c. Lista portofoliului de lucrări științifice relevante

Candidat abilitare IPA : prof. Dr. Florentina (RĂDOI) MATEI

1. Kouadio N.J., Zady A.L.O., Kra K.A.S., Diguță F.C., Niamke S., **Matei F.** (2024). In Vitro Probiotic Characterization of *Lactiplantibacillus plantarum* Strains Isolated from Traditional Fermented Dockounou Paste. *Fermentation*. 10(5):264. https://doi.org/10.3390/fermentation10050264
2. Dinu, L.-D.; Al-Zaidi, Q.J.; Matache, A.G.; **Matei, F.** (2024). Improving the Efficiency of Viability-qPCR with Lactic Acid Enhancer for the Selective Detection of Live Pathogens in Foods. *Foods*, *13*, 1021. https://doi.org/10.3390/foods13071021.
3. Al-Zaidi Q.J., Matache A.G., Dinu L.-D., Calzoni E., **Matei F.** (2024). Development of the lactic acid permeabilizer v-qPCR technique for *Salmonella spp.* detection and quantification. AgroLife Scientific Journal, 13(1), 9–14. https://doi.org/10.17930/AGL202411.
4. Coulibaly W.H., Kouadio N.R., Camara F., Diguta C., **Matei F.** (2023). Functional properties of lactic acid bacteria isolated from Tilapia (*Oreochromis niloticus*) in Ivory Coast. *BMC Microbiol* **23**, 152. https://doi.org/10.1186/s12866-023-02899-6.
5. Rădoi-Encea R-Ș, Pădureanu V., Diguță C.F., Ion M., Brîndușe E., **Matei F.** (2023). Achievements of Autochthonous Wine Yeast Isolation and Selection in Romania—A Review. Fermentation. 9(5): 407. https://doi.org/10.3390/fermentation9050407
6. Mogmenga I., Somda M.K., Ouattara C.A.T., Keita I., Dabiré Y., Diguță C.F., Toma R.C., Ezeogu L.I., Ugwuanyi J.O., Ouattara A.S., **Matei F** (2023). Promising Probiotic Properties of the Yeasts Isolated from *Rabilé*, a Traditionally Fermented Beer Produced in Burkina Faso. Microorganisms, 11(3):802. https://doi.org/10.3390/microorganisms11030802
7. Popescu P.A., Palade L.M., Nicolae I.C., Popa E.E., Miteluț A.C., Drăghici M.C., **Matei F.,** Popa M.E. (2022). Chitosan-Based Edible Coatings Containing Essential Oils to Preserve the Shelf Life and Postharvest Quality Parameters of Organic Strawberries and Apples during Cold Storage. *Foods*; 11(21):3317. https://doi.org/10.3390/foods11213317
8. Diguta C.F., Nitoi D.G., **Matei F.,** Luta G., Cornea C.P. (2020). The biotechnological potential of *Pediococcus* spp. isolated from Kombucha microbial consortium. Foods (MDPI), 9(12), 1780; https://doi.org/10.3390/foods9121780
9. Utoiu E., **Matei F**., Toma A., Diguta C.F., Stefan L.M., Manoiu S., Vrajmasu V.V., Moraru I., Israel-Roming F., Oancea A., Cornea C.P., Constantinescu- Aruxanderi D., Moraru A., Oancea F. (2018). Bee collected pollen with enhanced health benefits, produced by fermentation with a Kombucha consortium. Nutrients. 10 (10), 1365, p. 1-24; https://doi:10.3390/nu10101365.
10. Kaktcham P.M., Zambou Ngoufack F., Foko Kouam E.M., Ciobotaru O., **Matei F.**, Cornea C.P., Israel-Roming F. (2018). Antifungal activity of lactic acid bacteria isolated from peanuts, gari and orange fruit juice against food aflatoxigenic molds. Food Biotechnology, 32 (4), p.237-265. https://doi.org/10.1080/08905436.2018.1519443

fermentation

MDPI

Article

In Vitro Probiotic Characterization of *Lactiplantibacillus plantarum* Strains Isolated from Traditional Fermented Dockounou Paste

Natia Joseph Kouadio [1], Alalet Luc Olivier Zady [1], Kouassi Aboutou Séverin Kra [1], Filofteia Camelia Diguță [2,*], Sébastien Niamke [1] and Florentina Matei [2,3]

[1] Laboratory of Biotechnology Agriculture and Valorization of Biological Resource, UFR Biosciences, Felix Houphouet-Boigny University, Abidjan 22 BP 582, Côte d'Ivoire; jonatia2006@yahoo.fr (N.J.K.); zalo85@yahoo.fr (A.L.O.Z.); kra.severinemma@gmail.com (K.A.S.K.); niamkes@yahoo.fr (S.N.)
[2] Faculty of Biotechnologies, University of Agronomic Sciences and Veterinary Medicine Bucharest, 59 Marasti Blvd., 011464 Bucharest, Romania; florentina.matei@biotehnologii.usamv.ro
[3] Faculty of Food Industry and Tourism, Transilvania University of Brasov, 148 Castelului St., 500014 Brasov, Romania
* Correspondence: camelia.diguta@biotehnologii.usamv.ro

Abstract: This study aimed to evaluate the probiotic properties of 10 lactic acid bacteria (LAB) isolated from artisanal fermented plantain dockounou paste. A preliminary characterization of the LAB isolates was performed based on phenotypic and several biochemical properties, which was subsequently confirmed through 16S rRNA gene sequencing analysis, indicating that these isolates belonged to the species *Lactiplantibacillus plantarum*. With regard to safety criteria, the strains exhibited no alpha or beta hemolysis activity. Nevertheless, the majority of LAB strains demonstrated high sensitivity to the antibiotics tested. The results demonstrated that the majority of the strains exhibited remarkably high survival rates under simulated gastrointestinal conditions, such as pH = 1.5 (81.18–98.15%), 0.3% bile salts (68.62–100.89%), 0.4% phenol (40.59–128.24%), as well as 0.1% pepsin and pH = 2.5 (88.54–99.78%). The LAB strains demonstrated elevated levels of cell surface properties, indicative of the presence of a considerable defensive mechanism against pathogens. Intact LAB cells exhibited significant antioxidant abilities (48.18–83.58%). They also demonstrated a pronounced inhibitory effect on the growth of foodborne pathogens. Enzyme pattern analysis revealed that the LAB isolates produced both proteases and cellulases, as well as pectinase and/or amylase activity. The potential of the *L. plantarum* strains FS43, FS44, and FS48, as indicated by the results obtained from the standard *in vitro* assays, makes them suitable for further study as potential probiotics.

Keywords: fermented plantain dockounou paste; *Lactiplantibacillus plantarum*; probiotic properties; health benefits

check for updates

Citation: Kouadio, N.J.; Zady, A.L.O.; Kra, K.A.S.; Diguță, F.C.; Niamke, S.; Matei, F. *In Vitro* Probiotic Characterization of *Lactiplantibacillus plantarum* Strains Isolated from Traditional Fermented Dockounou Paste. *Fermentation* **2024**, *10*, 264. https://doi.org/10.3390/fermentation10050264

Academic Editor: Alessandra Pino

Received: 25 April 2024
Revised: 14 May 2024
Accepted: 17 May 2024
Published: 19 May 2024

Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Dockounou paste is an Ivorian traditional food made by combining senescent plantain flesh with maize or rice flour and baking or steaming it [1,2]. This food is produced through several processes, including peeling the senescent plantain fruit, scooping out the flesh, combining it with flour, fermenting, packing, frying, and processing it into flour [1,2].

Lactic acid bacteria, including those belonging to the genera *Lactococcus*, *Lactobacillus*, and *Streptococcus*, have been identified as playing a key role in the fermentation of traditional cereal-based foods and beverages worldwide [3–6]. Fermentation is the most significant of these processes since it improves the texture and organoleptic qualities of the food [4–6].

The potential health benefits of indigenous African fermented foods and beverages have not been adequately researched. It is a widely held view that the popularity of tradi-

tional fermented foods can be attributed to many factors, including the enhancement of taste, the conservation of food for a longer shelf life, and the improvement of nutritional value and digestive effectiveness. Some African cultural groups espouse the belief that fermented foods can be efficient in the treatment of illnesses, with a particular focus on digestive disorders. The indigenous microbial consortia present in fermented indigenous African foods are responsible for the beneficial properties of fermented foods mentioned above (reviewed by Achi and Asamudo [7]; Malongane and Berejena [8]). In recent years, numerous studies have been conducted with a focus on the isolation of LABs and yeasts from traditional fermented foods to identify potential starter cultures that can also exhibit valuable probiotic properties [3–6]. LAB species have been identified as the agents responsible for the manufacture of various Ivorian indigenous foods and beverages, such as *attiéké*, *attoupkou*, *placali* (fermented tubers), *baca*, *wômi*, *doklu* (fermented cereal-based foods), *adjuevan* (fermented fish), *bandji* and *tchapalo* (fermented beverages), and *dockounou* (fermented over-ripe fruits of plantain) [8–14]. Throughout the fermentation process of dockounou paste, Kouadio et al. [11] found different types of microorganisms predominating, such as yeasts, bacilli, and lactic acid bacteria. *L. plantarum* (64%), *Weissella cibaria* (22%), *L. fermentum* (7%), and *Pediococcus acidilactici* (7%) were mainly detected during the first 24 h of the production of doklu. *L. plantarum* (56%) dominated after 48 h of fermentation, and *L. fermentum* (100%) prevailed at the end of fermentation [12]. Mogmenga et al. [15] successfully isolated *Saccharomyces cerevisiae* with proven probiotic properties from a traditional fermented beer produced in Burkina Faso, known as Rabilé.

Also, the presence of *Lactobacillus* spp., *Bifidobacterium* spp., and *S. cerevisiae* yeasts has been identified in Romanian fermented beverages (socata and borș) [16,17], Romanian fermented dairy products [18–20], and Romanian fermented vegetables [18,21–23].

Probiotics are live microorganisms that assist the body in maintaining optimal health when consumed in appropriate quantities [24,25]. Lactic acid bacteria, such as *Bifidobacterium* and *Lactobacillus*, isolated from various sources, have been widely investigated using standard *in vitro* tests for key functional, probiotic, and technological properties [26–32]. The beneficial effects on health can be attributed to the synthesis of compounds that inhibit the proliferation of pathogens and compete with them for nutrients and adherence sites on the epithelial cells of the gut tract [29–32]. Probiotics can improve the immune system through macrophage activation, increased levels of immune globulins, increased natural killer cell activity, and/or increased levels of cytokines [30–32].

Furthermore, pharmaceutical probiotics are often expensive, and fermented foods and beverages represent a valuable alternative source of probiotics that may be more accessible to those who are unable to afford them, particularly in marginalized communities in Africa.

The objective of the present study was to assess the potential of LAB strains isolated from traditional fermented plantain dockounou paste as a probiotic *in vitro*. This was achieved by evaluating their functional properties (resistance in simulated gastrointestinal conditions and cell surface properties), probiotic properties (antibacterial, antioxidant, and enzyme activities), and safety properties (molecular identification, antibiotic sensitivity, and hemolysis activity).

2. Materials and Methods

2.1. LAB Strains and Growth Conditions

To evaluate their probiotic properties, 40 bacterial strains were isolated from fermented Dockounou paste [11]. The LAB isolates were preliminarily identified using conventional techniques, including colonial morphology, Gram staining, and biochemical reactions (catalase and oxidase tests). A total of 10 representative LAB isolates were selected for this research and preserved in cryotubes at a temperature of −20 °C in MRS broth, which was additionally supplemented with 20% glycerol. Before use, the stock LAB cultures were inoculated into MRS broth and incubated at 37 °C for 24 h.

2.2. Identification of LAB Strains via 16S rDNA Sequencing

Overnight LAB cultures were harvested through centrifugation at 5000× *g* for 10 min. The LAB cells were then employed to isolate bacterial DNA using the Zymo Research Quick-DNA™ Fungal/Bacterial Miniprep Extraction Kit (Zymo Research, Irvine, CA, USA), following the manufacturer's instructions. Finally, DNA quantification was conducted using the SpectraMax®® QuickDrop™ (Molecular Devices, San Jose, CA, USA). Polymerase chain reaction (PCR) amplification of the 16S ribosomal DNA (rDNA) region was performed using the primers 27F (5′ -AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′ -TACGGYTACCTTGTTACGACTT-3′). Amplified DNA fragments were then subjected to sequencing in both directions using the same primers by the Cellular and Molecular Immunological Application (CEMIA) sequencing service (Larissa, Greece). The newly obtained sequences were subjected to comparison with those already deposited in the NCBI database using the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi, latest accessed 4 December 2023) to identify the species level of the LAB strains based on the level of similarity. Furthermore, a phylogenetic tree was constructed using MEGA software version 11.0.13 [33] based on 16S rDNA genes to identify the bacterial species with the highest degree of similarity [34].

