plates) to values from 9 to 48 mm [83–85]. The amylase-producing capacity of both LAB and yeast strains depends on their source of isolation. Microorganisms obtained from starch- or amylose-rich environments tend to produce higher amounts of amylase than non-starch rich sources [86]. Consistently, Amapu et al. [84] reported 14 amylolytic LAB strains isolated from various substrates, including wet-milled cereals (sorghum, millet, corn, and acha), cassava flour, and tomato. Although isolates from corn flour exhibited the highest amylase activity (up to 1.10 U/mL) and hydrolysis halos (48 mm), the isolate from tomato (*L. brevis*) showed a large hydrolysis halo (42 mm) but the lowest reducing sugar concentration (0.30 mg/mL), suggesting limited enzymatic activity despite visual starch degradation. In contrast, the strain from corn (*L. plantarum* AMZ5) not only had the largest halo but also the highest concentration of reducing sugars (0.55 mg/mL), reinforcing the idea that starchy flours such as corn may serve as more effective inducers of amylase production. A similar pattern was observed for the strains isolated from cocoa mucilage in this study, which exhibited relatively low amylolytic activity, most likely due to the non-starchy nature of their isolation source.

The proteolysis process during sourdough fermentation is primarily influenced by the interaction between LAB and yeasts. However, studies have reported that the proteolytic activity of yeast strains isolated from sourdough is negligible compared to that of LAB [87–89]. Proteolysis contributes to enhancing both the technological and sensory qualities of bread by releasing peptides with antimicrobial properties and generating precursors for flavor and color compounds. These metabolites are key to the development and enhancement of the sensory profile of sourdough bread [90–93]. Moreover, the partial degradation of

gluten proteins modifies the viscoelastic properties of the dough, which can improve the bread volume and a soften crumb [94]. In addition, proteolysis delays the staling process by modifying the crumb structure and increasing moisture retention through changes in the water-binding capacity of the dough caused by protein breakdown [95].

**Table 2.** Enzymatic activities of yeasts and LAB from cocoa mucilage and coffee pulp.

| Strain                            | Halo Diameter (mm)         |                            |                            | Reference  |
|-----------------------------------|----------------------------|----------------------------|----------------------------|------------|
|                                   | Amylolytic Activity        | Proteolytic Activity       | Phytasic Activity          |            |
| Yeast                             |                            |                            |                            |            |
| <i>S. cerevisiae</i> YCTA5        | 1.36 ± 0.04 <sup>ab</sup>  | 34.15 ± 1.16 <sup>bc</sup> | 16.05 ± 1.19 <sup>a</sup>  | This study |
| <i>S. cerevisiae</i> YCTA9        | 1.43 ± 0.03 <sup>a</sup>   | 38.31 ± 1.15 <sup>a</sup>  | 15.85 ± 0.41 <sup>a</sup>  | This study |
| <i>S. cerevisiae</i> YCTA10       | 1.28 ± 0.03 <sup>b</sup>   | 36.51 ± 1.83 <sup>ab</sup> | 14.94 ± 1.04 <sup>a</sup>  | This study |
| <i>S. cerevisiae</i> YCTA12       | 1.37 ± 0.02 <sup>ab</sup>  | 37.78 ± 0.33 <sup>ab</sup> | 14.68 ± 0.24 <sup>a</sup>  | This study |
| <i>S. cerevisiae</i> YCTA13       | 1.39 ± 0.05 <sup>ab</sup>  | 31.98 ± 1.16 <sup>c</sup>  | 14.33 ± 0.21 <sup>a</sup>  | This study |
| <i>S. cerevisiae</i> YCTA14       | 1.37 ± 0.05 <sup>ab</sup>  | 39.86 ± 0.35 <sup>b</sup>  | 14.21 ± 0.66 <sup>a</sup>  | This study |
| <i>S. cerevisiae</i> YCTA15       | 1.37 ± 0.06 <sup>ab</sup>  | 15.97 ± 0.07 <sup>d</sup>  | 15.48 ± 0.38 <sup>a</sup>  | This study |
| <i>S. cerevisiae</i> YCTA16       | 1.40 ± 0.01 <sup>ab</sup>  | 36.26 ± 0.41 <sup>ab</sup> | 13.73 ± 0.31 <sup>a</sup>  | This study |
| <i>W. anomalus</i> YX1SR15        | 24.92 ± 0.02 <sup>ab</sup> | 25.08 ± 0.02 <sup>ab</sup> | 17.51 ± 0.11 <sup>ab</sup> | [32] *     |
| <i>W. anomalus</i> YX1SR23        | 20.04 ± 0.02 <sup>ab</sup> | 27.40 ± 0.04 <sup>ab</sup> | 15.82 ± 0.06 <sup>ab</sup> | [32] *     |
| LAB                               |                            |                            |                            |            |
| <i>Lpb. pentosus</i> LABCTA1      | 0.80 ± 0.01 <sup>B</sup>   | 27.80 ± 0.67 <sup>B</sup>  | 36.89 ± 2.14 <sup>B</sup>  | This study |
| <i>Leu. mesenteroides</i> LABCTA3 | 0.90 ± 0.01 <sup>A</sup>   | 40.00 ± 0.00 <sup>A</sup>  | 32.75 ± 0.04 <sup>B</sup>  | This study |
| <i>Lpb. plantarum</i> LABCTA6     | 0.81 ± 0.03 <sup>B</sup>   | 15.78 ± 1.09 <sup>C</sup>  | 48.48 ± 0.61 <sup>A</sup>  | This study |
| <i>Lpb. plantarum</i> LABCTA8     | 0.86 ± 0.01 <sup>AB</sup>  | 24.15 ± 1.31 <sup>B</sup>  | 46.00 ± 0.08 <sup>A</sup>  | This study |
| <i>Lpb. plantarum</i> LAB20B3HB   | 0.20 ± 0.01 <sup>ab</sup>  | 29.47 ± 0.31 <sup>ab</sup> | 22.00 ± 0.08 <sup>ab</sup> | [33] **    |

Amylolytic activity = low ≤ 1 mm; moderate 1 mm < halo ≤ 4 mm; high > 4 mm. Proteolytic activity = low ≤ 1 mm; moderate 1 mm < halo ≤ 5 mm; high > 5 mm. Phytasic activity = low ≤ 1 mm; moderate 1 mm < halo ≤ 15 mm; high > 15 mm. Different letters in the same column and the same group of microorganisms indicate significant differences (Tukey's HSD test,  $p < 0.05$ ). \* Data from Romero-Isaza [32]. \*\* Data from Vázquez-Vázquez [33].

The screen for proteolytic activity showed that the degradation halo varied among strains (Table 2). Although *S. cerevisiae* YCTA14 and YCTA9 exhibited the largest proteolytic halos, there were no significant differences between YCTA10, YCTA12, YCTA14, and YCTA16. In contrast, YCTA13 and particularly YCTA15 showed significantly lower proteolytic activity than YCTA14 and YCTA9. Among *Lactiplantibacillus* strains, *Lpb. pentosus* LABCTA1 and *Lpb. plantarum* LABCTA8 were not statistically different and showed intermediate degradation halos. Regarding the bacteria strains, *Leu. mesenteroides* LABCTA3 displayed the highest proteolytic activity, while *Lpb. plantarum* LABCTA6 exhibited the lowest value among bacterial strains. Contrary to the values commonly reported, recent studies have shown that strains of the species *S. cerevisiae* and LAB as *Lpb. plantarum* and *Limosilactobacillus fermentum* isolated from traditional sourdoughs have shown the ability to hydrolyze protein with a variable range from negligible to large halo diameters (>25 mm) [81,96,97]. In this study, LAB and yeast strains were isolated from coffee and cocoa fermentation, exhibiting varying proteolytic activities among yeast and LAB strains that ranged from approximately 15 to 40 mm; this reflects that the proteolytic activity is strain and source specific. Similar results have been reported for yeast and bacterial strains isolated from cocoa and coffee postharvest processes exhibiting proteolytic activity. Sukmawati et al. [98] screened 231 yeast isolates from fermented dried cocoa beans and identified three isolates producing proteolytic enzymes. Rodarten et al. [99] isolated 144 microorganisms from coffee fruit and found that 52.5% of bacteria and 2.6% of yeasts were capable of secreting proteases. Additionally, Muzaifa et al. [100] identified proteolytic

yeast strains isolated from Civet coffee, finding two strains of *Wickerhamomyces anomalus* and *Trichosporon asahii* species displaying protease activity. These findings highlight the importance of the strain isolation source and the potential application of allochthonous strains in starter co-culture formulation.

Finally, phytase-producing microorganisms play a key role in sourdough fermentation, as this enzyme is essential for breaking down phytic acid, an antinutritional factor that chelates essential minerals like iron, calcium, and zinc, reducing their bioavailability. Studies have demonstrated that sourdough fermentation activates both microbial and endogenous phytases, enhancing the bioaccessibility of minerals in whole-grain products [40,101]. The screening of phytase activity revealed notable differences among yeast and bacterial strains. Among yeasts, even though *S. cerevisiae* YCTA5 exhibited the highest phytase activity and *S. cerevisiae* YCTA16 displayed the lowest activity, the results obtained for YCTA5 to YCTA16 yeasts were not statistically different. Regarding bacterial strains, *Lpb. plantarum* LABCTA6 recorded the highest phytase activity, followed by *Lpb. plantarum* LABCTA8. *Lpb. plantarum* LABCTA6 and *Lpb. plantarum* LABCTA8 exhibited comparable phytase activity, with no statistically significant differences in halo diameters. Meanwhile, *Lpb. pentosus* LABCTA1 and *Leu. mesenteroides* LABCTA3 showed the lowest activity levels, but no statistical difference was observed between them, and both strains exhibited significantly lower activity than *Lpb. plantarum* LABCTA6 and LABCTA8. These results suggest that the isolated microorganisms may be efficient at phytate breakdown. Similarly, Palla et al. found that certain yeast strains display measurable phytase activity, indicating that yeasts may also contribute to phytic acid degradation in sourdough fermentation, although their enzymatic efficiency tends to be lower compared to LAB [102]. Additionally, Fekri et al. [103] isolated and identified phytate-degrading probiotic LAB and yeast strains from traditional sourdough, confirming that their enzymatic activity significantly improves whole wheat bread quality. Supporting this, Rizzello et al. [104] demonstrated that sourdough fermentation with selected LAB strains significantly increases phytase activity, leading to enhanced mineral bioavailability. These studies align with Gobetti et al. [105], who emphasized that the selection of phytase-producing strains improves mineral bioavailability and reduces anti-nutritional factors in the final product.

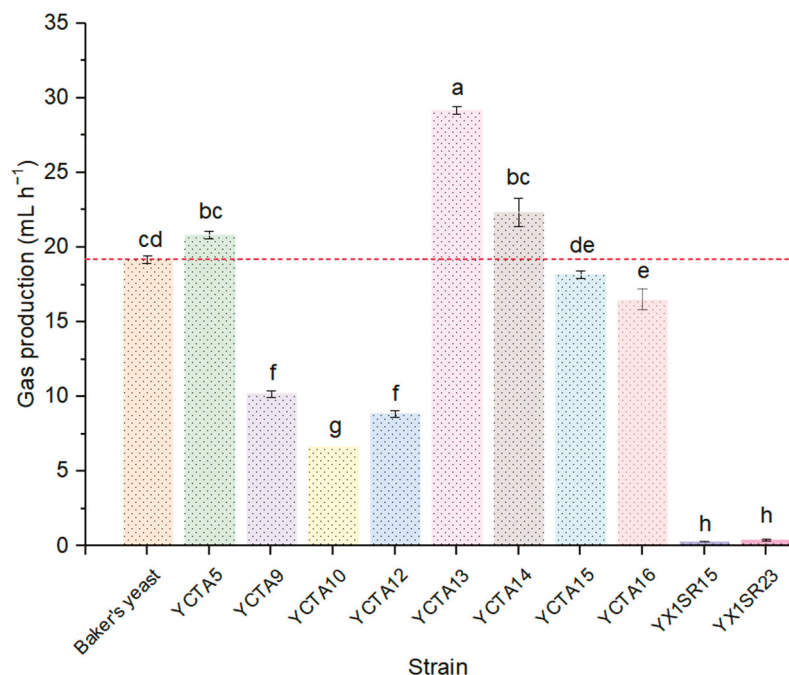
To drive the formulation of starter co-cultures, yeasts were assessed based on their CO<sub>2</sub> production, while LAB were evaluated according to their acidification capacity and EPS production. The integration of these results and the final selection of starter co-cultures are presented in the following sections.

### 3.2.2. Dough Fermentability by Yeast Isolated from Cocoa Mucilage and Coffee Pulp

The determination of leavening activity is directly related to the fermentability of bread dough by yeast strains, making it one of the most important selection criteria for a sourdough starter culture. The fermentability of dough by the evaluated yeast strains is shown in Figure 2.

Compared to commercial *S. cerevisiae*, which exhibited a leavening activity of 19.17 mL·h<sup>-1</sup>, the dough fermented with strain *S. cerevisiae* YCTA13 displayed the highest gas production at 29.17 mL·h<sup>-1</sup>, followed by *S. cerevisiae* YCTA14 (22.33 mL·h<sup>-1</sup>) and *S. cerevisiae* YCTA5 (20.83 mL·h<sup>-1</sup>). These results highlight the superior fermentative capacity of *S. cerevisiae* YCTA13. However, the *S. cerevisiae* YCTA14 and *S. cerevisiae* YCTA5 strains also demonstrated higher leavening activity than the commercial strain. In contrast, the *W. anomalus* YX1SR15 and YX1SR23 yeast strains, isolated from coffee fermentation, exhibited negligible leavening activity under the experimental conditions, despite their high enzymatic activity values, showing limited dough fermentability support through low CO<sub>2</sub> production. Similar results were reported by Sergeeva et al. [106] who evalu-

ated the breadmaking potential of *W. anomalus* CBSS605T and *T. delbrueckii* YIT3 strains, finding that both strains showed lower leavening ability in wheat dough compared to control *S. cerevisiae*, and that these strains needed more time to form the required structure of dough and bread crumb. This may be because *W. anomalus* has been described as a Crabtree-negative yeast, which leads to the low production of ethanol and CO<sub>2</sub> [107–109]. Thus, due to their limited leavening capacities, *W. anomalus* strains were discarded from the starter co-culture formulation.



**Figure 2.** Gas production rate of yeast strains isolated from cocoa mucilage and coffee pulp (mL·h<sup>-1</sup>), measured after 4 h of incubation at 30 °C. Baker's yeast (Commercial *S. cerevisiae*); YCTA5–YCTA16, *S. cerevisiae* YCTA5–YCTA16; YX1SR15, *W. anomalus* YX1SR15; YX1SR23, *W. anomalus* YX1SR23. The dotted red line represents the gas production rate of the commercial baker's yeast (*Saccharomyces cerevisiae*), which was used as a reference control for comparison. Different letters above the bars indicate significant differences (Tukey's HSD test,  $p < 0.05$ ).

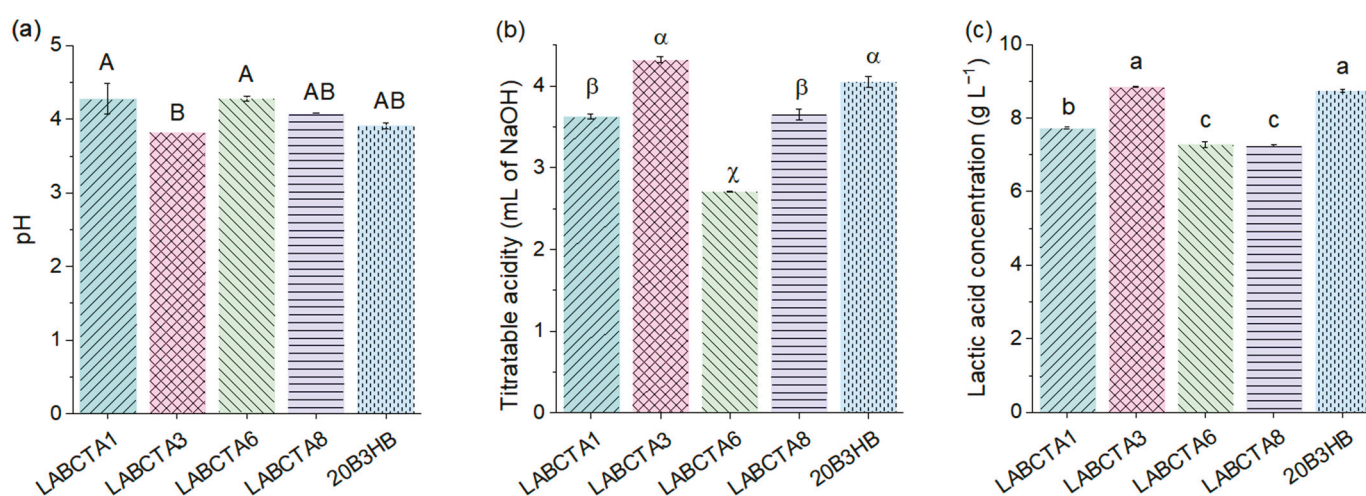
The results showed that *S. cerevisiae* YCTA5, YCTA13, and YCTA14 exhibited the highest CO<sub>2</sub> production among the evaluated strains. The results from YCTA5 and YCTA14 are not significantly different. This may be linked to their efficient metabolism of maltose and other fermentable sugars, as reported by Sánchez-Adriá et al. [110], who described the enhanced leavening activity and CO<sub>2</sub> production in yeast species as *S. cerevisiae*, *Kazachstania humilis* and *Torulaspora delbrueckii* when compared to commercial yeast. Similarly, Woo et al. [111] observed that a high gas-producing strain (*S. cerevisiae* BW1) improved gas retention and dough aeration in gluten-free sourdoughs. Ferraz et al. [112] demonstrated that higher yeast concentrations are related to increased gas production, which improve dough rise and bread texture. Selecting yeast strains with superior CO<sub>2</sub> production ensures a more efficient fermentation process and improved final product quality. Ogunsakin et al. [6] highlighted that yeast strains with CO<sub>2</sub> production and tolerance to acidic stress (pH 3.0–4.0) are ideal candidates for sourdough applications. Yeast isolates from cocoa fermentation have been reported to be acid-tolerant, as they can grow in acidic environments with lactic and acetic acid concentrations ranging from 0.5 to 2% (v/v) [113–115]. The cocoa-isolated yeast strains evaluated in this study were previously screened for acid stress tolerance. When comparing the specific growth rates ( $\mu$ ) of *S. cerevisiae* YCTA5, YCTA13, and YCTA14 in YPD broth as control (0.72, 1.68 and 0.65 h<sup>-1</sup>, respectively) with their performance under stress



conditions, pH 3.0 (0.58, 0.92, and 1.03 h<sup>-1</sup>), pH 4.0 (0.60, 0.60, and 0.65 h<sup>-1</sup>), and 2% *v/v* lactic acid (0.39, 0.56, and 0.48 h<sup>-1</sup>), it can be concluded that these strains are able to grow under the acidic conditions (pH 3.8–4.0) during sourdough fermentation [3]. This factor is expected to influence yeast performance positively in sourdough fermentation. In addition, Shen et al. [7] reported that yeast strains with high fermentability and enzymatic activity enhanced bread sensory properties. Based on gas production, enzymatic activity, and acid stress tolerance, the yeast strains *S. cerevisiae* YCTA5, YCTA13, and YCTA14 were selected for further analysis in co-culture with the chosen LAB strains, as co-culturing *S. cerevisiae* with LAB as *Lpb. plantarum* has been reported to improve bread quality characteristics such as leavening, volume, and texture [116].

### 3.2.3. Determination of Lactic Acid Production by LAB from Cocoa Mucilage and Coffee Pulp

The acid-producing capacity of LAB strains isolated from cocoa mucilage and coffee pulp was assessed by measuring pH, titratable acidity (TTA), and lactic acid concentrations in broth cultures after 16 h of fermentation at 30 °C (Figure 3). As shown in Figure 3a, *Leu. mesenteroides* LABCTA3 exhibited the lowest pH value at 3.82, followed by *Lpb. plantarum* 20B3HB at 3.91 and LABCTA8 at 4.07. Although these last two values were not significantly different from those of *Lpb. plantarum* LABCTA1 (4.28) and LABCTA6 (4.29), they suggest a strain-dependent acidification capacity. The pH results were supported by TTA values between 2.71 and 3.92 mL of NaOH (Figure 3b), with strains *Leu. mesenteroides* LABCTA3 showing the highest TTA value (4.33 mL of NaOH), which was significantly higher than *Lpb. plantarum* LABCTA6 (2.71 mL of NaOH). In terms of lactic acid production (Figure 3c), *Leu. mesenteroides* LABCTA3 also exhibited the highest concentration (8.84 g/L), followed by *Lpb. plantarum* 20B3HB (8.74 g/L), both of which were significantly higher than *Lpb. plantarum* LABCTA1 (7.28 g/L) and LABCTA8 (7.24 g/L). These results confirm a strong correlation between pH, TTA, and lactic acid production. The variability observed among strains in our results may be attributed to differences in metabolic pathways, fermentation rates, and stress adaptation mechanisms [7]. The correlation between pH, TTA, and lactic acid concentration features the efficiency of these strains in acid production, which is crucial for their potential application as sourdough starter culture strains.



**Figure 3.** Results of the analysis of fermented broths after 16 h at 30 °C in MRS broth: (a) pH values, (b) titratable acidity values (mL of NaOH), (c) lactic acid production of LAB (g/L). LABCTA1, *Lpb. pentosus* LABCTA1; LABCTA3, *Leu. mesenteroides* LABCTA3; LABCTA6, *Lpb. plantarum* LABCTA6; LABCTA8, *Lpb. plantarum* LABCTA8; 20B3HB, *Lpb. plantarum* 20B3HB. Different letters above the bars indicate significant differences (Tukey's HSD test, *p* < 0.05).



The acidification capacity of LAB influences dough pH, microbial stability, texture, shelf-life, and overall bread quality. A decrease in pH is essential for inhibiting undesirable microorganisms, extending shelf life, and enhancing the development of volatile compounds that contribute to the sensory profile of sourdough bread [4,5,117–119]. Additionally, the production of organic acids, such as lactic and acetic acids, has been associated with an improved crumb structure and gluten network stabilization. Wu et al. [120] reported that LAB with a high acidification capacity induced a lower dough pH, which enables gluten hydrolysis and depolymerization; these changes promote gluten network development and viscoelasticity. On the other hand, acetic acid, which is often produced by heterofermentative LAB, contributes to an improved crumb softness and bread shelf life due to its antifungal properties [121]. During sourdough fermentation, organic acids such as lactic and acetic acids contribute to delaying bread staling by modifying starch retrogradation and protein interactions. These acids reduce the rearrangement of linear amylose chains through acid hydrolysis, leading to chain shortening and decreased crystallization [122,123]. Similarly, in amylopectin, an acidic environment increases the proportion of shorter branches and disrupts the formation of double helices which drives amylopectin retrogradation [69,124,125]. Furthermore, carboxyl groups from organic acids can react with hydroxyl groups in starch to form ester bonds, resulting in chemically modified starches with a lower retrogradation capacity [123,125]. Regarding gluten, the acids reduce the dough pH below the isoelectric point, altering the gluten protein's surface charge and denaturing its structure; this promotes the formation of disulfide bond formation between gliadin and glutenin, enhancing dough cohesivity and extensibility [126,127]. The resulting protein network reduces water mobility and limits molecular rearrangements, thereby slowing bread retrogradation during storage [128]. With regard to our results, similar acidification patterns have been reported in studies evaluating the performance of *Lpb. plantarum* and *Leu. mesenteroides* in sourdough fermentation. For example, *Lpb. plantarum* strains can decrease the pH to values between 3.7 and 4.0 after 16–24 h of fermentation, which aligns with the results obtained in this study [129]. Likewise, *Leu. mesenteroides* has been reported to exhibit high lactic and acetic acid production due to its heterofermentative metabolism, contributing to the characteristic sourness and aromatic profile of sourdough. Additionally, *Leu. mesenteroides* has been associated with EPS production, which improves moisture retention in the crumb and enhances bread texture [8]. This introduces the next LAB selection criteria to be discussed, which will be crucial in determining the most suitable bacteria strains.

#### 3.2.4. Exopolysaccharide (EPS) Production by LAB from Cocoa Mucilage and Coffee Pulp

When selecting the LAB strains, it is important to consider EPS production, as it contributes to bread structure stabilization and has been widely reported in sourdough fermentation studies [130–134]. The results of the LAB screen are presented in Table 3, and only strain *Leu. mesenteroides* B4 exhibited EPS production among the strains isolated from cocoa fermentation.

EPSs interact with gluten and starch, enhancing viscoelasticity and preventing crumb staling [135]. They act as natural hydrocolloids, reducing the need for synthetic additives and improving overall bread quality [8]. The results obtained in this study align with those reported in the literature, as several LAB strains have been reported to produce EPSs in sourdough fermentation [134–136]. It is important to highlight that *Lpb. plantarum* 20B3HB, a previously screened sourdough starter culture candidate, exhibits EPS production as well [33]. Xu et al. [137] found that *Lpb. plantarum* and *Leuconostoc* species isolated from fermented foods exhibited high EPS production, positively influencing bread texture and shelf-life. Similarly, Menezes et al. [40] reported that sourdough fermented

with EPS-producing LAB improved crumb softness and delayed retrogradation. Among EPS-producing LAB species, *Leu. mesenteroides* is known for its production of heteropolysaccharides, primarily dextran, which enhances dough hydration, elasticity, and the sensory attributes of sourdough bread [134,137]. In contrast, *Lpb. plantarum* produces homopolysaccharides, which act as structural stabilizers in the dough matrix, improving gas retention and the crumb structure [40,134]. Variability in EPS production among LAB strains can be attributed to genetic differences in polysaccharide biosynthesis pathways, fermentation conditions, and carbohydrate utilization preferences [111]. Considering their EPS production, along with acidification capacity and enzymatic activity, the *Leu. mesenteroides* LABCTA3 and *Lpb. plantarum* 20B3HB strains were selected as candidates for inclusion in co-culture with the selected yeast strains for sourdough fermentation.

**Table 3.** EPS production screen of lactic acid bacteria strains after 48 h at 30 °C in MRS agar supplemented with 4% (*w/v*) sucrose.

| Bacteria Strain                   | EPS Production | Reference               | <i>Leu. mesenteroides</i> LABCTA3                                                   |
|-----------------------------------|----------------|-------------------------|-------------------------------------------------------------------------------------|
| <i>Lpb. pentosus</i> LABCTA1      | -              | This work               |  |
| <i>Leu. mesenteroides</i> LABCTA3 | +              | This work               |                                                                                     |
| <i>Lpb. plantarum</i> LABCTA6     | -              | This work               |                                                                                     |
| <i>Lpb. plantarum</i> LABCTA8     | -              | This work               |                                                                                     |
| <i>Lpb. plantarum</i> 20B3HB      | +              | Vazquez-Vazquez [33] ** |                                                                                     |

(+) Growth/production; (-) growth/absence of production. Representative image of EPS produced by *Leu. mesenteroides* LABCTA3. \*\* Data from Vazquez-Vazquez [33].

### 3.3. Sourdough Bread Quality

Six different mixed starter co-cultures were formulated based on the selection of the highest CO<sub>2</sub>-producing yeasts (*S. cerevisiae* YCTA5, YCTA13 and YCTA14 from cocoa mucilage) and two LAB strains with a highly acidification capacity and EPS production (*L. mesenteroides* LABCTA3 from cocoa mucilage and *Lpb. plantarum* 20B3HB from coffee pulp). Co-culture formulations are shown in Table 4. These co-cultures were designed to combine the metabolic properties of each strain in sourdough fermentation and to assess their impacts on the final bread parameters, such as the volume, height, and crumb firmness, which are linked to the microbial synergy during sourdough fermentation [138]. Microbial synergy refers to the cooperative interactions between yeasts and LAB that enhance fermentation and result in improved bread quality parameters [120,139].

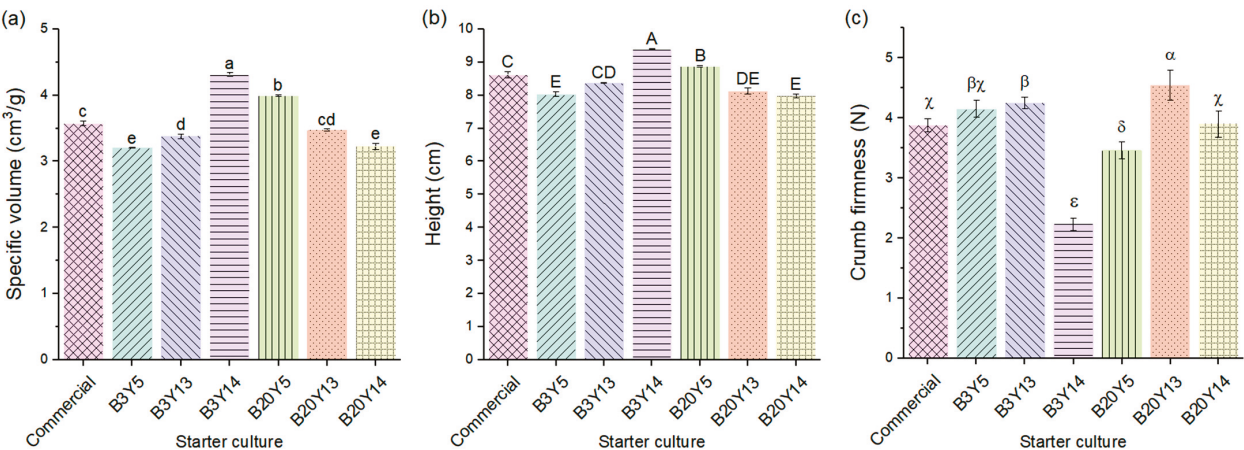
**Table 4.** Starter co-culture formulations of selected LAB and yeast strains.

| Starter Co-Culture | LAB Strain                        | Yeast Strain                |
|--------------------|-----------------------------------|-----------------------------|
| B3Y5               | <i>Leu. mesenteroides</i> LABCTA3 | <i>S. cerevisiae</i> YCTA5  |
| B3Y13              | <i>Leu. mesenteroides</i> LABCTA3 | <i>S. cerevisiae</i> YCTA13 |
| B3Y14              | <i>Leu. mesenteroides</i> LABCTA3 | <i>S. cerevisiae</i> YCTA14 |
| B20Y5              | <i>Lpb. plantarum</i> 20B3HB      | <i>S. cerevisiae</i> YCTA5  |
| B20Y13             | <i>Lpb. plantarum</i> 20B3HB      | <i>S. cerevisiae</i> YCTA13 |
| B20Y14             | <i>Lpb. plantarum</i> 20B3HB      | <i>S. cerevisiae</i> YCTA14 |

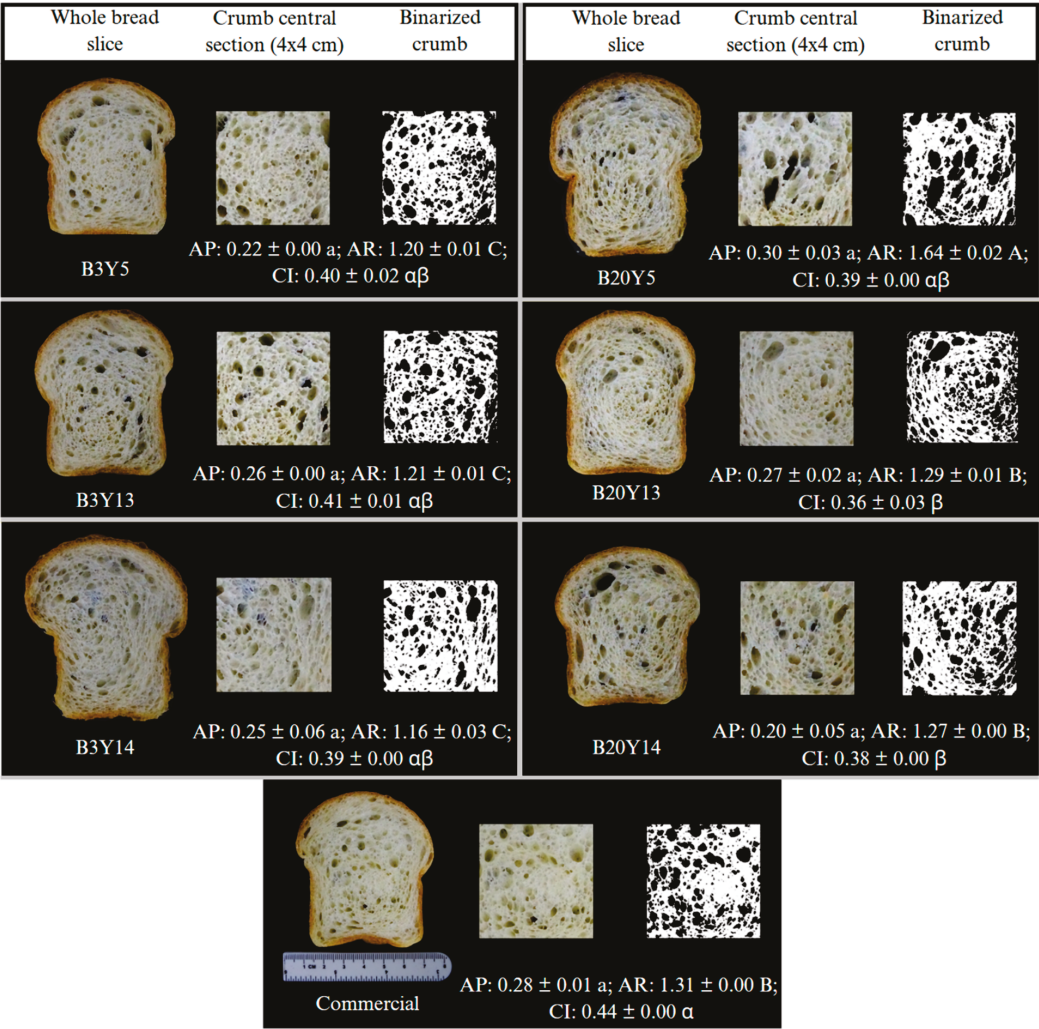
In this study, the analysis of bread quality parameters revealed statistically significant differences among the treatments (Figure 4). The B3Y14 co-culture showed the significantly highest specific volume (4.31 cm<sup>3</sup>/g) and loaf height (9.40 cm), and softest crumb (2.23 N). Starter co-culture B20Y5 also showed greater performance with a high specific volume (3.99 cm<sup>3</sup>/g) and loaf height (8.88 cm<sup>3</sup>/g), though statistically lower than B3Y14

(4.31 cm<sup>3</sup>/g) but higher than the commercial control (3.57 cm<sup>3</sup>/g). In contrast, co-cultures B3Y5 and B20Y14 exhibited the lowest specific volume (3.21 cm<sup>3</sup>/g and 3.23 cm<sup>3</sup>/g, respectively) and height (8.04 cm and 7.98 cm, respectively), with no significant difference between them, and both were significantly lower than the control (3.57 cm<sup>3</sup>/g, 8.63 cm), while B20Y14 had the lowest loaf height (7.98 cm). Regarding crumb firmness, B3Y14 yielded the softest crumb (2.23 N), which was significantly different from all other treatments, while B20Y13 (4.55 N), B3Y13 (4.25 N), and B3Y5 (4.15 N) resulted in the firmest crumbs, with no significant differences among them, but significantly firmer than the control (3.88 N) and B3Y14. The results for bread made with the B3Y14 and B20Y5 starters are explained by the microbial synergy between the highly acid-tolerant, CO<sub>2</sub>-producing yeasts and LAB strains with EPS production and enzymatic activity, leading to optimal gas retention, dough expansion, and crumb softness. In contrast, the B3Y5 and B20Y14 starter co-cultures showed a weak synergistic effect, likely due to competition for substrates, the production of inhibitory metabolites, or the absence of effective metabolic cross-feeding, as described by Ponomarova et al. [140] and Canon et al. [141]. These metabolic interactions may limit nutrient availability and metabolic cooperation between yeast and LAB, resulting in restricted dough rise and firmer crumbs, as described by Sun et al. [142]. The starter co-cultures B3Y13 and B20Y13 showed intermediate results, indicating partial functional compatibility that does not improve bread quality, which may be due to the low acid tolerance of the yeast strain. This observation is consistent with Carbonetto et al. [143], who found that in most sourdough yeast–LAB combinations, the presence of LAB reduced yeast populations, while LAB was rarely affected by yeasts. They emphasized that negative or neutral interactions seem to be dominant, often resulting from direct competition for substrates or the production of inhibitory metabolites. Furthermore, effective metabolic cross-feeding is not always established, and interactions are frequently competitive rather than cooperative, which affects fermentation and bread quality. The starter co-cultures B3Y13 and B20Y13 showed intermediate results, indicating partial functional compatibility that does not improve bread quality. The improvement in breadcrumb softness with the B3Y14 starter co-culture is consistent with Graça et al. [14], who used the incorporation of yogurt as a fermentation starter and thus obtained softer crumb structures and reduced staling. Yu et al. [10] demonstrated that spontaneous sourdoughs containing *Lpb. plantarum* and *Leuconostoc* species significantly improved bread porosity and overall structural quality. This result agrees with Woo et al. [111] and Elhariry et al. [116], who emphasized the cooperative fermentation effects of LAB and yeasts on stabilizing gas cells and enhancing crumb elasticity.

The results of the crumb alveolar analysis regarding alveolar porosity (AP), regularity/uniformity (AR), and alveolar circularity index (CI) exhibited differences between treatments (Figure 5). Porosity in bread crumb is an indicator of the retained gas during fermentation and baking. Although the highest AP was observed using co-culture B20Y5, no significant differences in alveolar porosity were detected between treatments. Porosity values are related to the gas cell structure, as alveoli are directly shaped by fermentation and gluten network stability. Low porosity values (<0.20) are associated with dense crumbs, while moderate porosity (0.20–0.35) indicates efficient gas retention and a stable crumb structure; in contrast, high porosity (>0.35) may result in large void formation or crumb collapse [144,145]. The results obtained in this study, ranging from 0.20 to 0.30, fit within the moderate range, which is desirable in sourdough bread, as it reflects a balanced interaction between microbial fermentation and dough structure, avoiding excessive expansion and crumb collapse.



**Figure 4.** Effect of different starter co-cultures on sourdough bread properties. (a) Specific volume (cm<sup>3</sup>/g), (b) height (cm), and (c) crumb firmness (N). Different letters above the bars indicate significant differences (Tukey's HSD test,  $p < 0.05$ ). For the starter co-culture nomenclature, see Table 4.



**Figure 5.** Crumb structure analysis of sourdough breads prepared with different starter co-cultures. From left to right: whole bread slice, central crumb section, and binarized crumb. Alveoli porosity (AP), regularity (AR), and circularity index (CI). For the starter co-culture nomenclature, see Table 4. Values sharing the same letter or symbol within each class are not significantly different (Tukey's HSD test,  $p > 0.05$ ). Images were scaled using a reference ruler during acquisition, and standardization was ensured across all treatments. The ruler in the slides was omitted from the final figure for clarity.

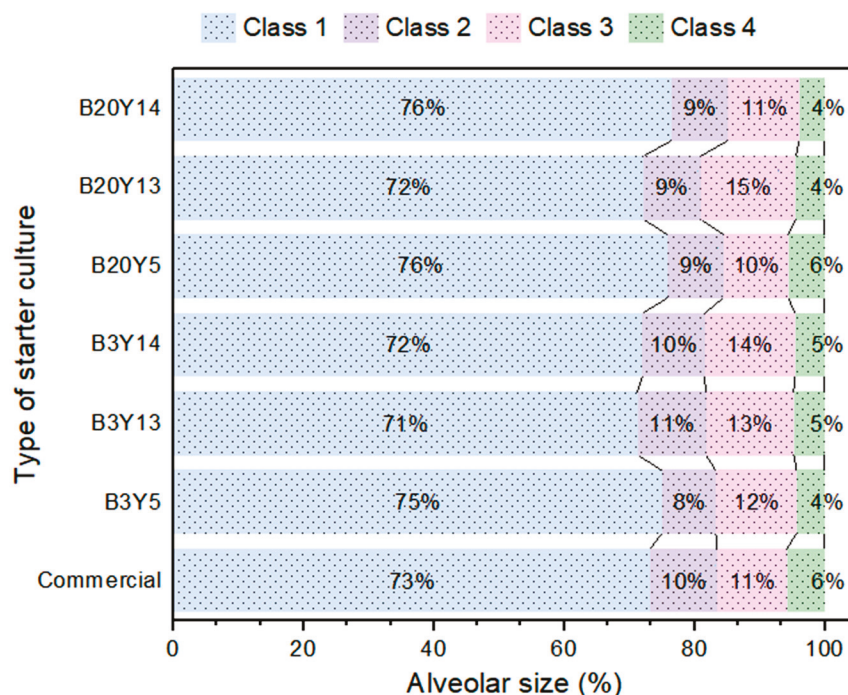


Regarding crumb alveolar regularity (AR), this parameter is associated with sensory perception and structural consistency, as it reflects crumb uniformity. Regularity was assessed through the coefficient of variation of Feret's diameter, calculated using Fiji/ImageJ. Significant differences were found among treatments ( $p < 0.05$ ). The B20Y5 co-culture exhibited the highest alveolar regularity value (1.64), indicating the most significant variation in alveolar diameter size and more heterogeneous crumb structure. These results align with Gonzales-Barron and Butler [42], who reported that higher gas cell variability is associated with crumb uniformity. In contrast, the use of co-culture B3Y14 showed the lowest alveolar regularity, which was significantly lower than B20Y5 and the commercial control (1.31), suggesting a uniform and consistent crumb. Similarly, B3Y5 and B3Y13 showed uniform crumb structures that were statistically comparable to B3Y14. The commercial starter presented an intermediate regularity that was not significantly different from B20Y13 and B20Y14. These results indicate that B3Y14 favored the formation of a homogeneous alveolar structure, which is desirable in sourdough bread, as a uniform alveolar distribution is linked to a better crumb structure and mouthfeel properties [145]. This uniformity may be attributed to the balanced metabolic activity of its microbial components, which favor even gas production and prevent the coalescence of alveoli, especially under controlled fermentation conditions [146].