2.3. In Vitro *Tolerance of LAB Strains to Simulated Gastrointestinal Conditions*

2.3.1. Resistance to Low pH

The influence of low pH (1.5) on the growth of LAB strains was explored using the method described by Carr et al. [35]. This involved inoculating fresh bacterial cultures into 6 mL of sterile MRS broth adjusted to pH 1.5 with 1 M HCl and incubating them at 37 °C for 3 h. The number of viable cells was then determined using the plate count agar method, with the results expressed as log CFU/mL. Survival rate (SR) was calculated using the following formula:

$$\mathrm{SR}\% = \frac{\mathrm{Log\ CFU/mL(finale)}}{\mathrm{Log\ CFU/mL(initial)}} \times 100, \quad (1)$$

where initial and final mean the viable cells (CFU/mL) at 0 h and after 3 h of incubation.

2.3.2. Resistance to Pepsin and Low pH

The capacity of LAB isolates to tolerate pepsin in acid media was tested following the method proposed by Diguță et al. [36], with few modifications. The simulated gastric juice was formulated by combining 0.128% sodium chloride (NaCl), 0.0239% potassium chloride (KCl), and 0.64% sodium bicarbonate ($NaHCO_3$) (Central Drug House, Mumbai, India) with 0.1% (*w*/*v*) pepsin (Himedia, Mumbai, India). The pH was then adjusted to 2.5. Overnight LAB cells were collected via centrifugation at 2000× *g* for 10 min and washed twice in sterile physiological saline (0.9% NaCl). The cells were then suspended in simulated gastric juice. Samples were taken at 0 and 3 h, serially diluted, and plated on MRS agar plates. Viable cells were quantified using the plate count method. The formula employed to calculate the survival rate in simulated gastric juice is identical to that used to assess acid pH resistance.

2.3.3. Tolerance to Bile Salts

The methodology proposed by Coulibaly et al. [28] was employed to assess the ability of LAB strains to withstand the effects of bile salts. The LAB strains were prepared as previously described. Cell pellets were inoculated in MRS broth supplemented with 0.3% (*w*/*v*) bile salts and then incubated for 4 h at 37 °C. Viable cells were quantified using

the plate count method, with results expressed as log CFU/mL. The survival rate was performed according to the following formula:

$$SR\% = \frac{\text{Log CFU/mL(final)}}{\text{Log CFU/mL(initial)}} \times 100, \quad (2)$$

where initial and final mean the viable cells (CFU/mL) at 0 h and after 4 h of incubation.

2.3.4. Phenol Tolerance

The ability of LAB isolates to survive in the presence of a phenol solution was investigated using a methodology described by Xanthopoulos et al. [37]. Overnight LAB cultures were inoculated in MRS broth containing 0.4% phenol and incubated for 24 h at 37 °C. Viable cells were subsequently quantified via the plate count method.

2.4. Safety Assessment

2.4.1. Hemolysis Test

The hemolytic potential of LAB isolates was performed by the methodology described by Yadav et al. [38]. Bacterial strains were inoculated onto blood agar plates (Oxoid, Basingstoke Hampshire, UK) supplemented with 5% (*w*/*v*) sheep blood and incubated at a temperature of 37 °C for 48 h. If the area around the bacterial growth was clear, it can be assumed that the bacterium had caused β-hemolysis. Conversely, if the area had turned greenish, this would indicate that the tested bacterium had produced α-hemolysis. It should be noted that γ-hemolysis is the term used to describe the absence of hemolysis or blood cell degradation when a microorganism is present.

2.4.2. Antibiotic Sensitivity

The antibiotic spectrum of the LAB isolates was evaluated using the disc diffusion method with antibiotics aligned with those recommended by the European Food Safety Authority (EFSA). This included chloramphenicol (30 µg/disc); tetracycline (30 µg/disc); erythromycin (10 µg/disc); lincomycin (10 µg/disc); ampicillin ((10 µg/disc); amoxicillin + clavulanic acid (20/10 µg/disc); penicillin (2 µg/disc); nitrofurantoin (300 µg/disc); trimethoprim + sulfamethoxazole (1/19 µg/disc). A 100 µL aliquot of each fresh LAB culture was spread onto MRS agar plates and allowed to dry. Subsequently, antibiotic discs were placed on the inoculated plates and incubated for 48 h at 37 °C. The diameter of the clear zone surrounding each disc was measured in millimeters to determine the antibiotic susceptibility of the isolates. Subsequently, the results were interpreted following the established guidelines set by the Clinical and Laboratory Standards Institute (CLSI) [39]. Isolates exhibiting diameters ≥20 mm were classified as susceptible (S), while those with diameters in the range of 15–20 mm were classified as intermediate (I), and those with diameters <15mm were classified as resistant (R).

2.5. Cell Surface Characteristics

2.5.1. Co-Aggregation Test

The co-aggregation capacities were evaluated using the methodology proposed by Collado et al. [40]. Overnight LAB cultures were harvested via centrifugation at 4000 rpm for 10 min and adjusted to a concentration of 10^8 CFU/mL using PBS buffer (VWR International, Rosny-sous-Bois, France). The target pathogens employed were *Escherichia coli* ATCC 8739 and *Salmonella enterica* serovar Typhimurium ATCC 14028. The indicator pathogens were cultivated in Luria–Bertani broth (Tulip Diagnostics (P) Ltd., Verna, Goa, India) overnight and prepared as described above. The LAB cell suspension (2 mL) was combined with a corresponding volume of pathogenic bacteria suspension. This mixture

was vortexed for 10 s to ensure thorough homogenization. The mixture was then incubated at 37 °C for 4 h. The co-aggregation capacity was expressed as follows:

$$\% \text{ co-aggregation} = \frac{\text{Amix0} - \text{Amix}}{\text{Amix0}} \times 100, \quad (3)$$

where Amix0 represents the absorbance of the bacterial mixture at t = 0, while Amix represents the absorbance of the same mixture after 4 h of incubation.

2.5.2. Cell Surface Hydrophobicity

The cell surface hydrophobicity was determined according to the method developed by Rosenberg et al. [41], with a few modifications. For this purpose, bacterial cells in the stationary phase were centrifuged at 10,000× *g* for 5 min, washed twice with phosphate-buffered saline (PBS: 130 mM sodium chloride, 10 mM sodium phosphate, pH 7.2), and then resuspended in PBS buffer. The absorbance (A0) was measured at 600 nm and standardized to 0.25 ± 0.05. A 3 mL portion of the cell suspension and 1 mL of hydrocarbon (xylene) were mixed and vigorously vortexed for 2 min. Following this, the suspension was incubated at 37 °C for one hour without the application of any shaking to facilitate the separation of the aqueous and organic phases. Subsequently, the aqueous phase was carefully gathered, and the absorbance was quantified at 600 nm (A1). The percentage of bacterial adhesion to the solvent was calculated using the following formula:

$$\% \text{ cell surface hydrophobicity} = \frac{\text{A1}}{\text{A0}} \times 100. \quad (4)$$

2.5.3. Auto Aggregation Test

The capacity of LAB isolates to aggregate spontaneously was evaluated using the methodology delineated by Rosenberg et al. [41]. The overnight culture was collected via centrifugation at 4000 rpm for 10 min at 4 °C, washed twice with PBS and resuspended in PBS buffer. The LAB suspensions were incubated at 37 °C for 24 h. The optical density of the bacterial suspension was measured at 600 nm. The percentage of auto-aggregation was calculated using the following equation:

$$\% \text{ auto-aggregation} = 1 - \frac{\text{At}}{\text{A0}} \times 100, \quad (5)$$

where At is the absorbance after 24 h, and A0 is the absorbance t = 0.

2.6. Study of the Probiotic Properties of Lactic Acid Bacteria

2.6.1. Antibacterial Activity

The antibacterial activity of LAB isolates was tested against four reference pathogens, including Gram-positive bacteria (*Listeria monocytogenes* ATCC 7644 and *Staphylococcus aureus* ATCC 33592) and Gram-negative bacteria (*Escherichia coli* ATCC 8739 and *Salmonella enterica* serovar Typhimurium ATCC 14028). This was conducted using the agar well diffusion method, as described by Balouiri et al. [42], with minor modifications. LAB isolates were cultivated in MRS broth at 37 °C for 48 h. Subsequently, the cell-free supernatants (CFSs) were obtained from the LAB isolates via centrifugation at 10,000 rpm for 10 min at 4 °C and filtration through sterile 0.22-micron Millipore filters (VWR International, Rosny-sous-Bois, France). An overnight pathogenic culture was prepared by adjusting the optical density at 600 nm (OD600 nm) to 0.2 ± 0.05 units (corresponding to a concentration of approximately 10^7 to 10^8 CFU/mL). The next stage was to transfer 1 mL of the pathogenic suspension to a sterile Petri plate (90 mm) and covered with 20 mL of TSA

(Scharlab S.L., Barcelona, Spain), which was cooled to 45 °C. The suspension was then homogenized gently until solidified. A sterile tip was employed in an aseptic manner to puncture 6 mm-diameter wells. Thereafter, a volume of 100 µL of the CFSs was added to each well. The plate was incubated at 37 °C for 24 h. The presence of a clear zone of 1 mm or more surrounding each well confirmed positive inhibition, which indicates antibacterial activity.

2.6.2. DPPH Free Radical Scavenging Activity

The DPPH free radical scavenging activity was assessed following the method outlined by Brand-Williams et al. [43]. Specifically, 0.2 mL of a freshly prepared solution of DPPH (0.2 mM) in ethanol was added to 0.8 mL of the sample solution (supernatant or intact cells). The reaction mixture was thoroughly vortexed and then incubated at room temperature in the dark for 30 min. The measurement of absorbance took place at 517 nm against an ethanol-containing blank. The DPPH solution-free sample was used as a positive control. The scavenging capacity is then calculated as follows:

$$\% \text{ Scavenger effect} = \frac{\text{Acontrol} - \text{Asample}}{\text{Acontrol}} \times 100. \quad (6)$$

2.6.3. Plate Screening of LAB Isolates for Hydrolytic Enzymes

The enzymatic patterns were assessed by inoculating LAB strains as spots on Luria agar (Tulip Diagnostics (P) Ltd., Verna, Goa, India) supplemented with different carbon sources such as 2% of soluble starch (amylase), 2% carboxymethylcellulose (cellulase), 2% olive oil (lipase), 2% and pectin (pectinase), as described by Coulibaly et al. [28] and Proca et al. [44] with slight modifications. To detect protease activity, skim milk (0.1% fat) was added in a 1:2 ratio with water (*v*/*v*) and 2% agar. The prepared plates were then incubated at 37 °C for 48 h. Amylase activity was revealed using Lugol solution (Tody Laboratories Int. S.R.L., Bucharest, România). A clear zone surrounding the bacterial isolate indicated a positive reaction. Cellulase activity was visualized as a clear halo surrounding bacterial growth by staining with a 0.1% Congo red solution (VWR Bdh Chemicals, Leuven, Belgium) and then washed with 1 M NaCl. A precipitation zone around the colonies was identified as lipase producers. Bacterial strains that developed a clear halo after flooding with Lugol solution indicated the presence of pectinase activity. Bacterial isolates that showed a clear zone of casein degradation were considered positive for protease production.

2.7. Statistical Analysis

The experiments were conducted in triplicate, and the results were expressed as the mean ± standard deviation. Excel 2016 was used for calculations, figures, and box plots. To select the best isolates, principal component analysis (PCA) and ascending hierarchical classification (AHC) were performed using XLStat (Version 2016) software.

3. Results

3.1. Identification of LAB Isolates

Ten distinct colonies were isolated from four dockounou-fermented pastes using classical methods. After growing on the MRS agar surface, the LAB isolates displayed smooth, round, cream-white colonies. Phenotypic characterization revealed rod-shaped cells that were Gram-positive and catalase-negative, as well as oxidase-negative (Table 1).

Sequence homology was demonstrated to range from 98% to 99% with 16S rDNA sequences from reference strains within the NCBI database. This led to the identification of the LAB isolates as belonging to the *Lactiplantibacillus plantarum*. A phylogenetic tree was created using MEGA (Molecular Evolution Genetic Analysis) software, version Xto display identities and relationships of representative and related standard strains (Figure 1). The partial 16S rDNA sequences from the LAB strains have been submitted to the NCBI database under accession codes PP196396 (*Lactiplantibacillus plantarum* FS43P4), PP196397 (*L. plantarum* FS44P4), PP196398 (*L. plantarum* FS45P4), PP196399 (*L. plantarum* FS46P4),

PP196400 (*L. plantarum* FS47P4), PP196401 (*L. plantarum* FS48P4), PP196402 (*L. plantarum* FS49P4), PP196403 (*L. plantarum* FS50P4), PP196404 (*L. plantarum* FS51P4), and PP196405 (*L. plantarum* FS65P4).

Table 1. Phenotypic characteristics of the LAB strains used in this study.

Isolates	Parameters					Origin of Isolate
	Shape	Gram Reaction	Cell	Catalase Test	Oxidase Test	
FS43	Smooth	+	rod-shape	-	-	Rice dockounou paste
FS51	Round	+	rod-shape	-	-	Rice dockounou paste
FS50	Smooth	+	rod-shape	-	-	Maize dockounou paste
FS49	Round	+	rod-shape	-	-	Maize dockounou paste
FS65	Smooth	+	rod-shape	-	-	Maize dockounou paste
FS48	Smooth	+	rod-shape	-	-	Maize dockounou paste
FS47	Round	+	rod-shape	-	-	Millet dockounou paste
FS46	Round	+	rod-shape	-	-	Millet dockounou paste
FS45	Smooth	+	rod-shape	-	-	Millet dockounou paste
FS44	Round	+	rod-shape	-	-	Cassava dockounou paste

Figure 1. Molecular phylogenetic analysis via maximum parsimony analysis of taxa.

3.2. Safety Criteria of LAB Strains

When assessing the safety of potential probiotic strains *in vitro*, antibiotic resistance and hemolytic activity are key criteria (refer to Table 2). All LAB strains that were tested were susceptible to the amoxicillin–clavulanic acid complex, nitrofurantoin, chloramphenicol, ampicillin, and sulfonamide–diaminopyrimidine complex. Only strain FS43 showed high susceptibility to the tetracycline class, while strains FS51, FS46, and FS44 showed intermediate susceptibility. Among the macrolide class, only strains FS50, FS47, and FS44 demonstrated resistance to lincomycin. Concerning erythromycin, strains FS50, FS47, and FS46 were susceptible when the diameter was over 20 mm, whereas strains FS65, FS48, FS45, FS44, FS51, and FS49 showed intermediate susceptibility. In the presence of penicillin, only strain FS65 was resistant, while the other strains showed either intermediate or complete sensitivity. The complex of sulphonamide and diaminopyrimidine exhibited intermediate sensitivity only in FS43, whereas the other strains were sensitive to this antibiotic. Additionally, none of the selected strains demonstrated hemolytic activity (refer to Table 2).

Table 2. Antibiotic sensibility and Hemolysis activity of LAB strains.

Strains / Class/Antibiotics		FS43	FS51	FS50	FS49	FS65	FS48	FS47	FS46	FS45	FS44
Chloramphenicol	CHL	20S	24S	24S	28S	24S	24S	26S	25S	24S	29S
Tetracyclin	T	20S	16I	14R	12R	12R	14R	12R	16I	14R	18I
Macrolides	E	12R	18I	20S	19I	18I	18I	21S	20S	18I	16I
	L	26S	24S	0R	26S	20S	26S	0R	28S	30S	0R
Beta-lactam	AM	20S	24S	26S	22S	20S	20S	24S	22S	22S	20S
	AMC	18S	20S	26S	30S	30S	28S	34S	32S	28S	26S
	P	16I	18I	18I	16I	14R	20S	20S	18I	20S	16I
Nitrofuran	F	22S	28S	22S	26S	22S	25S	28S	29S	28S	24S
Sulfamide + diaminopyrimidine	SXT	18I	20S	20S	22S	20S	22S	20S	20S	20S	20S
Hemolysis activity		γ	γ	γ	γ	γ	γ	γ	γ	γ	γ

CHL: Chloramphenicol (30 µg/disc); T: Tetracycline (30 µg/disc); E: Erythromycin (10 µg/disc); L: Lincomycin (10 µg/disc); AM: Ampicillin ((10 µg/disc); AMC: Amoxicillin + clavulanic acid (20/10 µg/disc); P: Penicillin (2 µg/disc); F: Nitrofurantoin (300 µg/disc); SXT: Trimethoprim + sulfamethoxazole (1/19 µg/disc). S: sensitive; I: intermediate; R: resistant; γ—gamma hemolysis.

3.3. Exploration of Probiotic Properties

3.3.1. Resistance to Simulated Gastrointestinal Tract Conditions

This study found that the survival of LAB strains varied depending on the stress conditions. Tolerance to high acidity levels (pH 1.5) in the stomach is a crucial prerequisite for LAB isolates to function as probiotics. As shown in Table 3, strain FS49 had the lowest survival rate (81.18 ± 1.05%), while strain FS65 had the highest (98.15 ± 0.95%). Furthermore, all LAB strains in this study demonstrated excellent growth at pH 2.5 and 0.1% pepsin, as shown in Table 3. The results indicate that LAB isolates exhibit remarkable resilience to harsh gastric conditions. Upon exposure to 0.4% phenol, the majority of strains (FS43, FS44, FS45, FS46, FS47, FS48, and FS49) demonstrated a survival rate exceeding 70%. Following exposure to 0.3% bile salts for 4 h, all LAB strains tested exhibited excellent survival rates, ranging from 68.62 ± 3.64% (FS46 strain) to 100.89 ± 3.52% (FS44 strain).

Table 3. Resistance of LAB strains to simulated gastrointestinal conditions.

	Survival Rate (%)			
Isolates	**pH 1.5**	**Pepsin (0.1%)/pH 2.5**	**Phenol (0.4%)**	**Bile Salts (0.3%)**
FS43	84.35 ± 0.85 ^{d}	91.58 ± 3.43 ^{def}	94.58 ± 3.70 ^{c}	82.25 ± 0.75 ^{c}
FS51	83.33 ± 0.91 ^{d}	89.98 ± 1.81 ^{ef}	40.59 ± 2.80 ^{g}	93.09 ± 1.25 ^{b}
FS50	87.05 ± 1.95 ^{c}	94.88 ± 2.41 ^{bcd}	44.12 ± 3.01 ^{fg}	99.58 ± 2.07 ^{a}
FS49	81.18 ± 1.05 ^{e}	91.45 ± 1.78 ^{def}	85.12 ± 6.60 ^{d}	79.63 ± 3.90 ^{cd}
FS65	98.15 ± 0.95 ^{a}	95.60 ± 0.84 ^{bc}	48.09 ± 2.80 ^{f}	77.791 ± 0.40 ^{de}
FS48	88.30 ± 0.91 ^{c}	99.78 ± 2.14 ^{a}	128.24 ± 1.08 ^{a}	77.49 ± 0.59 ^{de}
FS47	91.38 ± 0.88 ^{b}	92.63 ± 1.78 ^{cde}	112.64 ± 1.60 ^{b}	75.32 ± 2.15 ^{e}
FS46	90.74 ± 1.80 ^{b}	97.96 ± 0.84 ^{ab}	73.72 ± 0.78 ^{e}	68.62 ± 3.64 ^{f}
FS45	85.27 ± 0.57 ^{d}	92.44 ± 1.88 ^{cde}	90.22 ± 0.44 ^{cd}	74.18 ± 1.15 ^{e}
FS44	85.03 ± 0.98 ^{d}	88.54 ± 1.26 ^{f}	109.46 ± 0.51 ^{b}	100.89 ± 3.52 ^{a}

Each value represents the mean of three replicates, accompanied by their respective standard deviations. Values bearing the same lowercase letter (a, b, c, d, e, etc.) are not statistically significantly different at the 5% level within the same column.

3.3.2. Cell Surface Characteristics

The study of co-aggregation, auto-aggregation, and hydrophobicity offers insights into the adhesion and colonization behavior of LAB strains in the host intestinal tract. The co-aggregation rates of LAB strains with *S.* Typhimurium were significantly higher than those with *E. coli* (Figure 2). All LAB strains showed high co-aggregation rates (>55%)

against *S.* Typhimurium. In contrast, *E. coli* showed the lowest percent co-aggregation with FS47 (20.80 ± 1.22%) and the highest with FS46 (50.89 ± 1.00%). Additionally, the FS46 strain exhibited high co-aggregation with *S.* Typhimurium (71.78 ± 0.85%) (Figure 2).

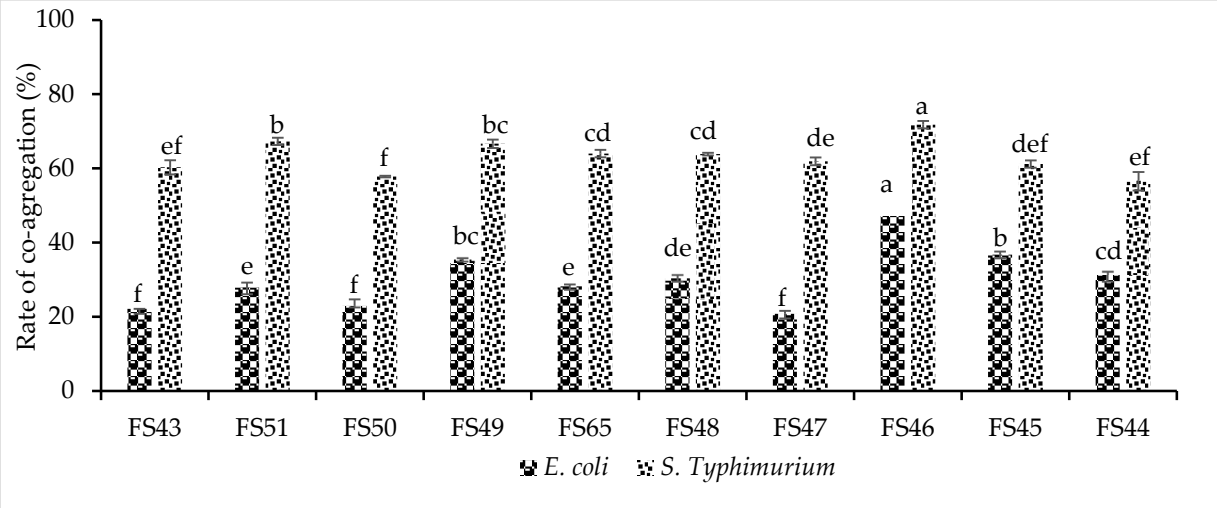


Figure 2. Co-aggregation rates of LAB strains with *E. coli* ATCC 8739 and *S.* Typhimurium ATCC 14028. Each value was expressed as the mean ± standard deviation (SD). Statistically significant differences ($p < 0.05$) were indicated by different lowercase letters above the error bars.

The functional ability of LAB strains to adhere to epithelial cells is closely linked to their auto-aggregation ability and cell surface hydrophobicity (Figure 3). The strains FS47, FS49, and FS44 demonstrated a relatively high hydrophobicity in xylene, exceeding 57%. Conversely, strain FS43 showed minimal hydrophobicity (35.23 ± 4.43%). All LAB strains demonstrated a significant level of auto-aggregation (>50%) after 24 h of incubation. Four LAB strains (FS47, FS48, FS45, and FS46) exhibited exceptionally high auto-aggregation, resulting in the formation of a visible precipitate at the bottom of the tubes.