Alveolar shape, quantified through the circularity index (CI), reflects the geometric regularity of gas cells within the bread crumb. Circularity values close to 1.0 indicate perfectly round alveoli [147]. Lower circularity values ( $<0.50$ ) have been consistently reported in sourdough breads compared to those leavened with commercial yeast ( $>0.80$ ) due to differences in fermentation kinetics, dough acidification, and gluten network development [147–149]. In our study, the commercial sourdough starter yielded the highest circularity, with only statistically significant differences ( $p < 0.05$ ) relative to B20Y13 and B20Y14. Intermediate values were observed in B3Y5, B3Y13, B3Y14, and B20Y5 (0.39–0.41), which did not differ significantly from the commercial reference. While breads made with baker's yeast alone typically present circularity values between 0.80 and 0.90, sourdough breads commonly show lower values, ranging from 0.45 to 0.70 depending on microbial diversity, dough rheology, and fermentation time [46,148]. The circularity values observed in this study ( $<0.45$ ) align with the structural behavior of sourdough bread, as mentioned before. These lower circularity values may result from metabolic interactions involving acidification,  $\text{CO}_2$  production, proteolysis, and EPS-producing strains. Such interactions influence gas cell expansion and stabilize or disrupt gluten matrices, as previously observed in sourdoughs fermented with *Leu. mesenteroides* and *S. cerevisiae* [95,140,142,150].

The alveolar area distribution was classified into five size classes (Figure 6). Class 1 alveoli ( $0.05\text{--}0.49\text{ mm}^2$ ), associated with a fine and homogeneous crumb, predominated in all treatments, accounting for more than 70% of the crumb structure in all cases. Intermediate-size alveoli (classes 2 and 3) ranged from 8 to 15%, contributing to a moderately open crumb structure [148], while large alveoli (classes 4 and 5), often considered structural defects [144,145], represented  $\leq 6\%$  of the total alveolar area. Statistical analysis using the Kruskal–Wallis test revealed no significant differences ( $p > 0.05$ ) among treatments within any of the alveolar size classes. Consequently, it was not possible to associate specific microbial consortia with distinct alveolar class distributions. These results suggest that although microbial combinations may influence the crumb microstructure in other ways (e.g., alveolar regularity and circularity), the overall proportion of each alveolar size remains relatively stable across treatments.





**Figure 6.** Percentage distribution of alveolar size in bread crumb produced with different starter co-cultures. Alveoli were classified by individual area into five classes: class 1 (0.05–0.49 mm<sup>2</sup>), class 2 (0.50–0.99 mm<sup>2</sup>), class 3 (1.00–4.99 mm<sup>2</sup>), class 4 (5.00–49.99 mm<sup>2</sup>), and class 5 (>50 mm<sup>2</sup>). For the starter co-culture nomenclature, see Table 4. No statistically significant differences were found among treatments within any alveolar class (Kruskal–Wallis test,  $p > 0.05$ ).

In conclusion, the image analysis of the crumb structure provided insights into the impact of different starter co-cultures on the alveolar structure in sourdough breads. While porosity values remained within a desirable moderate range, differences in alveolar regularity and the circularity index revealed notable variations in crumb uniformity and gas cell morphology. In particular, the use of the B3Y14 co-culture, composed of *Leu. mesenteroides* LABCTA3 and *S. cerevisiae* YCTA14, promoted a homogeneous alveolar distribution and balanced gas cell geometry.

#### 4. Conclusions

This study demonstrates that sourdough starter cultures formulated with yeast and LAB strains isolated from cocoa and coffee fermentations can enhance the technological and sensory qualities of sourdough bread. The selected *S. cerevisiae* YCTA13 strain showed superior leavening activity with 52.24% increase in CO<sub>2</sub> production when compared with commercial *S. cerevisiae*, improving dough aeration. *Leuconostoc mesenteroides* LABCTA3 displayed promising EPS production and acidification ability by reaching the lowest pH value (3.82), the highest titratable acidity (4.33 mL NaOH), and the highest lactic acid concentration (8.84 g/L), highlighting its potential as a functional LAB component in sourdough fermentation.

The B3Y14 co-culture (LABCTA3 + YCTA14) significantly improved the bread volume and height by 35.53 and 17.18%, respectively, compared to the commercial sourdough starter, and reduced crumb firmness by 59.66%. Additionally, B3YCTA14 reduced crumb firmness by 42.5%, resulting in a significantly softer texture. This co-culture also enhanced the crumb structure by increasing alveolar porosity by 3.8%, reducing regularity by 11.5%, and lowering the circularity index by 9.1% compared to the commercial control, reflecting a more uniform and typical sourdough morphology.

These findings highlight the importance of screening strains for selection based on functional properties, particularly CO<sub>2</sub> production, acidification and EPS production, to optimize sourdough performance through starter culture application. Furthermore, using microorganisms from sources other than sourdough opens new possibilities to find functional starter cultures. Future research should focus on deepening the understanding of microbial interactions and their impacts on bread quality, the aroma profile, and shelf-life.

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## Abbreviations

The following abbreviations are used in this manuscript:

|     |                      |
|-----|----------------------|
| LAB | Lactic acid bacteria |
| EPS | Exopolysaccharide    |
| AP  | Alveolar porosity    |
| AR  | Alveolar regularity  |
| CI  | Circularity index    |

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## Article

# Impacts of Sonication on Fermentation Process and Physicochemical, Microbiological and Sensorial Characteristics of Fermented Black Carrot Juice

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## Abstract

In recent years, ultrasound has been integrated into fermentation technology due to its activating effect on microorganisms, and the possible effects of ultrasound-assisted fermentation on the fermentation process, yield and quality of the final product have also attracted attention. This study aimed to reveal the effects of sonication applied before the fermentation on the fermentation process and the quality of fermented black carrot juice. The samples were sonicated at a frequency of 35 kHz and an amplitude of 60% for 0, 5, 15 or 30 min before the fermentation. During the fermentation, the pH, acidity, organic acid profile, ethanol and soluble solid content (SSC), color, turbidity, total lactic acid bacteria (LAB), total mesophilic aerobic bacteria (TMAB) and yeast counts were determined. The amount of SSC in the samples increased at the beginning of fermentation as the sonication time increased. Lactic, acetic and propionic acids were detected in the samples. The amount of lactic acid in all the samples treated with ultrasound was higher than in the control sample and the amounts of acetic acid, propionic acid and ethanol were lower. Ultrasound application caused an increase in the TMAB and yeast counts. A five-minute ultrasound application caused a decrease in the number of LAB, while 15- and 30-min applications caused an increase. Thirty minutes of ultrasound treatment resulted in the reddest fermented black carrot juices with the highest level of color saturation. The most appreciated sample in terms of taste, aroma and general acceptability was the sample subjected to a five-minute ultrasound application. As a result, ultrasound application before fermentation positively supports different quality parameters of fermented black carrot juice and the use of sonication in production can be recommended.

**Keywords:** fermented black carrot juice; sonication; lactic beverage; ultrasound-assisted fermentation

## 1. Introduction

Ultrasound is defined as sound waves with a frequency above the hearing limit of the human ear (20 kHz and above). It is also referred to as high-frequency pressure waves. Ultrasound within the 20 kHz–1 MHz range is characterized as low-frequency or high-power sound waves. Ultrasonic waves with a frequency greater than 1 MHz are classified as high-frequency sound waves and are frequently referred to as diagnostic sound waves [1]. Ultrasound technology finds application in a variety of fields such as enzyme inhibition,



microorganism inactivation, foaming, foam breaking, improving filtration efficiency, crystallization of fats and sugars, CO<sub>2</sub> degassing during fermentation, maturation and oxidation of alcoholic beverages, meat aging, humidification, cutting, waste treatment, precipitation of airborne dust, blending, freezing, melting, surface decontamination, emulsification and extraction of bioactive components [2–12]. In addition to microbial inactivation, ultrasound can also stimulate cell growth [13]. The use of appropriate ultrasound conditions favorably enhances the growth of microbial cells. Low-intensity sound waves create stable cavitations in liquid media and cause reversible damage to microbial cells. In this way, it accelerates the growth of microbial cells, changes their life phases and enables them to produce more metabolic products.

In previous studies, it has been reported that ultrasound application increases fibrinolytic enzyme production from *Bacillus sphaericus* MTCC 3672 by 1.48 times [14], accelerates the proliferation mechanism of *Saccharomyces cerevisiae* cells [15], shortens the fermentation time by up to 24 h and increases the amount of ethanol by 3.5 times [16,17], shortens fermentation time up to 36 h, increases the amount of riboflavin by 5 times in the production of riboflavin from *Ecemothecium ashbyii* [18], reduces the fermentation time of beverages such as wine, beer and sake produced with *S. cerevisia* by 50–64% [19] and reduces fermentation time in yogurt production by approximately 40% [20,21].

Although the mechanism by which ultrasound promotes the growth of microorganisms is not fully understood, it has been suggested that ultrasound provides faster passage of components, such as sugars, from plant tissue to the fermentation medium, increases cell biomass by improving the nutrient utilization of bacterial cells, accelerates intracellular and extracellular substance transfer due to increased cell membrane permeability, removes toxic compounds from the cell, and accelerates the entry of nutrients into the cell.

Fermented black carrot juice is a red-colored, cloudy and sour-tasting fermented lactic beverage. Its production involves the use of bulgur flour, turnip, water, sourdough, black carrot and salt as raw materials [22,23]. The beverage has its origins in the southeastern Anatolian provinces of Türkiye, particularly Adana, Mersin, Hatay and Kahramanmaraş, and has become a well-known and popular beverage throughout Turkey [24]. The beverage has been reported to exhibit antiproliferative activity and probiotic properties thanks to its phenolic compounds and microbial flora and has been reported to have positive effects on health [25,26]. Fermented black carrot juice contains *S. cerevisiae*, non-*Saccharomyces* yeasts, and both homo and heterofermentative lactic acid bacteria. The predominant genus among the lactic acid bacteria is *Lactobacillus*, comprising 89.63%, followed by *Leuconostoc* (9.63%) and *Pediococcus* (0.74%) [22]. The sugar necessary for the development of lactic acid bacteria is found in raw materials.

The fermentation process of black carrot juice typically requires 20 to 45 days, with the duration contingent on factors such as the chosen fermentation method and the ambient temperature. Although it is thought that fermentation is completed when acidity development stops in lactic acid fermentation, it is known that fresh fermented beverages are sensory-weak and need post-fermentation maturation for taste, aroma, color and texture development [27,28]. During maturation, a variety of biochemical reactions take place, leading to the formation of compounds that contribute to the development of color and aroma. Ultrasound is of great interest in fermentation technology as it promotes the growth of microorganisms, increases enzyme activity and metabolic performance, rapidly supplies the microorganism with the components it needs through extraction and enables post-fermentation maturation by promoting reactions such as oxidation, esterification, proteolysis and the Maillard reaction during maturation [27–30].

To the best of our knowledge, no study has been conducted on the effect of ultrasound applied to fermented black carrot juice before fermentation. This research was designed



to provide a new source for literature and to provide data for industrial production. The aim of this study was to determine the effects of ultrasound applications at different times before the fermentation on the fermentation process of fermented black carrot juice, as well as on the beverage's color, acidity, ethyl alcohol content, turbidity, total bacteria count, lactic acid bacteria count, yeast count and sensory properties.

## 2. Materials and Methods

### 2.1. Material

Black carrots, bulgur flour, baker's yeast (*Saccharomyces cerevisiae*), salt and drinking water were used in the production of fermented black carrot juice. Black carrots and bulgur flour were obtained from Gunseven Inc., Ereğli, Türkiye. Baker's yeast (Pak Gıda, Düzce, Türkiye), salt (Billur Salt Industry Co., İzmir, Türkiye) and water (Buzdağı, Seli Inc., Sakarya, Türkiye) were purchased from the local market.

### 2.2. Methods

#### 2.2.1. Fermentation and Sonication Methods

Bulgur (0.91%) and yeast (0.2%) were subjected to fermentation in a 1 L glass jar at ambient temperature for a 24-h period. Subsequently, black carrots (16.6%), which had been sliced into 1 cm thick and 10 cm long pieces after the removal of their heads and tails, salt (1.16%), and water were added. The jars were tightly closed. In this way, 24 samples with the same formulation were prepared and divided into 4 groups with 6 samples in each group. The initial group of six samples was not exposed to ultrasound, and these samples were considered the control group. The second group subjected to the ultrasound procedure was exposed for a duration of five minutes, while the third group was exposed for 15 min and the fourth group was exposed for 30 min. On the first day, a single jar from each group was selected for analysis. The remaining jars were subjected to a fermentation process at ambient temperature (25 °C). The fermentation process was periodically evaluated by analyzing samples collected from each group on days 4, 8, 12, 20 and 30. The ultrasound application was performed in an ultrasonic-assisted water bath (Elma, Transsonic TI-H-10, Singen, Germany). The jars, tightly sealed, were placed in the device and exposed to sonication at a frequency of 35 kHz and an amplitude of 60%. During the sonication process, the temperature was controlled and maintained at 25 °C by means of water circulation. The experiment was carried out in 3 replicates.

#### 2.2.2. Analysis Methods

##### pH, Soluble Solid Content, Total Titratable Acidity, Color and Turbidity Analyses

The pH of the fermented carrot juice samples was measured using a glass electrode pH meter (Inolab 720, WTW, Weilheim, Germany), while the soluble solid content values were determined with an Atago refractometer (HSR-500, Tokyo, Japan). The turbidity values were measured with a WTW turbidimeter (TURB 430 T, Weilheim, Germany), and the reflectance color values ( $L^*$ ,  $a^*$ ,  $b^*$ ,  $C^*$ , and  $h$ ) of the samples were measured with a spectrophotometer (CM-5, Konica Minolta, Osaka, Japan). All measurements were carried out at 20 °C. For the total titratable acidity analysis, 10 mL of the sample was taken, 20 mL of the pure water was added, and the pH was determined by titrating with 0.1 N NaOH until the pH was 8.2. The results are expressed as g lactic acid equivalent/100 mL [31].

##### Organic Acid Profile and Ethyl Alcohol Analyses

Organic acid profile and ethyl alcohol content of fermented black carrot juices were determined by HPLC (Agilent 1260, Agilent Technologies, Inc., Santa Clara, CA, USA). Prior to injection, the samples were diluted 1:2 (v/v) with ultrapure water and passed through a

0.45 µm pore diameter PVDF (polyvinylidene fluoride) filter. Separation was carried out on an Aminex HPX-87H column. Sulfuric acid (0.005 N) was used as mobile phase, and the flow rate was set to 0.6 mL/min. Ethanol was detected with a refractive index detector and organic acids were detected with a DAD detector set to 210 nm wavelength [32].

### Microbiological Analyses

Plate Count Agar was used for total mesophilic aerobic bacteria count, Potato Dextrose Agar for yeast count and De Man, Rogosa and Sharpe agar for lactic acid bacteria (LAB) count. Dilutions up to  $10^{-10}$  were prepared from fermented black carrot juices and 1 mL was taken to the media and inoculated according to the pour plating method. The inoculated petri dishes were incubated at 37 °C for 48 h for total mesophilic bacteria count, at 28 °C for 72 h for yeast count and at 37 °C for 48 h for lactic acid bacteria count. Microbiological counting results of fermented black carrot juices were given as log cfu/mL [23].

### Sensory Evaluation

Sensory analysis of fermented black carrot juice was carried out using a 0- to 9-point scale indicating color, aroma, taste, appearance and overall acceptability. The sensory analysis was conducted in accordance with ISO standard guidelines with a group of 15 volunteer panelists consisting of male and female non-smokers, aged between 25 and 50 years, from the Faculty of Agriculture, Selcuk University. The panelists conducted sensory evaluations of the samples taken on the 12th, 20th and 30th day of the fermentation period. The samples were presented to the panelists in 50 mL transparent glasses at 4 °C temperature [23].

### Statistical Analysis

The results were given as mean  $\pm$  standard deviation. To determine the effect of both ultrasound application and fermentation process, the data obtained were subjected to Analysis of Variance using MINITAB release 16.0 program. The Tukey Multiple Comparison Test was used to check whether the differences between group means were significant.

## 3. Results

Table 1 shows the pH, total titratable acidity, turbidity and soluble solid content values in the fermentation process of fermented black carrot juice samples. At the beginning of fermentation, the pH of the samples ranged from 5.85 to 6.17 and it was seen that the pH increased after 5 and 15 min of ultrasound application and the pH decreased with a 30 min ultrasound application. However, on the 30th day of fermentation, the lowest pH value was determined in the samples with ultrasound applied for 15 min.

**Table 1.** pH, total titratable acidity, soluble solid content and turbidity values of fermented black carrot juice samples.

| Sonication Time (min) | Fermentation Period (Day) | pH              | Total Titratable Acidity (g/L) | Soluble Solid Content (%) | Turbidity (NTU)    |
|-----------------------|---------------------------|-----------------|--------------------------------|---------------------------|--------------------|
| 0                     | 0                         | 5.85 $\pm$ 0.09 | 0.011 $\pm$ 0.000              | 1.40 $\pm$ 0.28           | 163.00 $\pm$ 12.73 |
|                       | 4                         | 3.72 $\pm$ 0.05 | 0.207 $\pm$ 0.003              | 2.00 $\pm$ 0.00           | 255.00 $\pm$ 0.00  |
|                       | 8                         | 3.44 $\pm$ 0.02 | 0.377 $\pm$ 0.018              | 2.40 $\pm$ 0.28           | 208.50 $\pm$ 31.80 |
|                       | 12                        | 3.52 $\pm$ 0.04 | 0.301 $\pm$ 0.018              | 1.80 $\pm$ 0.00           | 121.30 $\pm$ 74.60 |
|                       | 20                        | 3.36 $\pm$ 0.09 | 0.378 $\pm$ 0.0912             | 2.15 $\pm$ 0.21           | 223.50 $\pm$ 77.10 |
|                       | 30                        | 3.53 $\pm$ 0.00 | 0.359 $\pm$ 0.018              | 2.25 $\pm$ 0.21           | 162.50 $\pm$ 0.71  |

Table 1. Cont.

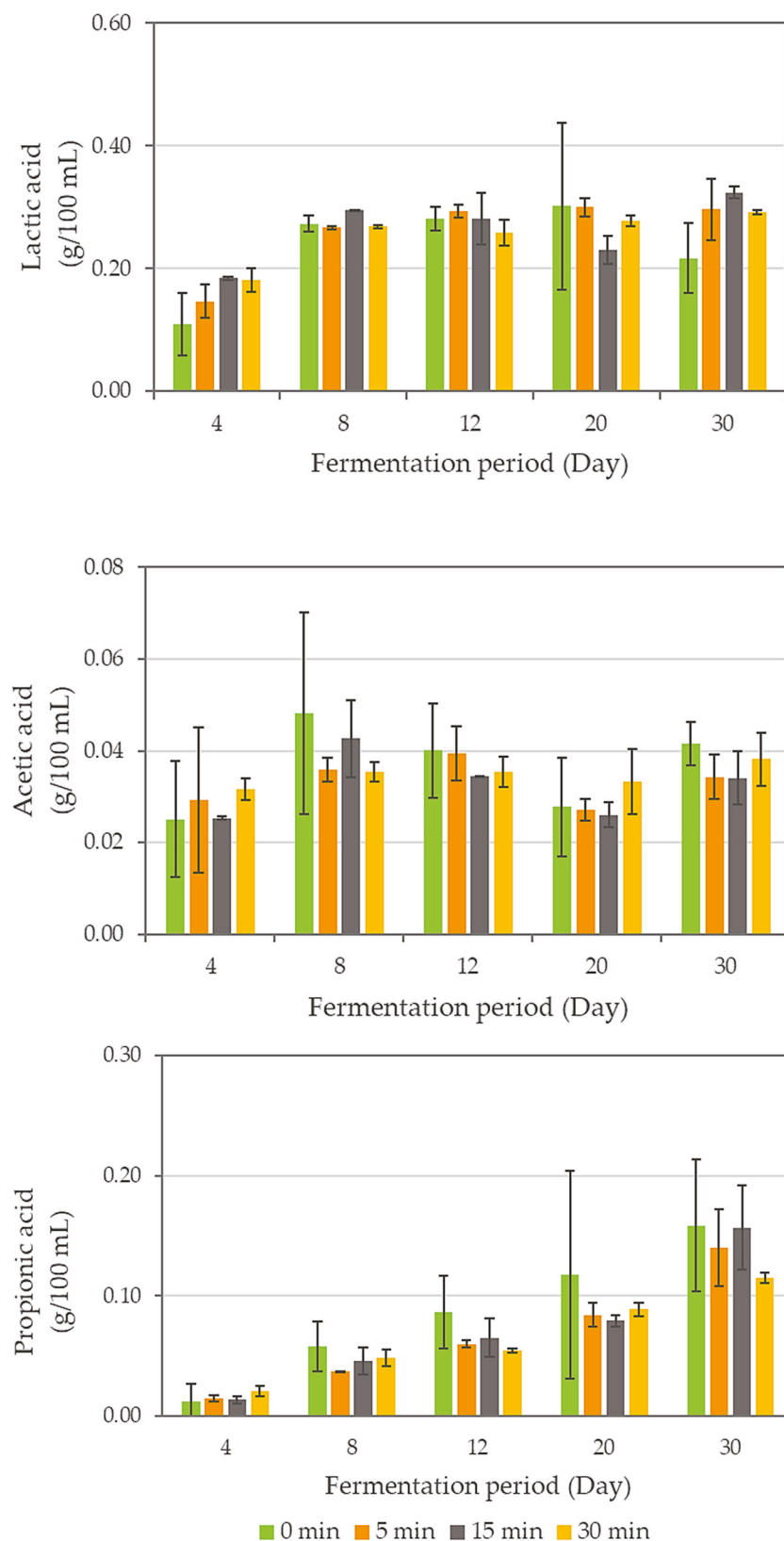
| Sonication Time (min) | Fermentation Period (Day) | pH          | Total Titratable Acidity (g/L) | Soluble Solid Content (%) | Turbidity (NTU) |
|-----------------------|---------------------------|-------------|--------------------------------|---------------------------|-----------------|
| 5                     | 0                         | 6.17 ± 0.45 | 0.008 ± 0.005                  | 1.40 ± 0.00               | 181.00 ± 17.00  |
|                       | 4                         | 3.61 ± 0.10 | 0.205 ± 0.087                  | 2.00 ± 0.00               | 271.00 ± 45.30  |
|                       | 8                         | 3.49 ± 0.02 | 0.268 ± 0.054                  | 1.80 ± 0.28               | 183.50 ± 2.12   |
|                       | 12                        | 3.48 ± 0.01 | 0.292 ± 0.066                  | 1.70 ± 0.42               | 167.50 ± 37.50  |
|                       | 20                        | 3.38 ± 0.01 | 0.407 ± 0.026                  | 2.20 ± 0.00               | 217.00 ± 7.07   |
|                       | 30                        | 3.50 ± 0.05 | 0.353 ± 0.061                  | 2.00 ± 0.57               | 190.00 ± 80.60  |
| 15                    | 0                         | 6.05 ± 0.20 | 0.006 ± 0.000                  | 1.70 ± 0.14               | 221.50 ± 7.78   |
|                       | 4                         | 3.62 ± 0.02 | 0.211 ± 0.048                  | 2.05 ± 0.07               | 271.00 ± 58.00  |
|                       | 8                         | 3.44 ± 0.02 | 0.364 ± 0.026                  | 2.40 ± 0.00               | 157.50 ± 40.30  |
|                       | 12                        | 3.45 ± 0.00 | 0.288 ± 0.036                  | 1.65 ± 0.21               | 208.50 ± 26.20  |
|                       | 20                        | 3.49 ± 0.06 | 0.299 ± 0.051                  | 1.85 ± 0.50               | 219.00 ± 65.10  |
|                       | 30                        | 3.43 ± 0.01 | 0.420 ± 0.059                  | 1.75 ± 0.35               | 148.00 ± 35.40  |
| 30                    | 0                         | 5.83 ± 0.34 | 0.009 ± 0.005                  | 1.75 ± 0.07               | 175.00 ± 31.10  |
|                       | 4                         | 3.56 ± 0.06 | 0.265 ± 0.003                  | 2.20 ± 0.28               | 253.50 ± 31.80  |
|                       | 8                         | 3.46 ± 0.01 | 0.314 ± 0.041                  | 2.20 ± 0.28               | 228.50 ± 26.20  |
|                       | 12                        | 3.56 ± 0.06 | 0.276 ± 0.110                  | 1.40 ± 0.00               | 203.50 ± 26.20  |
|                       | 20                        | 3.46 ± 0.00 | 0.332 ± 0.015                  | 1.80 ± 0.28               | 208.00 ± 4.24   |
|                       | 30                        | 3.53 ± 0.01 | 0.317 ± 0.005                  | 1.70 ± 0.14               | 208.00 ± 62.20  |

The soluble solid content of the fermented black carrot juices ranged from 1.40 to 1.75 at the beginning of fermentation and from 1.75 to 2.25 at the end of fermentation. The soluble solid content increased with 15- and 30-min ultrasound applications before fermentation, and the soluble solid content of these samples was lower during the fermentation.

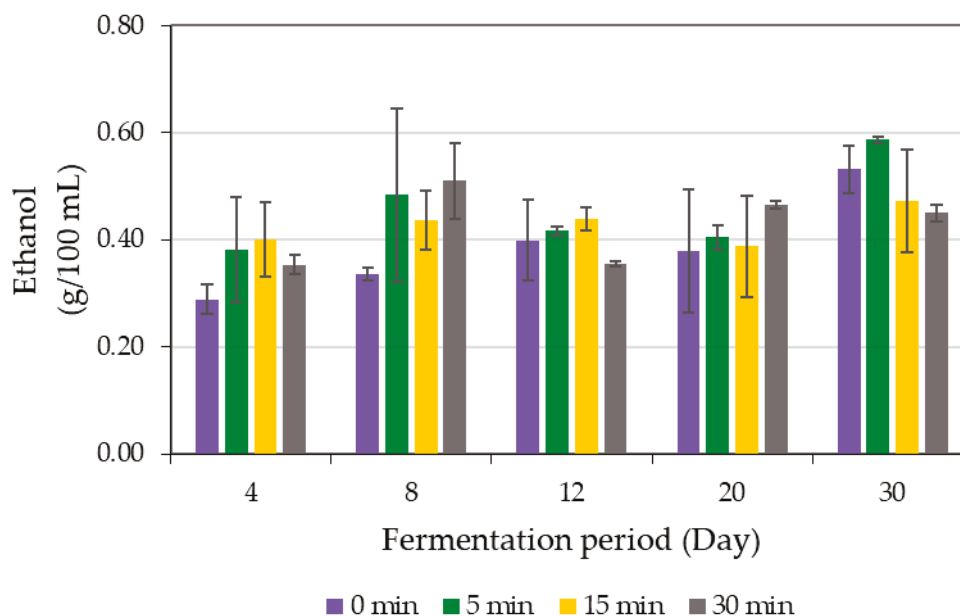
During the fermentation, the pH decreased, and the acidity and soluble solid content increased until the 30th day, although it showed fluctuations. Although the fermentation process had a statistically significant effect on the pH, acidity, turbidity, and soluble solid content, the effect of sonication on these parameters was statistically insignificant.

The organic acid content of fermented black carrot juice samples is illustrated in Figure 1, while the ethanol content is demonstrated in Figure 2. Lactic, acetic and propionic acids were detected in the samples. At the beginning of fermentation, the control sample had the lowest lactic acid content (0.110 mg/100 mL), while the sample treated with ultrasound for 15 min had the highest content (0.184 mg/100 mL). At the beginning of fermentation, the lowest lactic acid content was observed in the control sample (0.110 mg/100 mL), while the highest content was recorded in the sample exposed to 15 min of ultrasound (0.184 mg/100 mL). The highest amount of lactic acid was determined on the 20th day of fermentation in the control sample to be in the 5-min ultrasound-treated samples, and on the 30th day of fermentation in the 15-min ultrasound-treated sample. After 30 days of fermentation, it was found that the samples treated with ultrasound had a higher level of lactic acid compared to the control sample.

As in the case of lactic acid, it was determined that the amounts of acetic acid, propionic acid and ethanol in the samples treated with ultrasound at the beginning of fermentation were lower. As the fermentation process continued, a decrease in the levels of acetic and propionic acids were observed in the samples treated ultrasound. After 30 days of the fermentation, the lowest propionic acid was found in the samples that had been treated with 30 min of ultrasound, and the lowest acetic acid was found in the samples that had been exposed to 5 and 15 min of ultrasound. As the fermentation process approached its 30th day, a higher concentration of ethanol was observed in the sample subjected to 5 min of ultrasound treatment than that of the control sample. Conversely, a lower concentration of ethanol was detected in the samples that were exposed to 15 and 30 min of ultrasound treatment, in contrast to the control sample.



**Figure 1.** Lactic, acetic and propionic acid contents (g/100 mL) of fermented black carrot juice samples.



**Figure 2.** Ethanol contents of fermented black carrot juice samples.

Table 2 shows the total mesophilic aerobic bacteria, lactic acid bacteria and yeast count of fermented black carrot juices. The ultrasound treatment exhibited a significant impact on the microbial load and population within the fermentation medium. At the beginning of fermentation, an increase in the total number of bacteria and yeast was observed with ultrasound application, while ultrasound application caused a decrease in the number of lactic acid bacteria, except for 30 min of ultrasound application. As the fermentation progressed, the total number of bacteria decreased. This decrease was the lowest in the control sample and the highest in the sample treated with ultrasound for 5 min. During the fermentation, there was an increase on the 8th day and a decrease on the 12th day.

The lactic acid bacteria count was higher in the control sample than in the sample treated with ultrasound for 5 min, and lower than in the samples treated with ultrasound for 15 or 30 min. On the 12th day of fermentation, the highest lactic acid bacteria count was detected in the sample treated with ultrasound for 30 min. The yeast count decreased as the fermentation progressed. The lowest value was found in the control sample, while the highest value was found in the sample that was treated with ultrasound for 30 min. The lactic acid bacteria count of the control sample was higher than that of the sample treated with ultrasound for 5 min, and lower than the samples treated with ultrasound for 15 or 30 min. On the 12th day of fermentation, the highest lactic acid bacteria count was detected in the sample treated with ultrasound for 30 min. The yeast count decreased as the fermentation progressed. The lowest value was found in the control sample, while the highest value was found in the sample that was treated with ultrasound for 30 min.

Table 3 presents the color values of fermented black carrot juice samples. The  $L^*$ ,  $a^*$ ,  $b^*$ ,  $C^*$  and  $h$  represent brightness, redness, yellowness, color saturation and angle value, respectively, on the Hunter scale.

**Table 2.** Total Mesophilic Aerobic Bacteria (TMAB), Lactic Acid Bacteria Count (LAB) and yeast counts of fermented black carrot juice samples.

| Ultrasound Time (min) | Fermentation Period (Day) | TMAB (log cfu/mL)     | LAB (log cfu/mL)     | Yeast (log cfu/mL)    |
|-----------------------|---------------------------|-----------------------|----------------------|-----------------------|
| 0                     | 0                         | $9.19 \pm 0.06^{abc}$ | $7.81 \pm 0.39^{ab}$ | $7.17 \pm 0.01^{ab}$  |
|                       | 8                         | $9.15 \pm 0.01^{abc}$ | $8.37 \pm 0.06^a$    | $6.03 \pm 0.16^{bcd}$ |
|                       | 12                        | $8.10 \pm 0.23^{de}$  | $7.23 \pm 0.26^{bc}$ | $4.23 \pm 0.10^e$     |



Table 2. Cont.

| Ultrasound Time (min) | Fermentation Period (Day) | TMAB (log cfu/mL)          | LAB (log cfu/mL)            | Yeast (log cfu/mL)        |
|-----------------------|---------------------------|----------------------------|-----------------------------|---------------------------|
| 5                     | 0                         | 9.88 ± 0.06 <sup>a</sup>   | 6.03 ± 0.12 <sup>d</sup>    | 7.92 ± 0.05 <sup>a</sup>  |
|                       | 8                         | 9.10 ± 0.00 <sup>abc</sup> | 7.80 ± 0.22 <sup>ab</sup>   | 5.60 ± 0.05 <sup>cd</sup> |
|                       | 12                        | 7.15 ± 0.50 <sup>f</sup>   | 7.05 ± 0.361 <sup>bcd</sup> | 4.83 ± 0.38 <sup>de</sup> |
| 15                    | 0                         | 9.76 ± 0.09 <sup>ab</sup>  | 6.39 ± 0.05 <sup>cd</sup>   | 8.14 ± 0.11 <sup>a</sup>  |
|                       | 8                         | 8.98 ± 0.07 <sup>bc</sup>  | 8.50 ± 0.28 <sup>a</sup>    | 6.53 ± 0.01 <sup>bc</sup> |
|                       | 12                        | 7.41 ± 0.28 <sup>ef</sup>  | 7.75 ± 0.52 <sup>ab</sup>   | 5.05 ± 0.52 <sup>de</sup> |
| 30                    | 0                         | 9.62 ± 0.16 <sup>ab</sup>  | 7.61 ± 0.15 <sup>ab</sup>   | 8.15 ± 0.12 <sup>a</sup>  |
|                       | 8                         | 8.42 ± 0.19 <sup>cd</sup>  | 8.32 ± 0.22 <sup>a</sup>    | 6.27 ± 0.28 <sup>bc</sup> |
|                       | 12                        | 7.70 ± 0.22 <sup>def</sup> | 8.02 ± 0.13 <sup>ab</sup>   | 5.84 ± 0.56 <sup>cd</sup> |

Different letters in the same column indicate statistically significant differences between samples.

Table 3. Color parameters of fermented black carrot juice samples.

| Ultrasound Time (min) | Fermentation Period (Day) | <i>L</i> *   | <i>a</i> *   | <i>b</i> *    | <i>C</i> *    | <i>h</i>     |
|-----------------------|---------------------------|--------------|--------------|---------------|---------------|--------------|
| 0                     | 0                         | 70.83 ± 1.94 | 0.78 ± 0.14  | 5.02 ± 0.62   | 5.08 ± 0.63   | 81.16 ± 0.49 |
|                       | 4                         | 27.59 ± 3.21 | 60.36 ± 2.37 | 45.41 ± 4.26  | 75.55 ± 4.45  | 36.92 ± 1.51 |
|                       | 8                         | 22.72 ± 0.30 | 56.06 ± 0.24 | 38.15 ± 0.424 | 67.81 ± 0.44  | 34.24 ± 0.18 |
|                       | 12                        | 23.42 ± 0.00 | 56.46 ± 0.27 | 39.34 ± 0.042 | 68.82 ± 0.21  | 34.87 ± 0.16 |
|                       | 20                        | 24.74 ± 3.90 | 57.75 ± 3.85 | 41.56 ± 6.63  | 71.19 ± 6.98  | 35.62 ± 2.53 |
|                       | 30                        | 20.10 ± 2.62 | 52.48 ± 3.02 | 33.68 ± 4.43  | 62.38 ± 4.94  | 32.61 ± 1.93 |
| 5                     | 0                         | 68.30 ± 1.77 | 0.70 ± 0.15  | 4.11 ± 0.33   | 4.170 ± 0.35  | 80.47 ± 1.31 |
|                       | 4                         | 27.63 ± 3.16 | 60.73 ± 2.43 | 45.69 ± 4.26  | 76.02 ± 4.50  | 36.91 ± 1.47 |
|                       | 8                         | 25.01 ± 2.63 | 58.28 ± 2.37 | 41.98 ± 4.36  | 71.85 ± 4.47  | 35.71 ± 1.73 |
|                       | 12                        | 24.13 ± 3.63 | 57.16 ± 3.22 | 40.56 ± 6.17  | 70.13 ± 6.19  | 35.24 ± 2.60 |
|                       | 20                        | 21.71 ± 0.75 | 55.04 ± 0.85 | 36.37 ± 1.31  | 65.97 ± 1.43  | 33.45 ± 0.54 |
|                       | 30                        | 22.13 ± 4.33 | 54.78 ± 4.60 | 37.15 ± 7.42  | 66.24 ± 7.98  | 33.95 ± 3.10 |
| 15                    | 0                         | 68.77 ± 0.11 | 0.83 ± 0.04  | 4.905 ± 0.516 | 4.98 ± 0.52   | 80.38 ± 0.53 |
|                       | 4                         | 26.27 ± 2.92 | 59.41 ± 2.50 | 43.94 ± 4.67  | 73.91 ± 4.79  | 36.42 ± 1.76 |
|                       | 8                         | 20.24 ± 0.85 | 53.58 ± 0.90 | 33.87 ± 1.36  | 63.39 ± 1.48  | 32.30 ± 0.60 |
|                       | 12                        | 25.52 ± 0.19 | 58.85 ± 0.13 | 42.95 ± 0.24  | 72.85 ± 0.26  | 36.13 ± 0.09 |
|                       | 20                        | 22.32 ± 5.90 | 54.98 ± 6.55 | 37.38 ± 10.15 | 66.56 ± 11.12 | 33.87 ± 4.10 |
|                       | 30                        | 20.69 ± 6.17 | 53.16 ± 7.14 | 34.63 ± 10.68 | 63.55 ± 11.80 | 32.64 ± 4.64 |
| 30                    | 0                         | 68.25 ± 3.76 | 0.91 ± 0.23  | 5.56 ± 0.72   | 5.64 ± 0.76   | 80.83 ± 1.22 |
|                       | 4                         | 24.91 ± 1.85 | 58.21 ± 1.77 | 41.88 ± 3.11  | 71.72 ± 3.25  | 35.71 ± 1.20 |
|                       | 8                         | 23.23 ± 1.90 | 56.62 ± 1.98 | 38.99 ± 3.21  | 68.75 ± 3.45  | 34.52 ± 1.27 |
|                       | 12                        | 25.01 ± 5.47 | 57.91 ± 5.01 | 41.70 ± 8.88  | 71.42 ± 9.25  | 35.53 ± 3.46 |
|                       | 20                        | 23.27 ± 1.92 | 56.44 ± 1.92 | 39.05 ± 3.38  | 68.65 ± 3.50  | 34.65 ± 1.41 |
|                       | 30                        | 24.41 ± 3.06 | 56.83 ± 2.86 | 40.86 ± 5.09  | 69.51 ± 4.58  | 35.64 ± 2.02 |

At the initiation of fermentation, the *L*\*, *a*\*, *b*\*, *C*\* and *h* values of the control sample were 70.8, 0.78, 5.02, 5.08 and 81.16, respectively. Throughout the fermentation, the brightness (*L*\*) and *h* values of the fermented black carrot juices decreased, while the redness (*a*\*), yellowness (*b*\*) and color saturation (*C*\*) increased. At the beginning of fermentation, the *L*\* and *h* values of the samples decreased with ultrasound application, the redness values of the samples with 15- and 30-min ultrasound application increased and the yellowness and color saturation values of the samples with 30 min of ultrasound application were higher than the control sample. It was found that the reddest and most saturated color was obtained on the 30th day of fermentation of the sample treated with ultrasound for 30 min.

Figure 3 shows the radar plot of the sensory evaluation of the fermented black carrot juice samples on days 12, 20 and 30 of fermentation. On the 12th day of the fermentation, the sample that received a 30-min ultrasound treatment had the lowest sensory scores in

terms of taste, appearance and overall acceptability. In terms of color, aroma and overall acceptability, the samples with 5 and 15 min of ultrasound application scored higher than the control sample. On the 20th day of fermentation, the ultrasound-applied samples were more appreciated in terms of all sensory parameters except color. The panelists rated the fermented black carrot juice produced with a 5-min ultrasound application as the most acceptable in terms of taste, aroma and overall acceptability. In terms of appearance, the sample treated with 30 min of ultrasound was the most highly appreciated. In terms of color, the sample without ultrasound treatment was most highly rated. Ultrasound treatment had an overall positive effect on the sensory characteristics of the fermented black carrot juices.