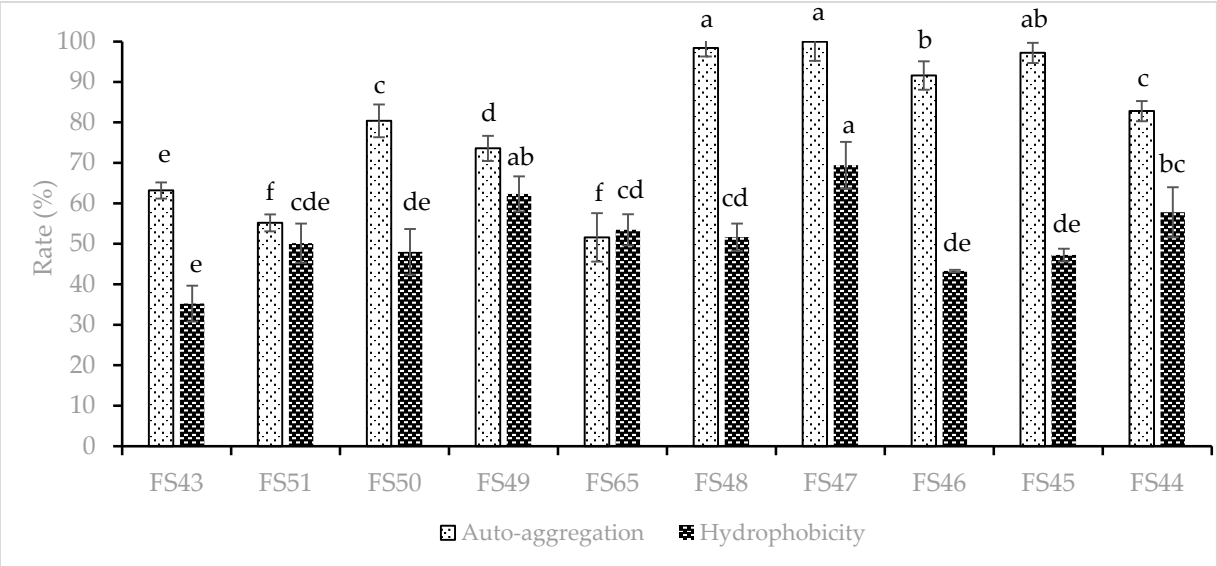


Figure 3. Auto-aggregation and hydrophobicity rates of LAB strains. Each value was expressed as the mean ± standard deviation (SD). Statistically significant differences ($p < 0.05$) were indicated by different lowercase letters above the error bars.

3.4. Exploration of Probiotic Properties

3.4.1. Antibacterial Spectrum

The antibacterial activity of the CFSs from the LAB strains against four pathogenic species is shown in Table 4. The LAB strains exhibited high antagonist activity against *S. aureus* ATCC 33592, *L. monocytogenes* ATCC 7644, and *S. enterica* serovar Typhimurium ATCC 14028, with inhibition diameters between 18 and 29 mm. Strains FS43, FS65, FS48, FS46, and FS44 demonstrated significant antibacterial activity against *E. coli* ATCC 8739. However, strains FS51, FS50, FS49, FS47, and FS45 demonstrated moderate antibacterial activity against *E. coli*, with inhibition diameters between 6 and 10 mm.

Table 4. Antimicrobial activity of LAB strains.

Isolates	*E. coli* ATCC 8739	*L. monocytogenes* ATCC 7644	*S. enterica* Serovar Typhimurium ATCC 14028	*S. aureus* ATCC 33592
FS43	+++	+++	+++	+++
FS51	++	+++	+++	+++
FS50	++	+++	+++	+++
FS49	++	+++	+++	+++
FS65	+++	+++	+++	+++
FS48	+++	+++	+++	+++
FS47	++	+++	+++	+++
FS46	+++	+++	+++	+++
FS45	++	+++	+++	+++
FS44	+++	+++	+++	+++

Legend: (-) the absence of a halo formation; (+) the presence of a halo measuring 1–5 mm in diameter; (++) the presence of a halo measuring 5–10 mm in diameter; (+++) the presence of a halo measuring >10 mm in diameter.

3.4.2. Antioxidant activity

The DPPH assay was used to evaluate the antioxidant capacity of both intact LAB cells and their supernatants (see Figure 4). Both the intact LAB cells and supernatants of the FS48 strain showed statistically significant antioxidant activity levels ($p < 0.05$). Except for the FS44 strain, the antioxidant activity levels detected in the intact cells were significantly higher than those in the free cell supernatants, as shown in Figure 4. Seven strains (FS46, FS45, FS49, FS43, FS65, FS47, and FS48) exhibited DPPH free radical scavenging rates ranging from 51.64% to 83.58%, while the remainder of strains showed rates below 50%. Overall, the CFS from strain FS65 showed the lowest antioxidant activity (13.87 ± 0.44%), while the CFS from strain FS48 showed the highest antioxidant activity (49.09 ± 0.88%).

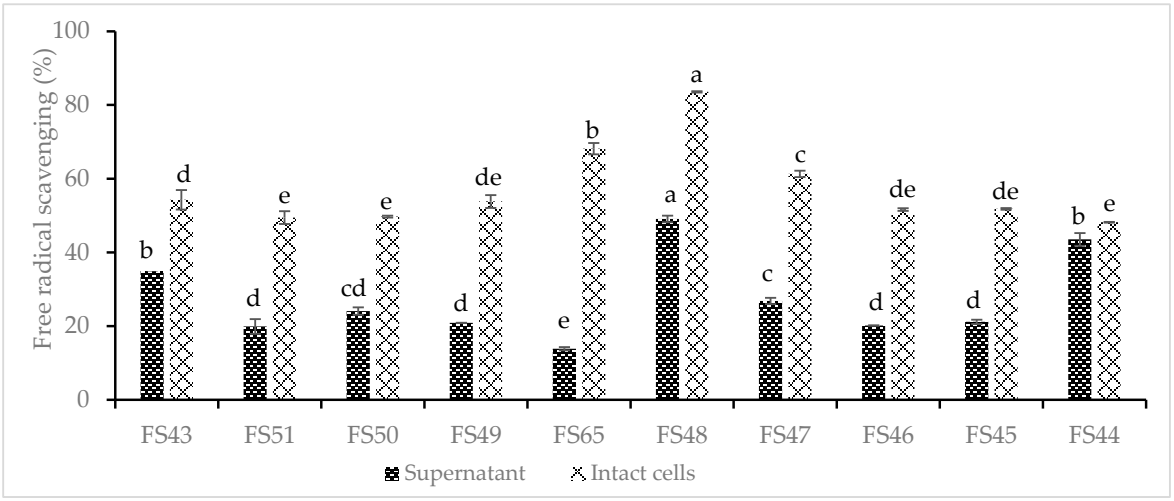


Figure 4. Antioxidant capacity of free cell supernatants and intact LAB cells evaluated via DPPH

assay. Each value was expressed as the mean ± standard deviation (SD). Statistically significant differences ($p < 0.05$) were indicated by different lowercase letters above the error bars.

3.4.3. Enzymatic Activity of LAB Strains

The LAB isolates were inoculated on selective media to detect amylase, cellulase, lipase, pectinase, and protease activities (refer to Table 5). The results showed that all LAB strains tested were positive for protease and cellulase activities. Out of the ten strains tested, only FS43 exhibited positive lipolytic activity. Eight LAB strains demonstrated positive pectinolytic activity. Furthermore, five strains (FS51, FS49, FS65, FS48, and FS45) showed positive amylase activity, while the others did not.

Table 5. Enzymatic activities of LAB strains.

Parameters / Strains	Enzymatic Activities				
	Proteolytic	**Lipolytic**	**Pectinolytic**	**Cellulolytic**	**Amylolytic**
FS43	+	+	+	+	-
FS51	+	-	+	+	+
FS50	+	-	+	+	-
FS49	+	-	-	+	+
FS65	+	-	+	+	+
FS48	+	-	-	+	+
FS47	+	-	+	+	-
FS46	+	-	+	+	-
FS45	+	-	+	+	+
FS44	+	-	+	+	-

The (+) symbol indicates that the enzymatic activity is positive, while the (-) symbol indicates that the enzymatic activity is negative.

3.5. Principal Component Analysis (PCA)

Principal component analysis (PCA) and hierarchical ascending classification (HAC) are powerful methods for synthesizing diverse information to enhance comprehension. The analysis results indicated that the combined influence of the two principal components (F1 and F2) explained 77.51% of the total variation. Of this, 65.03% was attributed to component F1 and 12.48% to component F2 (Figure 5). According to the PCA, based on the probiotic attributes of the LAB isolates, they could be divided into four distinct groups. The first group comprises strains FS43, FS44, and FS48 (positive correlation of both F1 and F2) and exhibited the highest values for hydrophobicity, phenol tolerance, antioxidant activity of the free cell supernatants, and intact cells (Figure 5). These three strains were classified as belonging to the same class based on the hierarchical ascending categorization of similarities (Figure 6). The second group consists of the FS50 and FS51 strains (positive side of F2 and negative side of F1) and exhibited high values for 0.3% bile salt tolerance. The third group comprises strains FS65, FS49, and FS46 (negative of both F1 and F2) and exhibited high values for pH 1.5 tolerance and co-aggregation ability with *S*. Typhimurium. In the fourth group, the strains FS45 and FS47 (positive side of F1 and negative side of F2, respectively) exhibited high values for pH 2.5 and 0.1% pepsin resistance, co-aggregation with *E. coli*, and auto-aggregation abilities, as well as a range of other probiotic properties. The results indicated that the strains FS43, FS44, and FS48 demonstrated the greatest probiotic potential.

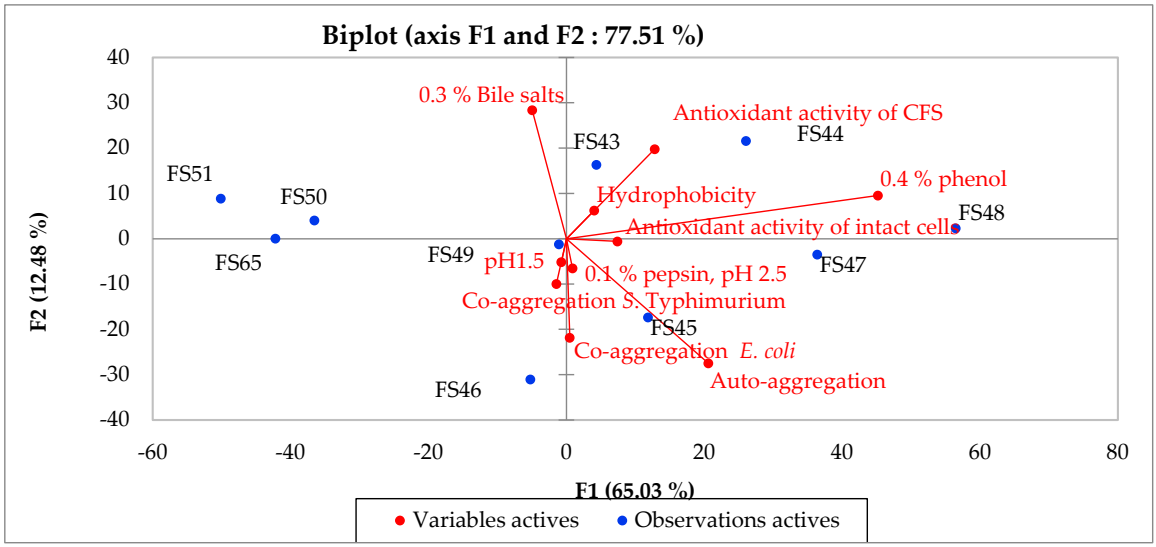


Figure 5. Principal component analysis of LAB strains according to probiotic properties.

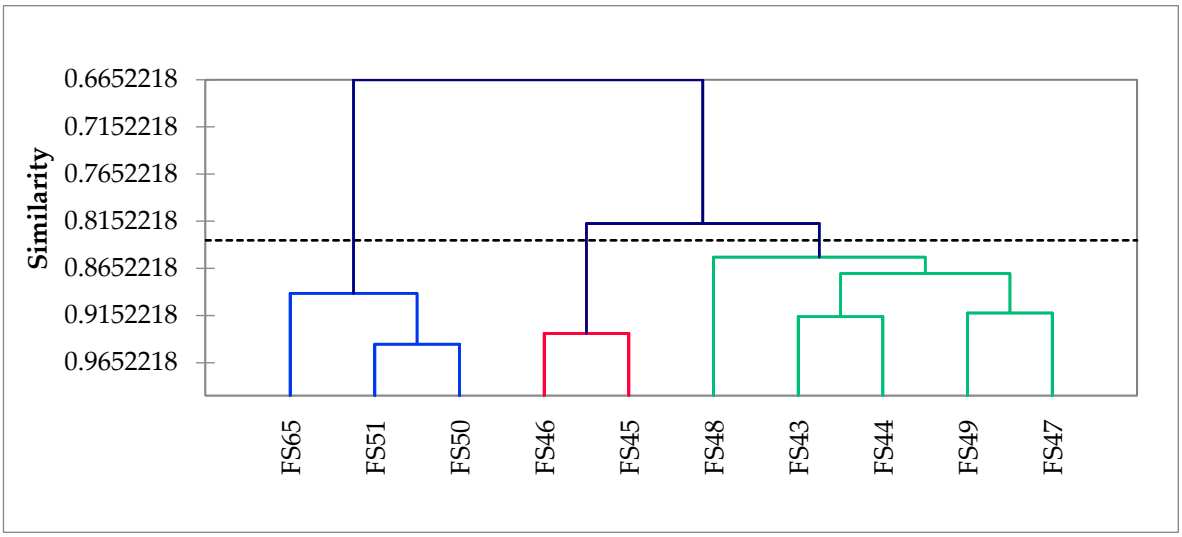


Figure 6. Hierarchical ascending classification of LAB strains according to probiotic properties. The dotted line delineates the cutoff point of this specific algorithm, which generates three clusters (indicated by distinct colors) based on probiotic characteristics that exhibit a high degree of similarity.