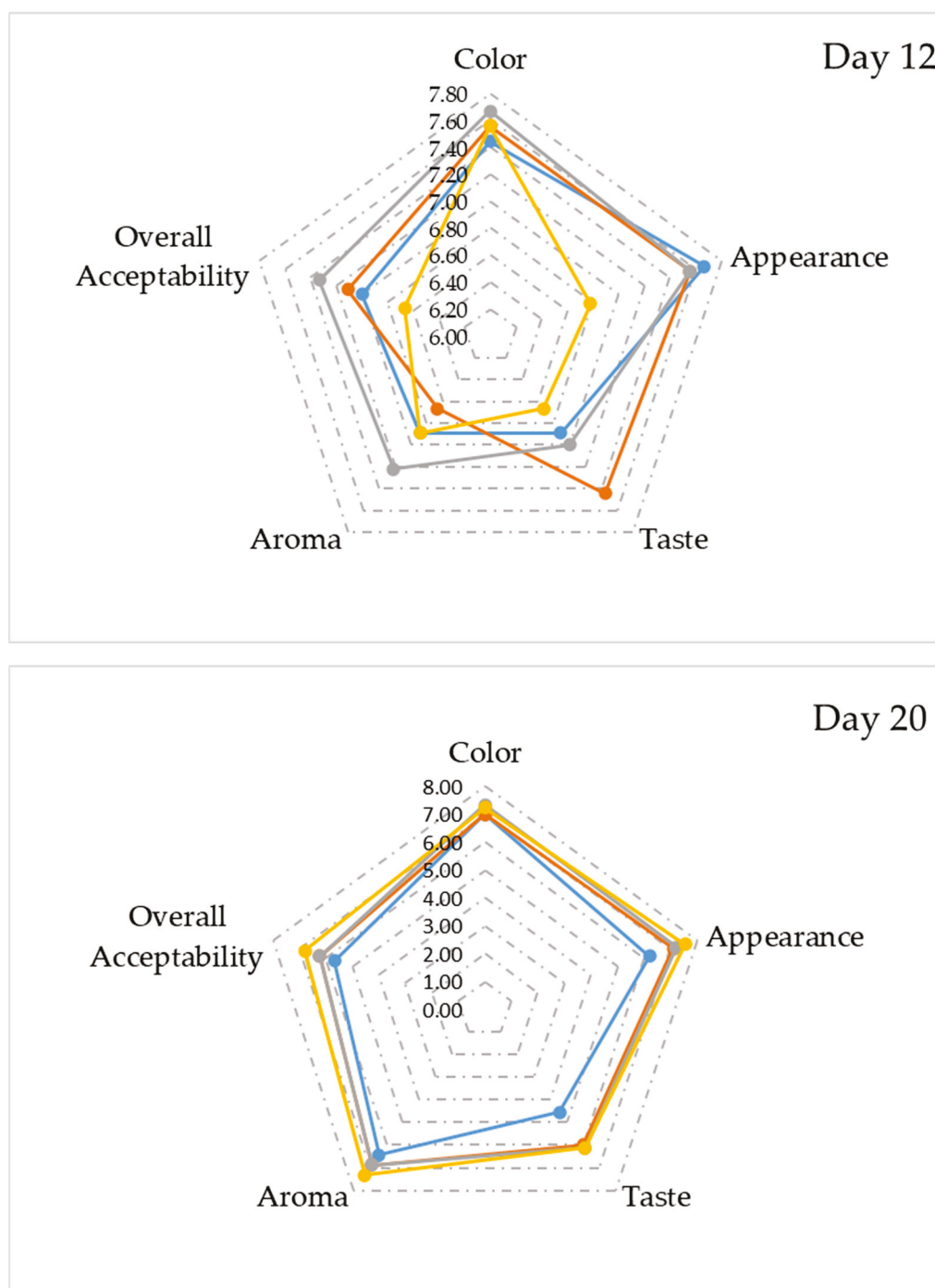
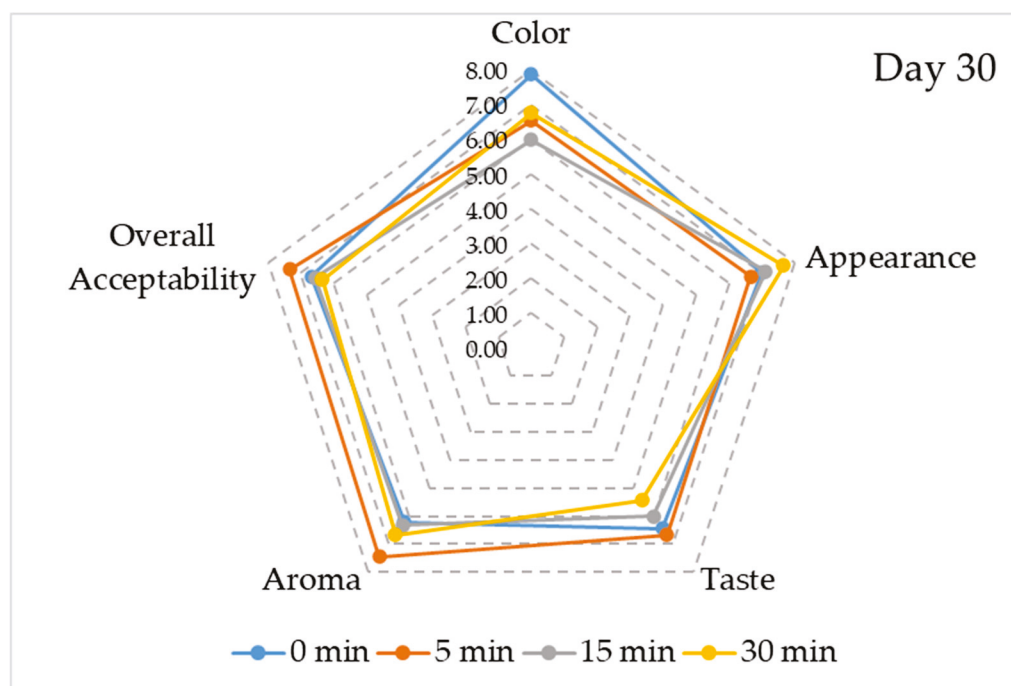


Figure 3. Cont.



**Figure 3.** Radar plot of sensory evaluation of fermented black carrot juice samples.

#### 4. Discussion

Fermented black carrot juice is a red-colored, cloudy beverage that is produced through the fermentation of black carrot, bulgur flour and baker's yeast in a brine solution at room temperature for a period of 2–4 weeks. The total titratable acidity ranges from 0.11% to 0.91%, and the pH ranges from 3.15 to 4.25 [24,33]. In the fermentation of fermented black carrot juice, *S. cerevisiae*, added to the medium, and natural microbiota, predominantly lactic acid bacteria originating from the raw materials utilized, play a crucial role. The amount of lactic acid bacteria in the fermented black carrot juice was found to range between 4.08 and 7.53 log cfu/mL, the total number of bacteria between 4.20 and 9.06 log cfu/mL, and the number of yeasts between 2.1 and 6.70 log cfu/mL [23,31,32,34]. The physicochemical, sensory and microbiological properties of the fermented black carrot exhibit variability depending on the fermentation conditions and the composition and properties of the raw materials utilized. In this study, the properties of the product obtained by sonication application vary among themselves but are within the range of values specified in the literature.

Sonication is an effective method of extracting bioactive compounds, particularly water-soluble components, from plant tissues [35,36]. During the fermentation of plant products, compounds within the tissue are transferred out of the cell by diffusion. The increase in the soluble solid content at the beginning of fermentation with sonication application may be attributed to the fact that the substance transfer, which should be by diffusion, is accelerated by sonication-assisted extraction. The fluctuation in the soluble solid content during the fermentation may be attributed to the transfer of water-soluble components from the raw materials to the medium, the breakdown of polymeric components into smaller building blocks due to the enzymatic activity, the transfer of components such as salt in the fermentation medium to the raw materials, the utilization of components in the medium by microorganisms and/or the production of new components [37]. Although statistically insignificant, the increase in pH at low sonication application times and the decrease at long application times may be attributed to the migration of components such

as salt into solid materials, especially carrots, and the free amino acids, pectic compounds and organic acids found in the structure of black carrots into the medium.

During the fermentation process of black carrot juice, a decrease in the pH and an increase in the acidity were observed. The decrease in the pH and the increase in the acidity can be attributed to the presence of organic acids, which are formed because of sugars being utilized within the environment due to microbial activity. The sugar required for the growth of lactic acid bacteria in fermented black carrot juice is found in the raw materials, especially in carrots and bulgur flour. The easier access of microorganisms to the components passing from tissues and cells to the fermentation liquid by sonication may have supported the growth of microorganisms.

*S. cerevisiae* and non-Saccharomyces yeasts (*P. kudriavzevii*, *P. fermentans*, *Candida oleophila*, *Kazachstania bulderi* and *Geotrichum candidum*) [34], as well as homofermentative and heterofermentative lactic acid bacteria (*Lactobacillus plantarum*, *L. paracasei* subsp. *paracasei*, *L. brevis*, *L. fermentum*, *Leu.mesenteroides* subsp. *mesenteroid*, *P. pentosaceaceus* and *L. delbrueckii* subsp. *delbrueckii*) [38] are involved in the fermentation process of fermented black carrot juice.

Lactic acid bacteria are classified as either homolactic or heterolactic. Homofermentative bacteria produce lactic acid from fermentable sugars, while heterofermentative bacteria produce secondary products, such as ethanol, acetic acid, diacetyl and carbon dioxide, in addition to lactic acid. Yeasts are capable of metabolizing fermentable sugars to alcohol under anaerobic conditions.

The sonication decreased the amount of lactic acid bacteria. However, the 30-min application did not differ statistically from the control sample. The sample sonicated for 5 min had a lower lactic acid bacteria count than the control sample both at the beginning of the fermentation and throughout fermentation, while the lactic acid bacteria count of the other ultrasound-applied samples was higher than the control sample. The 5-min sonication treatment of the samples may have exhibited an inhibitory effect on lactic acid bacteria, while the applications for 15 and 30 min may have had an activating effect. The higher levels of acetic and propionic acids in the sonicated samples could be due to the activation of heterofermentative lactic acid bacteria. The higher amount of ethyl alcohol in the initial stages of fermentation compared to the control sample may be attributed to the fact that yeast and heterofermentative bacteria are more active in the fermentation environment, as supported by the yeast and lactic acid bacteria numbers. The total bacterial count increased after the ultrasound application. However, throughout the fermentation, the count was lower in the sonicated samples than in the control sample.

The increase in fermentation efficiency is attributed to the stimulation of microorganisms with ultrasound and the consequent improvement of their metabolism [39]. In previous studies, it has been reported that the application of ultrasound shortened fermentation time, increased yield and accelerated cell proliferation in both alcohol fermentation with *S. cerevisiae* [15,19] and lactic acid fermentation with lactic acid bacteria [21,40].

The sensitivity of microorganisms to ultrasound varies according to their structure. It is noted that Gram-negative bacteria are more sensitive to ultrasonic cavitation than Gram-positives due to the weak structure of their cell membranes, and bacilli are more sensitive to ultrasonic cavitation than cocci due to the more superficial cell walls [13]. The acidity of the environment, the formation of ethyl alcohol, the diffusion of antioxidant components from tissues into the environment and the resistance of microorganisms to these components affect yeast and bacteria. The application of sonication may have affected the resistance of yeast and lactic acid bacteria. The presence of lower levels of propionic acid and acetic acid and higher levels of lactic acid in the sonicated samples compared to

the control sample suggests that sonication may have activated homofermentative lactic acid bacteria while inhibiting heterofermentative bacteria.

There are antagonistic and synergistic effects among *S. cerevisiae*, non-Saccharomyces yeast and homo and heterofermentative lactic acid bacteria. These interactions are basically attributed to the competition that occurs due to the consumption of the same nutrients by the microorganisms and the metabolites they produce supporting or inhibiting the growth of each other. In low-acid fermented foods, heterofermentative lactic acid bacteria increase the growth of *S. cerevisiae*, while *S. cerevisiae* generally inhibits the growth of heterofermentative lactic acid bacteria in higher alcohol environments. It has been shown that *Pichia* enhances heterofermentative lactic acid fermentation in a low-ethanol environment and increases the production of organic acids such as propionic acid and promotes lactic acid bacteria growth, while in a high-ethanol environment, it inhibits lactic acid bacteria growth and metabolism [41]. It is known that metabolites with antibacterial activity such as organic acids produced by heterofermentative lactic acid bacteria inhibit the growth and reproduction of non-Saccharomyces yeasts by secreting them. Since the sonication treatment showed activating or inactivating effects on *S. cerevisiae*, non-Saccharomyces, and homofermentative and heterofermentative lactic acid bacteria, it may have changed the population of these microorganisms and eliminated their antagonistic and synergistic interactions [41]. This approach may provide a possible explanation for the observed increase in lactic acid bacteria and yeast populations. Sonication affects enzyme activity and has been reported to increase the activity of certain enzymes. Under the right conditions, ultrasound can alter the structure of enzymes, speed up their contact with substrates, and enhance enzyme–substrate interactions, resulting in higher enzymatic activity [13].

In a previous study, 60 aroma compounds including 20 terpenes, 9 esters, 9 alcohols, 5 volatile acids, 6 volatile phenols, 5 lactones, 3 naphthalene, 2 carbonyl compounds and 1 C13-norisoprenoid have been detected in fermented black carrot juice [42]. The taste and aroma of the fermented black carrot juice originate from the raw materials used and fermentation and are additionally influenced by processing and storage conditions [42,43]. It has been reported that total aroma compounds decrease with pasteurization and storage [44]. The type of lactic acid bacteria in black carrot juice fermentation also causes changes in the flavor profile [42]. Fresh fermented foods that have just completed fermentation are sensorially weak, so they usually require a long maturation in terms of taste, aroma and color development. During the maturation of fermented foods, fermentation compounds that improve the sensory properties of fermented foods are formed through various biochemical reactions, primarily the Maillard reaction, esterification and proteolysis. It has been reported that acoustic cavitation in liquids with ultrasound application initiates these reactions and increases the reaction rates [28,39]. The acceleration of these reactions, as well as the differences in microbial diversity caused by sonication, may have affected the sensory properties of sonicated fermented black carrot juices. The  $a^*$  value, which is indicative of redness, is derived from the black carrots present in the formulation. The observation that the  $a^*$  value was lower in the samples sonicated at the beginning of fermentation than in the control sample can be attributed to the rapid transfer of anthocyanins from the carrots to the fermentation medium.

In general, it was observed that the sonicated samples were more turbid than the control sample at the beginning of fermentation and at the end of 30 days of fermentation. In a study, it was reported that ultrasound applied after fermentation increased the turbidity of fermented black carrot juice [32].

The components in a suspension, such as cellulose, protein, lipid, pectin and hemicellulose, cause turbidity. The increase in turbidity due to ultrasound application may be due to microbial enzyme activity, extraction of turbidity-causing components from the



tissue, colloidal disintegration and fragmentation of macromolecules into small pieces. The fluctuations that occur during fermentation can be attributed to the precipitation of the components causing turbidity by forming complexes and the simultaneous occurrence of the above-mentioned colloidal disintegration and release from the tissue [31].

## 5. Conclusions

In conclusion, applying ultrasound before the fermentation significantly affected the microbiological and sensory properties of the fermented black carrot juice. The ultrasound treatment increased the number of TMAB and yeast. The number of lactic acid bacteria differed according to the application time. A five-minute ultrasound application caused a decrease in the number of LAB, while 15 and 30 min applications caused an increase. The sensory properties of the beverage were found to develop faster with the ultrasound treatment. Taste, aroma and color development were observed to be significantly complete in the ultrasound-treated samples on the 20th day of fermentation. The ultrasound application before the fermentation was found to positively support the sensory, color and microbiological properties of the fermented black carrot juice, as well as accelerate the development of sensory properties. Therefore, sonication is recommended for producing the fermented black carrot juice, particularly to improve sensory characteristics and to accelerate maturation.

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## Article

# Development and Characterization of Pistachio Yogurt Analog: A Healthy, Sustainable, and Innovative Plant-Based Alternative

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## Abstract

Plant-based yogurts are increasingly recognized as sustainable and health-conscious alternatives to dairy-based products, driven by environmental, ethical, and nutritional motivations. Pistachio milk, derived from an efficient and resilient crop, emerges as a promising raw material for yogurt production, offering unique sensory qualities and a dense nutritional profile. Rich in unsaturated fatty acids, bioactive compounds, and essential nutrients, pistachios are ideal for fermentation with lactic acid bacteria (LAB). In this study, a novel pistachio-based yogurt analog (PBYA) was developed using lactic acid fermentation, with a yogurt commercial starter, of pistachio milk. The production process was optimized to create an additive-free, clean-label formulation without the use of stabilizers or thickeners. The physicochemical, microbiological, and sensory properties of the PBYA were evaluated over refrigerated storage. The final product exhibited high levels of protein (5.6%), fat (5.4–6.8%), and total solids (20.5–21.4%), along with desirable texture and flavor characteristics. Notably, PBYA presented significantly higher concentrations of total free amino acids (754 mg/L) compared to commercial soy (557 mg/L) and cow's milk yogurts (390 mg/L), particularly in essential amino acids such as lysine, methionine, and tryptophan. This enhanced free amino acid profile contributes to the product's functional and nutritional value. Sensory analysis revealed good acceptance of the product, with improvements in viscosity and firmness over time, likely due to microbial exopolysaccharide production. Overall, the findings highlight the feasibility and commercial potential of PBYA as a clean-label, plant-based fermented product that meets current consumer demands for sustainability, nutrition, and sensory quality.

**Keywords:** plant-based yogurt; sustainable food; amino acid; nut; lactic acid fermentation

## 1. Introduction

In recent years, growing concerns have driven the search for sustainable alternatives to traditional animal-derived products. Among these alternatives, plant-based dairy analogs, such as yogurt, have gained considerable attention as a sustainable option [1]. Replacing animal-based ingredients with plant alternatives can significantly reduce greenhouse gas emissions, water usage, and land consumption [2,3]. In this sense, raw plant-based materials, such as soy, oats, almonds, and other nuts, have a markedly lower environmental impact compared to animal dairy production. This shift not only addresses environmental concerns but also responds to the growing demand for plant-based diets that support human health and sustainability.

The pistachio tree is known for its resilience to drought, requiring significantly less water than many other crops [4]. The Mediterranean climate in regions like Spain, which is arid and semi-arid, is particularly suited for pistachio cultivation [4], making it an ideal crop for water-scarce areas. Moreover, it thrives with fewer chemical inputs, making them a viable option for environmentally friendly agricultural practices. As a result, pistachio production in Spain has been steadily increasing, and it is currently the leading European producer, reaching approximately 20,000 tons annually [5,6].

Beyond their environmental benefits, pistachios embody circular economy principles, as their by-products, such as shells, can be repurposed for bioenergy, animal feed, or even as biodegradable materials, further reducing waste and improving resource efficiency [7]. Additionally, pistachios are highly valued for their rich nutritional profile, including unsaturated fatty acids, vitamins, minerals, and bioactive compounds like phenolics, phytosterols, and tocopherols [8]. These compounds play an antioxidant, immune, and anti-inflammatory role, promoting the reduction in oxidative stress and having beneficial effects on the prevention of cardiovascular diseases, such as managing cholesterol levels, and preventing type-2 diabetes [9].

Recent studies highlight pistachios as a suitable matrix for fermentation by lactic acid bacteria (LAB), enhancing the release of health-promoting bioactive substances and improving the sensory and shelf-life characteristics of the final product [10,11].

Among the various plant-based options available, pistachio milk stands out as a particularly interesting and underexplored raw material for the production of dairy analogs. While much research has focused on other nut-based products like almond, cashew, and hazelnut yogurts [12–14], pistachio-based alternatives have received limited attention. However, the unique nutritional and sensory qualities of pistachios [15] present an exciting opportunity for developing innovative plant-based dairy products.

This study aims to explore the potential of using pistachio milk for plant-based yogurt production. The objective is to design and optimize a production method and evaluate the compositional, microbiological, and sensory properties of additive-free pistachio-based yogurt analog (PBYA). Through this investigation, pistachio is promoted as a sustainable crop with broader applications and pistachio milk is highlighted as a novel, environmentally friendly alternative for plant-based yogurt.

## 2. Material and Methods

### 2.1. Raw Materials

The raw material used was pistachio (*Pistacia vera* L., Kerman variety). It was purchased in its natural state—unpeeled, unsalted, and untoasted—from the local market.

Commercial yogurt starter culture (Ferlac, Type I yogurt culture, Abiasa, S.L., Pontevedra, Spain), containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* was used for the yogurt making.

Two commercial samples of plant-based yogurts were used to compare the results of the experimental PBYA in terms of free amino acids (FAA) profile and sensory characteristics. For this purpose, a soy-based yogurt analog (Hacendado, Valencia, Spain)—selected as it represents the most widely marketed plant-based alternative—and a traditional cow's milk yogurt (Hacendado, Valencia, Spain) were analyzed. The main composition of soy-based yogurt was 3.0% protein, 3.0% fat, 120 mg calcium/100 g, and 9% total solids, and for cow's milk yogurt, it was 3.5% protein, 3.5% fat, 120 mg calcium/100 g, and 12% total solids.

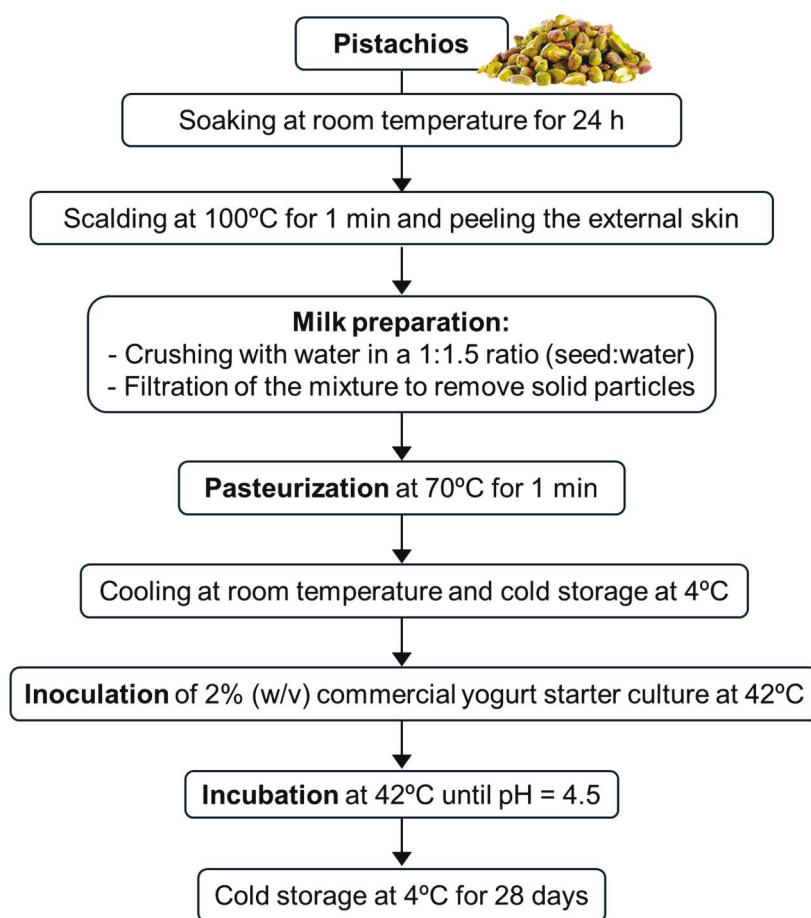


## 2.2. Production of Pistachio Milk and Pistachio-Based Yogurt Analog Manufacture

Pistachio milk was prepared following the method described by Al Zahrani and Shori [16] with modifications. Pistachios were soaked in water for 24 h to facilitate moisture absorption and subsequent processing. Following this, the seeds were blanched at 100 °C for 1 min and peeled. To obtain the pistachio milk, the nuts were ground with water at a 1:1.5 seed-to-water ratio using a countertop blender (Cecotec brand, Power Black Titanium 2600MAX Pro model, Valencia, Spain). This ratio was selected as it exhibited the best results in previous trials. The resulting mixture was filtered through a double fine-mesh cloth to remove solid residues. The pistachio milk obtained was then pasteurized at 70 °C for 1 min, cooled to room temperature, and stored refrigerated at 4 °C. The composition of pistachio milk obtained was 9.4% fat, 4.3% protein, 59 mg of calcium per 100 g, and 18% total solids. No additives, sugars, or stabilizers were added to the pistachio-based milk for yogurt manufacture.

For fermentation, the pistachio milk was heated at 42 °C and inoculated with 2% (*w/v*) of the commercial yogurt starter culture (Section 2.1). This culture was precultured in the pistachio milk following the manufacturer's instructions (5 g/100 L) and incubated for approximately 16 h at 37 °C until a population of 6 log CFU/mL was reached.

The inoculated milk was transferred to 30 mL glass containers with airtight lids and incubated at 42 °C until a pH of  $4.5 \pm 0.1$  was reached. The fermentation process was finalized by rapidly cooling the yogurt to 4 °C, and then it was stored refrigerated for 21 days. PBYA manufacture was carried out in duplicate one week later. A flowchart summarizing the entire pistachio yogurt production process is presented in Figure 1.



**Figure 1.** Flowchart of the PBYA production process.

Physicochemical, microbiological, and individual free amino acids and sensory analyses of the PBYA were conducted at different times during the 21-day refrigerated storage period. All the analyses were performed in duplicate, except the microbiological counts, which were carried out in triplicate.

### 2.3. Fermentation Kinetics

During the milk fermentation process, pH levels were initially monitored at 1 h intervals using a pH meter (Crison, Barcelona, Spain). As the pH approached 5.0, measurements were taken every 30 min. The maximum acidification rate ( $V_m$ ), expressed as absolute values (mpH units/min), was determined from the pH-time curves using the following formula:

$$V_m = \max\{d(ph)/dt\}$$

Additionally, key kinetic parameters were calculated to describe the fermentation process:  $T_m$ , the time when the maximum acidification rate occurred, and  $T_e$ , the time required to reach a pH of 4.6.

### 2.4. Microbiological Counts

Counts of total aerobic mesophilic bacteria were performed on plate count agar (PCA; Condalab, Madrid, Spain) under aerobic conditions at 37 °C for 48 h.

For the determination of the cell population of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* from the commercial starter, the pour plate method was used, and serial dilutions of the samples were spread onto MRS agar (Condalab, Madrid, Spain; pH = 5.42, adjusted with HCl 0.1 M) or M17 agar (Scharlab, Barcelona, Spain) enriched in lactose (10%) [17], respectively. Both cultures were incubated for 72 h at 37 °C [17].

Microbial counts in the three culture media were performed in triplicate.

### 2.5. Compositional Analysis

The pH was measured by using a digital pH-meter (Crison, Barcelona, Spain). Total solids (TS) were determined gravimetrically after oven drying, and titratable acidity (TA) was measured according to AOAC method [18]. Fat was assessed by the Gerber butyrometric method [18]. Total protein was determined by the Kjeldahl method [18], applying a conversion factor of 6.25 to convert total nitrogen to total protein, and calcium content was measured using a complexometric method [19]. Protein and calcium values were only determined in the samples of 1 day, as these components are generally stable throughout refrigerated storage and are not significantly affected by the fermentation process or storage duration.

### 2.6. Color Measurements

The color characteristics of the pistachio yogurts were assessed using a Minolta Chroma Meter CR-400 (Konica Minolta, Osaka, Japan), configured with a D65 light source and a 10° observation angle. The device was calibrated against a white reference plate, and the CIELab system was employed to evaluate the color coordinates  $L^*$ ,  $a^*$ , and  $b^*$ . The  $L^*$  value, ranging from 0 to 100, represents lightness, with 0 being black and 100 being white. The  $a^*$  value denotes the transition from red ( $+a^*$ ) to green ( $-a^*$ ), while the  $b^*$  value indicates the shift from yellow ( $+b^*$ ) to blue ( $-b^*$ ).

### 2.7. Free Amino Acid Content Analysis

For sample preparation, proteins were precipitated by mixing 3 mL of each yogurt sample with 3 mL of 12% trichloroacetic acid (TCA). The mixtures were then homogenized using an Ultra-Turrax T18 Basic (IKA-Werke GmbH & Co. KG, Staufen, Germany) at

14,000 rpm for 2 min to ensure complete protein denaturation. Following homogenization, samples were centrifuged at  $15,000 \times g$  for 15 min at 4 °C. The resulting supernatants were collected and stored at −20 °C until further analysis by reversed-phase high-performance liquid chromatography (RP-HPLC).

The quantification of 19 amino acids was performed by RP-HPLC using diethyl ethoxymethylenemalonate (DEEMM) derivatisation method [20]. Briefly, a mixture of 400 µL of the sample, 900 µL of 1 mol/L borate buffer pH 9.0, 300 µL of methanol, 20 µL of internal standard (1.0 g/L), and 30 µL of DEEMM was incubated at 30 °C in an ultrasonic bath for 30 min. The samples were then heated in an oven at 70 °C for 2 h to allow the complete degradation of excess DEEMM and reagent byproducts. After derivatization, the samples were filtered through regenerated cellulose esters 0.2 µm membranes (Agilent Technologies, Barcelona, Spain) coupled to a syringe into conical vials.

The analysis was performed on an Agilent 1200 HPLC (Agilent Technologies, Madrid, Spain) comprising an Agilent binary pump, an Agilent autosampler, and an Agilent photodiode array detector. Chromatographic separation was performed using a Zorbax Eclipse XDB C18 column particle (Agilent Technologies, Santa Clara, CA, USA) size 5 µm (250 mm × 4.6 mm) and an Agilent guard cartridge C18 particle size 5 µm (12.5 mm × 4.6 mm) thermostatted at 16 °C in an Agilent thermostatted column compartment. The mobile phase consisted of a 25 mmol/L acetate buffer pH 5.8 with 0.02% sodium azide (eluent A) and an 80:20 mixture of acetonitrile and methanol (eluent B). Samples (50 µL) were applied to the column and eluted at a flow rate of 0.9 mL/min according to the binary gradient used by Poveda [21].

The compounds of interest were identified based on their retention times and spectral characteristics at 280 nm, and they were quantified using the internal standard method.

## 2.8. Sensory Evaluation

Quantitative descriptive analysis (QDA) was conducted to evaluate the appearance, aroma, flavor, and texture attributes of the PBYA, following the methodology outlined by ISO 13299 standard [22] and as described in Ramos and Poveda [23]. The sensory panel consisted of eight trained assessors, aged 24 to 60, selected from the university staff. Their training in evaluating plant-based milks and yogurts was conducted in accordance with ISO 8586 standard [24], and they also had prior experience in assessing dairy products, particularly yogurt.

This study was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all participants involved in the study.

The panelists were asked to score the sample attributes, organized into four categories in the following order: visual (color and firmness); odor (dairy yogurt odor, nut odor, odor intensity and odor quality); flavor (dairy yogurt taste, sour, nut taste, bitter, flavor intensity and flavor quality); and texture in mouth (viscosity), using an unstructured 0 to 10 scale.

The samples were presented to the tasters coded with three-character tasting keys composed of two numbers and a central letter and were served randomly in 30 mL glass containers with airtight lids, each containing 20 mL of yogurt. The evaluations were conducted in duplicate across two separate sessions in a tasting room designed according to the recommendations of ISO 8589 standard [25].

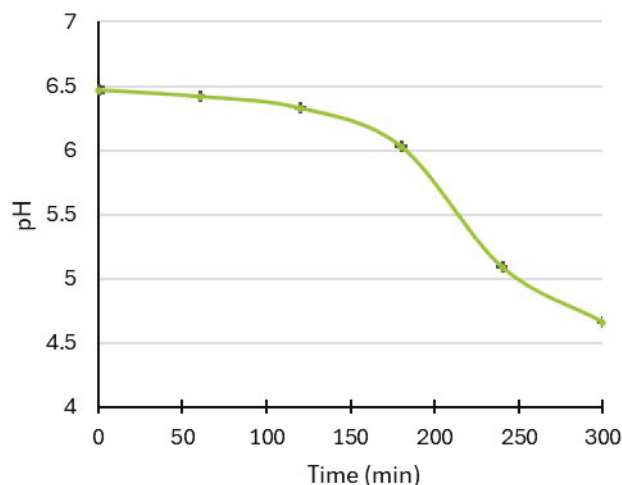
## 2.9. Statistical Analysis

A one-way analysis of variance (ANOVA) was applied to the results, using Tukey's test for the comparison of means ( $p < 0.05$ ). Normality, heteroscedasticity, and the independence of samples had been previously checked. These statistical treatments were performed using the IBM SPSS statistics package version 26.0 (SPSS Inc., Chicago, IL, USA).

### 3. Results and Discussion

#### 3.1. Fermentation Kinetics

The changes in pH of PBYA during fermentation can be observed in Figure 2. During the first 100 min, the pH remains relatively stable at approximately 6.5, suggesting an adaptation phase in which microorganisms acclimate to the medium and available nutrients. Subsequently, between 100 and 200 min, a gradual decline in pH is observed as LAB begin metabolizing the naturally occurring monosaccharides in pistachio nuts more efficiently, leading to lactic acid production.



**Figure 2.** Acidification profile of the PBYA.

Between 200 and 250 min, the acidification process accelerates significantly, as evidenced by a pronounced drop in pH, reflecting the peak metabolic activity of LAB and the rapid lactic acid synthesis. Beyond 250 min, the acidification rate slows down, with the pH approaching 4.6. This marks the end of fermentation. The observed acidification trend aligns with that reported for both dairy-based yogurts [26] and plant-based analogs [27]. This fermentation pattern is primarily driven by the metabolic interactions between the starter cultures, *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*. *L. delbrueckii* exhibits strong proteolytic activity, hydrolyzing proteins into peptides and amino acids that promote *S. thermophilus* growth. In return, *S. thermophilus* produces formic, folic, and pyruvic acids, as well as CO<sub>2</sub>, enhancing the metabolic activity of *L. delbrueckii* [28]. This behavior has also been previously observed in other studies on the production of sheep's and cow milk yogurts [23,29].

The acidification kinetics parameters are summarized in Table 1. The maximum acidification rate ( $V_m$ ) indicated a moderately high metabolic efficiency of LAB during the peak fermentation phase. This parameter is critical, as it dictates the rate at which pH decreases in the most active stage of the process.

**Table 1.** Kinetic acidification parameters of the PBYA.

| Parameters            |     |
|-----------------------|-----|
| V max (m unit pH/min) | 6.4 |
| T <sub>m</sub> (min)  | 240 |
| T <sub>e</sub> (min)  | 342 |

V max: maximum acidification rate; T<sub>m</sub>: time to reach V<sub>m</sub>; T<sub>e</sub>: time to reach the end of fermentation (pH 4.6).

The time required to reach the maximum acidification rate (T<sub>m</sub>) was influenced by key fermentation variables, including bacterial strain selection, inoculum size, temperature,

and overall fermentation duration, all of which significantly impact the pH dynamics of the final product [30].

Finally, the total fermentation time ( $T_e$ ) required to reach the final pH of 4.6 was 342 min. This intermediate duration is considered optimal, as shorter fermentation times may adversely affect the textural properties of the yogurt [31], while extended fermentation increases the risk of microorganism spoilage [30]. Notably, these values are comparable to those reported by Pereira et al. [28] for a fermented soy-based product and with those shown for cow's milk yogurts [29], underscoring the efficiency and stability of fermentation process in pistachio-based yogurt. These findings support the feasibility of PBYA as a high-quality plant-based alternative with a well-balanced acidification profile and fermentation time.

### 3.2. Microbial Counts

To ensure health benefits and support gut microbiota, it is essential to maintain an appropriate viable load of bacterial cultures in yogurt throughout shelf life. According to regulatory guidelines, yogurt must contain a minimum starter culture count of 7 log CFU/mL [32]. In this study, *Streptococcus thermophilus* (M-17) and *Lactobacillus bulgaricus* (MRS) counts in PBYA samples were monitored throughout storage (Table 2). The results indicate that cell counts in both MRS and M17 showed a slight decrease during the storage period but remained above the minimum-recommended viable cell levels for dairy yogurt ( $10^7$  UFC/g) throughout. The viability of bacterial cells during yogurt storage is influenced by factors such as acid accumulation, nutrient depletion, and the buildup of metabolic by-products. The observed decline in cell counts can be attributed to the increasing acidity of the medium and the nutrient limitation, leading to bacterial growth inhibition [33]. This trend is consistent with findings in other plant-based yogurts, such as those made from almonds or cashews [34], as well as for dairy-based yogurts [35]. Moreover, the final counts at the end of storage ( $8.1 \pm 0.2$  log CFU/mL) exceed the minimum legal threshold, indicating that the product remains viable for market introduction.

**Table 2.** Microbial counts of the PBYAs (log CFU/mL;  $n = 3$ ).

| Microbial Group                          | Period of Storage |                 |                 | <i>p</i> Value |
|------------------------------------------|-------------------|-----------------|-----------------|----------------|
|                                          | 1                 | 14              | 21              |                |
| <i>Streptococcus thermophilus</i> (M-17) | $8.6^a \pm 0.2$   | $8.4^b \pm 0.3$ | $8.1^c \pm 0.2$ | 0.0007         |
| <i>Lactobacillus bulgaricus</i> (MRS)    | $8.4^a \pm 0.1$   | $8.2^b \pm 0.1$ | $8.2^b \pm 0.2$ | 0.0085         |
| Total aerobic mesophilic bacteria (PCA)  | $8.8^a \pm 0.3$   | $8.1^b \pm 0.2$ | $8.2^b \pm 0.3$ | 0.0041         |

<sup>a-c</sup>: Means in the same row with different letters indicate statistically significant differences between the period of storage ( $p < 0.05$ ).

Additionally, to assess the hygienic quality of the pistachio milk prior to yogurt manufacture, counts on PCA were performed, yielding a value below 2.18 log CFU/mL. This indicates the good hygienic quality of the raw milk. Furthermore, counts on the same medium were carried out in the produced yogurts throughout the storage period. The results were very similar to those obtained using the selective media (Table 2), suggesting that the total aerobic bacteria predominantly correspond to the inoculated LAB, supporting the absence of significant contamination from external sources.



### 3.3. Compositional Analysis

The compositional analysis of the assessed PBYAs is presented in Table 3. The pH values decreased significantly during storage, ranging from 4.62 to 4.24, due to the residual metabolic activity of LAB strains, which continued producing organic acids, particularly lactic acid even at 4 °C. This ongoing acidification was reflected in a corresponding increase in TA, which rose significantly from 0.54 g lactic acid/100 g at the start of storage to 0.64 g lactic acid/100 g on day 21. These values of TA meet the *Codex Alimentarius* standards [32] for samples at 14 and 21 days of storage, which stipulate a minimum acidity of 0.6 g lactic acid/100 g. A similar trend in pH and TA was observed by Soumya et al. [34] on various plant-based yogurts, showing values comparable to those of PBYA. For instance, after 21 days of storage, coconut yogurt reached a TA of 0.58 g lactic acid/100 g, while oat yogurt exhibited a TA of 0.81 g lactic acid/100 g. However, these values remain lower than those reported for commercial dairy yogurts (1.035–1.38 g lactic acid/100 g) [36,37].

**Table 3.** Physico-chemical composition of PBYAs during refrigerated storage ( $n = 2$ ).

| Time           | pH                       | TA (%)                   | TS (%)     | Fat (%)                | Protein (%) | Calcium (mg/100 g) |
|----------------|--------------------------|--------------------------|------------|------------------------|-------------|--------------------|
| 1              | 4.62 <sup>a</sup> ± 0.01 | 0.54 <sup>b</sup> ± 0.03 | 20.5 ± 0.1 | 5.8 <sup>b</sup> ± 0.3 | 5.6 ± 1.7   | 71.9 ± 3.2         |
| 14             | 4.40 <sup>b</sup> ± 0.01 | 0.64 <sup>a</sup> ± 0.01 | 21.4 ± 0.8 | 5.4 <sup>b</sup> ± 0.6 | nd          | nd                 |
| 21             | 4.34 <sup>c</sup> ± 0.01 | 0.64 <sup>a</sup> ± 0.02 | 20.6 ± 0.0 | 6.8 <sup>a</sup> ± 0.3 | nd          | nd                 |
| <i>p</i> value | <0.0001                  | 0.0021                   | 0.080      | 0.0067                 | -           | -                  |

<sup>a–c</sup>: Means in the same column with different letters indicate statistically significant differences between the period of storage ( $p < 0.05$ ). nd: not determined.

Total solids, which reflect the dry matter content of yogurt, play a crucial role in determining product quality. A high total solids content reduces syneresis, thus improving structural stability. Additionally, it enhances viscosity, contributing to a creamier texture and better overall consistency [38]. The total solids content in PBYA ranged from 20.5 to 21.4% during cold storage. Compared to other plant-based yogurts, PBYA exhibits a similar total solids content to walnut yogurt (19.4%) [39] and groundnut yogurt (18.15%) [40]. Notably, it contains higher total solids than dairy yogurts, such as those made from cow's milk (11.39–16.49%) [40,41] or buffalo milk (14.48%) [38].

The results of the fat content of PBYAs showed no significant differences ( $p < 0.05$ ), although the highest result was found 21 days after storage (6.8%), being higher when compared to the content of cow (3.33%), sheep (4.33%) and goat (2.81%) yogurts [42]. High fat content is a desirable attribute as it improves the taste and texture of the final product [12]. Furthermore, pistachio fat is predominantly composed of monounsaturated and polyunsaturated fatty acids, particularly oleic and linoleic acids, which are known to support cardiovascular health, reduce inflammation, and contribute to a favorable lipid profile [43].

PBYAs had a protein content of 5.6%, higher than those found in dairy yogurts (4.4%) or other plant-based yogurts, such as soy (4%) or cashew (2%), and lower than oat yogurt (9%) or lentil yogurt (12%) [12]. This is to be expected, as pistachios contain a high amount of protein, resulting in a yogurt analog product with substantial protein levels. Proteins play a key role in determining physicochemical properties, including water retention and gel formation [12]. The calcium content in PBYA was 71.9 mg/100 g. This amount is lower compared to dairy yogurts (153.80 mg/100 g) [44], but similar to other plant-based products such as coconut yogurt (85 mg/100 g) [45]. As can be seen, calcium, a vital micronutrient, is present in significant and highly bioavailable quantities in dairy products. However,

relying on plant-based products can result in deficiencies of this essential nutrient. To address this issue, supplementation with critical nutrients is recommended [12]; however, as the primary objective of this study was to obtain a clean label product, the addition of calcium was not considered, and addressing this deficiency will be considered in a future study.

### 3.4. Color Measurement

Color plays a crucial role in food, as it is the primary characteristic that consumers perceive initially, affecting their acceptance, sensory attributes, and perceived safety, quality, and freshness of the product [39]. In the case of yogurt, color is a key indicator that affects quality, freshness, flavor expectations, commercial value, and product acceptability [46].

Table 4 displays the results of instrumental color analysis of the PBYA during the storage period. The obtained  $L^*$  values are high, indicating greater luminosity in the yogurts. Grasso et al. [36], who analyzed the color parameters of various plant-based yogurts, including soy, almond, coconut, and hemp yogurts, reported much lower  $L^*$  values, ranging from 60.2 to 64.2. According to these results, the PBYA exhibits a brighter color, appealing to consumers who prefer lightness ( $L^*$ ) in such type of product [47]. The values of  $L^*$ , and  $b^*$  reflect a slight tendency towards a loss of brightness and a decrease in the intensity of the yellow color over the 21 days of storage of the PBYA. These changes could be related to the oxidation of the natural pigments present in pistachio, as it has yellowish pigments such as flavonoids, which, as they are soluble in water, could be present in the final product [43]. However, the  $a^*$  values show stability in the green hue, suggesting that there were no significant alterations in the perception of the green freshness of the product during this period. Nevertheless, although measuring instruments can accurately detect slight variations in color parameters, these changes are often imperceptible to the human eye. This suggests that, from a practical and commercial perspective, the color of the yogurt remains stable during this time, which is a positive aspect for its acceptance and overall visual quality.

**Table 4.** Color parameters of the PBYAs during refrigerated storage ( $n = 2$ ).

| Sample         | $L^*$            | $a^*$          | $b^*$            |
|----------------|------------------|----------------|------------------|
| PBYA 1 d       | $83.1^b \pm 0.1$ | $-4.5 \pm 0.6$ | $36.9^a \pm 3.4$ |
| PBYA 14 d      | $83.7^a \pm 0.1$ | $-4.1 \pm 0.1$ | $33.6^b \pm 0.2$ |
| PBYA 21 d      | $82.1^c \pm 0.1$ | $-4.3 \pm 0.1$ | $33.5^b \pm 0.1$ |
| <i>p</i> value | 0.002            | 0.18           | 0.0005           |

<sup>a-c</sup>: Means in the same column with different letters indicate statistically significant differences between the period of storage ( $p < 0.05$ ).

### 3.5. Free Amino Acid Profile

Table 5 shows significant differences ( $p < 0.05$ ) in free amino acid (FAA) concentrations among pistachio-based yogurt, commercial soy yogurt, and commercial cow milk yogurt. PBYA exhibited the highest total FAA content ( $754 \pm 8$  mg/L), markedly surpassing that of soy ( $557 \pm 5$  mg/L) and cow milk yogurt ( $390 \pm 2$  mg/L). This superior FAA content in PBYA highlights its potential as a nutrient-dense, plant-based fermented product.

**Table 5.** Free amino acid content (mg/L) in PBYA at 14 days of storage and commercial soy yogurt analog and cow milk yogurt.

| Free Amino Acid          | PBYA                     | Commercial Soy Yogurt    | Commercial Cow Yogurt    | <i>p</i> Value |
|--------------------------|--------------------------|--------------------------|--------------------------|----------------|
| Essential amino acids    |                          |                          |                          |                |
| Histidine                | 11.2 <sup>a</sup> ± 1.6  | 12.2 <sup>a</sup> ± 4.8  | 4.15 <sup>b</sup> ± 0.81 | 0.017          |
| Isoleucine               | 9.15 <sup>c</sup> ± 0.98 | 21.6 <sup>a</sup> ± 0.8  | 15.3 <sup>b</sup> ± 0.5  | <0.001         |
| Leucine                  | 13.0 <sup>b</sup> ± 0.4  | 20.3 <sup>a</sup> ± 0.5  | 9.35 <sup>c</sup> ± 0.38 | <0.001         |
| Lysine                   | 67.8 <sup>a</sup> ± 2.3  | 28.7 <sup>b</sup> ± 1.4  | 4.03 <sup>c</sup> ± 0.07 | <0.0001        |
| Methionine               | 25.7 <sup>a</sup> ± 1.5  | 6.73 <sup>b</sup> ± 0.58 | 4.11 <sup>c</sup> ± 0.21 | <0.0001        |
| Phenylalanine            | 9.91 <sup>b</sup> ± 0.15 | 11.7 <sup>a</sup> ± 0.2  | 5.12 <sup>c</sup> ± 0.97 | <0.001         |
| Threonine                | 67.5 <sup>a</sup> ± 1.4  | 18.9 <sup>b</sup> ± 1.2  | 18.0 <sup>b</sup> ± 1.2  | <0.0001        |
| Tryptophan               | 54.9 <sup>a</sup> ± 2.6  | 16.8 <sup>c</sup> ± 1.9  | 20.3 <sup>b</sup> ± 1.1  | <0.0001        |
| Valine                   | 46.7 <sup>a</sup> ± 3.9  | 23.7 <sup>b</sup> ± 2.5  | 28.9 <sup>b</sup> ± 1.0  | <0.001         |
| Nonessential amino acids |                          |                          |                          |                |
| Alanine                  | 47.8 <sup>b</sup> ± 3.0  | 51.9 <sup>a</sup> ± 1.9  | 17.3 <sup>c</sup> ± 0.8  | <0.001         |
| Arginine                 | 25.2 <sup>b</sup> ± 0.4  | 32.0 <sup>a</sup> ± 0.2  | 1.57 <sup>c</sup> ± 0.01 | <0.0001        |
| Aspartic acid + Serine   | 51.5 <sup>a</sup> ± 1.3  | 38.1 <sup>b</sup> ± 0.7  | 25.6 <sup>c</sup> ± 0.11 | <0.0001        |
| Cystine                  | 27.9 <sup>a</sup> ± 1.6  | 17.2 <sup>b</sup> ± 0.9  | 26.4 <sup>a</sup> ± 0.23 | 0.002          |
| GABA                     | 112 <sup>a</sup> ± 1     | 71.4 <sup>c</sup> ± 1.1  | 83.6 <sup>b</sup> ± 1.3  | <0.0001        |
| Glutamate                | 129 <sup>b</sup> ± 2     | 134 <sup>a</sup> ± 1     | 91.3 <sup>c</sup> ± 1.7  | <0.0001        |
| Glycine                  | 8.34 <sup>a</sup> ± 1.3  | 5.18 <sup>b</sup> ± 0.69 | 3.17 <sup>c</sup> ± 0.08 | 0.001          |
| Proline                  | 31.7 <sup>b</sup> ± 0.2  | 37.2 <sup>a</sup> ± 0.6  | 28.8 <sup>c</sup> ± 0.9  | <0.001         |
| Tyrosine                 | 15.6 <sup>a</sup> ± 0.8  | 9.87 <sup>b</sup> ± 0.47 | 3.55 <sup>c</sup> ± 0.14 | <0.0001        |
| Total                    | 754 <sup>a</sup> ± 8     | 557 <sup>b</sup> ± 5     | 390 <sup>c</sup> ± 2     | <0.0001        |

<sup>a–c</sup>: Means in the same row with different letters indicate statistically significant differences between the different yogurts (*p* < 0.05).

Among the essential amino acids (EAAs), PBYA showed significantly higher levels of lysine, methionine, threonine, tryptophan, and valine as compared to soy yogurt and cow's milk yogurt. Notably, the lysine content in PBYA was over 16-fold higher than that in cow's milk yogurt and more than double that found in soy yogurt. Given that lysine is often the limiting amino acid in plant-based diets [46], its abundance in PBYA may offer a valuable nutritional advantage. Similarly, the elevated levels of methionine and tryptophan—critical for methylation processes and serotonin synthesis, respectively—suggest enhanced functional properties compared to commercial counterparts.

Soy yogurt showed higher isoleucine and leucine levels than the other two matrices. Nevertheless, PBYA presented a more balanced EAA profile overall. Conversely, cow's milk yogurt consistently exhibited the lowest values for nearly all EAAs, including lysine and methionine, aligning with previous findings that suggest reduced FAA accumulation in dairy-based yogurts post-fermentation [46].

The non-essential amino acids (NEAAs) profile further supports the superior bioactivity of PBYA. It contained the highest levels of  $\gamma$ -aminobutyric acid (GABA), a non-protein

amino acid recognized for its hypotensive, anxiolytic, and neuroprotective properties [48]. In comparison, GABA levels in soy and cow yogurts were significantly lower. The elevated GABA in PBYA is likely attributed to the microbial decarboxylation of glutamate during fermentation, a process well-documented in plant-based substrates rich in glutamic acid [49].

In addition, PBYA exhibited significantly higher values of glycine, tyrosine, and arginine, all of which contribute to metabolic regulation and antioxidant activity. Although soy yogurt contained more glutamate, which enhances umami flavor perception, PBYA followed closely, suggesting favorable sensory properties as well.

The superior amino acid profile of PBYA aligns with recent reports on the enhanced nutritional quality of nut-based fermented products [50,51]. For instance, Akbarian et al. [50] found comparable increases in bioactive amino acids in almond-based kefir, while Borrego-Ruiz et al. [51] reported lower FAA levels in oat-based yogurts compared to legume and nut fermentations. The results of this study reinforce the suitability of pistachio matrices for developing functional plant-based yogurts that not only meet but exceed the nutritional benchmarks set by conventional dairy alternatives.

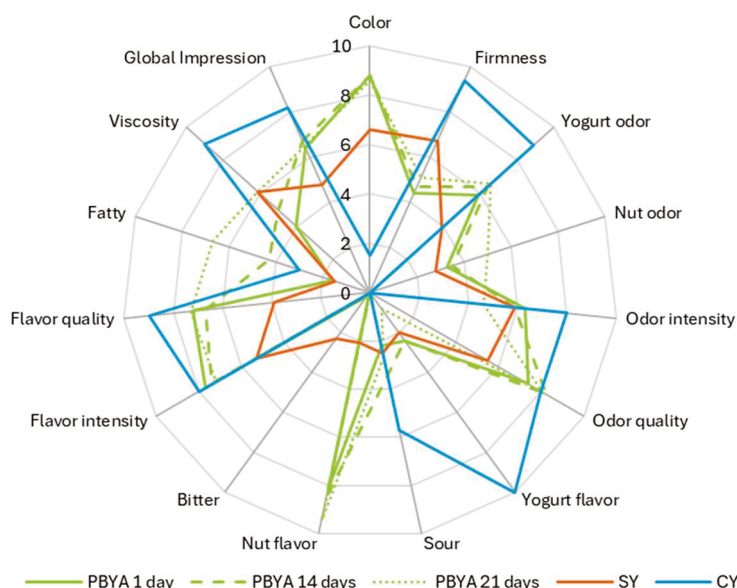
### 3.6. Sensory Evaluation

Yogurt, as a fresh ready-to-eat product, is valued for its sensory characteristics, which play a key role in consumer preference. Traditional yogurt is characterized by a smooth, creamy texture, mild tanginess, and refreshing flavor. Its consistency is typically viscous and homogeneous, free from syneresis, ensuring a pleasant mouthfeel [46]. These initial properties define the quality of yogurt and serve as a reference point before any changes occur during storage. In order to assess the acceptability of the PBYA, a sensory evaluation was performed, comparing the experimental PBYA of this study to that of the commercial cow yogurt and soy-based yogurt analog.

First, the three samples of PBYA showed a similar profile, with some small changes during the storage period (Figure 3). The samples were characterized by low firmness, moderate yogurt odor and flavor quality, and high color and nutty flavor scores. While some attributes in PBYA such as yogurt flavor and sourness declined over time—likely due to lipid oxidation and pH variation [52]—nut odor and nut flavor intensified, enhancing the characteristic profile of the pistachio matrix. Although the viscosity in mouth of PBYA was initially low, it is noteworthy that it increased significantly ( $p < 0.05$ ) with storage time, reaching the same value as for the soy yogurt analog at 21 days.

The three sensory profiles are clearly differentiated in Figure 3, with the profiles of PBYAs being very different from those of the commercial dairy and soy yogurts. Cow's milk yogurt clearly had the highest scores ( $p < 0.05$ ) for yogurt odor and flavor, as well as firmness and viscosity in the mouth. Soy yogurt was characterized by low odor and flavor intensity and the lowest ( $p < 0.05$ ) odor and flavor quality. It presented intermediate firmness and viscosity and very low yogurt odor and flavor. Compared to the commercial samples, the PBYA generally displayed intermediate characteristics ( $p < 0.05$ ). Notably, bitterness was completely absent in all PBYA samples, in contrast to the soy yogurt, which exhibited perceptible bitter notes. In terms of overall impression, the PBYA samples scored intermediate between the cow's milk yogurt and the soy milk yogurt analog, which received the lowest ( $p < 0.05$ ) scores.

These results underscore the potential of the obtained PBYA as a clean-label product with good sensory quality, even superior to that of commercial soy yogurt analog, with a good sensory acceptance, with odor and flavor characteristics close to those of dairy yogurt, and a viscosity similar to that of soy yogurt analog.



**Figure 3.** Sensory profile of PBYAs during the refrigerated storage period (1, 14, and 21 days), and cow and soy yogurts. PBYA: pistachio-based yogurt analog; SY: soy yogurt; CY: cow yogurt.

#### 4. Conclusions

The development of PBYA represents a significant innovation in plant-based alternatives, offering a unique, nutritious, and additive-free option for health-conscious consumers. Pistachio serves as an excellent base for the manufacture of plant-based yogurt, providing a rich, creamy texture, and distinct flavor in the final product. Nutritionally, PBYA is notable for its high content of unsaturated fats and its protein levels, which can even surpass those of conventional cow's milk yogurts, further enhancing its appeal as a functional food. The lactic fermentation process ensured optimal acidification and a stable, high-quality product, with lactic acid bacteria adapting well to the non-dairy base. Compositional analysis showed that PBYA is rich in total solids, fat, and protein, meeting the nutritional needs of consumers. Its bright color remained attractive throughout its shelf life, further enhancing its visual appeal, which is an important factor for consumer acceptance. Importantly, FAA analysis revealed significantly higher total FAA content in PBYA compared to commercial soy and cow milk yogurts. In particular, essential amino acids were present at significantly higher levels than in cow's milk yogurt, with lysine concentrations up to six times higher. Additionally, the elevated levels of  $\gamma$ -aminobutyric acid (GABA), glycine, tyrosine, and arginine highlight the functional and bioactive potential of this formulation. Sensory analysis revealed that, despite low firmness and moderate viscosity in the mouth, the product maintained an overall appealing flavor profile throughout the entire storage period, being better valued than a commercial soy yogurt analog. Further work is warranted to improve the texture of this product by using exopolysaccharide-producing LAB, thus avoiding the addition of texturizers or stabilizers, and also selected LAB strains, in order to improve the functional properties of the product.

These findings position pistachio-based yogurt analog as a competitive and health-promoting plant-based fermented product, suitable for consumers with dietary restrictions and for those seeking enhanced nutritional value in dairy alternatives. These results highlight the commercial viability of pistachio-based yogurt as a nutritious, visually attractive, and additive-free plant-based option, making it a strong contender in the growing market for clean-label alternatives.

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## Article

# Correlation Analysis Between Volatile Flavor Compounds and Microbial Communities During the Fermentation from Different Ages Mud Pits of Wuliangye Baijiu

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## Abstract

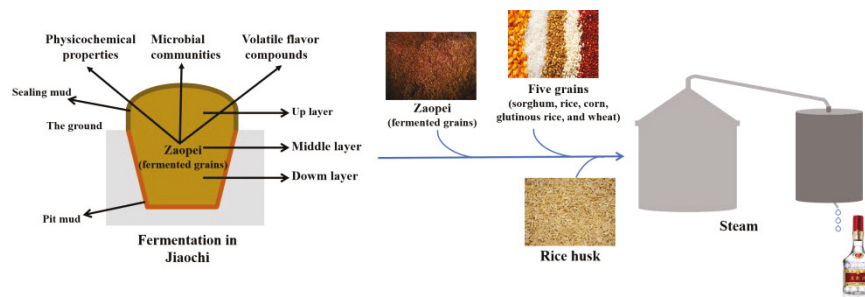
The continuous usage years of mud pits significantly impact the formation of volatile flavor compounds (VFCs) in Wuliangye Baijiu fermentation, yet their role in regulating initial microbial diversity and VFCs during fermentation remains unclear. This research analyzed the dynamic relationships among microorganisms, physicochemical characteristics, and VFCs from two different aged mud pits (continuous usage for 20 years: JC20 and 40 years: JC40). As fermentation advanced, VFCs increased, with JC40 showing a greater increment than JC20. Furthermore, the predominated microorganisms during fermentation of two pits were significantly different, resulting in distinct microbial successions. And the microbial diversity in JC40 was higher than JC20 at the fermentation anaphase from different layers of zaopei samples. Microbes were identified that highly correlated with physicochemical properties and VFCs, and the important microbial species differed between the two pits, proving the importance of mud pit continuous usage years for Wuliangye Baijiu production.

**Keywords:** Wuliangye Baijiu; continuous usage years of mud pit; microbial communities; physicochemical properties; volatile flavor compounds; correlation analysis

## 1. Introduction

As the leading enterprise, Wuliangye Baijiu ranks among the most favored Baijiu varieties in China with sales reaching CNY 89.1 billion in 2024. Renowned for its distinctive fermentation process and characteristic flavor profiles [1,2], it enjoys widespread consumption. The most notable feature of Wuliangye Baijiu lies in its fermentation within a specialized mud pit (also called JiaoChi), which created an ideal habitat for microbial proliferation [3]. The Fresh zaopei, meticulously crafted from a blend of sorghum, rice, corn, glutinous rice, wheat, rice husks, and baobaoqu powder, undergoes a 70-day fermentation within the sealed pit, a crucial process for developing its signature flavor compounds (Figure 1) [4,5]. Based on production experience, the higher-quality Wuliangye Baijiu is typically distilled from the down level zaopei from older pits. This observation underscores the fact that the continuous usage duration of mud pits has a substantial impact on the fermentation of zaopei at different layers [6–9]. It is commonly accepted that the longer the mud pit has been in use, the better the quality of Wuliangye Baijiu will be [10,11].

Consequently, it is essential to elucidate the relationship between the quality of Wuliangye Baijiu and the continuous used years of mud pit.



**Figure 1.** A brief schematic diagram of Wuliangye fermentation.

As previous research showed, older pits harbor a diverse consortium of functional microorganisms, including *Clostridium*, *Caproiciproducens*, *Methanobrevibacter*, *Aspergillus*, and *Candida*, while *Lactobacillus* dominates in younger mud pits [10,12,13]. These microbes actively participate in zaopei fermentation, shaping the composition of bacterial and fungal communities in complex ways [3,14,15].

A further study reported that *Monascus* and *Trichosporon* represent the exclusive dominant functional fungal species in the older mud pits. Moreover, eight core functional microbial genera have been identified, which are associated with the changes in volatile metabolites during the fermentation process [6]. A comprehensive body of research has delved into the intricate microbial diversity during strong flavor baijiu fermentation, as evidenced by the studies [16–18]. Notably, *Bacilli* emerge as the predominant microbial community during the early cultivation phase. Their relative abundance steadily escalates from approximately 65% to around 95% over the process of fermentation, trailed by Bacteroidetes and Clostridium. At the genus level, *Lactobacillus* exerts significant dominance, constituting 60–90% of the total bacterial population [3,19]. Another study has identified three bacterial communities—*Bacillus*, *Lactobacillus*, and *Acetobacter*—and six fungal genera—*Issatchenkia*, *Pichia*, *Candida*, *Aspergillus*, *Monascus*, and *Rhizopus*—as the key microbial components in zaopei [20]. Indeed, starch and other macromolecules are degraded and utilized with the help of microorganisms during fermentation, resulting in the accumulation of more than 3000 compounds in zaopei. However, the dynamic succession process during the fermentation of Wuliangye between compounds and microorganisms, as well as between microorganisms, still remains unclear.

So far, numerous studies have explored the VFCs in strong flavor baijiu. Analytical techniques such as gas chromatography/olfactometry have been employed to identify key VFCs in renowned baijiu brands, including Wuliangye, Yanghe, Jiananchun, and Gujinggong [21–23]. The VFCs of Wuliangye Baijiu were further analyzed by LiChrolut ENSPE fractionation coupled with comprehensive 2D gas chromatography and time-of-flight mass spectrometry (GC×GC-TOFMS), identifying about 500 compounds from over 3000 peaks [24]. In total, 75 aroma components were identified in Wuliangye-flavor raw liquor by headspace solid phase microextraction, liquid–liquid microextraction combined gas chromatography-mass spectrometry (HS-SPME/LLME-GC-MS) [25]. Despite these advancements, no report has been conducted to investigate the dynamic change in microorganisms and its effects on VFCs formation in the Wuliangye zaopei fermentation of mud pits with different continuously used years.

This study aimed to assess how continuous usage years of mud pits affect microbial communities and VFCs during zaopei fermentation. Zaopei samples from mud pits with two different continuous usage years were selected as research subjects. Physicochemical properties, VFCs, and microbial community structures during Wuliangye Baijiu



fermentation were monitored dynamically. Fermentation feature tests, headspace solid phase microextraction (HS-SPME) coupled with gas chromatography mass spectrometry (GC/MS) analysis, and metagenomic sequencing were used. Our results revealed that different continuous usage years of mud pits significantly influenced the microbial succession patterns and VFC profiles, thereby providing a more holistic understanding of microbial dynamics, as well as the changes in physicochemical characteristics and VFCs during Wuliangye fermentation.

## 2. Materials and Methods

### 2.1. Sample Collection

Two mud pits (4.3 m (L) × 3.3 m (W) × 2.3 m (H)) with 20 years (named JC20) and 40 years (named JC40) of continuous use from Wuliangye Yibin Co., Ltd. (Yibin, China), a representative China-based producer of strong-flavor baijiu, were selected for the fermentation of Wuliangye Baijiu. Zaopei samples were obtained from the two pits on the 0th, 5th, 15th, 20th, 27th, 34th, 41st, 48th, 55th, and 69th day of fermentation. Zaopei samples were obtained from three points (each point was regarded as a biological replication) in the upper, middle, and bottom layers of each pit using a hollow cylindrical sampler. All collected samples were pooled and thoroughly mixed in sterile plastic sampling bags immediately after collection to ensure uniformity. And then they were promptly transported to the laboratory and stored at −80 °C until they were needed for study.

### 2.2. Physiochemical Properties of Zaopei

A total of 5 key parameters (fermentation temperature, titratable acidity, moisture, total starch, and titratable reducing sugar) were detected to investigate the dynamic change in the two pits. The moisture percentage of zaopei was assessed via the gravimetric method, where samples were dried at 105 °C for a minimum of 3 h. The titratable acidity in zaopei was detected through titration with 0.1 M NaOH, using phenolphthalein as an indicator (endpoint at pH 8.2). Starch and titratable reducing sugar contents were analyzed using Fehling's reagent.

### 2.3. Analysis of VFCs in Zaopei

A total of 1 g of zaopei sample was taken into the headspace bottle and then the bottle cap was closed and immediately tightened. Volatile compounds were analyzed using an Agilent 7890A gas chromatograph interfaced with an Agilent 5977B inert mass spectrometer operating in electron ionization (EI) mode (Agilent, Santa Clara, CA, USA). Volatile components were extracted by a 2 cm 50/30 µm DVB/CAR/PDMS fiber (Supelco, Bellefonte, PA, USA). Prior to extraction, samples were thermally equilibrated at 50 °C for 5 min, followed by 45 min of extraction at the same temperature.

The SPME fiber was then thermally desorbed in the GC injection port (250 °C for 5 min, splitless mode). Compound separation was achieved using an HP-INNOWAX column (60 m length × 0.32 mm internal diameter, 0.5 µm stationary phase thickness) (Agilent, Santa Clara, CA, USA). The GC method employed helium as carrier gas (1 mL/min constant flow) with the following temperature program: initial hold at 40 °C for 3 min, followed by a 8 °C/min ramp to 250 °C, and a final isothermal hold for 5 min. Mass spectrometric detection was performed in electron impact ionization mode (70 eV) with the ion source maintained at 250 °C. A full-scan analysis covered 35–350 amu for compound characterization. Identification used NIST library matching (score ≥ 85), retention indices from n-alkanes, and comparison with reference standards. 4-Octanol (Sigma-Aldrich, St. Louis, MO, USA) at ~100 ppm served as the internal standard. Semi-quantification was based on the volatile compounds' relative peak areas to 4-octanol.

## 2.4. DNA Extraction

The PowerSoil® DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) was used for DNA extraction from 0.5 g homogeneous frozen samples following the manufacturer's protocols. The purity and concentration of DNA were evaluated with a NanoDrop 2000 UV spectrophotometer (Thermo Scientific, Waltham, MA, USA). All DNA samples were stored at  $-80\text{ }^{\circ}\text{C}$  until for study.

## 2.5. Statistical Analysis

Data were presented as the mean  $\pm$  standard deviation (SD). R language were used for data analysis. The Pearson correlation coefficient  $r^2$  was calculated using R language and the heat map of  $r^2$  was drawn by R 3.5.1. Differences were considered to be statistically significant at  $p < 0.05$ .

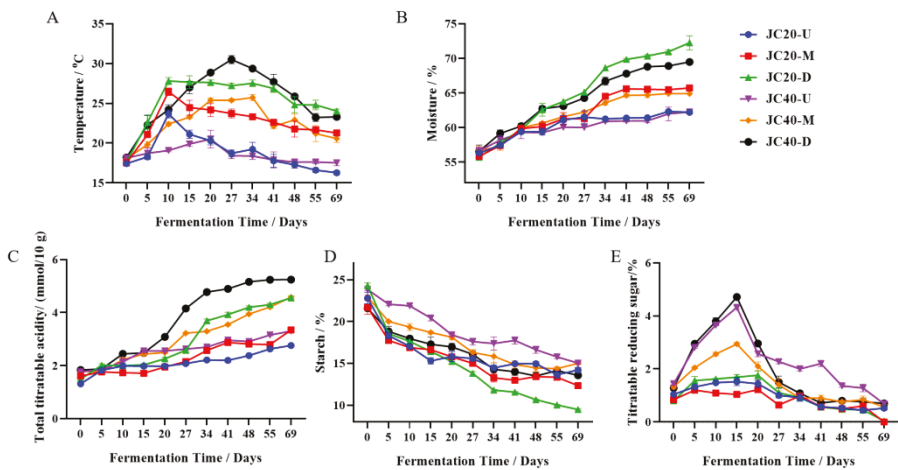
# 3. Results and Discussion

## 3.1. Dynamics of Fermentation Properties During Wuliangye Baijiu Making Process

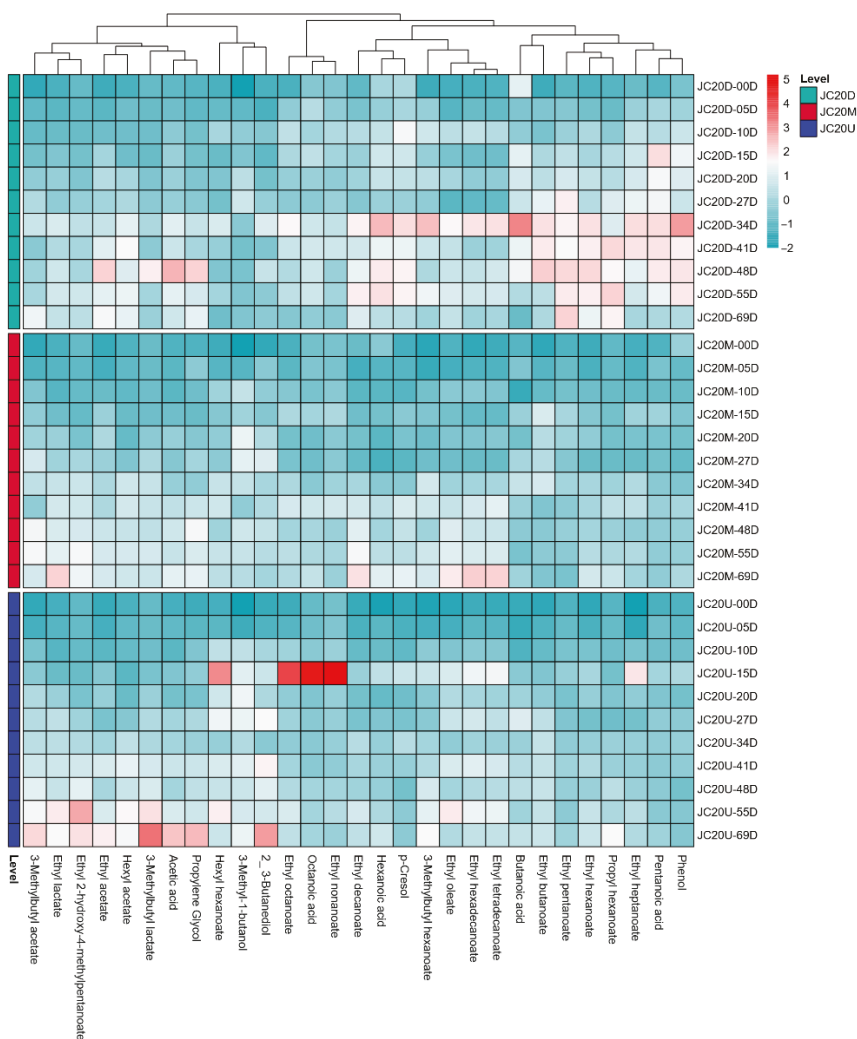
We systematically monitored the dynamic variations in fermentation temperature, moisture content, total starch, titratable reducing sugar, and titratable acidity throughout the fermentation processes of JC20 and JC40 (Figure 2). Initially, as Baijiu is produced in an open environment, the temperatures of JC20 and JC40 were comparable. As fermentation progressed, the temperature increased rapidly and gradually declined until the end. Notably, in both mud pits, the temperature gradient was consistent with traditional practices, where the lower layer zaopei registered the highest temperature, while the upper layer zaopei exhibited the lowest (Figure 2A). Regarding moisture content, a consistent upward trend was observed in both mud pits throughout the fermentation period. However, the lower layer zaopei of JC20 demonstrated a more pronounced increase, rising from  $55.70\% \pm 0.14$  to  $72.26\% \pm 1.00$ , compared to JC40's range of  $56.55\% \pm 0.92$  to  $69.49\% \pm 0.02$  (Figure 2B). The total titratable acids steadily accumulated during fermentation in both samples, reaching their peak concentrations at the end of the process (Figure 2C). Across all three layers, the zaopei from JC20 exhibited higher acidity levels than those from JC40, with the lower layer zaopei of both mud pits having the most significant acidity values:  $4.56 \pm 0.07$  for JC20 and  $5.25 \pm 0.05$  for JC40. The total starch content started high at the beginning of fermentation and steadily decreased until the final sampling point in both JC20 and JC40 (Figure 2D). Specifically, the middle and lower layers of zaopei in JC20 showed greater starch consumption compared to their counterparts in JC40. For the middle layers, the starch content decreased from  $21.7\% \pm 0.84$  to  $12.35\% \pm 0.25$  in JC20, contrasting with  $22.92\% \pm 0.29$  to  $15.00\% \pm 0.13$  in JC40; for the lower layers, the reduction was from  $24.30\% \pm 0.34$  to  $9.50\% \pm 0.10$  in JC20 versus  $21.59\% \pm 0.22$  to  $13.58\% \pm 0.13$  in JC40. The titratable reducing sugar content in JC40's zaopei across all three layers underwent a rapid increase followed by a sharp decline during fermentation. In contrast, the changes in JC20 were more subdued, characterized by a modest rise and subsequent decrease (Figure 2E).

## 3.2. Variation of Main VFCs in Fermentation Process

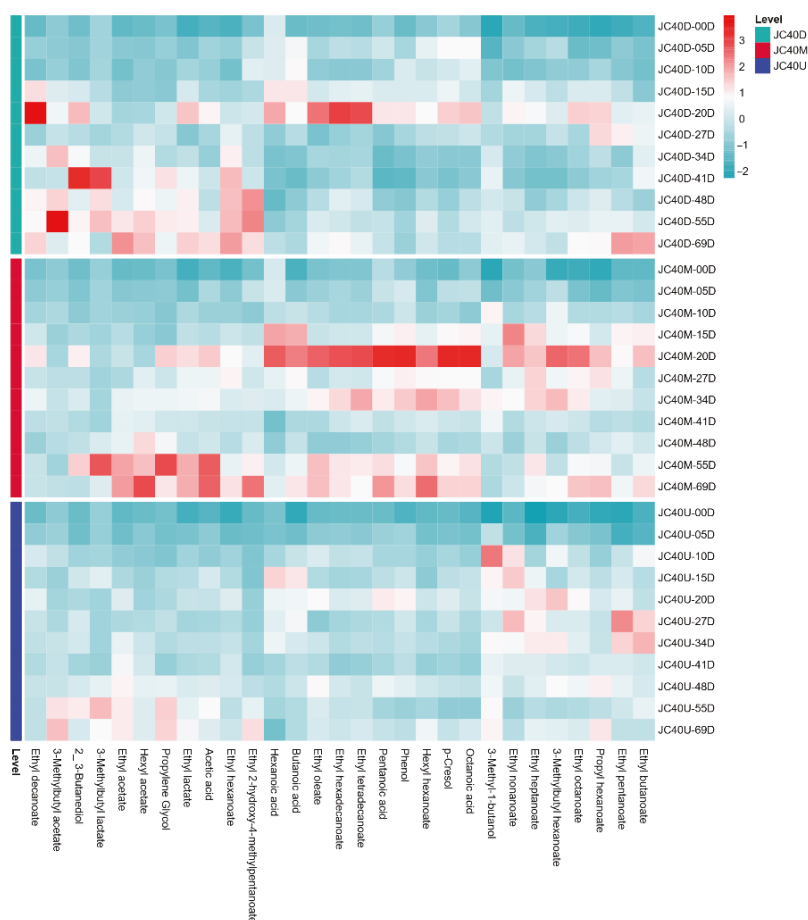
To explore the impact of mud pit continuous usage years on VFCs, HS-SPME/GC-MS was used to analyze zaopei from JC20 and JC40. A total of 29 main VFCs were semi quantified, including 19 esters, 3 alcohols, 5 acids, and 2 others (score  $> 85$ ). Heat map analysis (Figure 3) showed that metabolite profiles increased during fermentation, with greater variation in JC40 than in JC20. However, these flavor substances had distinct dynamic changes during fermentation.



**Figure 2.** Dynamics of zaopei fermentation properties during Wuliangye Baijiu fermentation. (A) Temperature, (B) moisture, (C) total titratable acidity, (D) starch, (E) titratable reducing sugar. The different color curve represent different layer zaopei samples (blue, red, green, purple, yellow, and black represent JC20-up, JC20-middle, JC20-down, JC40-up, JC40-middle, and JC40-down samples, respectively). Each data point is the mean of three measurements  $\pm$  SD.



**Figure 3.** Cont.



(B)

**Figure 3.** Heat map of changes in differential VFCs during the fermentation of JC20 (A) and JC40 (B).

Esters, crucial for the fruity aroma of Wuliangye Baijiu, were the most abundant VFCs in all zaopei samples. Notably, esters constituted the largest proportion of VFCs in all zaopei samples, accounting for 70.10–78.92% of the total VFCs at the end of fermentation (Table S1). Our results demonstrated that the concentrations of major esters steadily increased during fermentation, reaching their peak values at the conclusion of the process (Figure 3). Both the total ester content and most individual ester concentrations in JC40's three-layer zaopei samples were significantly higher than those in JC20. Among them, ethyl hexanoate, ethyl acetate, and ethyl lactate dominated the ester composition in all zaopei samples (Table S1). As a key determinant of Wuliangye Baijiu's quality, ethyl hexanoate content in the three-layer zaopei of JC40 at the end of fermentation was superior to that of JC20, following the order: JC40-D > JC40-M > JC40-U > JC20-D > JC20-M > JC20-U (Table S1). These results aligned with previous research [6], suggesting that longer mud pit continuous usage years lead to better quality Wuliangye Baijiu production.

Volatile acids are essential components among the VFCs of Wuliangye Baijiu, playing a pivotal role in its aroma profile. An optimal concentration of these acids endows the liquor with a harmonious and enduring pleasantness [26]. For example, hexanoic and acetic acids are major acids in strong flavor baijiu; suitable amounts enhance liquor aroma, but excessive levels disrupt its style [25]. In this study, five main volatile organic acids were identified in both JC20 and JC40 (Figure 3). Their concentrations exhibited an initial increase during the early fermentation phase, followed by either stabilization or a decline. This trend can likely be attributed to their involvement in esterification reactions, where they serve as key precursors for the synthesis of corresponding esters (Figure 3). Notably,

as the primary precursors for ethyl hexanoate, hexanoic acid levels in all three layers of JC40 zaopei were significantly higher than those in JC20 ( $p < 0.05$ , Table S1). This disparity in hexanoic acid content may well account for the elevated ethyl hexanoate levels observed in the zaopei of JC40 compared to JC20.

Alcohols, formed through glucose metabolism and the dehydrodecarboxylation of amino acids, act as essential precursors for ester formation. Higher alcohols are one of the important flavor compounds in Wuliangye Baijiu, yet excessive concentrations are unpalatable. In this study, three main volatile alcohols were identified (Figure 3). Notably, 3-methyl-1-butanol, a key constituent of higher alcohols, exhibited significantly higher concentrations in the three-layer zaopei samples of JC20 compared to JC40 at the end of fermentation ( $p < 0.05$ , Table S1).

### 3.3. Diversity and Differences in Microbial Community Composition During Fermentation

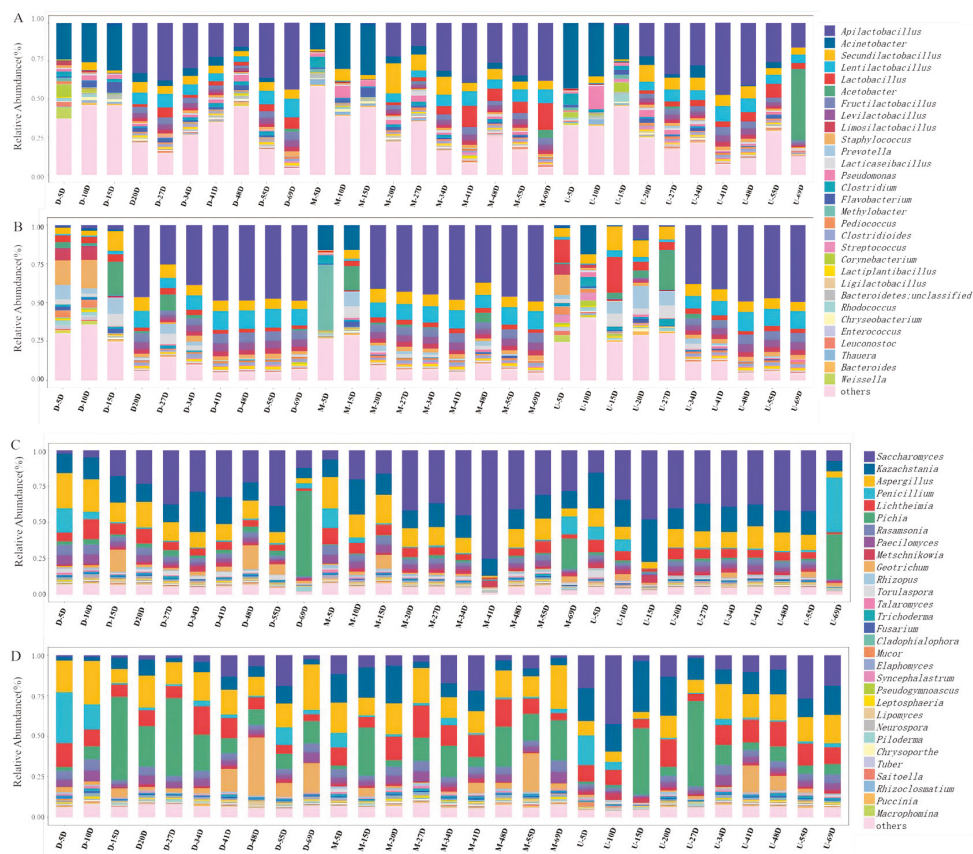
To explore the composition of microbial communities in different ages of mud pits, the fermentation process was monitored over a 70-day period. After quality control, a total of 1,179,351,053 high quality sequences were successfully retrieved for the bacterial communities across all samples. The number of sequences per sample ranged from 1,839,808 to 35,301,333. For eukaryote sequences, 23,505,524 high-quality reads were obtained, with the read counts per sample spanning from 10,582 to 3,541,662.

Subsequently, high-throughput sequencing was performed to assess microbial community succession during fermentation, with taxonomic classification conducted at both the phylum and genus levels. Analysis revealed the presence of four dominant bacterial phyla and four predominant fungal phyla, all exhibiting relative abundances above 0.1% (Figure S1). Among the bacterial, Firmicutes, Actinobacteria, Proteobacteria, and Bacteroidetes dominated in the early stages of fermentation in both mud pits. Their abundances then decreased while Firmicutes increased during fermentation with a relative abundance of more than 95.74% at the end of fermentation of JC40 and JC20 except the upper layer zaopei sample of JC20, which was 56.98% (Figure S1A,B). For fungi, Ascomycota was identified to be the predominant organism throughout the entire fermentation process. Moreover, at the end of fermentation, all zaopei samples had a relative abundance of Ascomycota exceeding 88.76% (Figure S1C,D).

At the genus level, the succession of microbial communities during the fermentation of JC20 and JC40 exhibited distinct characteristics. The distribution of microbial genera in JC40 was more balanced than that in JC20, potentially explaining why high quality Wuliangye Baijiu is commonly produced in older mud pits such as JC40. Initially, *Pantoea* and *Kosakonia* emerged as the predominant genera, while *Cronobacter* was dominant later. As fermentation advanced, the relative abundances of *Pantoea*, *Kosakonia*, and *Cronobacter* decreased, possibly due to the significant enrichment of lactic acid bacteria towards the end of the fermentation period.

Bacteria, a key functional microorganism, secretes numerous hydrolytic enzymes that aid in the formation of flavor compounds during Wuliangye Baijiu fermentation. In JC20, *Acinetobacter* was a major genus from day 5–15 but its proportion dropped rapidly as fermentation continued. *Apilactobacillus* became dominant on day 20 and remained so until the end of fermentation in both JC20 and JC40 (Figure 4A,B). Moreover, a notable divergence was observed in the dominant microbial communities of zaopei samples between JC40 and JC20 during the early fermentation stage (Figure 4B), which might be due to the differences in mud pit microorganisms and environmental microorganisms.





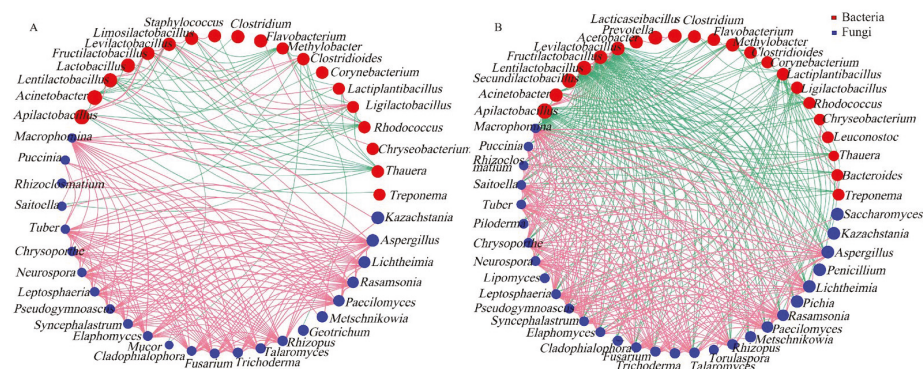
**Figure 4.** Bacterial (A,B) and fungal (C,D) community structure succession at the genus level. A and C (B,D) represent the results of JC20 (JC40).

Fungi play a crucial role in Wuliangye Baijiu fermentation, secreting diverse enzymes that drive the biosynthesis of flavor compounds [27]. Among them, *Saccharomyces* stands out as a pivotal microorganism for alcohol production. It demonstrates remarkable efficiency in sugar conversion, predominantly driving ethanol fermentation during the brewing of Wuliangye Baijiu. Our results indicate that in the upper and middle layer zaopei of JC20 and JC40, the relative abundance of *Saccharomyces* rose rapidly in the early and middle fermentation stages, consistent with the fact that the upper and middle layer zaopei have a higher yield than the lower layers. *Kazachstania* and *Aspergillus* also exhibited abundance at the beginning fermentation of JC20 and maintained relative stability as fermentation progressed (Figure 4C). For JC40, apart from the significant increase in *Saccharomyces*, the proportion of *Pichia* was also found to be increased during the fermentation (Figure 4D). Additionally, the relative abundances of *Aspergillus* and *Lichtheimia* maintained relative stability in each samples, highlighting their stable contribution to the fermentation ecosystem (Figure 4D).

### 3.4. Correlation Analyses Revealed the Relationships Between Different Microbes of Different Pits

Microbial interactions play a pivotal role in shaping the microbial composition, as they are intricately linked to fluctuations in microbial populations and have a direct impact on microbial metabolism during the fermentation process [28,29]. To elucidate these complex relationships, Pearson's correlation coefficients and *p* values were computed for the top 30 bacterial and fungal genera from JC20 and JC40 (Figure 5). The correlation analysis results revealed that in JC20, 19 bacterial and 24 fungal genera exhibited correlated behavior (Figure 5A), while in JC40, 22 bacterial and 29 fungal genera showed similar patterns of correlation (Figure 5B). Notably, across both JC20 and JC40, bacterial communities

generally displayed a negative correlation with fungal communities. Conversely, positive associations were observed among different fungal genera within each sample.