4. Discussion

Africa has a long history of producing traditional fermented foods in local dietary contexts, including those derived from cassava, maize, millet, tubers, wild legume seeds, and sorghum [6–9]. Additionally, fermented milk, meat products, and alcoholic beverages have been produced on the continent for centuries [6–9]. Given that a significant proportion of African foods are fermented by lactic acid bacteria, they can be isolated and studied for their multifunctional ability to ferment foods, impart distinct taste and high nutritional value, and ensure food safety over an extended period. Additionally, they can be studied for their probiotic properties, which could have beneficial health effects. This process also contributes to an enhanced health status in those who consume them, which is of great importance in the African context [8]. The review by Pereira et al. [26] provides a comprehensive overview of the criteria and the methodologies employed for the selection of probiotics, such as their ability to tolerate unfavorable conditions in the human digestive system, adhere to human body cells, exhibit antimicrobial and antioxidant

activities, synthesize enzyme, demonstrate antibiotic sensitivity, undergo hemolysis test, and fulfill other pertinent criteria.

This study aimed to assess the probiotic potential of 10 bacterial isolates obtained from the spontaneous fermentation of dockounou paste. The new isolates were preliminarily identified as lactic acid bacteria based on their colonial aspects (shape round or smooth), microscopic observations (bacilli, Gram-positive), and biochemical characteristics (negative oxidase and catalase). All LAB strains were identified as *L. plantarum* based on the high degree of homology observed between their 16S rDNA gene sequences and those deposited in the NCBI database. *Lactobacillus* spp. (particularly *L. plantarum* and *L. fermentum)* have been predominantly detected in various African fermented foods such as *doklu* (Ivorian maize dough) [12], Nigerian fermented foods [45–47], Maasai traditional fermented milk products [48], *ben-saalga* (Burkinabé fermented cereal gruel) [49]; *bushera* (Ugandan fermented cereal beverage) [50], *tchapalo* produced in Ivory Coast [51].

The paramount concern regarding probiotics must be their safety, which must not harm consumer health. The absence of hemolytic activity indicated that our LAB strains are unable to degrade blood constituents and, therefore, pose no pathological risk. Coulibaly et al. [28] also reported that *L. plantarum* and two other *Pediococcus* sp. isolated from the gut of Tilapia showed no hemolytic activity. The EFSA suggests that all microbial strains used as food or feed additives or probiotics should be subjected to testing to determine their sensitivity to different antibiotics [52]. Our findings have revealed that the majority of LAB strains demonstrated high or intermediate susceptibility to at least one antibiotic from the beta-lactam class (which inhibits cell wall synthesis) and the macrolide class (which inhibits protein synthesis). Furthermore, all strains demonstrated sensitivity to chloramphenicol, nitrofurans, and sulfonamide–diaminopyrimidine complex antibiotics. The overuse of antibiotics has resulted in the evolution and prevalence of resistant bacteria. Probiotics isolated from various dietary supplements or foods have demonstrated the ability to resist the effects of antibiotics [53–56]. Horizontal transfer of antibiotic-resistance genes from these probiotics to pathogenic bacteria residing in the intestinal tract could have significant health implications [54,55].

One of the key considerations when selecting LAB isolates as probiotics is their ability to withstand the challenges of the gastrointestinal tract. In our study, all LAB strains exhibited high survival rates, ranging between 81.18% and 91.38% in an acidic environment with a pH of 1.5. The LAB strains also demonstrated high survival rates, between 88.54% and 99.78%, in the presence of pepsin (0.1%) at a pH of 2.5. Furthermore, they exhibited high survival rates in the presence of bile salts (0.3%), ranging from 68.62 to 100.89%. Various strains of lactic acid bacteria demonstrated high survival rates, indicating their ability to withstand the acidic environment of the stomach (pH 1.5–3.0) and the small intestine (bile salts concentrations of 0.1–0.3%) [28,36,57]. Likewise, *Lactobacillus* spp. isolated from Shamita and Kocho (traditional Ethiopian beverages and foods) demonstrated that they can survive in extremely acidic conditions (greater than 80%) for 6 h [58]. Two *L. plantarum* strains, isolated from West African fermented cereals, exhibited comparable levels of acid resistance (pH 2.0) and tolerance to a bile salt concentration of 0.3% [47]. *L. plantarum* strains, isolated from traditional fermented milk of the Maasai in Kenya, demonstrated acid resistance at pH 2.0, with survival rates varying from 1% to 100% [48]. In a relatively recent study, Matei et al. [59] found that three strains of *Pediococcus pentosaceus* showed strong tolerance to high concentrations of bile salts (3% and 6%). It is widely accepted that the capacity of bacteria to resist phenol is an important indicator of their general viability. It has been observed that some bacterial species can synthesize harmful metabolites that can be deaminated by gut bacteria, creating toxic compounds with bacteriostatic properties such as phenol. Our results revealed that certain *L. plantarum* isolates exhibited greater resistance to phenol (0.4%) (73.72–128.24% viability) compared to others. For instance, *L. plantarum* strains varied in their ability to resist phenol, which had different effects on them [60]. *Lactobacillus* strains derived from traditional fermented foods, multigrain millet dosa batter, exhibited resistance to 0.2–0.8% phenols, maintaining viability at 53.3%–83.6% [61]. Fur-

thermore, in our study, three *L. plantarum* isolates demonstrated not only high resistance but also were able to grow in the presence of 0.4% phenol.

The interaction between microorganisms and epithelial cells is influenced by cell wall hydrophobicity [62–64]. This parameter can be employed to determine the adhesion capability of lactic acid bacteria to the solvent. High hydrophobicity indicates a strong interaction between LAB isolates and epithelial cells, resulting in enhanced pathogen exclusion. It is generally recommended to select a strain with a hydrophobicity of over 40% as a probiotic. The present study demonstrated that eight LAB strains exhibited hydrophobicity levels ranging from 47.23% to 69.43%. The results of this study are consistent with those of Yasmin et al. [65], who observed that *Bifidobacterium* strains exhibited high hydrophobicity when exposed to xylene. However, strains of *P. pentosaceus* and *L. plantarum* isolated from the gut of Tilapia exhibited a range of cell surface hydrophobicity in the presence of different organic solvents such as chloroform (9.4–87.2%), xylene (3.48–51.10%), and hexane (1.53–16.30%) [28]. This study demonstrated that *L. plantarum* strains exhibited a high degree of auto-aggregation (>50% at 24 h), indicating that they exhibited an adequate level of adherence, which is in agreement with previously reported results [65]. Also, the co-aggregation rate of LAB isolates was notably high with *S*. Typhimurium (57.80–71.78%), followed by *E. coli* (20.80–50.89%). Honey and Keerthi [66] observed that *L. plantarum* strains exhibited excellent auto-aggregation ability, with values ranging from 99.2% to 99.8%. The highest levels of co-aggregation with *S. typhi* were also noted, with values between 45.3% and 63.66% [66].

Lactic acid bacteria are capable of suppressing the growth of pathogenic microorganisms through the synthesis of antimicrobial metabolites (e.g., short-chain fatty acids, bacteriocins, hydrogen peroxide (H_2O_2), and others) and by competing with those for nutrients, as well as by adhering to epithelial cells [67,68]. The current study found that all LAB isolates demonstrated strong inhibitory effects against *S. aureus*, *L. monocytogenes*, and *S. enterica* serovar Typhimurium, with moderate effects against *E. coli*. These findings are consistent with prior research indicating the antagonist activity of *Lactobacillus* and *Pediococcus* strains against a wide range of bacterial pathogens, including those belonging to the Gram-negative and Gram-positive categories, which may contaminate food sources and cause intestinal illnesses [28,36,68–70].

Lactic acid bacteria have been extensively studied for their potential as probiotics, given their well-documented health benefits, which appear to be partially attributed to their antioxidant traits [71,72]. In the current study, most of the intact LAB cells exhibited high antioxidant activity, demonstrated by a high percentage reduction in DPPH with an average of 52% to 84%. The present study revealed a significant increase in antioxidant activity in intact cells compared to free-cell supernatants. These results contrast with those reported by Coulibaly et al. [28].

Lactobacilli have been identified as producers of enzyme complexes that enhance the digestibility of food and feed, as well as the activities of digestive enzymes and promote growth performance [73]. The evaluation of probiotic-producing enzymes can be conducted by cultivating candidate strains in culture media supplemented with precursors. These include casein, starch, carboxymethylcellulose, Tween 80 or olive oil, and sodium phytate, which are used to assess the activity of protease, amylase, cellulase, lipases, and phytases, respectively. The results of our study indicated that all LAB isolates possessed proteolytic and cellulolytic activities, with some also exhibiting pectinolytic or amylolytic activities. This result differs from the findings of Coulibaly et al. [28], in which the *P. pentosaceus* and *L. plantarum* strains isolated from the gut of Tilapia demonstrated lipase and β-galactosidase activity (with a few exceptions) but lacked amylase, cellulase, and protease activities. *L. fermentum* URLP18 isolated from *C. mrigala* demonstrated a high capacity for extracellular enzyme production, including amylase, protease, and lipase [74].

This study's results indicate that the isolation of lactic acid bacteria from naturally fermented and indigenous products is a topic worthy of further investigation. Nevertheless, strains FS43, FS44, and FS48 were identified as the most promising candidates for further

investigation, as they exhibited the greatest potential for beneficial effects, as indicated by principal component analysis (PCA) and hierarchical ascending classification (HAC). Artisanal fermented products are widely produced and consumed in traditional households and can be considered a valuable source of probiotics for improving the health of the Ivorian population.

5. Conclusions

The 10 lactic acid bacteria isolated from fermented plantain dockounou paste have been identified through 16S rDNA sequence analysis as *Lactiplantibacillus plantarum*. All LAB strains demonstrated high survival rate against pepsin (0.1%) and low pH (1.5 and 2.5) and exhibited high tolerance to bile salts (0.3%) and phenol (0.4%). Furthermore, all strains demonstrated high antioxidant activity and broad antibacterial activity against the main foodborne pathogens. The obtained findings indicated that three of the 10 LAB strains exhibited a high affinity towards xylene (more than 57%). All LAB strains exhibited high auto-aggregation properties, as well as strong degrees of co-aggregation with *S. enterica* serovar Typhimurium, followed by *E. coli*. Furthermore, the LAB isolates demonstrated no hemolytic activity and sensitivity to different antibiotic classes. The LAB isolates have been detected as valuable producers of bioactive multi-enzymes. Strains FS43, FS44, and FS48 exhibited multifunctional properties, suggesting that further *in vivo* research is required to assess their suitability as probiotics or for the development of innovative functional foods.

Author Contributions: Conceptualization, N.J.K., K.A.S.K., S.N. and F.C.D.; methodology, N.J.K., A.L.O.Z., K.A.S.K. and F.C.D.; software, N.J.K., K.A.S.K. and S.N.; validation, N.J.K., F.C.D., and F.M.; formal analysis, K.A.S.K., A.L.O.Z., and F.C.D.; investigation, N.J.K. and F.C.D.; resources, F.C.D. and F.M.; data curation, K.A.S.K., A.L.O.Z., and S.N.; writing—original draft preparation, N.J.K., A.L.O.Z. and S.N.; writing—review and editing, F.C.D. and F.M.; visualization, F.C.D. and F.M.; supervision, S.N., F.C.D. and F.M.; project administration, F.C.D. and F.M.; funding acquisition, N.J.K., K.A.S.K. and F.C.D. All authors have read and agreed to the published version of the manuscript.

Funding: The authors are grateful to AUF (Agence Universitaire de la Francophonie) for the scholarship granted to Natia Jo-seph Kouadio through the Eugen Ionescu program in its 2021 session at the University of Agronomic Sciences and Veterinary Medicine of Bucharest, Romania.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All obtained datasets are included in this publication.