**Figure 5.** Microbial association networks were constructed for samples JC20 (A) and JC40 (B). Bacterial and fungal nodes are represented by red and blue circles, respectively. Significant positive correlations ( $r > 0.7, p < 0.05$ ) are denoted by red lines. Significant negative correlations ( $r < -0.7, p < 0.05$ ) are indicated by green lines.

Delving deeper into the findings, in JC20, *Levilactobacillus*, *Fructilactobacillus*, *Apilactobacillus*, and *Clostridioides* were strongly positively correlated ( $|r| > 0.9, p < 0.05$ ). In contrast, *Clostridioides* negatively correlated with *Methylobacter*, *Acinetobacter*, and several other genera ( $|r| > 0.7, p < 0.05$ ). Multiple *lactobacillus* related genera were negatively correlated with *Methylobacter* ( $|r| > 0.7, p < 0.05$ ), except for *Rhodococcus*, which showed a strong positive correlation with it ( $|r| > 0.9, p < 0.05$ ) (Figure 5A). Regarding JC40, *Clostridioides* negatively correlated with *Methylobacter*, *Flavobacterium*, *Acinetobacter*, *Lipomyces*, *Thauera*, and *Rhodococcus* ( $|r| > 0.7, p < 0.05$ ), but no genera showed a strong positive correlation with *Clostridioides* ( $|r| > 0.9, p < 0.05$ ). Seven bacterial genera (*Levilactobacillus*, *Fructilactobacillus*, *Lentilactobacillus*, *Apilactobacillus*, *Ligilactobacillus*, *Lactiplantibacillus*, and *Clostridioides*) displayed significant negative correlation with *Methylobacter* ( $|r| > 0.7, p < 0.05$ ), while *Acinetobacter* and *Clostridium* were positively correlated with *Methylobacter* ( $|r| > 0.9, p < 0.05$ ) (Figure 5B). For fungi, in JC20, *Aspergillus* correlated positively with 16 fungal genera and *Rhizopus* with 17 ( $|r| > 0.9, p < 0.05$ ) (Figure 5A). For JC40, *Aspergillus* positively correlated with 15 fungi ( $|r| > 0.9, p < 0.05$ ) but negatively with five bacteria ( $|r| > 0.7, p < 0.05$ ). Similarly, *Rhizopus* had positive links with 15 fungi and negative ones with five bacteria under the same significance. *Saccharomyces* and *Pichia* were positively correlated with *Torulaspora* and *Piloderma* ( $|r| > 0.9, p < 0.05$ ), respectively (Figure 5B). Overall, bacteria–fungi correlations were mostly negative ( $|r| > 0.7, p < 0.05$ ) (Figure 5).

The results of high throughput sequencing revealed that the dominant microbial genera in JC20 and JC40 samples evolved with fermentation time and environmental changes. This suggests that certain genera, such as *Secundilactobacillus*, *Acinetobacter*, *Limosilactobacillus*, and *Flavobacterium*, may struggle to adapt to rising ethanol concentrations and increased acidity. These environmental shifts result from the selective pressure exerted by *Apilactobacillus* and *Saccharomyces*. Moreover, there were three bacteria (*Limosilactobacillus*, *Lactobacillus*, *Staphylococcus*) and one fungi (*Geotrichum*) that showed correlated behavior only in JC20, while six bacteria (*Bacteroides*, *Leuconostoc*, *Prevotella*, *Acetobacter*, *Secundilactobacillus*, and *Lacticaseibacillus*) and six fungi (*Pichia*, *Penicillium*, *Saccharomyces*, *Talaromyces*, *Piloderma*, and *Lipomyces*) showed correlated behavior only in JC40. Correlation analyses indicated that microbial community interactions in JC40 were more diverse and stronger than in JC20, which was in line with the typical characteristics of the brewing process of “the older the pit, the better the baijiu” [30]. Additionally, as dominant fungal genera, *Rhizo-*

*pus* and *Saccharomyces* genera demonstrate robust fermentation, decomposition capabilities, and resistance to acid and ethanol [29,31,32].

### 3.5. Correlations Between Microorganisms with Physicochemical Properties

Environmental factors govern the changes in microbial community structures. To elucidate the relationships between microbial communities and physicochemical properties during the fermentation of JC20 and JC40, RDA was conducted (Figure 6). The results demonstrated that the microbial community dynamics were significantly influenced by variables such as total starch content, titratable acid levels, moisture, titratable reducing sugar, and fermented temperature. In terms of bacterial communities, in JC20 fermentation, *Acinetobacter*, *Clostridium*, and *Corynebacterium* were associated with total starch and titratable reducing sugar and *Lactococcus*, *Acetobacter*, *Staphylococcus*, and *Pediococcus* had significantly positive correlation with titratable acids of JC20 (Figure 6A). As for JC40, *Acinetobacter*, *Clostridium*, *Methylobacter*, *Pediococcus*, and *Staphylococcus* were only correlated with total starch over the fermentation of JC40 (Figure 6B). And *Fructilactobacillus* and *Apilactobacillus* had significantly positive correlation with titratable acids (Figure 6B). According to previous studies, these bacteria can produce acids by using carbohydrates [33–35].

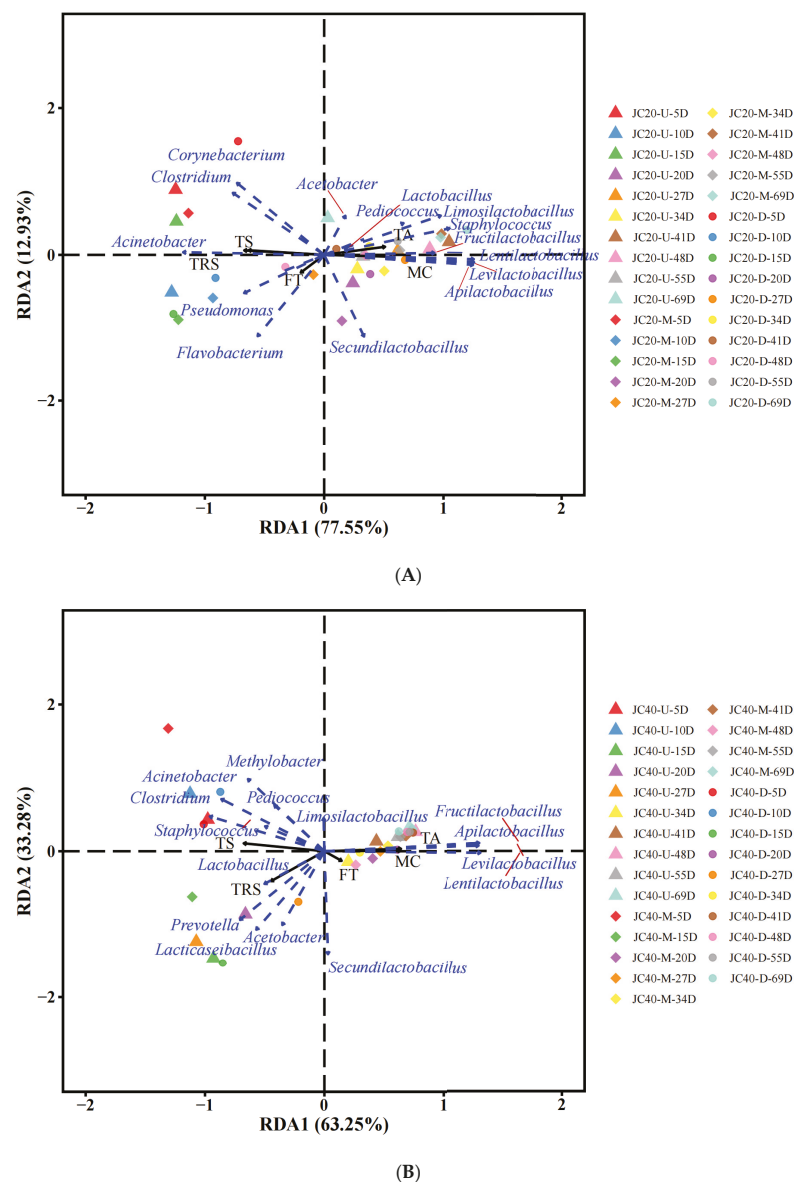
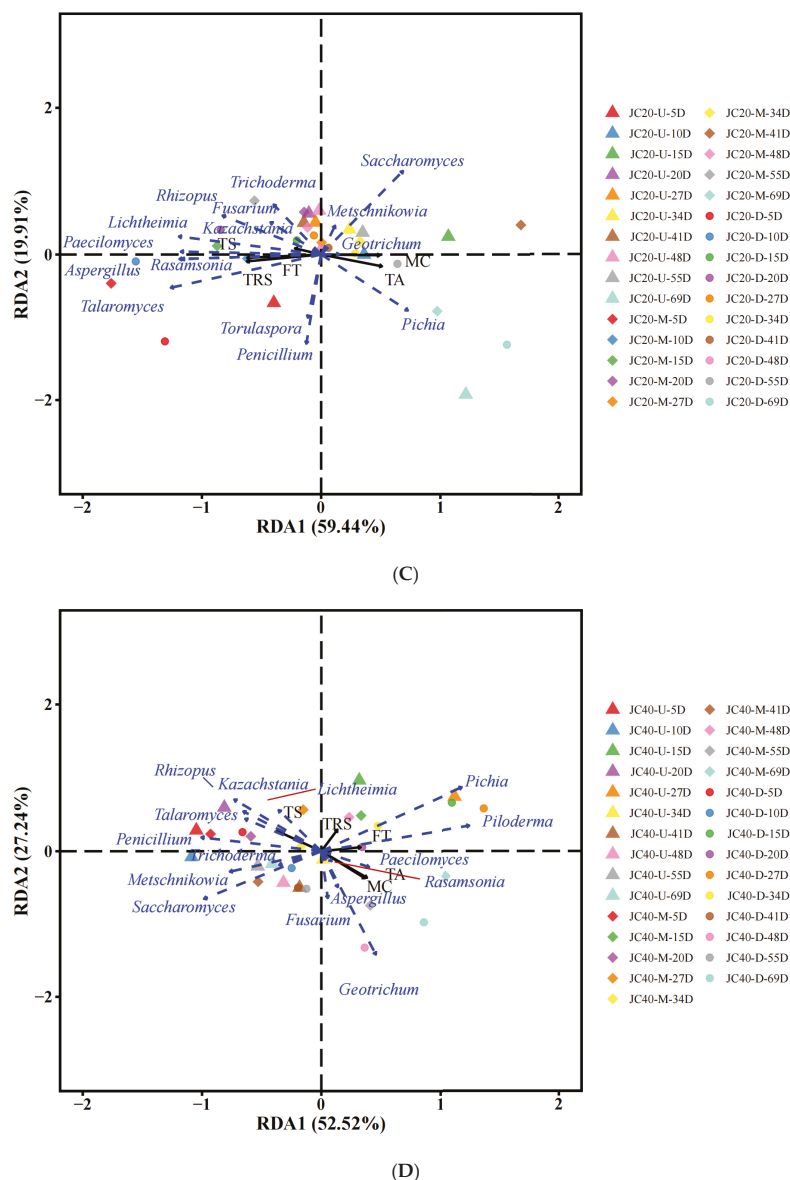


Figure 6. Cont.



**Figure 6.** Microbial RDA analysis (top 15 species) and fermentation characteristics. Correlation analysis between fermentation parameters and bacterial genera (A,B) or fungal genera (C,D). (A,C) represent the results of JC20; (B,D) represent the results of JC40. TS: total sugar, TA: titratable acid, MC: moisture content, TRS: titratable reducing sugar, FT: fermented temperature.

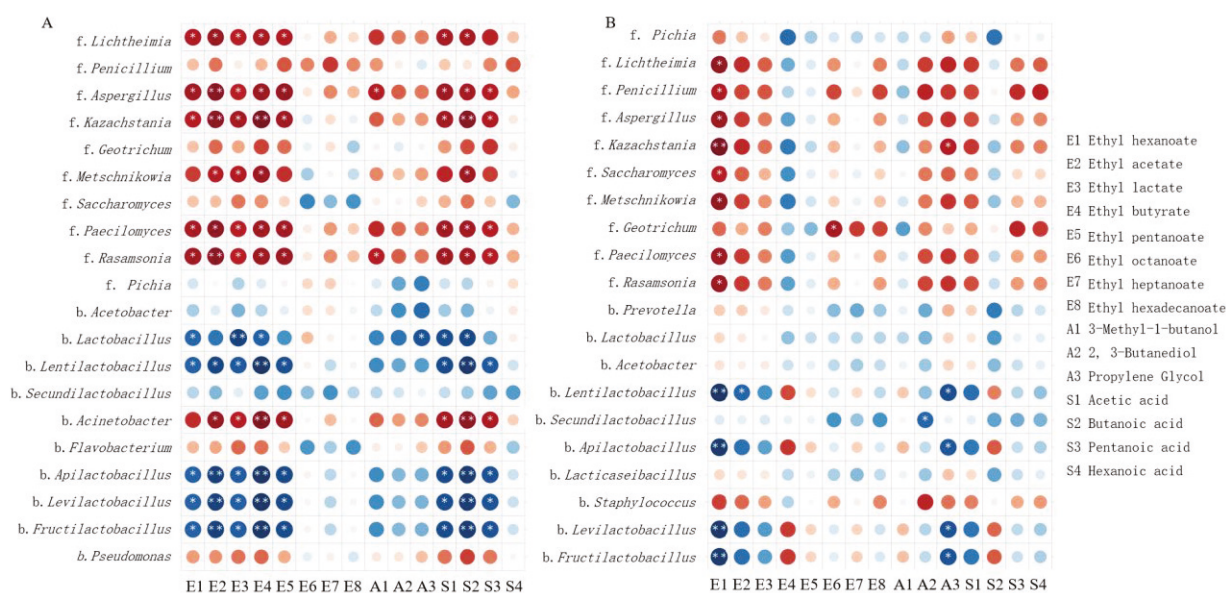
For fungi, *Aspergillus* plays a central role in the saccharification, fermentation, and esterification processes that are fundamental to Chinese Baijiu production [36–38]. In this study, in JC20, *Aspergillus* was linked to titratable acids (Figure 6C), while in JC40, it correlated with titratable reducing sugar and temperature (Figure 6D). Furthermore, *Rhizopus* and *Kazachstania* were related to the total starch content in both JC20 and JC40. *Rhizopus* is known for its strong amylase-producing ability during Wuliangye Baijiu fermentation [29,39], and can break down starch into fermentable sugars [40], consistent with our findings. Moreover, *Pichia* exhibited significant positive associations with titratable reducing sugar and temperature, highlighting its potential role in responding to key environmental factors during the fermentation process.

### 3.6. Correlations Between Microorganisms with VFCs

In the production of Chinese Baijiu, understanding the interactions between microorganisms and VFCs during fermentation is crucial, given the complexity of the Baijiu ferment-



tation system, which involves numerous microorganisms and chemical compounds [38,41]. Therefore, Pearson's correlation coefficient was used to reveal the interaction between microorganisms (top 10 bacteria and top 10 fungi) and 15 main VFCs in this study (Figure 7).



**Figure 7.** Correlation analysis of microbiota and VFCs during JC20 (A) and JC40 (B) fermentation. Pearson's rank correlations between microorganisms (top 10 bacteria and top 10 fungi) and 19 main VFCs. Circle size represents correlation strength (large circles for strong, small for weak), and the color scale indicates correlation type: dark blue for perfect positive correlation (1), dark red for perfect negative (−1). Significant correlations are marked: \* for  $|r| > 0.7$  ( $p < 0.01$ ), \*\* for  $|r| > 0.9$  ( $p < 0.01$ ).

The correlation analysis result revealed that nearly all top 10 fungal species in both JC20 and JC40 fermentation pits played pivotal roles in synthesizing main VFCs, including ethyl hexanoate [E1], ethyl acetate [E2], and ethyl lactate [E3]. These fungi also contributed to the formation of 2,3-butanediol [A2], along with organic acids such as acetic acid [S1] and pentanoic acid [S3]. To the best of our knowledge, *Aspergillus* facilitates esterase production [38,42], and *Paecilomyces*, *Rasamsonia*, and *Kazachstania* produce glucoamylase to break down glucose and generate various VFCs such as acids, esters, and alcohols [33]. Our findings in Figure 7 supported these observations. However, differences emerged in the production of other VFCs. For JC20, the synthesis of ethyl butyrate [E4], ethyl valerate [E5], 3-methyl-1-butanol [A1], and butanoic acid [S2] positively correlated with most fungal species (Figure 7A), while for JC40, ethyl caprylate [E6], ethyl palmitate [E8], and hexanoic acid [S4] were positive correlated with most fungal microorganisms (Figure 7B).

In contrast, most bacteria showed weak relation to the formation of VFCs during the fermentation process, and some bacteria such as *Acetobacter*, *Lactobacillus*, *Lentilactobacillus*, *Secundilactobacillus*, *Apilactobacillus*, *Levilactobacillus*, and *Fructilactobacillus* showed negative correlation with VFCs synthesis of JC20 (Figure 7A). Similarly, in JC40, *Lentilactobacillus*, *Secundilactobacillus*, *Apilactobacillus*, *Levilactobacillus*, and *Fructilactobacillus* showed negative correlations with the production of the majority of VFCs (Figure 7B). As Figure 5 showed, it might be because bacteria showed a negative correlation with fungi.

The correlation analysis between VFCs and microorganisms offers valuable insights into the microbial species driving VFC biosynthesis. This knowledge can be leveraged to precisely regulate VFC production during Wuliangye Baijiu fermentation. However, it is crucial to acknowledge that fermentation is a multifaceted process influenced by numerous factors. For instance, introducing or increasing the abundance of specific microbial strains may have far-reaching effects on the entire fermentation ecosystem. Only by taking a



holistic approach and considering all variables involved in fermentation can we achieve consistent VFC production, uphold the quality of zaopei, and ultimately produce superior Wuliangye Baijiu.

#### 4. Conclusions

This investigation examined microbial succession patterns and associated biochemical transformations in fermentation pits with distinct operational histories (20-year [JC20] vs. 40-year [JC40] continuous use) from the Wuliangye production system.

In this study, two different mud pits (JC20 and JC40), that were used continuously for many years, from Wuliangye were selected to monitor the dynamic changes and the relation among the microbial and physiochemical properties and VFCs. The results showed a positive association between different fungi genera while it presenting a generally negative correlation between different bacteria genera or between fungi and bacteria genera from both two pits. Moreover, fungal microorganisms (such as *Aspergillus*, *Paecilomyces*, *Rasamsonia*, and *Kazachstania*) were found to make greater contributions to the synthesis of main VFCs during the fermentation process of both two pits. Additionally, the diversity of microorganisms successfully identified of three different layers zaopei samples from JC40 at the fermentation anaphase were significantly higher than that of JC20, which also resulted in the production of higher quality Wuliangye from JC40. The results offer key insights into microbiota that drive the generation of VFCs in Wuliangye production.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fermentation11070388/s1>, Table S1: Detection results of VFCs at the end of fermentation. Figure S1: Bacterial (A,B) and fungal (C,D) community structure succession at the phylum level. A and C (B and D) represent the results of JC20 (JC40).

**Author Contributions:** Y.L. (Yanping Lu): Conceptualization, Methodology, Validation, Data curation, Visualization, Project administration, and Writing—Original draft preparation. Y.L. (Yalan Li): Methodology, Validation, and Investigation. B.W.: Methodology and Validation. Y.L. (Yuzhu Li): Methodology, J.S.: Resources. J.Z.: Conceptualization and Writing—Reviewing and Editing. D.Z.: Supervision. Y.X.: Supervision. All authors have read and agreed to the published version of the manuscript.

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## Article

# Processing Factors and Risk Assessment of Pesticide Residues in Wine

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**Abstract:** The presence of pesticide residues in wine raises concerns about consumer health and regulatory compliance. This study evaluates the behaviour of seven pesticides (boscalid, penconazole, tebufenozide, kresoxim-methyl, trifloxystrobin, chlorpyrifos and lambda-cyhalothrin) during key stages of vinification, including clarification, fermentation, and racking, using liquid chromatography–tandem mass spectrometry. Penconazole, kresoxim-methyl and trifloxystrobin were not detected at the beginning of the vinification stage. Boscalid, chlorpyrifos and lambda-cyhalothrin showed a reduction of 100% whilst, a concentration below the limit of quantification at the end of the vinification stage. However, tebufenozide showed the lower elimination rate (88.1%), presenting a concentration at the end of the vinification process of 21.71 µg/kg. Experimental data confirm that pesticide concentrations progressively decline due to a combination of physicochemical transformations, adsorption onto solids, and biochemical processes. The clarification and racking stages are particularly important for reducing residues, primarily by removing suspended solids that adsorb pesticides. Fermentation also contributes to this reduction through microbial metabolism and enzymatic hydrolysis. The effectiveness of these stages depends on the pesticide’s solubility, chemical stability, and interactions with components in the wine matrix. Additionally, a risk assessment based on Acceptable Daily Intake and Estimated Daily Intake was performed to evaluate potential consumer exposure, showing lower risk for all pesticides studied. The findings emphasise the importance of optimising clarification and racking procedures, selecting effective fining agents, and refining filtration techniques to further enhance pesticide removal.

**Keywords:** ADI; EDI; pesticide residues; risk assessment; wine safety; winemaking process

## 1. Introduction

Viticulture is a globally significant agricultural sector, with annual wine production in 2023 surpassing 235 million hectolitres [1]. The use of pesticides is crucial for safeguarding grapevines against fungal diseases, insect pests, and weeds, which helps maintain both crop yield and quality. However, the presence of pesticide residues in wine has raised significant safety concerns due to potential health risks and stringent regulatory controls [2–4].

Good Agricultural Practices (GAP) aim to minimise pesticide residue levels by following recommended safety intervals and dosages. Assessing pesticide dissipation under GAP conditions offers a thorough understanding of how residues behave during winemaking and their potential implications for wine safety. In this sense, chronic exposure to certain

pesticide compounds has been linked to endocrine disruption, neurotoxicity, and carcinogenicity. This has prompted international food safety authorities, including the European Food Safety Authority (EFSA) and the United States Environmental Protection Agency, to establish Maximum Residue Limits (MRLs) for wine and related products [5,6].

The dissipation of pesticide residues throughout vinification is influenced by multiple physicochemical and biochemical mechanisms, including hydrolysis, oxidation, microbial metabolism, adsorption, and volatilisation [7,8]. The extent of residue reduction depends on the pesticide's solubility, chemical stability, and interaction with wine matrix components. For instance, hydrophobic pesticides tend to adsorb onto grape solids, including skins, seeds, and yeast lees, which are removed during pressing, racking, and clarification, while hydrophilic compounds persist in the liquid phase and are more likely to be retained in the final product [9].

Several commonly used pesticides in viticulture, including boscalid, penconazole, tebufenozide, kresoxim-methyl, trifloxystrobin, chlorpyrifos or lambda-cyhalothrin, have been studied for their dissipation behaviour in wine [7,10]. The behaviour of these pesticides in wine production varies based on their water solubility, volatility, and affinity for solid or liquid phases within the wine matrix. For instance, boscalid, tebufenozide, and kresoxim-methyl are moderately to highly hydrophobic (partition coefficient  $\log p > 3$ ), which encourages their adsorption onto grape solids and aids in their removal during the clarification and racking stages. Trifloxystrobin is also hydrophobic but tends to persist during fermentation. This persistence is likely due to its high organic carbon/water partition coefficient ( $K_{oc}$ ) and resistance to microbial degradation. In contrast, chlorpyrifos and lambda-cyhalothrin have very low water solubility and are primarily removed through adsorption. However, some residues are still detectable in the later stages of winemaking. Penconazole displays intermediate properties and shows moderate dissipation throughout the vinification process. In this sense, factors such as pH, ethanol content, temperature, and interactions with yeast and fining agents can affect the degradation kinetics and distribution of each compound during winemaking [11–13].

Among vinification processes, clarification and racking are crucial for reducing pesticide residues. These stages involve removing solid particles where pesticides may be adsorbed, which significantly lowers residue levels. Fermentation also aids in pesticide dissipation, mainly through enzymatic degradation, hydrolysis, and adsorption onto yeast biomass. However, its effectiveness can vary based on factors such as fermentation temperature, ethanol concentration, and the choice of yeast strain [14–16]. Some studies suggest that ethanol improves the breakdown of certain pesticide residues, while other studies emphasise the importance of yeast cell walls in adsorbing and immobilising these compounds. The  $\log p$  value of each pesticide influences its affinity for organic or aqueous phases, which in turn affects its behaviour during fermentation [17].

In addition to fermentation, techniques for clarification and stabilisation further reduce pesticide residues. Fining agents, such as bentonite, gelatine, and activated carbon, can help to precipitate suspended pesticide residues, while filtration techniques, including microfiltration and ultrafiltration, can improve removal efficiency [1,18]. The degree of pesticide elimination differs between red and white wines. Red wines generally show a greater reduction in pesticide levels, primarily due to longer maceration periods that improve the adsorption of pesticides onto grape solids. Furthermore, the polyphenols found in red wines interact with pesticides, affecting their stability and the speed at which they degrade [12,19].

Processing factor (F) is a useful metric for assessing how vinification affects pesticide dissipation. An F value less than 1 indicates a net reduction in pesticide concentration due



to the winemaking processes, while an F value greater than 1 suggests an increase in concentration. This increase often results from volume reduction or phase partitioning [20,21].

From a regulatory standpoint, ensuring consumer safety necessitates rigorous monitoring of pesticide residues in wine. Advances in analytical chemistry have greatly enhanced detection capabilities, with LC-MS/MS and GC-MS being commonly used to assess residue dissipation during vinification [22–27]. In this sense, to evaluate the potential health risks from residual pesticides, regulatory agencies determine the Acceptable Daily Intake (ADI). This defines the maximum amount of a pesticide that can be consumed daily over a lifetime without causing adverse effects [28]. The Estimated Daily Intake (EDI) is utilised to assess whether wine consumption surpasses established safety limits.

The assessment of health risks of each pesticide is calculated by dividing the EDI of each residue by their respective ADI. Some studies indicate that, even in worst-case exposure scenarios, the EDI/ADI ratio remains within acceptable safety thresholds. This reinforces the idea that pesticide residues in wine present minimal toxicological risks, provided that regulatory limits are adhered to [3,12,13]. However, despite natural reductions during vinification, some pesticides still persist at trace levels, requiring additional mitigation strategies in order to ensure consumer safety necessities and to provide additional data on the behaviour of each pesticide regulated.

This study aims to investigate the dissipation of pesticide residues (boscalid, penconazole, tebufenozide, kresoxim-methyl, trifloxystrobin, chlorpyrifos, and lambda-cyhalothrin) across different winemaking stages, providing insights into the mechanistic pathways influencing residue reduction and their implications for food safety and regulatory compliance, performing an evaluation of the potential health risk of each pesticide studied.

## 2. Materials and Methods

### 2.1. Materials and Reagents

The pesticides selected for this study are boscalid, and kresoxim-methyl (Collis: boscalid 20% + kresoxim-methyl 10% *w/v*), penconazole (Topas 100 EC: penconazole 10% *w/v*), trifloxystrobin (Flint: 50% trifloxystrobin *w/v*), tebufenozide (Mimic: tebufenozide 24% *w/v*), chlorpyrifos (Dursban: chlorpyrifos 48% *w/v*), and lambda-cyhalothrin (Karate Zeon: lambda-cyhalothrin 10% *w/v*), which are commonly used in viticulture in Spain and frequently detected in grape treatments [29]. In addition, these pesticides were chosen based on their prevalence in grape protection programs, persistence, and regulatory relevance [7].

To better understand the behaviour of each pesticide during the vinification process, their key physicochemical properties are summarised in Table 1. This includes water solubility, the octanol–water partition coefficient (log P), molecular weight, and  $K_{oc}$ .

**Table 1.** Physicochemical properties of the selected pesticides used in the study.

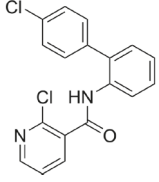
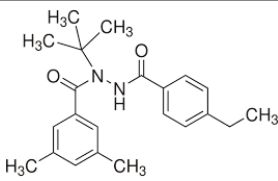
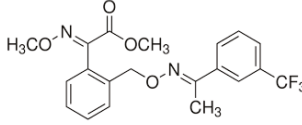
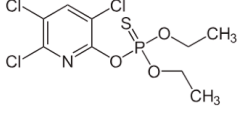
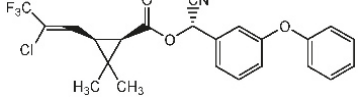
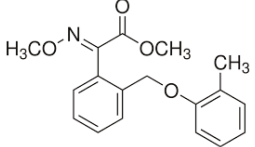
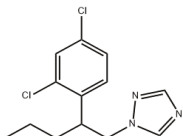
| Pesticide | Structure                                                                           | Water Solubility<br>(mg/L at 25 °C) | Log P | Molecular Weight<br>(g/mol) | $K_{oc}$ (mL/g) |
|-----------|-------------------------------------------------------------------------------------|-------------------------------------|-------|-----------------------------|-----------------|
| Boscalid  |  | 4.6                                 | 2.96  | 343.2                       | 643             |

Table 1. Cont.

| Pesticide          | Structure                                                                           | Water Solubility<br>(mg/L at 25 °C) | Log P | Molecular Weight<br>(g/mol) | K <sub>oc</sub> (mL/g) |
|--------------------|-------------------------------------------------------------------------------------|-------------------------------------|-------|-----------------------------|------------------------|
| Tebufenozide       |    | 0.413                               | 4.27  | 352.5                       | 716                    |
| Trifloxystrobin    |    | 0.61                                | 5.40  | 408.9                       | 5150                   |
| Chlorpyrifos       |    | 1.4                                 | 4.70  | 350.6                       | 6070                   |
| Lambda-cyhalothrin |    | 0.005                               | 7.00  | 449.9                       | 180,000                |
| Kresox-im-methyl   |   | 2.0                                 | 3.40  | 313.4                       | 1260                   |
| Penconazole        |  | 150                                 | 3.72  | 284.8                       | 959                    |

Note. Water solubility, log P, molecular weight, and K<sub>oc</sub> values were obtained from the Pesticide Properties Database (PPDB) [30] and manufacturer safety data sheets.

## 2.2. Field Trials

The experimental vineyard was located in the Region of Murcia (Spain). This area is characterised by an arid climate and an extensive viticultural tradition. The study was carried out on *Vitis vinifera* L. Monastrell grapevines, a variety widely cultivated in south-eastern Spain. The vineyard was divided into eight experimental plots (10 × 10 m each), with seven treated plots and one untreated control plot. The control plot was located at a sufficient distance to avoid contamination and to ensure comparable soil and climatic conditions.

Applications were made according to each pesticide's pre-harvest interval (PHI) at 7, 10, 14, 15 and 21 days before harvest [29]. Each pesticide formulation was applied according to the manufacturer's recommendations and independently to ensure that its respective PHI was met on the day of sampling. Treatments were applied in a range of relative humidities between 70% and 80% and an ambient temperature ranging between 24 and 28 °C using a Maruyama MS073D backpack sprayer (Auburn, WA, USA) equipped with a 2 mm nozzle. No rainfall was recorded on the days when the treatment was applied.

Each pesticide treatment was applied in seven separate plots and pesticide residues were analysed individually per treatment. The pesticide residue values reported in this study represent the mean concentration of all samples analysed from the treated plots.

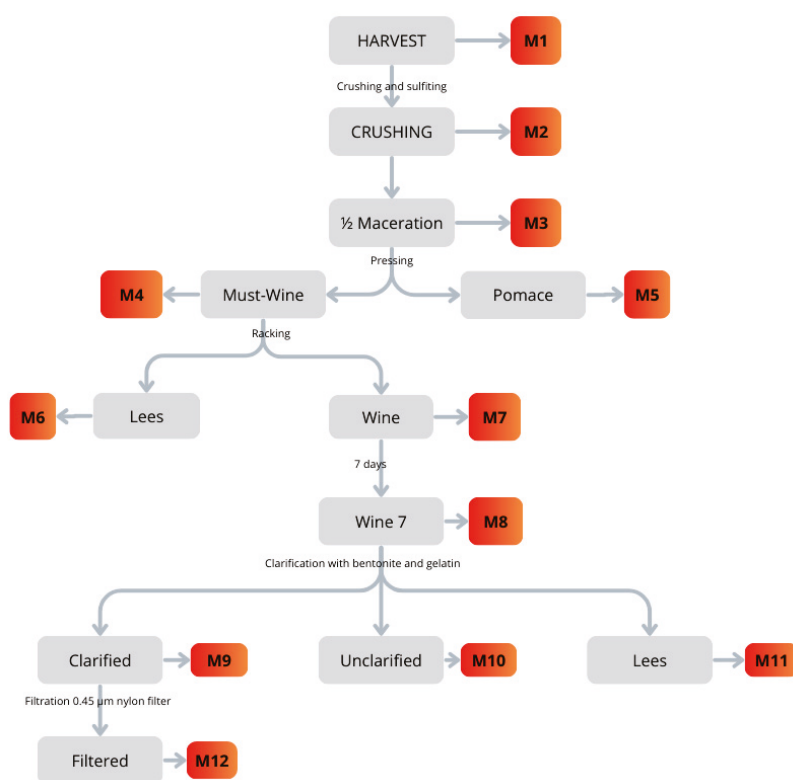
Grape samples were collected at the specific PHI of each pesticide to ensure that residue levels reflected real commercial conditions. For each treatment, 15 kg of grapes were randomly harvested from the treated plots and packed in opaque polyethylene bags

to prevent degradation by light. Samples were kept at room temperature during transport to the laboratory to ensure minimal physical damage.

On arrival, the grapes were homogenised with dry ice under controlled conditions to obtain a representative analytical sample. All samples were immediately frozen at  $-20\text{ }^{\circ}\text{C}$  until analysis. To ensure data reliability, pesticide residues were quantified within two weeks of freezing, with all extractions and analyses performed on the same day to minimise degradation.

### 2.3. Processing Factors

The vinification process was carried out at a controlled laboratory scale, simulating the industrial conditions used in commercial wineries. Figure 1 illustrates the flowchart of the vinification process, showing the main stages of vinification and the monitoring points for the reduction or concentration of pesticide residues at each stage, from harvest to final filtration.



**Figure 1.** Vinification Process Flowchart.

The process began with the grape harvest (M1), where Monastrell (*Vitis vinifera*) grapes were selected and harvested under controlled conditions. Pesticide residue concentrations were quantified in the harvested grapes to establish baseline levels.

Following harvest, the grapes were crushed (M2), and the resulting must was analysed for pesticide residues. The first processing factor (F1) was determined by comparing the residue concentrations in the crushed must (M2) to those in the harvested grapes (M1). This stage is crucial as it begins the mechanical breakdown of the grape structure, which influences pesticide dispersion.

The next step was maceration (M3), where the must remained in contact with the skins to extract phenolic compounds and colour. Pesticide residues were measured again, and the second processing factor (F2) was calculated by comparing the residue levels in the must after maceration (M3) with those in the crushed must (M2). This stage improves

extraction and the effect of maceration on pesticide residue levels was studied by following changes in residue concentrations.

After maceration, the must was fermented (M4) in stainless steel tanks at 24–28 °C for 10–14 days. Commercial *Saccharomyces cerevisiae* yeast strains were used to convert sugars to ethanol, and during fermentation microbial metabolism and enzymatic hydrolysis contributed to the degradation of pesticide residues. Samples were taken at various intervals during fermentation and the third processing factor (F3) was calculated by comparing the pesticide levels in the fermented wine (M4) with those in the must before fermentation (M3). This step is critical in assessing how fermentation affects pesticide degradation.

After fermentation, the wine was separated from the solid residues [racking (M5)]. At this point, the wine was decanted, and the remaining solids (pomace) were pressed to extract additional liquid. The fourth processing factor (F4) was calculated by comparing the pesticide residue concentrations in the wine after racking (M5) with those before racking (M4), highlighting the role of solid particle removal in pesticide dissipation.

After racking, the wine underwent clarification (M6) where clarifying agents, such as bentonite and gelatine, were added to promote precipitation of suspended solids and further reduce pesticide residue concentrations. The fifth processing factor (F5) was calculated by comparing the residue concentrations in the clarified wine (M6) with those in the wine before clarification (M5).

After clarification, the wine was filtered (M7) through a 0.45 µm membrane to remove any remaining particles. The sixth processing factor (F6) was calculated by comparing the pesticide concentrations in the filtered wine (M7) with those in the clarified wine (M6). Filtration is an important step in ensuring that residual pesticide molecules are removed from the wine before bottling.

After filtration, the wine underwent cold stabilisation (M8), where it was chilled at 0–4 °C to prevent tartaric acid precipitation for seven days. Although the main purpose of this step is to improve the stability of the wine, it may also affect the degradation of pesticide residues. The seventh processing factor (F7) was calculated by comparing the pesticide concentrations in the cold-stabilised wine (M8) with those in the filtered wine (M7).

After cold stabilisation, the wine was sent to clarification to obtain clarified (M9) and filtered (M12) wine. The wine from M8 was also sent for clarification to obtain unclarified wine (M10), where the lees were also obtained (M11). Residue concentrations were measured at these stages and the eighth, ninth and tenth processing factors (F8, F9 and F10, respectively) were calculated to assess the total residue reduction from harvest (M1) to final bottling (M9 and M10). The global processing factor ( $F_{\text{global}}$ ) was also calculated (M12/M1). These stages contribute to any final adjustments in pesticide residue levels through physical interactions with the wine matrix.

#### 2.4. Extraction and Analysis of Pesticide Residues

The extraction of pesticide residues was carried out using the QuEChERS multi-residue method [31,32]. Briefly, a 10 mL sample of wine was placed into a 50 mL polypropylene centrifuge tube, followed by the addition of 10 mL of acetonitrile. The tube was sealed and manually shaken vigorously for one minute while placed in an ice bath. A buffered salt mixture (4 g of anhydrous magnesium sulphate, 0.5 g of disodium citrate sesquihydrate, and 1 g of trisodium citrate dihydrate) was added. The tube was hand-shaken vigorously for another minute and centrifuged at 3500 rpm for 5 min. The resulting extract was acidified (pH ≈ 4.3) with 5% formic acid and directly injected into the chromatographic system. In the analytical sequence, duplicate samples were included at the beginning and

end as quality control measures to ensure sample stability, and they met the established acceptance and rejection criteria.

Pesticide residues were analysed using an Agilent Infinity Liquid Chromatograph, model 1260, coupled to a triple quadrupole mass spectrometer (Agilent Triple Quad LC/MS 6410B) operating in dynamic MRM scan mode (Agilent Technologies, Palo Alto, CA, USA). Chromatographic separation was performed on an Agilent Poroshell 120 EC-C18 column (3 mm × 100 mm, 2.7 µm) thermostated at 40 °C, with a flow rate of 0.6 mL/min. The injection volume consisted of 5 µL of sample plus 95 µL of mobile phase. The mobile phase consisted of acetonitrile with 0.1% of formic acid (phase A) and water with 0.1% of formic acid and 2 mM ammonium formate (phase B). The elution gradient started at 20% phase A, increasing linearly to 100% A in 10 min, holding for 6 min, before returning to the initial composition within 1 min. The total analysis time was 14 min, with an additional 5 min for stabilisation [31,32].

Quantification and identification of all pesticides analysed were performed using multiple reaction monitoring with an electrospray ionisation source in positive mode. The nebuliser gas was synthetic air at 40 psi, with a flow rate of 9 L/min. Ionisation voltage was set at 5500 V, and solvent evaporation was carried out with synthetic air at 350 °C.

## 2.5. Validation of the Analytical Method

The analytical method was validated following the guidelines outlined in the European Union document SANTE/11312/2021 v.2 on analytical quality control and validation procedures for pesticide residue analysis in food and feed [33]. The validation included an assessment of potential interferences in the quantification of the target compounds, evaluation of detector response linearity in matrix extracts, calculation of Relative Standard Deviation (RSD) for response factors, back-calculated concentration and correlation coefficients ( $R^2$ ), as well as recovery at two concentration levels of the limit of quantification (LOQ) (LOQ and 10LOQ) under repeatability and reproducibility conditions. Additionally, a series of quality control checks were implemented in each analysis sequence to ensure method robustness.

Each analytical sequence included the following: calibration curve, solvent blank, matrix extract, duplicate test samples, fortified samples at the lowest concentration level, and control standards. Detector response linearity was determined in triplicate using blank matrix extracts (must and wine) fortified with the selected pesticides at five concentrations (1, 10, 50, 100 and 500 µg/kg). To identify each pesticide, the limit of detection (LOD) was defined as the lowest concentration yielding a signal-to-noise (S/N) ratio greater than 3, while the limit of quantification (LOQ) was the lowest concentration with an S/N ratio greater than 10 and acceptable accuracy and precision.

Repeatability and reproducibility of the method were evaluated using six consecutively analysed fortified samples at LOQ and 10LOQ levels for all pesticides. The acceptance criterion was that the RSD should be  $\leq 20\%$ . Reproducibility was assessed by processing samples on six different days under identical conditions. Accuracy was determined by recovery experiments, where fortified samples at LOQ and 10LOQ levels were analysed, and results were compared with known standards analysed within the same sequence. The acceptance criterion for accuracy required mean recovery rates between 70% and 120%, with an RSD  $\leq 20\%$ .

## 2.6. Dietary Risk Assessment

The dietary risk assessment was conducted to evaluate the potential health risks associated with the consumption of wine containing pesticide residues. The Estimated Daily Intake (EDI) of pesticide residues was calculated following the methodology proposed



by EFSA [3,23] and considering national wine consumption patterns in Spain. The Risk Quotient (RQ) was determined as the ratio between the estimated intake of each pesticide and its respective Acceptable Daily Intake (ADI), as defined by international regulatory agencies such as EFSA and FAO/WHO [28]. The equations used for risk assessment were Equations (1) and (2) as follows:

$$\text{EDI (mg/kg bw)} = (\text{C} \times \text{R})/\text{kg bw} \quad (1)$$

$$\text{RQ} = \text{EDI}/\text{ADI} \quad (2)$$

where C represents the estimated national consumption of wine, R is the concentration of pesticide residues in wine (mg/L), and kg bw represents the mean adult body weight in Spain, considered as 60 kg for standard calculations [28]. ADI indicates the maximum amount of a pesticide that can be ingested daily over a lifetime without appreciable health risk [3].

$\text{RQ} \leq 1$  indicates an acceptable risk to human health. Whereas  $\text{RQ} > 1$  indicates that the risk of a pesticide to humans is unacceptable, and higher RQ values indicate higher risks [34,35].

### 2.7. Statistical Analysis

Descriptive statistical parameters, including mean, standard deviation and coefficient of variation, were calculated for all pesticide residues measured at different vinification stages. Data processing and statistical analyses were performed using IBM SPSS Statistics (version 24.0).

To evaluate the correlation of the variables used in the pesticides studied, as well as the relationship with the ADI, the software was Rstudio IDE Desktop 2023.06.01 and R 4.4.2 was used [36]. Different R libraries were used depending on the analysis performed: for example, data import into R was performed using the Readxl library, and graph analysis was performed using ggplot2, rstatix, patchwork, dplyr and tidyverse.

## 3. Results and Discussion

### 3.1. Validation

The analytical method for pesticide residue detection was validated following the SANTE guidelines [33]. Both LOD and LOQ were determined for each pesticide, together with recovery rates and repeatability. The method showed an excellent performance with LOD values in the range of 0.3 µg/kg to 0.8 µg/kg and LOQ values in the range of 1.0 µg/kg to 2.1 µg/kg. Recovery rates for all pesticides ranged from 89% to 108%, and the repeatability was between 1.5% and 6.8%, meeting with the criteria set by the SANTE guidelines [33]. Further details of the validation parameters are shown in Table 2.

**Table 2.** Validation data for pesticide analysis in wine samples (LOD, LOQ, recovery, and RSD).

| Pesticide          | LOD (µg/kg) | LOQ (µg/kg) | Recovery (%) | RSD (%) (Repeatability) | RSD (%) (Reproducibility) |
|--------------------|-------------|-------------|--------------|-------------------------|---------------------------|
| Boscalid           | 0.5         | 1.5         | 89–102       | 1.5                     | 3.1                       |
| Kresoxim-methyl    | 0.4         | 1.2         | 87–103       | 2.1                     | 3.6                       |
| Penconazole        | 0.3         | 1.0         | 90–108       | 2.3                     | 4.0                       |
| Trifloxystrobin    | 0.6         | 1.8         | 90–104       | 2.8                     | 5.3                       |
| Tebufenozide       | 0.5         | 1.5         | 88–106       | 3.2                     | 5.7                       |
| Chlorpyrifos       | 0.8         | 2.0         | 92–108       | 3.9                     | 6.8                       |
| Lambda-cyhalothrin | 0.7         | 2.1         | 91–106       | 2.0                     | 5.5                       |

### 3.2. Pesticide Residues Behaviour

Pesticide residues were quantified at each stage of the winemaking process, from the harvested grapes (M1) to the final wine products (M12), paying particular attention to those with the highest and lowest dissipation rates.

Table 3 shows the residue concentrations of each pesticide studied (boscalid, penconazole, tebufenozide, kresoxim-methyl, trifloxystrobin, chlorpyrifos and lambda-cyhalothrin) at each vinification stage (M1 to M12).

**Table 3.** Residual pesticide values ( $\mu\text{g/L}$ ) at different vinification stages of vinification (M1–M12).

| Sample      | Boscalid | Penconazole | Tebufenozide | Kresoxim-methyl | Trifloxystrobin | Chlorpyrifos | Lambda-cyhalothrin |
|-------------|----------|-------------|--------------|-----------------|-----------------|--------------|--------------------|
| M1          | 43.68    | <10         | 182.76       | <10             | <10             | 25.66        | 22.45              |
| M2          | 24.30    | <10         | 90.62        | <10             | <10             | 11.02        | 13.47              |
| M3          | 29.12    | <10         | 95.22        | <10             | <10             | 21.46        | 17.06              |
| M4          | 20.25    | <10         | 58.18        | <10             | <10             | 29.08        | 12.29              |
| M5          | 108.02   | <10         | 354.91       | <10             | <10             | 65.85        | 42.65              |
| M6          | 37.26    | <10         | 98.21        | <10             | 17.45           | 26.39        | 21.61              |
| M7          | <10      | <10         | 24.23        | <10             | 22.79           | 10.00        | <10                |
| M8          | <10      | <10         | 22.79        | <10             | <10             | <10          | <10                |
| M9          | <10      | <10         | 21.58        | <10             | <10             | <10          | <10                |
| M10         | <10      | <10         | 22.47        | <10             | <10             | <10          | <10                |
| M11         | 22.20    | <10         | 47.67        | <10             | <10             | <10          | <10                |
| M12         | <10      | <10         | 21.71        | <10             | <10             | <10          | <10                |
| % Reduction | 100      | -           | 88.1         | -               | -               | 100          | 100                |

Penconazole and kresoxim-methyl were not detected at any vinification stage. Trifloxystrobin was not detected at the beginning of the vinification process; however, it was detected in M6 (17.45  $\mu\text{g/L}$ ) and M7 (22.79  $\mu\text{g/L}$ ) vinification stages, likely due to the fact that these are the stages of pressing and concentration, and that the coefficient of variation and the recovery have an uncertainty of about 40%. Chlorpyrifos and lambda-cyhalothrin were detected at the beginning of the vinification stage; however, they were eliminated throughout all vinification stages, showing a 100% elimination rate.

Boscalid showed the greatest reduction during the vinification process, with a concentration below the LOQ at the last stage of vinification. The initial concentration in the harvested grapes (M1) was 43.68  $\mu\text{g/kg}$ , which decreased to <10  $\mu\text{g/kg}$  by the final filtration stage (M12), showing a reduction of 100%. In contrast, tebufenozide showed the smaller reduction. Its initial concentration in the harvested grapes (M1) was 182.76  $\mu\text{g/kg}$  and, although it decreased during vinification, the final concentration at M12 was 21.71  $\mu\text{g/kg}$ , reflecting the lower dissipation (88.1%). In this sense, it can be said that the stages that showed the higher elimination rates were M5, M6 and M11.

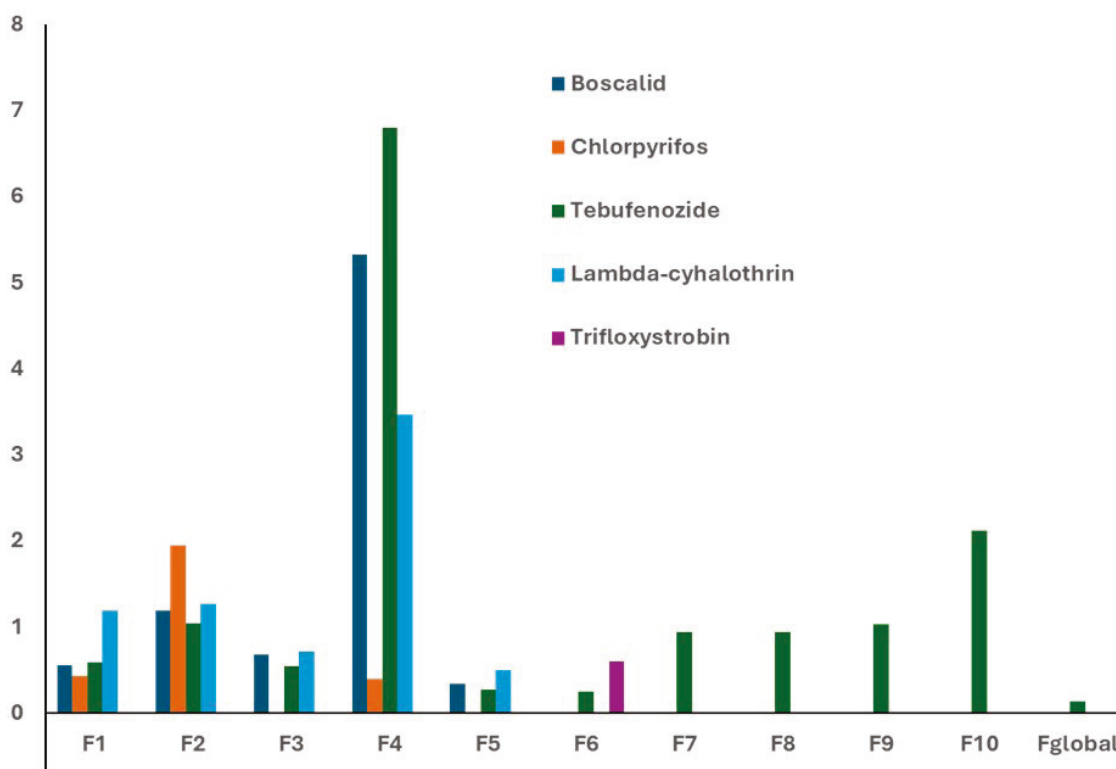
Similar results have been found in other studies on wine and other foods, both from our research group and from others. These results depend on the greater or lesser elimination of the physicochemical characteristics of the pesticide studied [12,13,16,37].

Figure 2 shows the corresponding processing factors ( $F_1$ – $F_{10}$  and  $F_{\text{global}}$ ) which help to quantify removal efficiency at each stage of vinification, providing a comprehensive overview of how each step contributes to pesticide removal. As can be seen in Figure 2, reductions in pesticide residues were observed during stages involving the removal of solids, such as crushing, maceration and racking, particularly  $F_4$ ,  $F_5$  and  $F_{\text{global}}$ . These stages are particularly effective because they remove byproducts such as pomace, lees and marc, which absorb significant amounts of pesticide residues.

These results indicate that there is no toxicological risk in the final wine product, as nearly all the pesticide residues found in the grapes were removed during the alcoholic fermentation and clarification processes. However, the wine byproducts—such as po-

mace, lees, and marc—that can be used in other industries, may present a risk due to the accumulation of pesticides in these materials.

Microbial activity during fermentation also contributed to the dissipation of pesticide residues. This finding is consistent with the work of Lozowicka et al. [38], which emphasised the role of microbial metabolism in pesticide breakdown during the fermentation process. Furthermore, the EFSA report highlighted filtration as a highly effective method for removing pesticide residues during the final stages of winemaking [3].



**Figure 2.** Variation of processing factors during grape vinification process.

It can be concluded that the vinification process effectively reduced pesticide residues at all stages, with key steps such as crushing, maceration, fermentation and filtration playing a crucial role in the removal of pesticide residues from the wine.

### 3.3. Risk Assessment

Risk evaluation assessment for human health aims to estimate the nature and likelihood of adverse effects from pesticide exposure through wine consumption. The chronic risk relating to ingestion of the pesticides studied (boscalid, penconazole, tebufenozide, kresoxim-methyl, chlorpyrifos and lambda-cyhalothrin) was assessed by comparing pesticide residue levels in the wine with the EDI (calculated based on the average daily wine consumption, according to national dietary guidelines [33]), and the ADI (sourced from the EU Pesticides Database [39] and EFSA [3], with the lowest ADI values considered in cases of joint toxicological actions) for each pesticide.

Table 4 shows EDI, ADI, and RQ values calculated for wine under GAP conditions. These values reflect the potential health risk from wine consumption based on average exposure.

As can be observed, on the one hand, the mean RQ values were all below 5% of the ADI, except for chlorpyrifos in wine, which reached 7.5% of the ADI showing a RQ of 0.0070. However, in all instances, the RQ values remained well below the safety threshold

of 1, indicating a very low potential risk for human health. For other fruits using the same pesticides, similar results have been reported elsewhere [37].

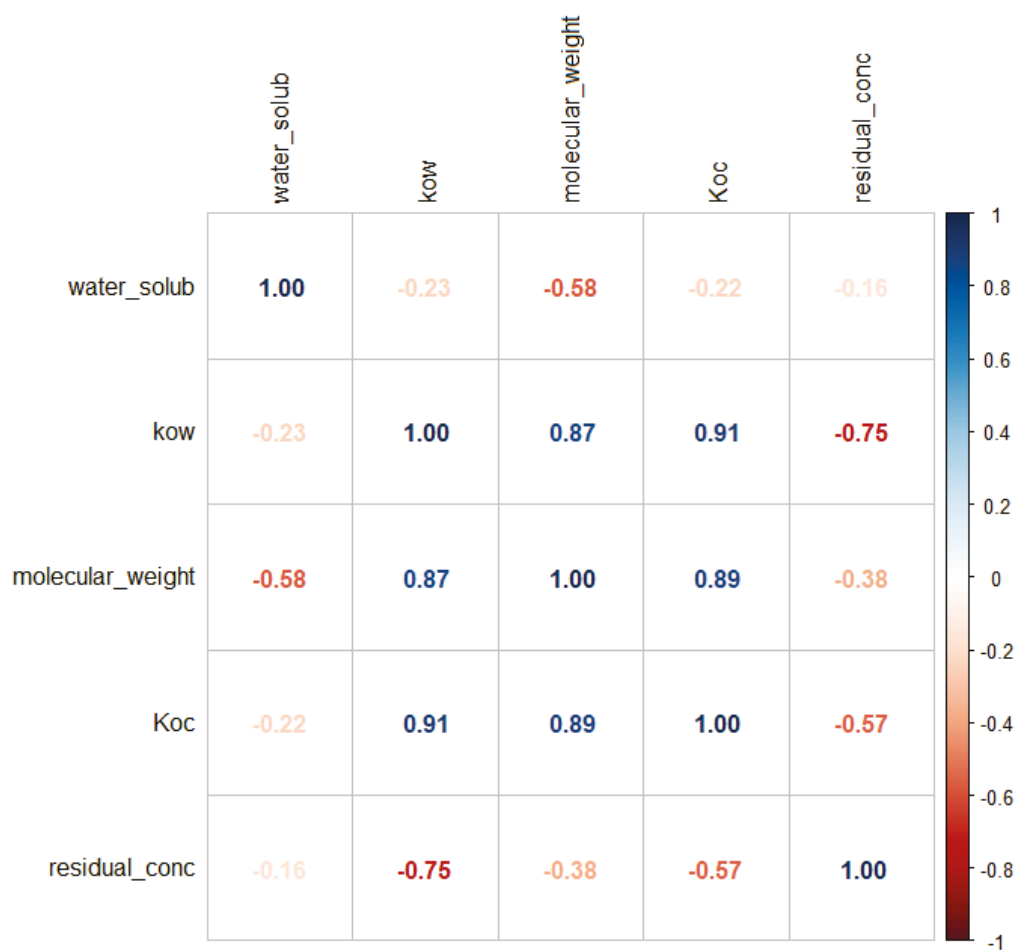
On the other hand, wine byproducts, such as pomace, lees, and marc, may present a higher risk due to pesticide accumulation, suggesting that they could pose a greater risk if used in applications like animal feed.

**Table 4.** Estimated Daily Intake (EDI), Acceptable Daily Intake (ADI), and Risk Quotient (RQ) for pesticide residues in wine under GAP conditions.

| Pesticide          | Residual Concentration (µg/L) | EDI (µg/kg bw/day) | ADI (µg/kg bw/day) | RQ     |
|--------------------|-------------------------------|--------------------|--------------------|--------|
| Boscalid           | 8.3                           | 0.027              | 160                | 0.0002 |
| Tebufoenozide      | 21.71                         | 0.072              | 30                 | 0.0024 |
| Chlorpyrifos       | 2.1                           | 0.007              | 1                  | 0.0070 |
| Lambda-cyhalothrin | 1.2                           | 0.004              | 5                  | 0.0008 |
| Kresoxim-methyl    | 6.7                           | 0.0220             | 100                | 0.0002 |
| Penconazole        | 4.3                           | 0.010              | 50                 | 0.0003 |
| Trifloxystrobin    | 7.5                           | 0.0250             | 100                | 0.0002 |

Note. Wine intake: 0.2 L/day. EDI: Estimated Daily Intake = (residue × consumption)/body weight (assumed 60 kg). ADI: Acceptable Daily Intake. RQ: Risk Quotient = EDI/ADI. Residual concentrations represent average values in wine under GAP conditions. A Risk Quotient (RQ) < 1 indicates negligible dietary risk.

Further, a correlation analysis of the variables used for the pesticides studied (water solubility,  $k_{ow}$ , molecular weight,  $k_{oc}$ , residual concentration), and the correlation matrix is shown in Figure 3.



**Figure 3.** Correlation matrix of the variables used for the pesticides studied.

As expected, there is a significant correlation of residual concentration with  $k_{ow}$  and  $k_{oc}$ , and to a lesser extent with molecular weight and insignificantly with water solubility, as reported in previous studies [40,41].

Applying a multiple regression model using all variables (including those from the health risk analysis), the following model was obtained with a significant  $p$ -value of 0.00306 ( $<0.05$ ) and an adjusted  $R^2$  value of 1:

$$EDI = 4.92 \times 10^{-4} - 1.71 \times 10^{-6} \times \text{water solubility} - 8.78 \times 10^{-5} \times K_{ow} - 1.40 \times 10^{-9} \times K_{oc} + 2.81 \times 10^{-3} \times \text{residual concentration}$$

As can be observed, this model is totally independent of the molecular weight variable and the variable that most influenced the model is the residual concentration, according to Equation (1), and as reported elsewhere [42].

## 4. Conclusions

This study confirms that the winemaking process plays a significant role in the degradation of pesticide residues, mainly through physicochemical transformations, microbial metabolism and adsorption to solid matrices. The effectiveness of each winemaking step varies according to the chemical nature of the pesticide, with fermentation, clarification, racking and filtration contributing to residue reduction to different extents.

The results show that fermentation contributes to pesticide degradation via microbial enzymatic activity and adsorption to yeast biomass. However, the efficiency of this process depends on the hydrophobicity of the pesticide, as hydrophilic compounds tend to persist in the aqueous phase. Clarification and racking further reduce pesticide residues by removing suspended solids that retain pesticides, with the efficiency of fining agents, such as bentonite and gelatine, varying depending on the molecular structure of the pesticides involved. Filtration completes the removal process, particularly for pesticides with a high adsorption affinity for colloidal organic matter, ensuring that residual contaminants are minimised.

The  $F_{total}$  calculations confirm that vinification generally results in net pesticide dissipation, with most residues having  $F$  values below 1, indicating that reduction mechanisms outweigh potential concentration effects.

Risk assessment-based EDI and ADI confirms that pesticide concentrations in the final wine remain well below the safety limits set by regulatory authorities. These results reinforce the role of winemaking as a natural mitigation process that enhances consumer safety.

Despite the reductions observed, certain pesticide residues show limited degradation, particularly those with high water solubility and chemical stability, highlighting the need for additional mitigation strategies. Optimisation of microbial metabolism and yeast strain selection could further improve pesticide degradation during fermentation. In addition, evaluation of the interaction of fining agents with pesticide residues could improve clarification efficiency, while further development of filtration techniques, such as nanofiltration or adsorption-based methods, could provide more effective residue removal. Furthermore, extending studies to assess the effect of maceration time, fermentation temperature and storage conditions on pesticide stability would provide a more comprehensive understanding of dissipation patterns in different oenological scenarios.

These findings provide a scientific basis for improving winemaking processes to minimise pesticide residues and ensure compliance with increasingly stringent food safety regulations, while maintaining wine quality. The integration of optimised processing techniques will be crucial in reducing chemical contaminants in wine production and increasing consumer confidence in the safety of the final product.

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M.Á.C. and J.O.; Investigation, S.C., M.Á.C. and J.O.; Methodology, S.C., M.Á.C. and J.O.; Project administration, M.Á.C. and J.O.; Resources, M.Á.C. and J.O.; Software, S.C., J.M.V.-d.-B., M.Á.C., P.A.-M. and J.O.; Supervision, M.Á.C. and J.O.; Validation, S.C., J.M.V.-d.-B., M.Á.C., P.A.-M. and J.O.; Visualization, S.C., J.M.V.-d.-B., M.Á.C., P.A.-M. and J.O.; Writing—original draft, S.C., J.M.V.-d.-B., M.Á.C., P.A.-M. and J.O.; Writing—review & editing, S.C., J.M.V.-d.-B., M.Á.C., P.A.-M. and J.O. All authors have read and agreed to the published version of the manuscript.

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## Article

# Ultrasound-Assisted Kinetics of Alcoholic Fermentation at Varying Power Levels for the Production of Isaño Wine (*Tropaeolum tuberosum*)

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**Abstract:** Ultrasound is considered a physical technology that can be applied at various stages of food processing to optimise resources and reduce production time. In this study, the influence of ultrasonic treatments at different power levels (0 W, 100 W, 200 W, and 300 W) on three Isaño genotypes (yellow, yellow with purple eyes, and purple) during alcoholic fermentation was investigated. The main parameters assessed were yeast growth kinetics, ethanol production, and substrate consumption in an ultrasonic bath operating at a frequency of 50 kHz. The findings demonstrated enhanced yeast growth, accelerated substrate consumption, and increased ethanol production compared to the control (untreated sample). However, the impact of ultrasound on fermentation decreased as the power level increased. Notably, an ultrasonic power of 100 W applied over 84 h of fermentation resulted in the highest ethanol yield (10.36% *v/v*) in the purple Isaño genotype. In conclusion, ultrasonic treatment is a promising approach to improve the fermentation process of Isaño, potentially enabling its development as a functional beverage with both nutritional and therapeutic properties.

**Keywords:** alcoholic fermentation; Isaño; kinetics; *Saccharomyces cerevisiae*; ultrasound

## 1. Introduction

The mashua or Isaño (*Tropaeolum tuberosum*) is a tuberous plant belonging to the family Tropaeolaceae [1]. This annual herbaceous plant is currently cultivated in the high Andean regions of Peru, Bolivia, Ecuador, Venezuela, Colombia, and Argentina [2,3]. There are several genotypes, the most common being yellow, purple, and yellow with purple eyes. Consumed as a staple food by Andean populations, mashua (Isaño) is also recognised for its medicinal properties [4]. Traditionally, it has been used in the treatment of venereal diseases, skin and respiratory disorders, urinary and prostate disorders, and for its hepatoprotective

and nephroprotective effects. Additionally, it exhibits anticancer activity and functions as a diuretic agent [5–7]. These benefits are attributed to its rich composition of vitamins, amino acids, minerals, fibres, polyphenols, flavonoids, anthocyanins, antioxidants, fatty acids, glucosinolates, and alkamides [7–12]. Despite its potential, the industrial use of Isaño remains limited [13]. However, recent studies have investigated its use in food products, including bakery products [14], sausages [15], and beverages [16]. Previous research suggests that a promising approach to using Isaño is its transformation into a fermented alcoholic beverage known as “Isaño wine”, due to its high starch content ( $41.35 \pm 1.60\%$  dry basis) [17].

Traditionally, wine is produced from grapes [18]. However, wines have also been made from other fruits and tubers, including pineapple, nanche, pomegranate, papaya, and oca [19,20]. Driven by this diversity, our research group aims to explore the potential of an Andean crop for the production of a fermented beverage with consumer benefits. The fermentation of Isaño presents several challenges, particularly the conversion of starch into fermentable sugars and yeast inhibition due to the presence of glucosinolates and phenolic compounds. To overcome these obstacles, a physical, chemical, or enzymatic pretreatment, or a combination of these, must be applied to hydrolyse the starch and release simple sugars, such as glucose and maltose, which can be efficiently utilised by yeasts in winemaking [21]. Traditional wine fermentation is a process that typically requires long maturation times and significant storage space [22]. To optimise this process and reduce production time, several innovative technologies have been applied, including pulsed electric fields, gamma radiation, high pressure, and ultrasound [23]. While different approaches have been investigated, their practical application depends on various factors, such as equipment availability, process efficiency, and impact on microbial activity [24,25].

Among these techniques, ultrasound has emerged as a particularly promising approach in the food industry, as it not only accelerates fermentation but also improves the technological and functional properties of food products [26,27]. This technology is based on the cavitation effect, which consists of the implosion of air bubbles generated by ultrasonic waves at frequencies above the human hearing threshold ( $>16$  kHz). This phenomenon facilitates the physical or chemical modification of food properties. Several studies have reported on the use of ultrasound in fermentation processes, highlighting that its effect depends on the growth phase of the yeast, as well as the frequency and power of the ultrasound applied. It has been reported that the application of ultrasound in the fermentation of cider with *Hansenia* sp., at a frequency of 40 kHz and a power of 100 W, accelerated the growth of the yeast, reaching values up to 10 times higher than those of the control group [28]. Similarly, in beer fermentation with *Saccharomyces cerevisiae*, ultrasonic treatment at 40 kHz and 120 W increased the glucose fermentation rate by 2.3 and 2.5 times at temperatures of 30 °C and 20 °C, respectively [29]. Other studies reported that the use of 40 kHz and 160 W improved ethanol production by 13.18% [30], while the use of 23 kHz and 120 W increased it by 19.33% [31]. On the other hand, treatment with low-frequency ultrasound in the range of 20–30 kHz and with power levels of 23.4–26.7 W reduced fermentation time by 6–9 h compared to controls [32]. Taken together, these results demonstrate that the use of ultrasound in fermentation reduces process time and improves product quality characteristics [26]. Furthermore, it is regarded as a green, sustainable, innovative, cost-effective, rapid, portable, and easy-to-use technology, and its non-destructive nature makes it highly suitable for fermentation processes [33]. However, its effectiveness depends on the type and frequency of ultrasound, the type of antinutrient, the duration of exposure, and the composition of the food. Therefore, the aim of this experimental study was to investigate the effect of ultrasound power and Isaño genotypes on fermentation kinetics parameters.



## 2. Materials and Methods

### 2.1. Plant Material

Isaño genotypes (yellow, yellow with purple eyes, and purple) were cultivated in the agroecological zone of Yunguyo, Puno, Peru, at an altitude of 3880 m a.s.l., with latitude coordinates 15°38'30" S and longitude 69°49'50" W. Samples of each genotype were collected using a random sampling method and transported to the laboratory for further processing. Prior to processing, they were washed with tap water to remove any surface contaminants, then dried at room temperature and stored in a dark place at  $25 \pm 2$  °C with a relative humidity of  $68.1 \pm 1.5\%$ .

### 2.2. Winemaking

The production of Isaño wine followed the procedure described by Nnaemeka et al. [34], with some modifications. Initially, the tubers were selected, with any damaged ones removed, washed with tap water, and disinfected with a sodium hypochlorite solution at 200 ppm. Subsequently, 100 g of Isaño tubers were cooked at 85 °C for 30 min. After cooking, the tubers were mixed with 900 g of water and crushed in a blender (Oster brand, model BLST4655-053, Mexico City, Mexico) for 5 min. The resulting mixture (must) was transferred to a 1 L glass bioreactor (PHIWE, Göttingen, Germany) equipped with seven connections and a heating coil for hot water circulation to maintain a constant temperature, and immersed in an ultrasonic bath fitted with a multi-frequency ultrasound generator (QX Ultrasonic, Beijing, China). The samples were then treated with  $\alpha$ -amylase enzyme (LD Carlson Co., Kent, OH, USA) at a concentration of 0.21 g/L, with the pH adjusted to 5.5, and incubated at 37 °C for 60 min. Following this treatment, the samples were cooled, and the pH was adjusted to 3.25 using a citric acid solution to deactivate the  $\alpha$ -amylase enzyme. Subsequently, the enzyme amyloglucosidase (Brewhaus brand, Fort Worth, TX, USA) was added at a concentration of 0.01 g/L, and the mixture was incubated at 60 °C for 6 h. After the enzymatic treatment, the soluble solids were adjusted to 16 °Brix, and the pH was set to 4.5. The hydrolysed must was inoculated with 0.20 g/L of *Saccharomyces cerevisiae* EC-1118 (LALVIN® brand, Lallemand Inc., Montreal, QC, Canada), which had been previously activated by dissolving it in the must at 30 °C with continuous stirring for 30 min. The bioreactor was connected to a container filled with water through a tube, allowing the release of CO<sub>2</sub> while retaining water vapour and volatile alcohols. Additionally, 5 mL needles and syringes were inserted into one of the reactor's channels to collect samples every 12 h without opening the bioreactor. Fermentation was carried out at 25 °C for 84 h at an ultrasonic frequency of 50 kHz, with variable powers of 100, 200, and 300 W, and an energy density of 6 W/cm<sup>2</sup>. A mixture without ultrasonic treatment was included as a control. The collected samples were centrifuged and analysed to determine ethanol concentration, biomass, and reducing sugars. All experiments were performed in triplicate, and the results are presented as the mean  $\pm$  standard deviation.

### 2.3. Analytical Determinations

#### 2.3.1. Determination of Biomass

Microbiological analysis was carried out following the methodology described by Zhang et al. [31], with some modifications. Serial dilutions were prepared by mixing 1 mL of the Isaño wort sample with 9 mL of sterile distilled water to obtain the 10<sup>-1</sup> dilution. From this, 1 mL was taken and mixed with an additional 9 mL of distilled water to prepare the 10<sup>-2</sup> dilution, and so on. The diluted samples were properly homogenised before being plated onto Petri dishes containing OGYE Agar, a selective medium for yeasts and moulds. The plates were incubated at 20–25 °C for 48 h. Yeast colonies were counted using a colony counter.

### 2.3.2. Quantification of Ethanol Concentration

As described by Flores-Mendoza et al. [35], an oxidising solution was prepared by dissolving 0.85 g of potassium dichromate in 15 mL of distilled water. Then, 25 mL of concentrated sulphuric acid was measured, and the potassium dichromate solution was gradually added while maintaining the solution in a cold-water bath. The final volume was adjusted to 100 mL. Ethanol solutions were prepared at known concentrations, as shown in Table 1. A stock solution was made by dissolving 7 mL of 99.9% pure ethanol in 100 mL of distilled water. This stock solution was then diluted to the desired concentrations (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mL in 50 mL of water), resulting in the final ethanol concentrations.

**Table 1.** Quantities and concentrations for the preparation of standard solutions.

| Amount of Ethanol | Concentration (g/L) |
|-------------------|---------------------|
| 0 mL/50 mL        | 0                   |
| 0.5 mL/50 mL      | 0.5518              |
| 1.0 mL/50 mL      | 1.1034              |
| 1.5 mL/50 mL      | 1.6552              |
| 2.0 mL/50 mL      | 2.2069              |
| 2.5 mL/50 mL      | 2.7587              |
| 3.0 mL/50 mL      | 3.3104              |

Then, 2 mL of each standard solution was added and reacted with 4 mL of potassium dichromate solution at 75 °C for 30 min, followed by cooling with cold water to stop the reaction. The absorbance was measured using a spectrophotometer (Genesys 20, Thermo Scientific, Mississauga, ON, Canada) at 587 nm. The same procedure was applied to the sample, where the standard ethanol solution was substituted with the sample.

### 2.3.3. Determination of Reducing Sugars

Reducing sugars were analysed according to the procedure described by Burgos [36]. The tests were performed by weighing 1 g of 3,5-dinitrosalicylic acid (DNS), 1.6 g of NaOH, and 43.8 g of Na-K tartrate. NaOH was dissolved in 50 mL of distilled water, followed by the addition of Na-K tartrate and DNS, while protecting the reagent from light with aluminum foil. Next, the solution was made up to 100 mL with distilled water in a volumetric flask and left to stir overnight in an amber flask. The glucose standard solution was prepared with the following concentrations: 0, 0.5, 0.7, 0.9, 1.1, 1.3, 1.5, 1.7, and 2.0 g/L. To each screw-cap tube, 0.25 mL of each solution and 0.25 mL of DNS reagent were added, and the tubes were covered with aluminum foil to protect the reaction from light. The tubes were placed in a thermostatic bath at 92 °C for 5 min. The reaction was then stopped by cooling the tubes in ice for 5 min. Afterwards, 2.5 mL of distilled water was added to each tube, followed by shaking. The absorbance was measured at 540 nm using a spectrophotometer. For the fermentation samples, centrifugation was performed at 1700 g for 10 min, and the same procedure was followed, replacing the standard glucose solution with the sample.

### 2.3.4. Determination of °Brix

The °Brix value was determined using a tabletop Milwaukee MA871-BOX digital refractometer (Milwaukee Instruments, Inc., Rocky Mount, NC, USA). One or two drops of the must were placed on the prism, and the reading was taken immediately [37].

### 2.3.5. Determination of Kinetic Parameters

The experimental data were fitted using the Monod model (Equation (1)):

$$\mu = \mu_{\max} \frac{S}{(K_s + S)} \quad (1)$$

where  $\mu$  represents the specific growth rate ( $\text{h}^{-1}$ ),  $\mu_{\max}$  is the maximum growth rate ( $\text{h}^{-1}$ ),  $K_s$  is the saturation constant ( $\text{g/L}$ ), and  $S$  is the limiting substrate concentration ( $\text{g/L}$ ).

To determine the coefficients of the Monod equation, the rate of change in substrate concentration ( $ds/dt$ ) was first calculated. Subsequently, the specific growth rate ( $\mu$ ) as a function of time was determined. The slope of the straight line obtained by linearising the Monod equation (by plotting  $1/S$  vs.  $1/\mu$ ) was calculated using Microsoft Excel. The maximum cell growth rate and the saturation constant during fermentation were estimated based on the °Brix data.

### 2.3.6. Fermentation Process Performance

The biomass yield  $Y_{X/S}$  ( $\text{g biomass} \cdot \text{g substrate}^{-1}$ ) and product yield  $Y_{P/S}$  ( $\text{g ethanol} \cdot \text{g substrate}^{-1}$ ) were calculated according to Equations (2) and (3), respectively:

$$Y_{X/S} \left( g_{\text{biomass}} * g_{\text{substrate}}^{-1} \right) = \frac{X_f - X_0}{S_0 - S_f} \quad (2)$$

$$Y_{P/S} \left( g_{\text{ethanol}} * g_{\text{substrate}}^{-1} \right) = \frac{P_f - P_0}{S_0 - S_f} \quad (3)$$

where  $X_0$  and  $X_f$  are the initial and final biomass concentrations,  $P_0$  and  $P_f$  are the initial and final ethanol concentrations, and  $S_0$  and  $S_f$  are the initial and final substrate concentrations, respectively.

### 2.4. Experimental Design

The data were statistically analysed using analysis of variance (ANOVA) under a Completely Randomised Design. Table 2 presents the factors and levels used in the process. The means were compared using Tukey's test at a significance level of 0.05, with the statistical analysis performed using Infostat software (version 2020, Universidad Nacional de Córdoba, Argentina).

**Table 2.** Experimental design for Isaño fermentation.

| Experiment N° | Isaño Genotype | Power (W) |
|---------------|----------------|-----------|
| 1             | Yellow         | 0         |
| 2             | Yellow         | 100       |
| 3             | Yellow         | 200       |
| 4             | Yellow         | 300       |
| 5             | Yellow-purple  | 0         |
| 6             | Yellow-purple  | 100       |
| 7             | Yellow-purple  | 200       |
| 8             | Yellow-purple  | 300       |
| 9             | Purple         | 0         |
| 10            | Purple         | 100       |
| 11            | Purple         | 200       |
| 12            | Purple         | 300       |

### 3. Results and Discussion

#### 3.1. Behaviour of the Biomass Concentration of *Saccharomyces Cerevisiae* During Ultrasound-Assisted Fermentation in Three Isaño Genotypes

Figure 1 illustrates the growth kinetics of *Saccharomyces cerevisiae* during ultrasound-assisted fermentation of yellow, yellow with purple eyes, and purple Isaño must over a fermentation period of 0 to 84 h. Rapid yeast growth was observed between 2 and 60 h of fermentation, followed by a deceleration phase between 60 and 84 h in the ultrasound-treated samples. In contrast, the control sample (0 W) did not exhibit this deceleration by the end of the 84 h period.

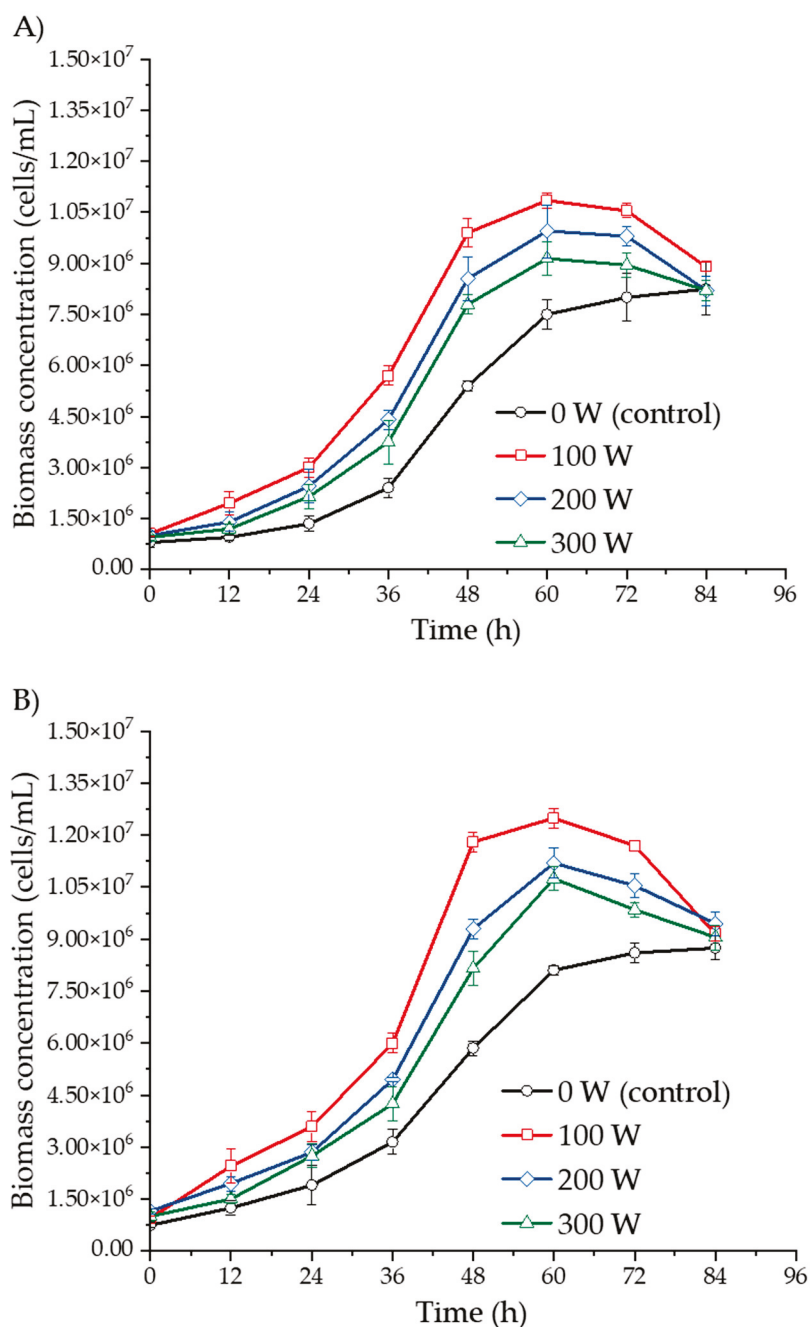
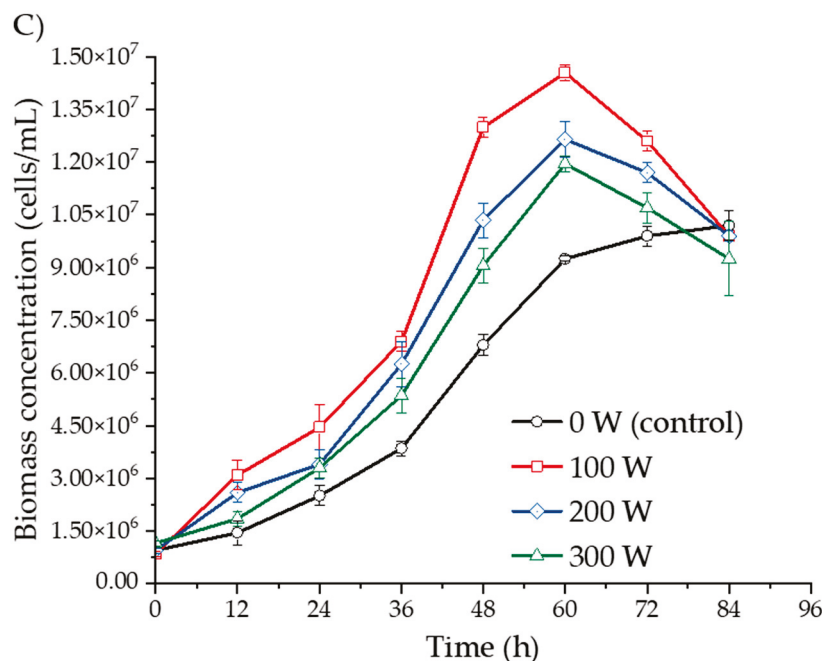


Figure 1. Cont.



**Figure 1.** Biomass concentration kinetics of *S. cerevisiae* in three Isaño genotypes: (A) yellow, (B) yellow with purple eyes, and (C) purple.

In Figure 1A, biomass concentrations in ultrasound-treated yellow Isaño must were significantly higher than those in the control samples. Similar trends were observed in yellow Isaño must with purple eyes (Figure 1B) and the purple Isaño must (Figure 1C). Across all samples, Isaño must provided a favourable environment for yeast growth during fermentation, attributed to its high starch content of  $41.35 \pm 1.60\%$  on a dry basis [17]. The increase in biomass can primarily be explained by glucose consumption derived from starch, as yeasts metabolise sugars to produce ethanol and other metabolites [38]. Moreover, yeast growth is influenced by additional factors such as sugar concentration, fermentation temperature, inoculum density, nitrogen and oxygen availability, the presence of inhibitory or stimulating substances from yeast or bacterial growth, fungicide residues, and the addition of sulphur dioxide [39]. In the purple Isaño must, ultrasound at 100 W demonstrated the most efficient promotion of *S. cerevisiae* growth, followed by 200 W, 300 W, and the control (0 W) at 60 h of fermentation. The higher biomass observed in purple Isaño must may be attributed to its superior nutritional and functional properties, including a high antioxidant content [40]. Additionally, ultrasound has been shown to enhance the extraction of phenolic compounds, which could further contribute to biomass growth [41]. These findings are consistent with prior studies that highlight the positive effects of ultrasound on fermentation. For instance, Al Daccache et al. [28] reported that ultrasound accelerated growth kinetics by 52% during the logarithmic phase, with a 10-fold increase in the biomass growth rate compared to the untreated sample. Similarly, Klomklieng and Prateepasen [42] reported that ultrasound enhanced ethanol production and reduced fermentation time by 6 to 9 h compared to the control. The observed increase in biomass concentration could be attributed to the stimulation of cell division induced by low-power ultrasound [39]. However, Cao et al. [43] cautioned that high-power ultrasound may cause cellular damage and even cell death. Huang et al. [44] suggested that ultrasound promotes microbial growth by disaggregating cell clusters and increasing membrane permeability, thereby enhancing cellular functions and component activity. This increased permeability facilitates the exchange of intracellular and extracellular materials, accelerating the material and energy metabolism of *S. cerevisiae* [26,31]. Taken together, these results highlight the beneficial



effects of ultrasound, particularly at moderate power levels, on the growth and metabolism of *S. cerevisiae* during fermentation, ultimately reducing fermentation time.