Acknowledgments: Our thanks go to the Ivorian authorities of Felix Houphouet-Boigny University who authorized us to carry out this postdoctoral study in Bucharest, Romania. We thank the technician Badea Florentina from the Laboratory of Microbiology of the Faculty of Biotechnologies for his help, which was of great use to us.

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

References

1. Kra, K.A.S.; Akoa, E.E.F.; Mégnanou, R.M.; Yéboué, K.; Akpa, E.E.; Niamké, S.L. Physicochemical and nutritional characteristics assessments of two different traditional foods prepared with senescent plantain. *Afr. J. Food Sci.* **2013**, *7*, 51–55.
2. Akoa, E.E.F.; Kra, K.A.S.; Mégnanou, R.M.; Kouadio, N.J.; Niamké, L.S. Optimization of dockounou manufacturing process parameters. *Sustain. Agric. Res.* **2014**, *3*, 67–75.
3. Carboni, A.D.; Martins, G.N.; Gómez-Zavaglia, A.; Castilho, P.C. Lactic acid bacteria in the production of traditional fermented foods and beverages of Latin America. *Fermentation* **2023**, *9*, 315. [CrossRef]
4. Petrova, P.; Petrov, K. Lactic acid fermentation of cereals and pseudocereals: Ancient nutritional biotechnologies with modern applications. *Nutrients* **2020**, *12*, 1118. [CrossRef]
5. Sionek, B.; Szydłowska, A.; Küçükgöz, K.; Kołożyn-Krajewska, D. Traditional and new microorganisms in lactic acid fermentation of food. *Fermentation* **2023**, *9*, 1019. [CrossRef]
6. Kayitesi, E.; Onojakpor, O.; Moyo, S.M. Highlighting the impact of lactic-acid-bacteria-derived flavours or aromas on sensory perception of african fermented cereals. *Fermentation* **2023**, *9*, 111. [CrossRef]
7. Achi, O.K.; Asamudo, N.U. Cereal-based fermented foods of Africa as functional foods. *Bioact. Mol. Food* **2018**, 1–32.

8. Malongane, F.; Berejena, T. Exploring the microbiome present in fermented indigenous African foods and their potential impact on human health. *J. Agric. Food Res.* **2024**, *16*, 101101. [CrossRef]
9. Assohoun, M.C.N.; Djéni, T.N.; N'Guessan, F.K.; Koussemon, M. Preliminary study on antimicrobial properties of lactic acid bacteria involved in the fermentation of corn dough during *doklu* processing in Côte D'Ivoire. *Food* **2012**, *6*, 65–70.
10. Soro-Yao, A.A.; Kouakou, B.; Koffi-Nevry, R.; Djè, K.M. Microbiology of Ivorian fermented products: A review. *Asian J. Agric. Food Sci.* **2013**, *1*, 37–47.
11. Kouadio, N.J.; Goualie, G.B.; Ouattara, G.H.; Kra, K.A.S.; Niamke, L.S. Microorganisms associated with traditional plantain-based food "dockounou" during spontaneous fermentation. *Food Environ. Saf.* **2014**, *13*, 276–282.
12. Assohoun-Djeni, N.M.C.; Djeni, N.T.; Messaoudi, S.; Lhomme, E.; Koussemon-Camara, M.; Ouassa, T.; Chobert, J.M.; Onno, B.; Dousset, X. Biodiversity, dynamics and antimicrobial activity of lactic acid bacteria involved in the fermentation of maize flour for *doklu* production in Côte d'Ivoire. *Food Control* **2016**, *62*, 397–404. [CrossRef]
13. Mokoena, M.P.; Mutanda, T.; Olaniran, A.O. Perspectives on the probiotic potential of lactic acid bacteria from African traditional fermented foods and beverages. *Food Nutr. Res.* **2016**, *60*, 29630. [CrossRef] [PubMed]
14. Adesulu-Dahunsi, A.T.; Dahunsi, S.O.; Ajayeoba, T.A. Co-occurrence of *Lactobacillus* species during fermentation of african indigenous foods: Impact on food safety and shelf-life extension. *Front. Microbiol.* **2022**, *13*, 684730. [CrossRef] [PubMed]
15. Mogmenga, I.; Somda, M.K.; Ouattara, C.A.T.; Keita, I.; Dabiré, Y.; Diguță, C.F.; Toma, R.C.; Ezeogu, L.I.; Ugwuanyi, J.O.; Ouattara, A.S.; et al. Promising Probiotic Properties of the Yeasts Isolated from *Rabilé*, a Traditionally Fermented Beer Produced in Burkina Faso. *Microorganisms* **2023**, *11*, 802. [CrossRef] [PubMed]
16. Grosu-Tudor, S.; Stefan, I.; Stancu, M.; Cornea, C.; De vuyst, L.; Zamfir, M. Microbial and nutritional characteristics of fermented wheat bran in traditional Romanian borş production. *Rom. Biotechnol. Lett.* **2018**, *24*, 440–447. [CrossRef]
17. Constantin, E.A.; Constantinescu-Aruxandei, D.; Matei, F.; Shaposhnikov, S.; Oancea, F. Biochemical and microbiological characterization of traditional Romanian fermented drinks—Socata and borș—A review. *AgroLife Sci. J.* **2023**, *12*, 53–61. [CrossRef]
18. Ștefan, I.R.; Grosu-Tudor, S.S.; Zamfir, M.; Cornea, C.P. Screening for S-layer production by some lactobacilli from home-made fermented foods. *Sci. Bull. Ser. F Biotechnol.* **2016**, *XX*, 167–171.
19. Angelescu, I.R.; Zamfir, M.; Stancu, M.M.; Grosu-Tudor, S.S. Identification and probiotic properties of lactobacilli isolated from two different fermented beverages. *Ann. Microbiol.* **2019**, *69*, 1557–1565. [CrossRef]
20. Grosu-Tudor, S.S.; Zamfir, M.; Van der Meulen, R.; De Vuyst, L. Isolation of novel homopolysaccharide-producing lactic acid bacteria from Romanian raw milk and fermented dairy products. *Eur. Food Res. Technol.* **2013**, *237*, 609–615. [CrossRef]
21. Petruț, Ș.M.; Sârbu, I.; Corbu, V.M.; Pelinescu, D.; Iftime, I.; Dimov, T.V. Screening of lactic acid bacteria from spontaneously fermented products of Romania. *Rom. Biotechnol. Lett.* **2019**, *24*, 254–260. [CrossRef]
22. Grosu-Tudor, S.S.; Zamfir, M. Functional properties of lactic acid bacteria isolated from Romanian fermented vegetables. *Food Biotechnol.* **2013**, *27*, 235–248. [CrossRef]
23. Cornea, C.P.; Israel Roming, F.; Sicuia, O.A.; Voaideș, C.; Zamfir, M.; Grosu-Tudor, S.S. Biosurfactant production by *Lactobacillus* spp. strains isolated from Romanian traditional fermented food products. *Rom. Biotechnol. Lett.* **2016**, *21*, 11312–11320.
24. FAO/WHO. *Guidelines for the Evaluation of Probiotics in Food*; Food and Agriculture Organization of the United Nations and World Health Organization Working Group Report; FAO/WHO: London, ON, Canada, 2002.
25. Hill, C.; Guarner, F.; Reid, G.; Gibson, G.R.; Merenstein, D.J.; Pot, B.; Morelli, L.; Canani, R.B.; Flint, H.J.; Salminen, S.; et al. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* **2014**, *11*, 506–514. [CrossRef] [PubMed]
26. De Melo Pereira, G.V.; de Oliveira Coelho, B.; Magalhães Júnior, A.I.; Thomaz-Soccol, V.; Soccol, C.R. How to select a probiotic? A review and update of methods and criteria. *Biotechnol. Adv.* **2018**, *36*, 2060–2076. [CrossRef] [PubMed]
27. Chou, L.S.; Weimer, B. Isolation and characterization of acid- and bile-tolerant isolates from strains of *Lactobacillus acidophilus*. *J. Dairy Sci.* **1999**, *82*, 23–31. [CrossRef] [PubMed]
28. Coulibaly, W.H.; Kouadio, N.R.; Camara, F.; Diguță, C.; Matei, F. Functional properties of lactic acid bacteria isolated from Tilapia (*Oreochromis niloticus*) in Ivory Coast. *BMC Microbiol.* **2023**, *23*, 152. [CrossRef] [PubMed]
29. Meena, K.K.; Taneja, N.K.; Jain, D.; Ojha, A.; Kumawat, D.; Mishra, V. *In vitro* assessment of probiotic and technological properties of lactic acid bacteria isolated from indigenously fermented cereal-based food products. *Fermentation* **2022**, *8*, 529. [CrossRef]
30. Ayivi, R.D.; Gyawali, R.; Krastanov, A.; Aljaloud, S.O.; Worku, M.; Tahergorabi, R.; Silva, R.C.D.; Ibrahim, S.A. Lactic Acid Bacteria: Food Safety and Human Health Applications. *Dairy* **2020**, *1*, 202–232. [CrossRef]
31. Picard, C.; Fioramonti, J.; Francois, A.; Robinson, T.; Neant, F.; Matuchansky, C. Review article: *Bifidobacteria* as probiotic agents–physiological effects and clinical benefits. *Aliment. Pharmacol. Ther.* **2005**, *22*, 495–512. [CrossRef]
32. Chen, J.; Chen, X.; Ho, C.L. Recent development of probiotic bifidobacteria for treating human diseases. *Front. Bioeng. Biotechnol.* **2021**, *9*, 770248. [CrossRef] [PubMed]
33. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Mol Biol Evol.* **2018**, *35*, 1547–1549. [CrossRef] [PubMed]
34. Vauterin, L.; Vauterin, P. Computer-aided objective comparison of electrophoresis patterns for grouping and identification of microorganisms. *Eur. Microbiol.* **1992**, *1*, 37–41.