### 3.2. Ethanol Production Behaviour in Ultrasound-Assisted Fermentation of Three Isaño Genotypes

Figure 2 shows the effect of ultrasound-assisted fermentation at different power levels on the ethanol content in the must of three Isaño genotypes. In Figure 2A, it is evident that ethanol production in the yellow Isaño must treated with ultrasound increased significantly compared to the control sample (0 W). A similar pattern was observed in the yellow-purple Isaño fermentation must (Figure 2B) and the purple Isaño must (Figure 2C). In the purple Isaño must, the ethanol concentration reached  $10.36 \pm 0.33\%$  (v/v) at 84 h with an ultrasonic power of 100 W. At power levels of 200 W, 300 W, and in the control (0 W), the ethanol content in the purple Isaño must was  $8.70 \pm 0.41\%$ ,  $8.18 \pm 0.07\%$ , and  $7.07 \pm 0.38\%$  (v/v), respectively. In comparison, the yellow-purple and yellow Isaño musts reached  $9.27 \pm 0.58\%$  (v/v) and  $8.51 \pm 0.60\%$  (v/v), respectively. These results are consistent with previous studies. Klomklieng and Prateepasen [42] demonstrated that low-power ultrasound improves ethanol production and reduces fermentation time by 6 to 9 h compared to the control. Similarly, Choi et al. [30] reported that the use of indirect ultrasound (ultrasonic bath) at a power of 160 W and a frequency of 40 kHz in barley-based beer fermentation enhanced ethanol production and shortened fermentation time. Pulidindi et al. [29] also found that ultrasound at 120 W and 40 kHz improved ethanol production. Finally, Al Daccache et al. [28] highlighted that low-intensity ultrasound significantly increases biomass growth, glucose consumption, and ethanol production during apple juice fermentation. Ultrasound treatment increases ethanol production in *S. cerevisiae* by promoting yeast growth through enhanced membrane permeability and enzymatic activities [45]. Ultrasound intensity affects cellular ultrastructure. Previous studies have shown that, in samples without ultrasonic treatment, cells remain intact and maintain a uniform morphology, with the cytoplasm and organelles homogeneously distributed, along with a clearly visible nuclear region. However, when ultrasonic treatment is applied at 102.8 W for 10 min, the cellular structure becomes irregular, with no visible organelles in the cytoplasm, the presence of vacuoles, and signs of plasmolysis in some cells. At a higher intensity of 288 W over the same duration, significant damage is observed, with evidence of plasmolysis [46].

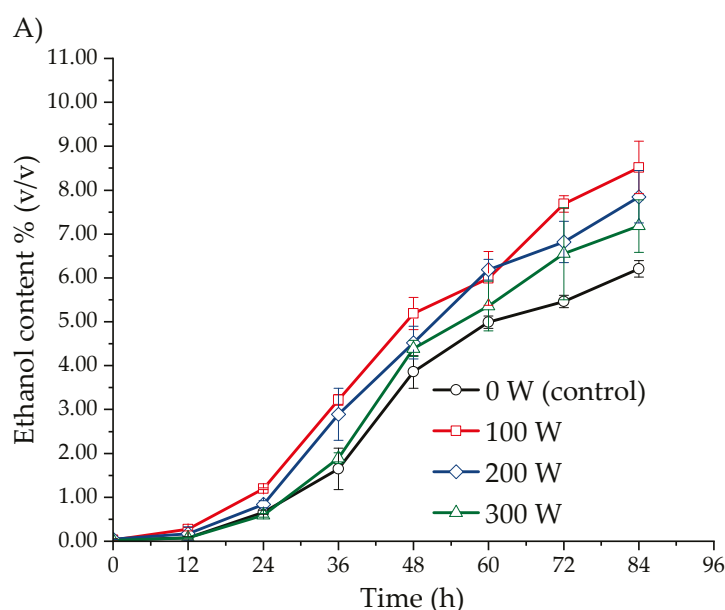
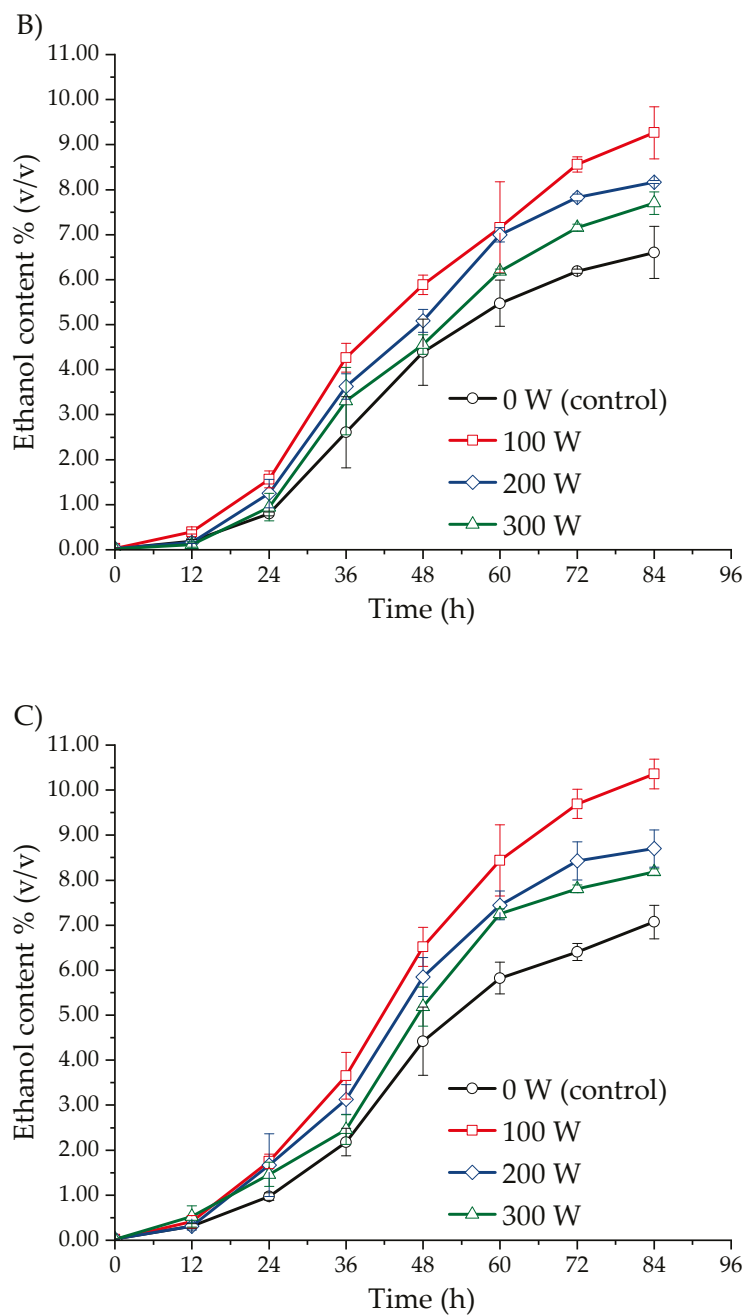


Figure 2. Cont.



**Figure 2.** Ethanol production kinetics in three Isaño genotypes: (A) yellow, (B) yellow with purple eyes, and (C) purple.

The higher ethanol production observed in the purple Isaño must, compared to the yellow and yellow-purple genotypes, can be attributed to its unique nutritional and functional properties. Purple Isaño is a rich source of antioxidants, such as flavonoids and polyphenols, with an antioxidant capacity eight to ten times greater than that of yellow Isaño [40,47]. These characteristics provide a favourable environment for the growth of *Saccharomyces cerevisiae* during fermentation [38]. Consequently, the results demonstrate that ultrasonic treatments during fermentation not only increase ethanol production but also optimise the process by reducing the time required to reach significant concentrations of this metabolite.

### 3.3. Behaviour of Reducing Sugars in the Ultrasound-Assisted Fermentation Process Evaluated in Three Isaño Genotypes

Figure 3 shows the effect of ultrasound-assisted fermentation, applied at different power levels, on the reducing sugar content in the fermentation must of three Isaño genotypes over a period of 0 to 84 h. The samples subjected to ultrasonic treatment exhibited a greater reduction in reducing sugar content compared to the non-sonicated samples over the same time period. The reducing sugar content progressively decreased as fermentation time increased, with the most significant reduction rate observed at an ultrasonic power of 100 W, followed by 200 W, 300 W, and finally 0 W. This trend was consistent across all three Isaño genotypes. In the case of purple Isaño, the reducing sugar content reached  $2.62 \pm 0.03$  g/L at 84 h. These findings correlate with the increase in biomass concentration and ethanol content observed during fermentation (Figures 2 and 3). Furthermore, the effects observed with ultrasound treatments align with previous studies, which have reported that ultrasound improves fermentation efficiency by reducing sugar content more rapidly, increasing ethanol production speed, and shortening the overall fermentation time [29]. One potential explanation for these observations is that ultrasound may facilitate the transfer of oxygen in the liquid medium, thereby promoting faster sugar consumption after its application [31]. The must provides a favourable environment for microbial growth, enabling yeast to metabolise the available sugars during fermentation. As the process progresses, the reduction in available sugars, coupled with the increase in ethanol concentration, eventually limits yeast population growth and halts fermentation [38]. In conclusion, the application of ultrasound was beneficial for the growth and fermentation activity of *Saccharomyces cerevisiae*. Among the power levels tested, the 100 W treatment proved to be the most effective, outperforming the 200 W and 300 W treatments.

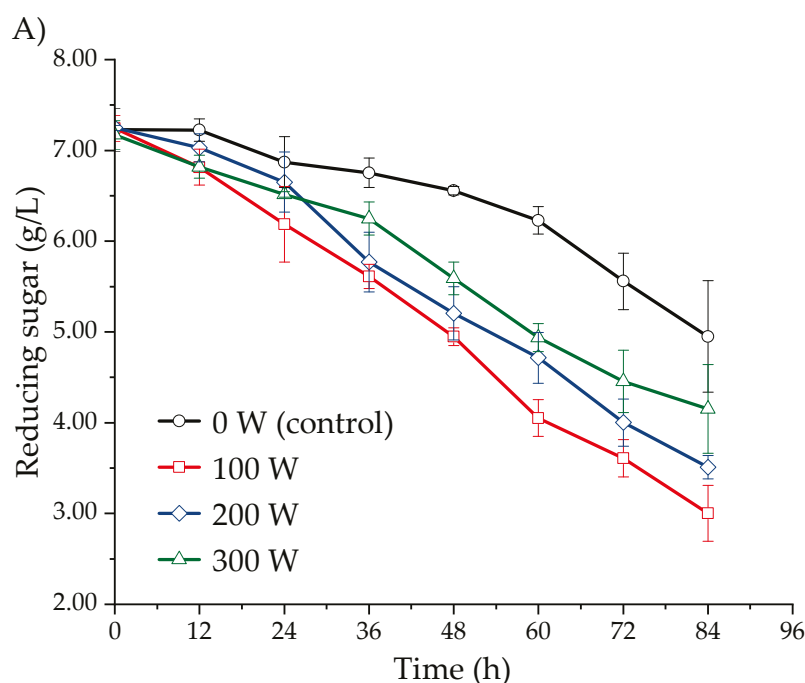
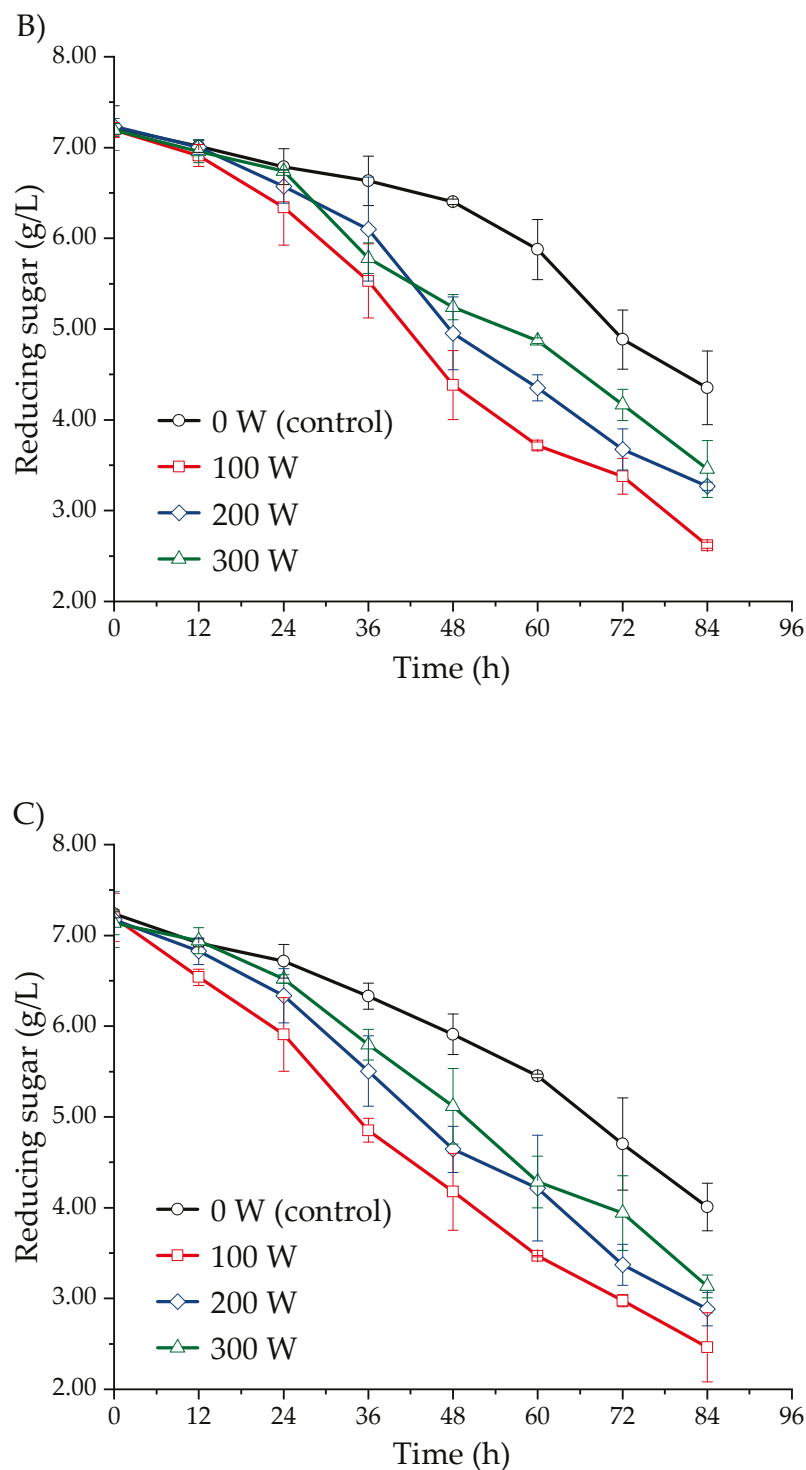


Figure 3. Cont.



**Figure 3.** Kinetics of reducing sugars, evaluated in three Isaño genotypes: (A) yellow, (B) yellow with purple eyes, and (C) purple.

### 3.4. Behaviour of Soluble Solids in the Fermentation Process

Figure 4 presents the effect of ultrasound, applied at different power levels, on the soluble solids content (°Brix) in the fermentation must of *S. cerevisiae* from three Isaño genotypes, observed over a period of 0 to 84 h. The ultrasonicated samples exhibited a greater decrease in soluble solids content compared to the non-sonicated samples. The soluble solids content decreased as fermentation time progressed, with the most rapid reduction occurring at an ultrasonic power of 100 W, followed by 200 W, 300 W, and finally the control (0 W). This trend was consistent across all three Isaño genotypes evaluated. In

the case of purple Isaño, the °Brix decreased from 16.0 to  $4.3 \pm 0.3$  at 84 h of fermentation. The decrease in °Brix is associated with the consumption of sugars by *S. cerevisiae* during the fermentation process, which continues until the sugars are exhausted and microbial activity ceases [38,48]. However, high-power ultrasound may have an adverse effect on cell growth, which could explain the slower rate of reduction in °Brix observed at higher power levels [30]. Based on the results obtained, it can be concluded that ultrasound treatment at 100 W significantly enhances the fermentation process, with purple Isaño showing the best fermentation performance.

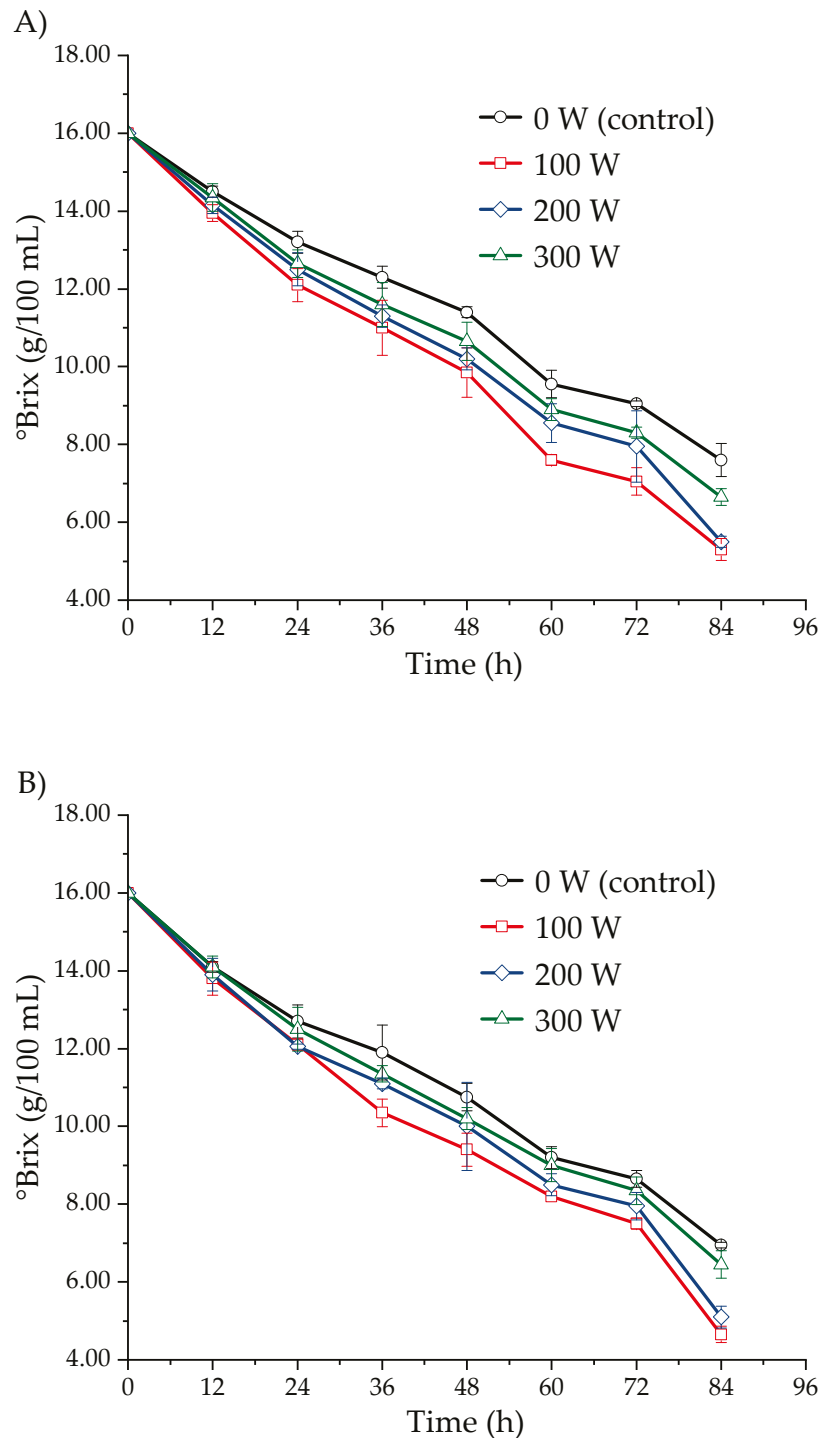
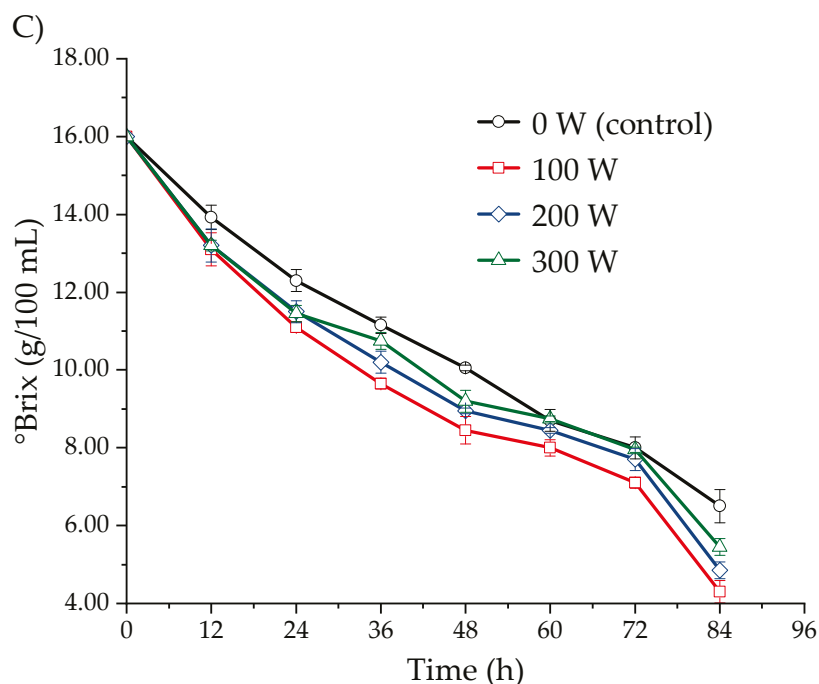


Figure 4. Cont.





**Figure 4.** Kinetics of the decrease in soluble solids (°Brix) during fermentation at 25 °C, evaluated in three Isaño genotypes: (A) yellow, (B) yellow with purple eyes, and (C) purple.

### 3.5. Application of the Monod Kinetic Model Based on the °Brix Parameter in Ultrasound-Assisted Fermentation of Yeast

Table 3 presents the kinetic parameters of maximum cell growth rate ( $\mu_{\max}$ ) and saturation constant ( $K_s$ ) of the Monod model, along with the biomass yield ( $Y_{x/s}$ ) and product yield ( $Y_{p/s}$ ) for the ultrasound-assisted fermentation process. These parameters were evaluated in three Isaño genotypes (yellow, yellow with purple eye, and purple) under different ultrasonic power levels (0 W, 100 W, 200 W, and 300 W). In the sample without ultrasonic treatment (control), the  $\mu_{\max}$  values were  $0.007 \pm 0.004 \text{ h}^{-1}$ ,  $0.017 \pm 0.001 \text{ h}^{-1}$ , and  $0.028 \pm 0.007 \text{ h}^{-1}$ , while the  $K_s$  values were  $13.829 \pm 11.115 \text{ g/L}$ ,  $30.773 \pm 13.535 \text{ g/L}$ , and  $27.801 \pm 9.927 \text{ g/L}$  for the yellow, yellow-purple, and purple fermentation musts, respectively. Comparatively, these  $\mu_{\max}$  values are higher than those reported by Llacsá and Cucho [49] for quinoa chicha fermentation ( $0.004 \text{ h}^{-1}$ ) and similar to those obtained by Pari [50] for banana peel fermentation ( $0.0278 \text{ h}^{-1}$ ).

When applying ultrasonic treatment,  $\mu_{\max}$  showed significant variations depending on the power level and genotype. For yellow Isaño,  $\mu_{\max}$  increased from  $0.006 \pm 0.003 \text{ h}^{-1}$  to  $0.029 \pm 0.013 \text{ h}^{-1}$  as the power decreased from 300 W to 100 W. In the case of yellow Isaño with purple eyes,  $\mu_{\max}$  initially decreased, followed by an increase from  $0.167 \pm 0.190 \text{ h}^{-1}$  to  $0.211 \pm 0.257 \text{ h}^{-1}$ . For purple Isaño, the growth was more consistent, with  $\mu_{\max}$  increasing from  $0.015 \pm 0.001 \text{ h}^{-1}$  to  $0.064 \pm 0.059 \text{ h}^{-1}$  as the power increased from 100 W to 300 W. Specifically, it was observed that the depletion of soluble solids occurred more rapidly at 100 W in the purple Isaño must, with  $\mu_{\max}$  of  $0.015 \pm 0.001 \text{ h}^{-1}$  and  $K_s$  of  $16,088 \pm 20,360 \text{ g/L}$ . The  $Y_{p/s}$  was  $0.899 \pm 0.016$ , and  $Y_{x/s}$  ranged between  $7.60 \times 10^5 \pm 8.79 \times 10^4$  and  $9.84 \times 10^5 \pm 6.81 \times 10^3$ .

Overall, biomass yields per gram of glucose consumed were significantly higher in ultrasound-treated cultures (100, 200, and 300 W) compared to the control (0 W). These results are consistent with those reported by Al Daccache et al. [28], who found that the maximum growth rate in ultrasound-assisted samples was up to 10 times higher than in untreated samples. Similarly, studies on yeast have shown that ultrasound treatment increases ethanol production and biomass yield. For instance, He et al. [45] observed a

30.79% increase in ethanol production and a 24.10% increase in *Saccharomyces cerevisiae* biomass at 28 kHz ultrasonic frequency, which they attributed to improved cell membrane permeability and increased enzymatic activity. Another study by Yang et al. [51] reported that ultrasonic treatment at 28 kHz, 180 W/L for 24 h increased ethanol yield by 34.87%, which was associated with changes in gene expression and cellular metabolism. In conclusion, ultrasonic treatments improve cell membrane permeability by generating micropores that facilitate substrate transfer and stimulate cell growth. These effects are reflected in increased biomass and ethanol yields in the three Isaño genotypes studied.

**Table 3.** Kinetic parameters of the Monod model and the yield of the fermentation process.

| Power of Ultrasound              | $\mu_{\max}$ (h <sup>-1</sup> ) | K <sub>s</sub> (g/L)         | Y <sub>x/s</sub>                                              | Y <sub>p/s</sub>           |
|----------------------------------|---------------------------------|------------------------------|---------------------------------------------------------------|----------------------------|
| Yellow genotype                  |                                 |                              |                                                               |                            |
| 0 W (Control)                    | 0.007 ± 0.004 <sup>b</sup>      | 13.829 ± 11.115 <sup>a</sup> | 8.91 × 10 <sup>5</sup> ± 3.85 × 10 <sup>4</sup> <sup>a</sup>  | 0.741 ± 0.012 <sup>a</sup> |
| 100 W                            | 0.029 ± 0.013 <sup>a</sup>      | 43.130 ± 43.698 <sup>a</sup> | 7.37 × 10 <sup>5</sup> ± 8.04 × 10 <sup>3</sup> <sup>bc</sup> | 0.797 ± 0.041 <sup>a</sup> |
| 200 W                            | 0.007 ± 0.004 <sup>b</sup>      | 10.928 ± 13.538 <sup>a</sup> | 6.92 × 10 <sup>5</sup> ± 2.72 × 10 <sup>4</sup> <sup>c</sup>  | 0.751 ± 0.057 <sup>a</sup> |
| 300 W                            | 0.006 ± 0.003 <sup>b</sup>      | 10.036 ± 14.116 <sup>a</sup> | 7.71 × 10 <sup>5</sup> ± 1.10 × 10 <sup>4</sup> <sup>b</sup>  | 0.762 ± 0.051 <sup>a</sup> |
| Yellow genotype with purple eyes |                                 |                              |                                                               |                            |
| 0 W (Control)                    | 0.017 ± 0.001 <sup>a</sup>      | 30.773 ± 13.535 <sup>a</sup> | 8.94 × 10 <sup>5</sup> ± 2.45 × 10 <sup>4</sup> <sup>a</sup>  | 0.736 ± 0.056 <sup>a</sup> |
| 100 W                            | 0.211 ± 0.257 <sup>a</sup>      | 279.88 ± 365.26 <sup>a</sup> | 7.16 × 10 <sup>5</sup> ± 7.93 × 10 <sup>3</sup> <sup>d</sup>  | 0.807 ± 0.044 <sup>a</sup> |
| 200 W                            | 0.110 ± 0.128 <sup>a</sup>      | 157.38 ± 150.15 <sup>a</sup> | 7.68 × 10 <sup>5</sup> ± 1.61 × 10 <sup>4</sup> <sup>c</sup>  | 0.754 ± 0.008 <sup>a</sup> |
| 300 W                            | 0.167 ± 0.190 <sup>a</sup>      | 240.41 ± 288.28 <sup>a</sup> | 8.43 × 10 <sup>5</sup> ± 1.60 × 10 <sup>4</sup> <sup>b</sup>  | 0.805 ± 0.019 <sup>a</sup> |
| Purple genotype                  |                                 |                              |                                                               |                            |
| 0 W (Control)                    | 0.028 ± 0.007 <sup>a</sup>      | 27.801 ± 9.927 <sup>a</sup>  | 9.84 × 10 <sup>5</sup> ± 6.81 × 10 <sup>3</sup> <sup>a</sup>  | 0.749 ± 0.005 <sup>b</sup> |
| 100 W                            | 0.015 ± 0.001 <sup>a</sup>      | 16.088 ± 20.360 <sup>a</sup> | 7.87 × 10 <sup>5</sup> ± 3.53 × 10 <sup>3</sup> <sup>b</sup>  | 0.899 ± 0.016 <sup>a</sup> |
| 200 W                            | 0.038 ± 0.008 <sup>a</sup>      | 44.400 ± 29.197 <sup>a</sup> | 8.17 × 10 <sup>5</sup> ± 1.18 × 10 <sup>3</sup> <sup>b</sup>  | 0.793 ± 0.031 <sup>b</sup> |
| 300 W                            | 0.064 ± 0.059 <sup>a</sup>      | 74.833 ± 79.340 <sup>a</sup> | 7.60 × 10 <sup>5</sup> ± 8.79 × 10 <sup>4</sup> <sup>b</sup>  | 0.766 ± 0.001 <sup>b</sup> |

The results are presented as the SD of the means. Means with different superscripts (a, b, c, d) in the same column are significantly different,  $p < 0.05$ .

## 4. Conclusions

This study shows that ultrasonic treatment significantly improves the fermentation process of *Saccharomyces cerevisiae* in musts from yellow, yellow with purple eyes, and purple Isaño genotypes compared to untreated samples. When the power was increased from 100 W to 300 W, the rates of yeast growth, ethanol production, and substrate consumption improved considerably. Optimal fermentation efficiency was achieved with an ultrasonic power of 100 W, a frequency of 50 kHz, and a fermentation duration of 84 h, particularly in the purple Isaño must. Under these conditions, ethanol production reached 10.36% ( $v/v$ ), with a maximum microbial growth rate ( $\mu_{\max}$ ) of 0.015 h<sup>-1</sup> and a saturation constant (K<sub>s</sub>) of 16.088 g/L.

These results confirm the potential of ultrasound technology to optimise the production of Isaño wine, a crop well-adapted to the extreme conditions of the Peruvian Altiplano. This product could be positioned as a functional beverage with nutritional properties and potential health benefits.

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## Article

# Influence of Kefir Grain Concentration on the Nutritional, Microbiological, and Sensory Properties of Camel Milk Kefir and Characterization of Some Technological Properties

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**Abstract:** This study aimed to manufacture kefir from camel milk using an extensive production system with different amounts of kefir grains, as well as to highlight their nutritional, sensorial, and technological characteristics. During processing, the pasteurization of camel milk, the addition of three doses of kefir grains (2%, 5%, and 10%), and incubation for 18 h were carried out. The microbiological and nutritional properties of the camel milk, kefir grains, and resulting kefirs were assessed. The sensory evaluation and technological processes involved in the production of the selected kefir were then carried out. The results showed that the chemical composition of the camel milk was as follows: fat:  $41.7 \pm 3.18$  g/L; protein content:  $37.82 \pm 0.66$  g/L; ash:  $8.92 \pm 0.61$  g/L; dry matter:  $114.21 \pm 0.11$  g/L; and lactose:  $41.3 \pm 0.21$  g/L. Kefir grains were acidic and moist and contained low fat content ( $0.02 \pm 0.01$ ). The total aerobic flora in camel milk was  $FAMT 4.77 \times 10^4$  CFU/mL. The bacterial load of lactic acid bacteria in the camel kefir prepared with 10% kefir grains was  $5.1 \pm 0.6 \log_{10}$  CFU/mL, while the yeast and mold load was  $4.24 \pm 0.83 \log_{10}$  CFU/mL. The amount of kefir grains present had a significant effect ( $p < 0.05$ ) on pH, acidity, and viscosity and improved the protein content, resulting in higher nutritional quality. According to a sensory evaluation, the ranking test showed that the best camel kefir can be produced by the addition of 2% kefir grains. It was the most appreciated by 73% of the tasters based on its physicochemical, microbiological, and sensory characteristics. All obtained camel kefirs were able to fulfill the *Codex Alimentarius* requirements, ensuring their safety and quality, with overall improvements in taste, texture, and acceptability. A phenotypic and morphological study of lactic acid bacteria isolated from the selected kefir (CK 2%, 18H) showed that these bacteria are Gram+, citrate+, catalase−, shell−, and rod-shaped. All the strains isolated showed good lipolytic and proteolytic activity, with areas of proteolysis between 8 and 15 mm. These strains were also revealed to have antibacterial activity and good acidifying and texturizing effects.

**Keywords:** camel milk; kefir; physicochemical characteristics; technology; microbiological activity; sensory evaluation

## 1. Introduction

Camels are pseudoruminant mammals that are highly adapted to desert breeding systems [1]. In Tunisia, camel farming is conventionally extensive, a method perfectly

suited to the biology of the species, and is concentrated in the southern areas of the country. Camel milk is a vital part of the daily diet of desert dwellers [2]. In pastoral areas, the camel is able to graze on a great variety of plants, including halophyte grasses, bushes, and trees, leading to lower pressure on the floristic biodiversity of the arid lands [3]. Camels are able to provide highly nutritious milk via the exploitation of the limited grazing space in difficult regions, unlike other herbivorous ruminants [4]. Indeed, camel milk is famous for its benefits due to its nutritional quality and medicinal properties [5]. It could soon become a new superfood due to its high nutritional value, easy digestibility, and low fat content [6]. It is mainly consumed raw, pasteurized, or fermented to extend its shelf life. Kefir, a traditional fermented milk product believed to have come from the Caucasus region, is one of these fermented products. It is produced by the fermentation of milk from various animals (goats and cows). Its organoleptic parameters include an acidic taste and a yeasty aroma [7]. The beverage is produced by fermenting milk with kefir grains (KGs), which are a kind of white to yellow, gelatinous starter culture. The grains vary in size (varying from 0.3 to 3.5 cm in diameter) and are composed of a microbial symbiotic mixture of lactic acid bacteria ( $10^8$  CFU/g), yeast ( $10^6$ – $10^7$  CFU/g), and acetic acid bacteria ( $10^5$  CFU/g) that stick to a polysaccharide matrix [8]. After successive fermentations, kefir grains can break up to generate new grains [9]. Kefir is the product that remains after removing the kefir grains [7].

Codex Stan 243-2003 [10] recommends that milk kefir have at least 2.8% protein, below 10% fat, and at least 0.6% lactic acid. Even though this product has therapeutic value [11], the use of camel milk for the preparation of kefir remains limited. The processing of camel milk is commonly perceived as challenging, and the quality of the end products is often deemed inferior compared to their counterparts derived from cow milk [12,13]. In recent years, there has been an increasing interest in the commercial use of kefir, since it can be marketed as a natural, safe, and inexpensive food that is easily produced at home. In Tunisia, knowledge of this beverage, its benefits, and the kefir manufacturing process is not very widespread. This drink differs from other fermented dairy products because it is not the result of the metabolic activity of a single or a few microbial species [14]. The composition of the grains and the production process influence the properties of the kefir product. In fact, the characteristics of kefir depend on the ratio of kefir grains to milk [15]. There is a lack of detailed characterization of camel milk kefir produced with varying kefir grain concentrations, particularly regarding its physicochemical, microbiological, and sensory properties. This study aimed to determine some of the physicochemical, microbiological, and sensory characteristics of camel kefir using various grain amounts. Finally, the isolation of lactic acid bacteria from the selected kefir and the analysis of their technological properties will undoubtedly add value to the products.

## 2. Materials and Methods

### 2.1. Camel Milk and Kefir Grains (KGs)

The camel milk used in this study was a mixture from the total milking of 10 healthy, multiparous, and mid-lactating dairy camels (*Camelus dromedarius*) raised in an extensive Saharan breeding system. The samples were produced on a native camel farm in south-eastern Tunisia (Medenin; Halg Gemel, Gabes; Zarat, Tataouine; Kerchawa). Kefir grains (KGs) exhibit a notable physical resemblance to cauliflower, featuring a distinctive shape and measuring approximately 1–3 cm in length. They typically possess an off-white or cream color. The KGs used in this study were collected from private consumers and stored at 4 °C until use.

## 2.2. Manufacture of Camel Milk Kefir

In accordance with [16], 2%, 5%, and 10% kefir grains (*w/v*) were added to pasteurized camel milk (heated to 63 °C for 30 min) [17]. The mixture was then incubated for 18 h at 25 °C. After fermentation, the kefir grains were separated from the fermented milk through filtration and could be used to initiate further fermentation with milk. This experiment was repeated five times.

## 2.3. Physicochemical Characteristics (Milk, Kefir, and KGs)

The pH values of the various samples of raw milk, kefir, and kefir grains were determined using a pH meter (Jenway 3510 type pH meter, Cole-Parme, Vernon Hills, IL, USA). The titratable acidity was determined in triplicate according to [18].

The viscosity (cP) of milk and kefir was measured using a Brookfield-type viscosimeter (model DV-E, AMETEK Brookfield, Middleborough, MA, USA) with an accuracy of  $\pm 1\%$ . The rotational speed was set to 100 rpm. The dry matter (DM) of milk, kefir, and KGs, expressed in g/L, was determined by drying samples at 105 °C for 24 h until a constant weight was achieved using an oven (Binder, Bohemia, NY, USA). The ash content was determined by incinerating milk, kefir, and KGs for 4 h at 550 °C in an electric muffle furnace [18].

The fat content of milk and kefir was measured using the Gerber method with the Neusal method [19]. The fat content of KGs was determined using the Soxhlet method after boiling the samples with HCl to obtain bound lipid fractions [20].

The fat content was calculated according to the following formula:

$$\text{Fat (g/L)} = (m_2 - m_1) \times 100/m_0 \quad (1)$$

with  $m_0$  = mass of sample in grams;  $m_1$  = mass of empty extraction flask; and  $m_2$  = mass of extraction flask after drying.

The protein content of milk and kefir was measured using the Bradford method [21]. The lactose content of milk and kefir was determined by HPLC using a mixture of Carrez I (0.5 mol/L aqueous potassium ferrocyanide), Carrez II (0.5 mol/L aqueous zinc acetate), and acetonitrile [22].

## 2.4. Microbiological Analysis of Camel Milk and Kefir Grains (KGs)

The microbiological analysis used is in accordance with [18]. Yeast and mold (YM) loads were enumerated in Sabouraud Chloramphenicol medium (Pronadisa Micro and Molecular Biology) and incubated for 4 to 5 days at 25 °C. An aliquot (0.1 mL) of the dilution was evenly spread on the agar surface using a sterile spreader.

The lactic acid bacteria (LAB) were cultured in MRS (Man, ROGOSA, Sharpe Agar) [23] (37 °C for 24–48 h).

The bacterial CFU was determined by selecting colonies that grew from 25 to 250 colonies in a Petri dish as follows:

$$\text{Colony/gram (CFU/mL)} = \text{the number of colonies} \times (1/\text{dilution factor}) \quad (2)$$

## 2.5. Sensory Analysis

The sensory characteristics of the kefir sample were assessed using the hedonic test [24] with the participation of 100 untrained tasters (male and female, aged 21 to 55 years). Samples of camel kefir, coded with three random numbers and varying in inoculation doses, were randomly assigned in 30 mL portions. These were then presented in 50 mL glasses for evaluation. The tasters were given drinking water between every two samples. The considered attributes are the salinity, viscosity, odor, acidity, and color of the drinks,

scored using the nine-point hedonic scale [25]. The sensory score was coded from 1 to 9, with the following definitions: dislike extremely, dislike very much, dislike moderately, dislike slightly, neither like nor dislike, like slightly, like moderately, like very much, and like extremely. Finally, according to a ranking test, the tasters indicated the most acceptable camel kefir. The data from the sensory evaluation were subjected to One-Way ANOVA using SPSS (Version 20).

## 2.6. Isolation of Lactic Acid Bacteria (LAB) from Selected Kefir

Strain isolation from camel kefir (CK, 2%, 18H) was performed by preparing serial dilutions of the samples. A 0.1 mL sample of the dilution was spread on de Man, Rogosa, and Sharpe (MRS) agar. The LAB isolates were incubated (37 °C for 48 h). The isolates were identified by morphological characteristics on selective media, and the biochemical tests used were the Gram reaction, catalase test, citrate test, and Sherman Milk Culture test. Only Gram-positive bacteria with catalase-negative responses were found. Citrate utilization in the presence of carbohydrates was studied on Simmons citrate medium (Fluka Biochemica). The presence of blue coloration indicated a positive reaction. The growth of strains in NaCl (4, 6.5%) at different temperatures (10–45 °C) and at two different pH values (4.2, 9.6) was determined. During the test, representative colonies were stored in glycerol culture at −20 °C and kept to investigate their technological aspects and antibacterial activities. Cultures were also kept on an MRS agar slant at 4 °C and streaked every 4 weeks [26].

The test of Sherman Milk Culture indicates the ability of bacteria to grow in the presence of methylene blue, which is blue in a highly oxidizing medium and colorless in a reduced medium. Each culture to be tested was inoculated into skimmed milk with 0.1% and 0.3% methylene blue. After incubation for 24 to 48 h at 37 °C, observations of methylene blue reduction and milk coagulation were noted. *Lactococci* reduce methylene blue with coagulation; however, thermophilic streptococci are sensitive to this dye [27].

## 2.7. Technological Aspects of Selected Kefir

### **Acidifying activity**

The acidifying activity of the strains was measured according to the method described by [28]. This evaluation involved monitoring the pH changes in the cultures over time while simultaneously determining total acidity through titration with sodium hydroxide at regular intervals (0, 2, 4, 6, and 24 h). Precultures were prepared using pasteurized camel milk and strains obtained from MRS medium agar plates. These precultures were used to inoculate 100 mL of pasteurized camel milk in a 250 mL Erlenmeyer flask, which was incubated at 37 °C for 24 h.

### **Proteolytic activity**

To determine the proteolytic activity of LAB, MRS agar supplemented with 10% skim milk was poured, solidified, and then dried. Sterile Whatman paper discs were deposited on the surface of the agar. Each disc received a volume of 20 µL of a young culture. After incubation at 37 °C for 24 h, proteolysis was indicated by clear zones around the discs [29].

### **Lipolytic activity**

To determine the lipolytic activity, the strains were inoculated on agar spots in Tween-80 (1, 3, 5%) [30]. Incubation was carried out at 25 °C for 72 h. Strains with an opaque area due to the formation of esters with calcium-liberated fatty acids were considered positive [31].

### Exopolysaccharide production

The cultures were streaked on modified MRS (m-MRS; glucose replaced with 100 g/L sucrose) [32], incubated at the optimal growth temperature for 24 h, and then tested for slime formation using the inoculated loop method [33]. Formed colonies were dragged up using a metal loop, and the strains were considered positive slime producers if the length of the slime was above 1.5 mm [34].

### 2.8. Antimicrobial Activity Tests

The antibacterial activity of camel kefir strains was determined using the agar well method in MH agar against pathogenic strains [35]. The pathogenic strains used were *Pseudomonas*, *Escherichia coli*, *Micrococcus*, *Klebsiella pneumoniae*, *Staphylococcus*, and *Streptococcus* sp. These bacterial strains are widely utilized in antibacterial studies, as they encompass both Gram-positive and Gram-negative bacteria, including opportunistic and pathogenic species. Additionally, their clinical significance lies in their frequent association with human infections.

All test bacteria were cultured in Mueller–Hinton broth (MHB; Difco) and incubated at 37 °C for 24 h. Twenty microliters of each kefir strain was directly dropped onto the surface of the MHA. The Petri dishes were incubated for 24 h at 37 °C, and the inhibition zone was observed. The presence of a clear zone at the site of strain inoculation was considered total inhibition. All activity tests were performed in triplicate.

### 2.9. Statistical Analysis

The study was designed with a randomized complete design (RCD) with three levels of kefir grains (2%; 5%; 10%) with five replications. The statistical analysis was carried out by the SPSS software (20). The chemical composition, sensory quality, and biological activities of each strain were assessed by analysis of variance (ANOVA; variation in the dose) using the Duncan test ( $p < 0.05$ ).

## 3. Results and Discussion

### 3.1. Characterization of Camel Milk and KGs

The physicochemical and microbiological composition of camel milk and kefir grains are given in Table 1.

**Table 1.** Physicochemical and microbiological characteristics (mean  $\pm$  Standard Deviation) of camel milk and kefir grains.

| Parameters                     | Camel Milk n = 9  | Kefir Grains n = 7 |
|--------------------------------|-------------------|--------------------|
| pH                             | 6.77 $\pm$ 0.11   | 3.9 $\pm$ 0.70     |
| Acidity °D                     | 17.36 $\pm$ 0.34  | 135.00 $\pm$ 1.20  |
| Viscosity (cP)                 | 2.56 $\pm$ 0.22   | -                  |
| Chemical composition           | (g/L)             | (g/kg)             |
| Fat                            | 41.70 $\pm$ 3.18  | 0.02 $\pm$ 0.01    |
| (DM)                           | 114.21 $\pm$ 0.11 | 122.79 $\pm$ 0.02  |
| Ash                            | 8.92 $\pm$ 0.61   | 7.90 $\pm$ 0.81    |
| Protein                        | 37.82 $\pm$ 0.66  | 44.50 $\pm$ 0.10   |
| Lactose                        | 41.3 $\pm$ 0.21   | -                  |
| LAB (log <sub>10</sub> CFU/mL) | 3.55 $\pm$ 0.22   | 8.48 $\pm$ 0.15    |
| YM (log <sub>10</sub> CFU/mL)  | 3.75 $\pm$ 0.36   | 6.63 $\pm$ 0.19    |

DM: dry matter; LAB: lactic acid bacteria; YM: yeast and molds.



The pH and acidity of camel milk ( $6.77 \pm 0.11$  and  $17.36 \pm 0.34$ , respectively) were found to be similar to the values reported by Fguiri [36]. The acidity level is influenced by microbial flora that produces lactic acid during milking at room temperature [37] and high vitamin C content [38]. The concentrations of fat, proteins, and lactose in camel milk were also consistent with previous studies [39–41]. Various factors, such as geographical location, feeding conditions, lactation stage, age, health, environment, milking practices, and weather conditions, can significantly affect the composition of camel milk [42].

The lactic acid bacteria load in camel milk from the extensive system was  $3.55 \pm 0.22 \log_{10}$  CFU/mL, while the load of yeasts and molds was  $3.75 \pm 0.36 \log_{10}$  CFU/mL. According to [43], the highest bacterial load in camel milk was observed in the extensive system ( $1.43 \pm 0.87 \log_{10}$  CFU/mL for LAB and  $6.36 \pm 2.44 \log_{10}$  CFU/mL for MY), possibly influenced by environmental factors, milking conditions, and transport time from milking to laboratory analysis. Other studies have also suggested that the breeding system can impact the quality of cow's milk [44].

The KGs were yellowish-white in color with an acidic pH, with [45] reporting similar pH values (3.5 and 4.0). The fat content of kefir grains is very low, ranging from 0.02 to 0.01 g/kg. These results align with those reported by [46], which showed a fat content of 0.06% to 2% of the grain weight. The microbial load of KGs in YM was  $6.63 \pm 0.19 \log_{10}$  CFU/g, equivalent to  $4.3 \pm 2.3 \times 10^6$  CFU/g, and in LAB, it ranged from  $8.48 \pm 0.15 \log_{10}$  CFU/g, equal to  $3 \pm 1.2 \times 10^8$  CFU/g. The grains consisted of a symbiotic microbial mixture of lactic acid bacteria ( $10^8$  CFU/g) and yeast ( $10^6$ – $10^7$  CFU/g) [47]. These grains contain lactic acid bacteria (LAB) and various yeasts combined with casein and complex sugars in a polysaccharide matrix [16].

### 3.2. The Effect of the KG Dose on the Composition of Kefir

The dose of kefir grains (KGs) was a crucial key factor influencing the physicochemical and microbiological composition of kefir. In this study, various KG doses were analyzed to determine their impact on essential quality parameters, such as acidity, pH, microbial count, and overall composition. The composition of kefir produced with three different KG doses is presented in Table 2.

**Table 2.** The effect of dose on the quality of camel kefir (mean  $\pm$  Standard Deviation).

| Parameters                 | 2%                | 5%                | 10%               |
|----------------------------|-------------------|-------------------|-------------------|
| pH                         | $5.1 \pm 0.4^a$   | $4.8 \pm 0.38^b$  | $4.5 \pm 0.35^c$  |
| Acidity °D                 | $89.95 \pm 5.4^c$ | $99.4 \pm 16.5^b$ | $112.03 \pm 2^a$  |
| Viscosity                  | $6.24 \pm 0.57^c$ | $7.88 \pm 0.84^b$ | $10.83 \pm 1.8^a$ |
| Chemical composition (g/L) |                   |                   |                   |
| Fat                        | $41.0 \pm 7.1^a$  | $40.27 \pm 7.1^a$ | $40 \pm 7.05^a$   |
| Protein                    | $22.04 \pm 1.3^b$ | $25.34 \pm 3.6^b$ | $31.1 \pm 5.4^a$  |
| DM                         | $114.1 \pm 7.7^a$ | $113.8 \pm 7.8^a$ | $113.7 \pm 7.8^a$ |
| Ash                        | $8.99 \pm 0.49^a$ | $8.97 \pm 0.53^a$ | $8.97 \pm 0.48^a$ |
| Lactose                    | $12.66 \pm 1^a$   | $12.2 \pm 0.3^a$  | $11.3 \pm 1.3^a$  |
| LAB ( $\log_{10}$ CFU/mL)  | $5.32 \pm 0.24^a$ | $5.33 \pm 0.25^a$ | $5.10 \pm 0.6^a$  |
| YM ( $\log_{10}$ CFU/mL)   | $3.98 \pm 0.25^a$ | $4.53 \pm 0.64^a$ | $4.24 \pm 0.83^a$ |

a, b, c: Means within the same row with different superscript letters show significant statistical differences ( $p < 0.05$ ); DM: dry matter; LAB: lactic acid bacteria; YM: yeast and molds.

The dose of KGs had a significant effect on physical parameters. Acidity and viscosity increased proportionally to the kefir grain dose. Conversely, the pH of kefir exhibited a relationship with the amount of grains used as inoculum. These findings align with previous research [48], which showed a pH decrease in cow milk kefir after 24 h of incubation. This pH reduction is primarily attributed to the production of organic acids,

ethanol, CO<sub>2</sub>, and other volatile compounds [49]. The viscosity of kefir increased with higher doses of grains, as it led to a higher concentration of lactic acid bacteria (LAB) and yeasts, accelerating protein coagulation. This resulted in a thicker milk matrix. Some LAB strains also produced exopolysaccharides (EPSs) like kefiran, which further increased viscosity, improved stability, strengthened the gel network, and enhanced texture [50].

No significant differences were observed in the fat, DM, ash, or lactose content of kefir when varying the dose of KGs. The chemical composition of cow kefir consists mainly of water (90%), followed by sugars (6%), fats (3.5%), proteins (3%), and ash (0.7%) [51]. The fermentation process converts lactose into lactic acid, resulting in a decrease in pH and a thicker consistency. Camel kefir may be a suitable option for individuals with lactose intolerance [52].

The use of different doses of KGs did not show any significant effect on the bacterial load (LAB and YM). According to a study by [48], kefir from a cow contains 10<sup>8</sup> CFU/mL of lactic acid bacteria, 10<sup>6</sup> CFU/mL of yeast, and 10<sup>6</sup> CFU/mL of acetic acid bacteria. The symbiotic interaction between LAB and yeasts plays a critical role in kefir's unique composition, influencing both texture and flavor [53]. However, excessive yeast activity may lead to excessive CO<sub>2</sub> and alcohol production, potentially affecting the sensory characteristics of kefir [54].

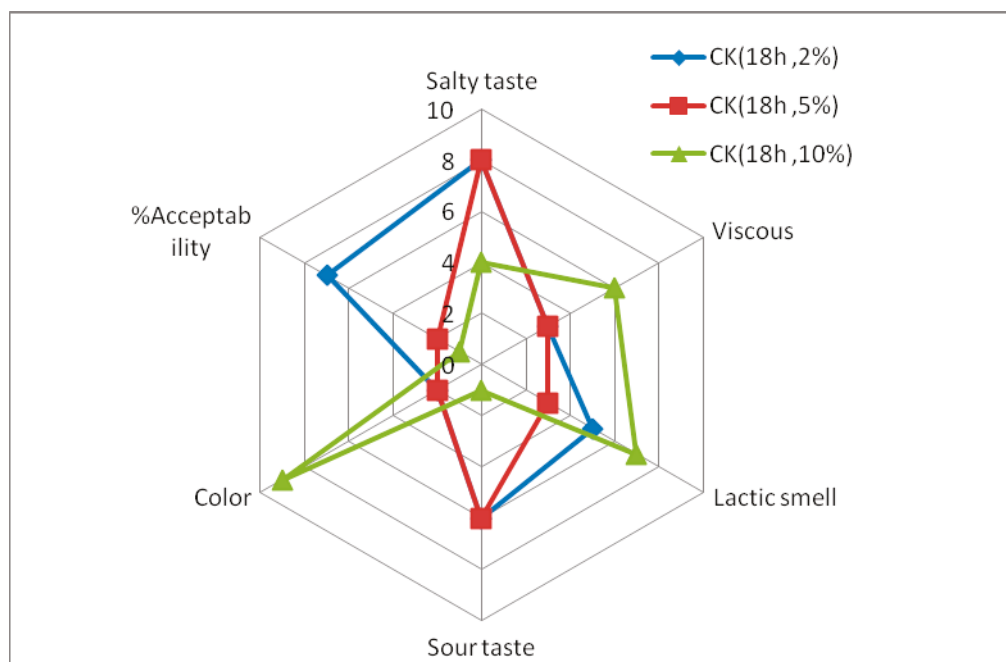
All camel kefirs from the extensive system match the description of typical kefir in the *Codex Alimentarius*. They contain at least 2.8% protein (from 2.2 to 3.11 ± 5.4%), 0.6% lactic acid (from 0.89 to 1.12%), and less than 10% fat (~4%). The total number of microorganisms in the final product must be at least 10<sup>7</sup> (CFU)/mL (5.32 log<sub>10</sub> CFU/mL) and the number of yeasts at least 10<sup>4</sup> CFU/mL (4.53 log<sub>10</sub> CFU/mL) [52]. Maintaining an optimal microbial load is essential for sensory quality. Controlled fermentation improves texture, flavor, and aroma, while excessive microbial growth deteriorates sensory properties and reduces shelf life. A balanced microbial load improves product stability, enhances sensory appeal, and ensures safety [55].

### 3.3. Sensory Evaluation of Camel Kefirs (CKs)

CK (2%, 18 h) was the most acidic with a pH of 6.3, showing pronounced acidity, attributed to lactic acid production during fermentation (18 h). Camel kefir (10%, 18 h) exhibited increased viscosity and a strong lactic odor, indicative of lactic acid bacteria presence in kefir grains [56]. The evaluated CK (2%, 18 h) scored above 2.5 for all attributes. Camel kefir with 2% and 5% CK (5%, 18 h) had a saltier taste (8.08 ± 2.51), with CK (5%, 18 h) exhibiting the weakest color. The kefirs scored above 3 for salinity, influenced by a diet of halophytic plants rich in salts [57].

The results of the classification test revealed that camel kefir (2%, 18 h) was preferred by 73% of testers. Organoleptic analysis indicated that CK (2%, 18 h) received higher scores for all evaluated attributes, demonstrating overall great acceptance (Figure 1). Sensory evaluation revealed that CK (2%, 18 h) scored higher in taste, texture, and appearance, indicating the highest level of acceptability.

In a study by [58], it was concluded that camel kefir (2%, 18 h) was the most preferred by tasters when assessing the sensory quality of camel kefir from a semi-intensive system.



**Figure 1.** Sensory evaluation of camel kefir. CK (2%, 18 h), CK (5%, 18 h), and CK (10%, 18 h): camel kefir with 18 h of incubation and 2, 5, and 10% KGs.

### 3.4. Physiological and Biochemical Tests

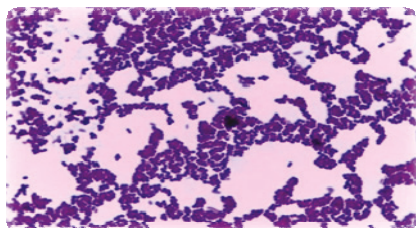
The strains were identified through physiological and biochemical tests; the results are presented in Table 3. Out of thirty-eight lactic acid bacteria strains isolated and purified from camel kefir, eighteen were selected. These strains are Gram-positive, unable to produce catalase, and exhibit various morphologies. They appear purple under the microscope due to their peptidoglycan-rich cell walls [59].

**Table 3.** Physiological and morphological profiles of bacterial strains isolated from selected camel kefir.

| Strains          | Catalase | Gram | Forms        | Citrate |
|------------------|----------|------|--------------|---------|
| SK <sub>1</sub>  | -        | +    | Cocci        | -       |
| SK <sub>2</sub>  | -        | +    | Cocci        | -       |
| SK <sub>3</sub>  | -        | +    | Coccobacilli | -       |
| SK <sub>4</sub>  | -        | +    | Cocci        | -       |
| SK <sub>5</sub>  | -        | +    | Spherical    | -       |
| SK <sub>6</sub>  | -        | +    | Cocci        | -       |
| SK <sub>7</sub>  | -        | +    | Cocci        | -       |
| SK <sub>8</sub>  | -        | +    | Spherical    | -       |
| SK <sub>9</sub>  | -        | +    | Cocci        | -       |
| SK <sub>10</sub> | -        | +    | Cocci        | -       |
| SK <sub>11</sub> | -        | +    | Spherical    | -       |
| SK <sub>12</sub> | -        | +    | Cocci        | -       |
| SK <sub>13</sub> | -        | +    | Cocci        | -       |
| SK <sub>14</sub> | -        | +    | Cocci        | -       |
| SK <sub>15</sub> | -        | +    | Cocci        | -       |
| SK <sub>16</sub> | -        | +    | Cocci        | -       |
| SK <sub>17</sub> | -        | +    | Spherical    | -       |
| SK <sub>18</sub> | -        | +    | Rounded      | -       |

SK<sub>1</sub> → SK<sub>18</sub>: lactic strains isolated from camel kefir; +: positive test; -: negative test.

After performing Gram staining (Figure 2), the bacteria were observed by a microscope at a magnification of 100×. All bacteria were Gram-positive, indicated by their purple color, and exhibited various forms, such as cocci and coccobacilli (spherical). Following the catalase test, no bubbles were produced, indicating that all the bacteria were catalase-negative. The lack of catalase activity is a characteristic of lactic acid bacteria, although some species may acquire this activity.



**Figure 2.** Microscopic observation of lactic acid bacteria with magnification (G: 10X100).

Table 4 presents the various tests employed to identify and characterize microbial strains based on their physiological and biochemical properties. This included strain codes and growth conditions, such as temperature tolerance, pH range, and NaCl tolerance.

**Table 4.** Physiological and biochemical characterization of isolated strains.

| Isolated Strains | Growth at Different Temperatures |       |       | Growth at Different pH |     | Growth at Different [NaCl] |    |    |
|------------------|----------------------------------|-------|-------|------------------------|-----|----------------------------|----|----|
|                  | 10 °C                            | 39 °C | 45 °C | 4.2                    | 9.6 | 4%                         | 6% | 9% |
| SK <sub>1</sub>  | +                                | +     | +     | +                      | +   | +                          | +  | +  |
| SK <sub>2</sub>  | +                                | +     | +     | +                      | +   | +                          | +  | +  |
| SK <sub>3</sub>  | +                                | +     | +     | +                      | +   | +                          | +  | +  |
| SK <sub>4</sub>  | +                                | +     | +     | +                      | +   | +                          | +  | +  |
| SK <sub>5</sub>  | +                                | +     | +     | +                      | +   | +                          | +  | +  |
| SK <sub>6</sub>  | +                                | +     | +     | +                      | +   | +                          | +  | +  |
| SK <sub>7</sub>  | +                                | +     | +     | +                      | +   | +                          | +  | +  |
| SK <sub>8</sub>  | +                                | +     | +     | +                      | +   | +                          | +  | +  |
| SK <sub>9</sub>  | +                                | +     | +     | +                      | +   | +                          | +  | +  |
| SK <sub>10</sub> | +                                | +     | +     | +                      | +   | +                          | +  | +  |
| SK <sub>11</sub> | +                                | +     | +     | +                      | +   | +                          | +  | +  |
| SK <sub>12</sub> | +                                | +     | +     | +                      | +   | +                          | +  | +  |
| SK <sub>13</sub> | +                                | +     | +     | +                      | +   | +                          | +  | +  |
| SK <sub>14</sub> | +                                | +     | +     | +                      | +   | +                          | +  | +  |
| SK <sub>15</sub> | +                                | +     | +     | +                      | +   | +                          | +  | +  |
| SK <sub>16</sub> | +                                | +     | +     | +                      | +   | +                          | +  | +  |
| SK <sub>17</sub> | +                                | +     | +     | +                      | +   | +                          | +  | +  |
| SK <sub>18</sub> | +                                | +     | +     | +                      | +   | +                          | +  | +  |

SK<sub>1</sub> → SK<sub>18</sub>: lactic strains isolated from camel kefir; +: positive test.

By using physiological and biochemical tests, the isolated strains were classified into two groups: mesophilic and thermophilic strains (Table 4). Mesophilic strains can thrive at moderate temperatures, typically between 20 and 45 °C, while thermophilic strains show optimal growth at higher temperatures, usually above 45 °C. This classification offers valuable information on the adaptability of the strains and their potential uses in various environmental conditions.

All isolated strains were capable of growing in MRS broth at temperatures of 10 °C, 39 °C, and 45 °C (Table 4), indicating their thermophilic nature. The ability to thrive at

different temperatures suggests that the strains are well adapted to warmer environments, a common trait of thermophilic microorganisms.

Furthermore, all the strains exhibited growth at pH 4.2 and pH 9.6, as well as in hypersaline media with 4%, 6%, and 9% NaCl. These findings confirm the robustness of the isolated strains and their ability to survive in challenging conditions, such as extreme pH and high salinity.

All results of the NaCl growth test were positive, indicating that the strains exhibited high salinity tolerance. This is significant, as it suggests that the isolates may belong to the genus *Enterococcus*, known for its ability to grow at 45 °C and tolerate salinity levels up to 6.5% and a pH of 9.6, consistent with the findings of this study. In contrast, *Lactococci* do not thrive under such extreme conditions, providing additional support for the identification of the isolates as *Enterococcus* species [60].

#### Growth and Activity of Isolated Strains in Sherman's Milk

Table 5 presents the results of methylene blue reduction and milk coagulation tests for each isolated strain grown in Sherman's milk. The observations were recorded for cultures supplemented with methylene blue at concentrations of 0.1% and 0.3%.

**Table 5.** Cultivation of lactic acid strains in Sherman's milk.

| Lactic Strains   | Methylene Blue      |                     |
|------------------|---------------------|---------------------|
|                  | 0.1%                | 0.3%                |
| SK <sub>1</sub>  | Coagulation of milk | Coagulation of milk |
| SK <sub>2</sub>  | Coagulation of milk | Coagulation of milk |
| SK <sub>3</sub>  | Coagulation of milk | Coagulation of milk |
| SK <sub>4</sub>  | Coagulation of milk | Coagulation of milk |
| SK <sub>5</sub>  | Coagulation of milk | Coagulation of milk |
| SK <sub>6</sub>  | Coagulation of milk | Coagulation of milk |
| SK <sub>7</sub>  | Coagulation of milk | Coagulation of milk |
| SK <sub>8</sub>  | Coagulation of milk | Coagulation of milk |
| SK <sub>9</sub>  | Coagulation of milk | Coagulation of milk |
| SK <sub>10</sub> | Coagulation of milk | Coagulation of milk |
| SK <sub>11</sub> | -                   | -                   |
| SK <sub>12</sub> | -                   | -                   |
| SK <sub>13</sub> | -                   | -                   |
| SK <sub>14</sub> | -                   | -                   |
| SK <sub>15</sub> | -                   | -                   |
| SK <sub>16</sub> | -                   | -                   |
| SK <sub>17</sub> | -                   | -                   |
| SK <sub>18</sub> | -                   | -                   |

SK<sub>1</sub> → SK<sub>18</sub>: lactic strains isolated from camel kefir; -: negative test.

During incubation, some tested isolates caused significant coagulation of Sherman's milk, regardless of the methylene blue concentration. However, no reduction of methylene blue was observed for the others, as indicated by the lack of discoloration in the medium. This suggests a negative reaction in terms of methylene blue reduction for these tested strains.

The lactic acid bacteria strains (SK<sub>1</sub> to SK<sub>10</sub>) isolated from camel kefir showed their ability to grow in and acidify Sherman's milk, resulting in coagulation at both 0.1% and 0.3% methylene blue concentrations.

The lactic acid bacteria strains SK<sub>11</sub> to SK<sub>18</sub> did not induce coagulation of Sherman's milk or show any capacity to reduce methylene blue at either tested concentration. The absence of coagulation suggests that these strains either lack sufficient acidifying activity or



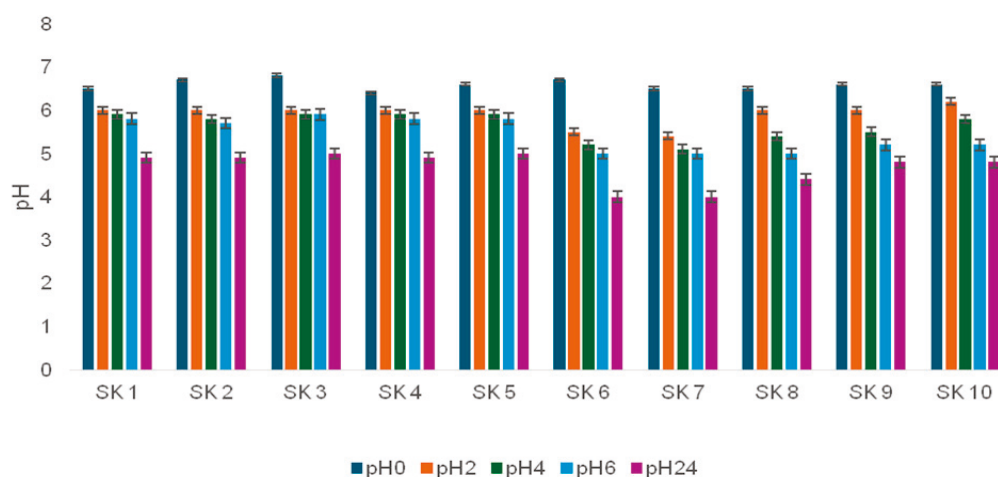
do not grow effectively in this medium. These strains also failed to reduce methylene blue significantly, indicating that their population density in the medium was not high enough to trigger a visible color change. Due to their inability to produce visible technological changes, strains SK<sub>11</sub> to SK<sub>18</sub> were not subjected to further technological evaluations. Consequently, they were excluded from subsequent phases of this study, as they do not exhibit the essential functional properties required for further analysis.

### 3.5. Technological Activities of Lactic Acid Strains Isolated from Selected Kefir

The evaluation of the technological properties of lactic acid bacteria (LAB) strains isolated from kefir made with camel milk (KC, 2%, 18 h) was essential for selecting appropriate starter cultures for the standardized production of dairy products. This assessment encompassed various factors that impacted the fermentation process and the quality of the final product. The key technological properties typically assessed included the acidification rate, proteolytic and lipolytic activity, and gas production.

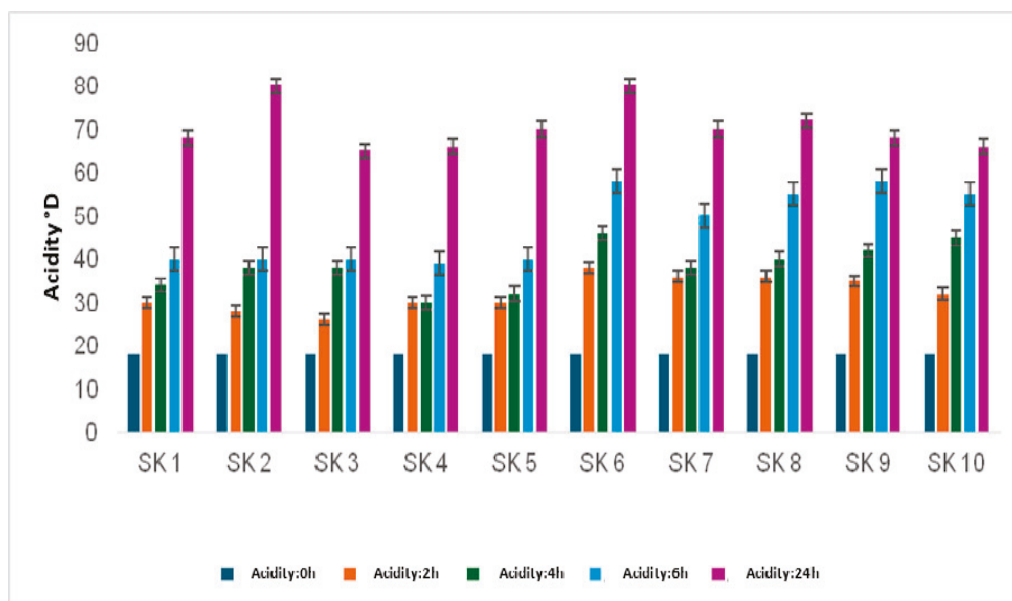
#### 3.5.1. Acidifying Activity

The acidifying activity of LAB strains was studied to control both pH and acidity levels during fermentation. The strains were evaluated based on their acid production capacity to determine the most suitable starter culture. Figure 3 illustrates the acidifying activity of the strains, as indicated by the pH decrease. The results depicted in Figure 3 show that the pH decreased from 6.7 to 5.55 within the first two hours of incubation. After 24 h of incubation, the pH values ranged between 4.03 and 5.02. According to a previous study, strains are classified as fast, medium, or slow acidifiers based on the pH reaching 0.4 U in 3, 3 to 5, and >5 h, respectively [34]. In our study, all isolated strains exhibited varying levels of acidifying activity. Strains SK6 and SK7 showed the highest acidifying activity, with pH values of 3.7 and 3.6, respectively, after 24 h of fermentation. These strains were classified as acidifying and selected for further testing. Fast-acidifying strains like this are ideal candidates in the dairy fermentation processes as starters.



**Figure 3.** Evaluation of the pH of isolated bacteria. SK1 → SK10: lactic strains isolated from camel kefir.

Differences in pH reduction between genera, between species, and sometimes between strains of the same species have been mentioned by many authors, including Luquet [61]. Acidity increased during fermentation, and there was variability in the acidification rate between the different strains used (Figure 4). After 24 h of incubation, the results obtained allowed all the strains studied to be classified into the categories of lactic acid bacteria with acidity between 64.8 °D and 81.9 °D.



**Figure 4.** Changes in acidity over time in kefirs inoculated with different strains. SK1 → SK10: lactic strains isolated from camel kefir.

The results show that all isolated bacterial strains can lower pH and increase acidity, but the degree of acidification varies. This aligns with Lu-quet's [61] findings, highlighting differences not only between bacterial genera but also among species and even within individual strains. While all strains contributed to acid production, some induced a more significant pH decrease, indicating variations in their ability to ferment sugars and produce organic acids like lactic acid and acetic acid. These variations may be due to genetic factors, enzyme activity, and adaptability to different substrates [62].

Some strains grow faster and can acidify the medium more quickly, while others have a greater affinity for some sugars, affecting their acid production. These differences suggest the possibility of selecting particular bacterial strains for specific uses, such as in fermented dairy products and probiotics. A better grasp of strain-specific acidification can lead to improved fermentation conditions and higher product quality.

### 3.5.2. Proteolytic Activity

The results of proteolytic activity are summarized in Table 6. All strains produced clear zones around the discs, with diameters ranging from 8 to 15 mm, indicating that all strains exhibit proteolytic activity. According to Veuillemand (1986) [29], a strain is considered proteolytic if it produces a lysis zone with a diameter between 5 and 15 mm. These findings confirm the proteolytic nature of lactic acid bacteria. Previous research has shown that lactic acid bacteria lack the ability to synthesize certain amino acids but possess a complex bacterial proteolytic system that enables them to thrive in protein-rich environments with low free amino acids, such as milk [63]. In conclusion, all strains studied demonstrated proteolytic activity, supporting their adaptation to environments abundant in proteins and deficient in free amino acids.

**Table 6.** Technological activities of lactic acid strains isolated from selected kefir.

| Camel Lactic Strains | Diameter of Proteolysis Zone (mm) | Diameter of Opaque Area (mm) |             |             | Exopolysaccharide Production | Gas Release |
|----------------------|-----------------------------------|------------------------------|-------------|-------------|------------------------------|-------------|
|                      |                                   | 1% Tween 80                  | 3% Tween 80 | 5% Tween 80 |                              |             |
| KS <sub>1</sub>      | 8                                 | 12                           | 16          | 5           | ±                            | ±           |
| KS <sub>2</sub>      | 9                                 | 7                            | 10          | 8           | ±                            | ±           |
| KS <sub>3</sub>      | 10                                | 9                            | 12          | 6           | +                            | +           |
| KS <sub>4</sub>      | 5                                 | 6                            | 8           | 7           | +                            | +           |
| KS <sub>5</sub>      | 11                                | 5                            | 9           | 6           | +                            | +           |
| KS <sub>6</sub>      | 7                                 | 6                            | 7           | 5           | +                            | +           |
| KS <sub>7</sub>      | 12                                | 7                            | 10          | 6           | +                            | +           |
| KS <sub>8</sub>      | 15                                | 8                            | 10          | 5           | +                            | +           |
| KS <sub>9</sub>      | 14                                | 9                            | 9           | 7           | +                            | +           |
| KS <sub>10</sub>     | 13                                | 10.2                         | 9           | 8           | +                            | +           |

SK<sub>1</sub> → SK<sub>10</sub>: lactic strains isolated from camel kefir; +: positive test; ± positive/negative test.

### 3.5.3. Lipolytic Activity

The lipolytic activity of lactic acid bacteria strains was analyzed, and the results are presented in Table 6. It was found that a substrate concentration of 3% Tween 80 provided optimal conditions for lipolytic activity in all tested strains.

Different genera of lactic acid bacteria exhibited varying levels of lipolytic activity, with *lactococci* and *enterococci* exhibiting significant activity when 1% olive oil was used as a substrate [64]. This indicates that these bacteria have strong lipase or esterase activity, allowing them to effectively break down lipids.

The lipolytic potential of these bacteria likely influences the flavor and texture development of fermented dairy products, as lipid hydrolysis leads to the release of free fatty acids, which impact sensory properties [65].

### 3.5.4. Texturizing Activity

The results of the production of exopolysaccharides by lactic isolates are illustrated in Table 6. All strains studied were capable of growing on a hypersucrose medium and forming sticky colonies, indicating the production of exopolysaccharides (EPSs). The production of EPSs by lactic acid bacteria is a beneficial phenomenon for many industrial food processes [66]. The ability of lactic acid bacteria to produce EPSs is important for enhancing texture, preventing syneresis, and increasing viscosity in finished products [67]. Factors influencing EPS production include the strain, growth rate, temperature, culture medium, acidification rate, pH, and oxygen levels in the culture medium [68]. Lactic acid bacteria synthesize exocellular polysaccharides (sugar polymers) during growth, which are used to improve the texture and viscosity of products like kefir [69].

### 3.5.5. Gasogenic Activity

The gasogenic activity test yielded positive results for all tested strains, except for KS<sub>1</sub> and KS<sub>2</sub> (Table 6). KS<sub>1</sub> and KS<sub>2</sub> exclusively metabolized lactose into lactic acid without producing CO<sub>2</sub>, indicating their classification as strictly homofermentative lactic acid bacteria. In contrast, the remaining eight strains (KS<sub>3</sub> to KS<sub>10</sub>) produced both lactic acid and CO<sub>2</sub>. The presence of CO<sub>2</sub>, observed as gas bubbles in the Durham tube, is a characteristic trait of heterofermentative bacteria. This test effectively distinguished between homofermentative and heterofermentative strains based on their ability to generate CO<sub>2</sub> from glucose. This differentiation was crucial, as gas production served as a metabolic indicator for the identification and classification of lactic acid bacteria, which are essential for their technological applications in fermentation processes [70].

Through the evaluation of bacteria, we identified LAB strains that not only facilitated the fermentation process but also contributed to the desired characteristics of camel kefir (2%, 18H).

### 3.6. Antibacterial Properties of Lactic Acid Bacteria Isolated from Camel's Kefir

This test aimed to assess the sensitivity of six pathogenic strains against 10 lactic acid bacteria strains isolated from camel milk kefir (2%, 18 h) (Table 7). All lactic acid strains showed strong antibacterial activity against pathogens, including *Pseudomonas*, *Escherichia coli*, *Micrococcus*, *Klebsiella pneumoniae*, *Staphylococcus*, and *Streptococcus*. Among them, camel kefir strain 4 (KS4) showed the most significant antibacterial activity, while strain KS<sub>2</sub> showed lower activity compared to the others. According to a study by [71], the antibacterial properties of lactic acid bacteria are attributed to the production of various antibacterial metabolites, such as bacteriocins, hydrogen peroxide, organic acids, and diacetyl.

**Table 7.** Antibacterial activity of the 10 strains of lactic acid bacteria against 6 pathogenic strains.

|                  | <i>Pseudomonas</i>      | <i>E. coli</i>          | <i>Micrococcus</i>      | <i>Klepsiella</i>       | <i>Staphylocoque</i>    | <i>Streptocoque</i>     |
|------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| KS <sub>1</sub>  | 9 ± 0.4 <sup>ab</sup>   | 6.4 ± 0.5 <sup>b</sup>  | 4.1 ± 1.6 <sup>ab</sup> | 6.1 ± 1 <sup>ab</sup>   | 3.9 ± 0.7 <sup>c</sup>  | 4.3 ± 1.4 <sup>b</sup>  |
| KS <sub>2</sub>  | 4.2 ± 1.1 <sup>c</sup>  | 3.8 ± 0.8 <sup>c</sup>  | 4.1 ± 1.1 <sup>ab</sup> | 3.6 ± 2 <sup>b</sup>    | 3.7 ± 1.4 <sup>bc</sup> | 4 ± 1.2 <sup>b</sup>    |
| KS <sub>3</sub>  | 7.2 ± 0.3 <sup>b</sup>  | 3.9 ± 1.1 <sup>c</sup>  | 5.9 ± 0.8 <sup>a</sup>  | 6 ± 1.2 <sup>ab</sup>   | 4.4 ± 1.5 <sup>b</sup>  | 4.5 ± 1 <sup>ab</sup>   |
| KS <sub>4</sub>  | 9.9 ± 0.2 <sup>a</sup>  | 10 ± 0.5 <sup>a</sup>   | 3 ± 0.5 <sup>b</sup>    | 4.4 ± 1.3 <sup>b</sup>  | 4.1 ± 2 <sup>b</sup>    | 6.4 ± 1 <sup>a</sup>    |
| KS <sub>5</sub>  | 5.9 ± 1 <sup>b</sup>    | 4.9 ± 0.4 <sup>bc</sup> | 5 ± 1 <sup>a</sup>      | 3.9 ± 0.5 <sup>b</sup>  | 4 ± 1.6 <sup>b</sup>    | 4 ± 2.2 <sup>ab</sup>   |
| KS <sub>6</sub>  | 6 ± 0.5 <sup>b</sup>    | 5.1 ± 1.5 <sup>b</sup>  | 5 ± 0.9 <sup>a</sup>    | 4.1 ± 0.7 <sup>b</sup>  | 4 ± 2.1 <sup>b</sup>    | 4.1 ± 1.3 <sup>b</sup>  |
| KS <sub>7</sub>  | 9.8 ± 0.34 <sup>a</sup> | 5 ± 2 <sup>b</sup>      | 4.2 ± 1.2 <sup>ab</sup> | 4.1 ± 1.4 <sup>b</sup>  | 6 ± 0.6 <sup>ab</sup>   | 6 ± 1 <sup>a</sup>      |
| KS <sub>8</sub>  | 6.5 ± 1.3 <sup>b</sup>  | 5.3 ± 1 <sup>b</sup>    | 3 ± 1.5 <sup>b</sup>    | 6.3 ± 0.8 <sup>ab</sup> | 4.9 ± 0.8 <sup>ab</sup> | 5 ± 1.5 <sup>ab</sup>   |
| KS <sub>9</sub>  | 9 ± 0.7 <sup>ab</sup>   | 4 ± 0.5 <sup>bc</sup>   | 4.8 ± 0.8 <sup>a</sup>  | 6 ± 0.7 <sup>ab</sup>   | 8.2 ± 0.6 <sup>a</sup>  | 4.6 ± 1.7 <sup>ab</sup> |
| KS <sub>10</sub> | 7 ± 1.6 <sup>b</sup>    | 5 ± 0.43 <sup>bc</sup>  | 6.3 ± 1 <sup>a</sup>    | 8 ± 1.3 <sup>a</sup>    | 6.7 ± 0.9 <sup>a</sup>  | 4 ± 2 <sup>ab</sup>     |
| P                | 0.01                    | 0.01                    | 0.01                    | 0.02                    | 0.01                    | 0.03                    |

a, b, c: Means in the same row with different letters are statically different ( $p < 0.05$ ); SK<sub>1</sub> → SK<sub>10</sub>: lactic strains isolated from camel kefir.

Research has shown that kefir exhibits antibacterial properties. Antimicrobial peptides and bioactive molecules, such as lysozymes, act as the first line of defense against pathogen colonization [72]. Both bacteria and yeasts isolated from kefir have demonstrated antimicrobial activity against enteropathogenic bacteria and spoilage fungi in both in vivo and in vitro studies. Additionally, kefir's microbiota and associated compounds have exhibited promising antimicrobial effects [73].

## 4. Conclusions

In recent decades, the popularity of kefir has been increasing, with numerous nutritional benefits attributed to this fermented beverage. However, in Tunisia, fermented camel milk, known as camel kefir, is not widely recognized. This study aimed to produce and characterize camel kefirs with varying levels of kefir grains. The results of the research indicated a significant impact of the kefir grain dosage on the quality of the camel beverage. Furthermore, the quality of the camel kefirs produced met safety standards set by regulations. Among the different formulations tested, camel kefir with 2% kefir grains and fermented for 18 h aligned with *Codex Alimentarius* requirements and was preferred by the taste testers.

The technological aspects of ten strains of lactic acid bacteria isolated from camel kefir showed promising potential, including acidification, proteolysis, lipolysis, thickening, gas production, and antibacterial activities. These findings suggest that lactic acid bacteria from

camel kefir have favorable technological characteristics. Encouraging the consumption and commercialization of camel milk kefir could be beneficial. Further research is needed to assess the shelf life of this dairy product.

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## Abbreviations

The following abbreviations are used in this manuscript:

|                |                                                            |
|----------------|------------------------------------------------------------|
| MRS            | de Man, Rogosa, and Sharpe                                 |
| LAB            | Lactic acid bacteria                                       |
| GK             | Kefir grain                                                |
| DM             | Dry matter                                                 |
| YM             | Yeast and molds                                            |
| CK (2%, 18 h)  | Camel kefir with 2% kefir grains and 18 h incubation time  |
| CK (5%, 18 h)  | Camel kefir with 5% kefir grains and 18 h incubation time  |
| CK (10%, 18 h) | Camel kefir with 10% kefir grains and 18 h incubation time |
| SK1–SK10       | Lactic strains isolated from selected camel kefir.         |

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