35. Carr, F.J.; Chill, D.; Maida, N. The lactic acid bacteria: A literature survey. *Crit. Rev. Microbiol.* **2002**, *28*, 281–370. [CrossRef] [PubMed]
36. Diguță, C.F.; Nițoi, G.D.; Matei, F.; Luță, G.; Cornea, C.P. The Biotechnological potential of *Pediococcus* spp. isolated from Kombucha microbial consortium. *Foods* **2020**, *9*, 1780. [CrossRef] [PubMed]
37. Xanthopoulos, V.; Litopoulou-Tzanetaki, E.; Tzanetakis, N. Characterization of *Lactobacillus* isolates from infant faeces as dietary adjuncts. *Food Microbiol.* **2000**, *17*, 205–215. [CrossRef]
38. Yadav, R.; Puniya, A.K.; Shukla, P. Probiotic Properties of *Lactobacillus plantarum* RYPR1 from an Indigenous Fermented Beverage Raabadi. *Front. Microbiol.* **2016**, *7*, 1683. [CrossRef] [PubMed]
39. CLSI. *CLSI Supplement M100S. Performance Standards for Antimicrobial Susceptibility Testing*, 26th ed.; Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2016.
40. Collado, M.C.; Meriluoto, J.; Salminen, S. Measurement of aggregation properties between probiotics and pathogens: *In vitro* evaluation of different methods. *J. Microbiol. Methods* **2007**, *71*, 71–74. [CrossRef] [PubMed]
41. Rosenberg, M.; Gutnick, D. and Rosenberg, E. Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol. Lett.* **1980**, *9*, 29–33. [CrossRef]
42. Balouiri, M.; Sadiki, M.; Ibnsouda, S.K. Methods for *in vitro* evaluating antimicrobial activity: A review. *J. Pharm. Anal.* **2016**, *6*, 71–79. [CrossRef]
43. Brand-Williams, W.; Cuvelier, M.; Berset, C. Use of a free radical method to evaluate antioxidant activity. *LWT* **1995**, *28*, 25–30. [CrossRef]
44. Proca, I.G.; Diguta, C.F.; Jurcoane, S.; Matei, F. Screening of halotolerant bacteria producing hydrolytic enzymes with biotechnological applications. *Sci. Bull. Ser. F Biotechnol.* **2020**, *XXIV*, 197–204.
45. Sanni, A.; Morlon-Guyot, J.; Guyot, J.P. New efficient amylase-producing strains of *Lactobacillus plantarum* and *L. fermentum* isolated from different Nigerian traditional fermented foods. *Int. J. Food Microbiol.* **2002**, *72*, 53–62. [CrossRef] [PubMed]
46. Adesulu-Dahunsi, A.T.; Sanni, A.I.; Jeyaram, K.; Banwo, K. Genetic diversity of *Lactobacillus plantarum* strains from some indigenous fermented foods in Nigeria. *LWT* **2017**, *82*, 199–206. [CrossRef]
47. Oguntoyinbo, F.A.; Narbad, A. Multifunctional properties of *Lactobacillus plantarum* strains isolated from fermented cereal foods. *J. Funct. Foods* **2015**, *17*, 621–631. [CrossRef]
48. Mathara, J.M.; Schillinger, U.; Kutima, P.M.; Mbugua, S.K.; Guigas, C.; Franz, C.; Holzapfel, W.H. Functional properties of *Lactobacillus plantarum* strains isolated from Maasai traditional fermented milk products in Kenya. *Curr. Microbiol.* **2008**, *56*, 315–321. [CrossRef] [PubMed]
49. Omar, N.B.; Abriouel, H.; Lucas, R.; Martínez-Cañamero, M.; Guyot, J.-P.; Gálvez, A. Isolation of bacteriocinogenic *Lactobacillus plantarum* strains from Ben Saalga, a traditional fermented Gruel from Burkina Faso. *Int. J. Food Microbiol.* **2006**, *112*, 44–50. [CrossRef] [PubMed]
50. Muyanja, C.; Narvhus, J.A.; Treimo, J.; Langsrud, T. Isolation, characterisation and identification of lactic acid bacteria from bushera: A Ugandan traditional fermented beverage. *Int. J. Food Microbiol.* **2003**, *80*, 201–210. [CrossRef] [PubMed]
51. Aka, S.; N'Guessan, K.F.; Nanga, Y.Z.; Loukou, Y.G.; Mazabraud, A.I.; Djè, K.M. Characterization of *Lactobacillus* species isolated from mash, sour wort and *tchapalo* produced in Côte d'Ivoire. *Food* **2010**, *4*, 49–54.
52. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Rychen, G.; Aquilina, G.; Azimonti, G.; Bampidis, V.; De Lourdes Bastos, M.; Bories, G.; Chesson, A.; Cocconcelli, P.S.; Flachowsky, G.; et al. Guidance on the characterisation of microorganisms used as feed additives or as production organisms. *EFSA J.* **2018**, *16*, e05206.
53. Sharma, P.; Tomar, S.K.; Goswami, P.; Sangwan, V.; Singh, R. Antibiotic resistance among commercially available probiotics. *Food Res. Int.* **2014**, *57*, 176–195. [CrossRef]
54. Wong, A.; Ngu, D.Y.S.; Dan, L.A.; Ooi, A.; Lim, R.L.H. Detection of antibiotic resistance in probiotics of dietary supplements. *Nutr. J.* **2015**, *14*, 95. [CrossRef] [PubMed]
55. Li, T.; Teng, D.; Mao, R.; Hao, Y.; Wang, X.; Wang, J. A critical review of antibiotic resistance in probiotic bacteria. *Food Res. Int.* **2020**, *136*, 109571. [CrossRef] [PubMed]
56. Dou, W.; Abdalla, H.B.; Chen, X.; Sun, C.; Chen, X.; Tian, Q.; Wang, J.; Zhou, W.; Chi, W.; Zhou, X.; et al. ProbResist: A database for drug-resistant probiotic bacteria. *Database* **2022**, *2022*, baac064. [CrossRef] [PubMed]
57. Lei, V.; Jakobsen, M. Microbiological characterization and probiotic potential of koko and koko sour water, African spontaneously fermented millet porridge and drink. *J. Appl. Microbiol.* **2004**, *96*, 384–397. [CrossRef] [PubMed]
58. Negasi, A.; Fassil, A.; Asnake, D. *In vitro* evaluation of lactic acid bacteria isolated from traditional fermented Shamita and Kocho for their desirable characteristics as probiotics. *Afr. J. Biotechnol.* **2017**, *16*, 594–606. [CrossRef]
59. Matei, B.; Salzat, J.; Diguță, C.F.; Cornea, C.P.; Luta, G.; Utoiu, E.R.; Matei, F. Lactic acid bacteria strains isolated from Kombucha with potential probiotic effect. *Rom. Biotechnol. Lett.* **2018**, *23*, 13592–13598.
60. Xanthopoulos, V.; Hatzikamari, M.; Adamidis, T.; Tsakalidou, E.; Tzanetakis, N.; Litopoulou-Tzanetaki, E. Heterogeneity of *Lactobacillus plantarum* isolates from Feta cheese throughout ripening. *J. Appl. Microbiol.* **2000**, *88*, 1056–1064. [CrossRef]
61. Divyashree, S.; Ramu, R.; Sreenivasa, M.Y. Evaluation of new candidate probiotic *Lactobacillus* strains isolated from a traditional fermented food- multigrain-millet dosa batter. *Food Biosci.* **2024**, *57*, 103450. [CrossRef]
62. Krausova, G.; Hyrslova, I.; Hynstova, I. *In Vitro* Evaluation of adhesion capacity, hydrophobicity, and auto-aggregation of newly isolated potential probiotic strains. *Fermentation* **2019**, *5*, 100. [CrossRef]

63. Kos, B.; Susković, J.; Vuković, S.; Simpraga, M.; Frece, J.; Matosić, S. Adhesion and Aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *J. Appl. Microbiol.* **2003**, *94*, 981–987. [CrossRef] [PubMed]
64. García-Cayuela, T.; Korany, A.M.; Bustos, I.; de Cadinanos, L.P.G.; Requena, T.; Peláez, C.; Martínez-Cuesta, M.C. Adhesion abilities of dairy *Lactobacillus plantarum* strains showing an aggregation phenotype. *Food Res. Int.* **2014**, *57*, 44–50. [CrossRef]
65. Yasmin, I.; Saeed, M.; Khan, W.A.; Khaliq, A.; Chughtai, M.F.J.; Iqbal, R.; Tehseen, S.; Naz, S.; Liaqat, A.; Mehmood, T.; et al. *In vitro* probiotic potential and safety evaluation (hemolytic, cytotoxic activity) of *Bifidobacterium* strains isolated from raw camel milk. *Microorganisms* **2020**, *8*, 354. [CrossRef] [PubMed]
66. Honey, C.C.; Keerthi, T.R. Probiotic potency of *Lactobacillus plantarum* kx519413 and kx519414 isolated from honey bee gut. *FEMS Microbiol. Lett.* **2018**, *365*, fnx285.
67. Mani-López, E.; Arrioja-Bretón, D.; López-Malo, A. The impacts of antimicrobial and antifungal activity of cell-free supernatants from lactic acid bacteria *in vitro* and foods. *Compr. Rev. Food Sci. Food Saf.* **2022**, *21*, 604–641. [CrossRef]
68. Rocchetti, M.T.; Russo, P.; Capozzi, V.; Drider, D.; Spano, G.; Fiocco, D. Bioprospecting antimicrobials from *Lactiplantibacillus plantarum*: Key factors underlying its probiotic action. *Int. J. Mol. Sci.* **2021**, *22*, 12076. [CrossRef]
69. Gómez, N.C.; Ramiro, J.M.P.; Quecan, B.X.V.; de Melo Franco, B.D.G. Use of potential probiotic lactic acid bacteria biofilms for the control of *Listeria monocytogenes*, *Salmonella* Typhimurium, and *Escherichia coli* O157:H7 biofilms formation. *Front. Microbiol.* **2016**, *7*, 863. [CrossRef]
70. Arena, M.P.; Silvain, A.; Normanno, G.; Grieco, F.; Drider, D.; Spano, G.; Fiocco, D. Use of *Lactobacillus plantarum* strains as a bio-control strategy against food-borne pathogenic microorganisms. *Front. Microbiol.* **2016**, *7*, 464. [CrossRef]
71. Feng, T.; Wang, J. Oxidative stress tolerance and antioxidant capacity of lactic acid bacteria as probiotic: A systematic review. *Gut Microbes* **2020**, *12*, 1801944. [CrossRef]
72. Kullisaar, T.; Zilmer, M.; Mikelsaar, M.; Vihalemm, T.; Annuk, H.; Kairane, C. and Kilk, A. Two antioxidative lactobacilli strains as promising probiotics. *Int. J. Food Microbiol.* **2002**, *72*, 215–224. [CrossRef]
73. Maske, B.L.; Pereira, G.V.D.; Vale, A.D.; Neto, D.P.D.; Karp, S.G.; Viesser, J.A.; Lindner, J.D.; Pagnoncelli, M.G.; Soccol, V.T.; Soccol, C.R. A review on enzyme-producing lactobacilli associated with the human digestive process: From metabolism to application. *Enzym. Microb. Technol.* **2021**, *149*, 109836. [CrossRef] [PubMed]
74. Govindaraj, K.; Samayanpaulraj, V.; Narayanadoss, V.; Uthandakalaipandian, R. Isolation of lactic acid bacteria from intestine of freshwater fishes and elucidation of probiotic potential for aquaculture application. *Probiotics Antimicrob. Proteins* **2021**, *13*, 1598–1610. [CrossRef] [PubMed]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

foods

MDPI

Article

Improving the Efficiency of Viability-qPCR with Lactic Acid Enhancer for the Selective Detection of Live Pathogens in Foods

Laura-Dorina Dinu [1,*], Quthama Jasim Al-Zaidi [1], Adelina Georgiana Matache [1] and Florentina Matei [1,2]

[1] Faculty of Biotechnology, University of Agricultural Sciences and Veterinary Medicine, 011464 Bucharest, Romania; alzaidy2004.n3@gmail.com (Q.J.A.-Z.); adelinamatache21@gmail.com (A.G.M.); florentina.matei@biotehnologii.usamv.ro (F.M.)

[2] Faculty of Food Industry and Tourism, Transilvania University of Brasov, 500015 Brasov, Romania

* Correspondence: laura.dinu@biotehnologii.usamv.ro

Abstract: Pathogenic *Escherichia coli* are the most prevalent foodborne bacteria, and their accurate detection in food samples is critical for ensuring food safety. Therefore, a quick technique named viability-qPCR (v-qPCR), which is based on the ability of a selective dye, such as propidium monoazide (PMA), to differentiate between alive and dead cells, has been developed. Despite diverse, successful applications, v-qPCR is impaired by some practical limitations, including the ability of PMA to penetrate the outer membrane of dead Gram-negative bacteria. The objective of this study is to evaluate the ability of lactic acid (LA) to improve PMA penetration and, thus, the efficiency of v-qPCR in detecting the live fraction of pathogens. The pre-treatment of *E. coli* ATCC 8739 cells with 10 mM LA greatly increased PMA penetration into dead cells compared to conventional PMA-qPCR assay, avoiding false positive results. The limit of detection when using LA-PMA qPCR is 1% viable cells in a mixture of dead and alive cells. The optimized LA-PMA qPCR method was reliably able to detect log 2 CFU/mL culturable *E. coli* in milk spiked with viable and non-viable bacteria. Lactic acid is cheap, has low toxicity, and can be used to improve the efficiency of the v-qPCR assay, which is economically interesting for larger-scale pathogen detection applications intended for food matrices.

Keywords: quantitative PCR; propidium monoazide (PMA); lactic acid; detection of viable pathogens; food matrix

check for updates

Citation: Dinu, L.-D.; Al-Zaidi, Q.J.; Matache, A.G.; Matei, F. Improving the Efficiency of Viability-qPCR with Lactic Acid Enhancer for the Selective Detection of Live Pathogens in Foods. *Foods* **2024**, *13*, 1021. https://doi.org/10.3390/foods13071021

Academic Editors: Youqiang Xu and Yu Zheng

Received: 23 February 2024
Revised: 22 March 2024
Accepted: 25 March 2024
Published: 27 March 2024

Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Pathogen detection is a crucial aspect of maintaining public health, preventing outbreaks, and ensuring the safety of food, soil, water, and other biological samples. According to the World Health Organization, more than 1.5 million individuals worldwide have passed away in the last three years from illnesses brought on by various foodborne infections [1]. *Escherichia coli* is a high-risk food contaminant and can be an indicator of fecal contamination in different foods [2,3]. The conventional method for identifying bacteria is based on culture cultivation, which typically takes two to three days or longer to yield complete results. Culture-based approaches have drawbacks, such as being labor-intensive, lengthy, and susceptible to contamination by germs that are not the target. Both colony-based detection and polymerase chain reaction (PCR) are widespread methods used for the detection of *E. coli* in complex samples such as bodily fluids or food matrices [4,5]. Molecular biology methods, PCR, and quantitative PCR (qPCR) are the most significant contemporary technologies for pathogen detection and identification; however, they cannot discern between living and dead cells, thus leading to false positive results.

Among various molecular biology methods employed for pathogen detection, viable quantitative PCR (v-qPCR) has emerged as a powerful technique that offers high sensitivity and specificity. Viable qPCR combines the principles of qPCR, a sensitive technique for amplifying DNA, with the ability to differentiate between live and dead microorganisms [6–8]. Traditional qPCR detects the presence of DNA regardless of whether the microorganism is

viable or not. However, viable qPCR goes a step further by incorporating viability dyes, such as propidium monoazide (PMA), ethidium monoazide (EMA), or commercially available reagent D, to selectively target and inhibit the amplification of DNA from non-viable cells while allowing amplification from viable cells [8–10]. DNA intercalating dyes are able to enter dead cells without damaging their membrane integrity and intercalate with DNA using their photo-inducible azide groups, preventing PCR-induced DNA amplification [7,8]. Viable qPCR assays with PMA have been successfully optimized to detect major foodborne pathogens, including *E. coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes* [11–17].

Despite many successful applications, some practical limitations that impact the accuracy of v-qPCR data have been reported, especially concerning the complexity of the sample matrix, the length of the qPCR amplicon, and the ability of some bacterial strains to avoid dye uptake [18,19]. To increase the PMA uptake without compromising the viability of live cells, different enhancers have been used. Thus, surfactant sodium deoxycholate (DOC) has been used to enhance PMA uptake and improve the efficiency of v-qPCR bacterial detection for *Vibrio vulnificus* killed via freezing or prolonged refrigeration, *E. coli* subjected to mild or pasteurizing heat treatments and heat-killed cells of *Salmonella enterica* serovar *Typhimurium* [20–23]. However, the co-incubation of Gram-positive *L. monocytogenes* cells with PMA and deoxycholate produces a strong undesired uptake of viability dye by live bacterial cells, suggesting that the effect is strain- and Gram-specific [22]. Additionally, DOC has been proven to enhance the PMA-qPCR results for infectious virus detection in water samples or in a multiplex PMA-PCR developed to detect *Salmonella* spp., *Shigella* spp., and *Staphylococcus aureus* in food products [24,25]. Therefore, other studies used sodium dodecyl sulfate (SDS) to detect viable *Staphylococcus aureus* and *E. coli* in spiked UHT milk samples [26,27]. While maintaining the viability of living cells, SDS has been shown to increase the permeability of dead cells to PMA [28]. Sodium lauroyl sarcosinate (sarkosyl) is a milder detergent than SDS and has proven to be more effective than DOC at boosting PMA signals in the v-qPCR detection of viable *E. coli* from a mixture of alive cells and cells inactivated by heat, lactic acid, or peroxyacetic acid [29]. Recently, SDS-PMA RT-qPCR was developed, a rapid method used to detect SARS-CoV-2 viral particles from biological samples [30]. Similarly, combining PMA and surfactant (sarkosyl 0.025% or triton X-100 0.5%) treatments increased PMA permeability to dead cells and improved v-qPCR detection of *S. aureus* [31]. Moreover, a multiplex PMA-qPCR assay with sarcosyl pre-treatment was successfully applied to detect *Legionella pneumophila*, *S. typhimurium*, and *S. aureus* from water samples [32].

Therefore, this study aimed to contribute to the efforts improving v-qPCR efficiency when testing lactic acid (LA), a new type of PMA-penetrating enhancer. Sublethal concentrations of LA up to 10 mM have been proven to permeabilize the outer membrane of Gram-negative bacteria, causing leakage, damaging the cytoplasmic membrane, and altering the molecular structure [33,34]. Experiments were performed with model Gram-negative bacteria *Escherichia coli* in order to evaluate the ability of LA to improve PMA penetration and, thus, the efficiency of the v-qPCR assay when detecting the alive fraction of bacteria in culture. Moreover, the LA-PMA-qPCR method was applied to the rapid and accurate detection of viable *E. coli* cells in spiked milk.

2. Materials and Methods

2.1. Bacterial Strain and Culture Conditions

The bacterial strain used in this study, *E. coli* ATCC 8739, was cultured in 10 mL of tryptic soy broth–SB medium (Acumedia, San Bernardino, CA, USA) at 37 °C for 24 h until the stationary phase was achieved. Six and seven serial tenfold dilutions of each culture were prepared and spread onto duplicated tryptic soy agar–TSA media (Scharlau, Spain). The plates were incubated at 37 °C for 24 h, and then the colonies were counted, and the number of viable *E. coli* were determined.

2.2. Testing the Sublethal Effect of Lactic Acid on the Strain E. coli ATCC 8739

The sublethal effect of LA was tested using tubes with 3 mL of TSB media with 15 mM, 20 mM, and 30 mM of LA inoculated with 1 mL of overnight *E. coli* culture. After 24 h of incubation at 37 °C, the optical density (OD_{600}) was determined. The experiment was performed twice, and the average value was calculated.

2.3. Preparation of E. coli Suspensions and Heat Treatment

To obtain dead *E. coli* cells, the overnight cultures were heated at 80 °C for 45 min, and then the loss of cell viability was checked by spreading 1 mL of the heat-treated cell suspensions onto TSA media and incubating the plates for 24 h at an optimal growth temperature. To study signal reduction, aliquots (400 µL/each) of live cells and others with heat-killed cells (400 µL/each) were prepared. The limit of detection study was performed with cell mixtures that contain a defined ratio of viable (100%, 50%, 10%, 1%, 0.1%, and 0%) and dead cells, with a total volume of 400 µL/each.

2.4. Pre-Treatment of Cell Suspensions with Lactic Acid

A 30% (*v*/*v*) L-(+)-lactic acid stock solution (Sigma, St Saint Louis, MO, USA) was used to prepare 5 to 30 mM LA solutions with a pH = 5–5.5. Some of the cell aliquots and alive–dead mixtures (400 µL/each) were incubated with 400 µL of LA for 30 min and centrifuged at 150 rpm at room temperature before PMA treatment.

2.5. PMA Treatment and Cross-Linking

In this study, 1 mg of PMA (Biotium, Hayward, CA, USA) was dissolved in 98 µL of sterile distilled water to create a stock solution of 20 mM and stored in the dark at −20 °C. Treatment with PMA was performed based on the manufacturer's instructions, adding 1 µL of stock PMA in 400 µL aliquots to obtain a final concentration of 50 µM. The tubes containing the cell aliquots and mixtures were incubated in the dark at room temperature and centrifuged at 150 rpm for 10 min. Light exposure was performed using a 1000 W halogen light source (Omnilux, Napa, CA, USA) placed 20 cm away from the sample tubes. During the photolysis step, the tubes were kept on ice to avoid excessive heating and occasional mixing. After 5 min of light exposure, cells were harvested via centrifugation (12,000 rpm, for 2 min) and washed twice with sterile distilled water to remove trace amounts of PMA solution that might interfere with free DNA during extraction. The pellet was resuspended in 200 µL of distilled water for DNA isolation.

2.6. Extraction of DNA and qPCR

For all samples (LA-PMA-treated, PMA-treated, and non-treated), genomic DNA was extracted from the bacterial cell pellet using a Quick DNA Fungal/Bacterial Miniprep kit (ZymoResearch, Irvine, CA, USA), following the manufacturer's instructions. Briefly, the resuspended bacterial cells (200 µL) were mixed with 750 µL of BashingBead buffer and mechanically disintegrated using Minibead beater equipment (Biospec Products, Bartlesville, OK, USA) for 2 min. Then, the DNA was isolated after washing. DNA concentration and purity were checked with a QuickDrop Micro-Volume Spectrophotometer (Molecular Devices, San Jose, CA, USA). For all experiments, 1 µL of extracted DNA (up to 50 ng DNA), serving as the template for qPCR assay, was added to 24 µL of a mixture containing 12.5 µL of Maxima SYBR Green/ROX qPCR MasterMix (ThermoScientific, Waltham, MA, USA), 0.5 µL of each primer (forward and reverse primer), and 10.5 µL of PCR-grade water. The following primers were used for the detection of DNA-targeted highly conserved regions of the *uivA-7* gene: forward primer uidA-7-F 5′-GGGATAGTCTGCCAGTTCAGTT-3′ and reverse primer uidA-7-R-deg 5′-GATGTCACDCCGTATGTTATTG-3′ [35]. Quantitative PCR was performed using the Rotor-Gene 6000 5plex HRM (Qiagen-Corbett Life Science, Sydney, Australia) instrument, and software was used to generate the standard curve and for microbial quantification. The 3 steps with the melting program were as follows: denaturation at 95 °C for 10 min, followed by 40 cycles of 15 s denaturation at 95 °C, 30 s

annealing at 60 °C, and 20 s elongation at 72 °C, based on the product size (83 bp). In all cases, negative control amplification was included using 1 µL of PCR-grade water (Thermo Fisher Scientific, Waltham, MA, USA) instead of a DNA template. The specificity of qPCR amplification was confirmed by running dissociation curves, and no unspecific products were formed.

For the limit of detection study, the mean of the threshold cycle (Ct) values was used to plot against the natural logarithm of viability (%), and the coefficient of regression (R^2) values of the corresponding trend line were calculated.

For signal reduction analysis after PMA treatment, delta Ct (dCtPMA) was used for live and dead cells treated or not with PMA, as follows:

$$dC_{tALIVE\text{-}PMA} = C_{tALIVE,\ PMA} - C_{tALIVE,\ non\text{-}PMA}$$

$$dC_{tNON\text{-}VIABLE\text{-}PMA} = C_{tNON\text{-}VIABLE,\ PMA} - C_{tNON\text{-}VIABLE,\ nonPMA}$$

For signal reduction analysis after LA-PMA treatments, delta Ct (dCtLA-PMA) was used for live and dead cells treated or not with lactic acid, as follows:

$$dC_{tALIVE\text{-}LA\text{-}PMA} = C_{tALIVE,\ LA\text{-}PMA} - C_{tALIVE,\ PMA}$$

$$dC_{tNON\text{-}VIABLE\text{-}LA\text{-}PMA} = C_{tNON\text{-}VIABLE,\ LA\text{-}PMA} - C_{tNON\text{-}VIABLE,\ PMA}$$

The expected results for dC_{tALIVE} should be close to zero (± 2), while for $dC_{tNON\text{-}VIABLE} > 4$.

2.7. Artificially Inoculated Food Assays

Ultra-high temperature (UHT) sterilized milk was purchased from the local market and used for spiking studies. For standard curve preparation, the overnight culture of *E. coli* ATCC 8739 (1 mL) was inoculated into 1 mL of milk to reach a final concentration of 10^7 CFU/mL. After the 10 mM LA and PMA treatment, genomic DNA was isolated, and serial dilutions of DNA were used to construct the standard curve. UHT milk was first confirmed to be negative for *E. coli* via plate count on TSA medium and via qPCR analysis. The three milk samples were inoculated with *E. coli* as follows: sample 1—10^2 CFU/mL alive cells; sample 2—10^2 CFU/mL dead cells; and sample 3—a mixture of 10^2 CFU/mL viable and 10^2 CFU/mL non-viable cells. All of the samples were pre-treated with 10 mM LA, followed by PMA. Then, the genomic DNA was isolated, and qPCR was amplified, as mentioned above.

2.8. Statistical Analysis

Statistical analysis was calculated using the IBM SPSS Statistics 23 software package (IBM Corporation, Armonk, NY, USA). The analysis of variance was performed with ANOVA at 95% significance ($p = 0.05$). For qPCR analysis, the average results obtained from two different experiments with triplicates are shown, and the results were compared with ANOVA. The counts obtained after plating were log-transformed, and then the average results and standard deviation were calculated. All of the experiments were performed twice with duplicates or triplicates, and the samples were analyzed within 24 h.

3. Results

3.1. Effect of LA and PMA Treatments on qPCR Amplification from Viable and Non-Viable Bacterial Cell Suspensions

First, the effect of the PMA treatment on real-time PCR-based detection of culturable and heat-treated *E. coli* ATCC 8739 was evaluated, and the results are shown in Figure 1. Viable bacterial cell suspensions were treated or untreated with PMA prior to DNA isolation and qPCR. The delta $C_{tALIVE\text{-}PMA}$ for the viable suspensions is 0.375, a lower value than 2, which proved that PMA did not inhibit the DNA amplification from alive *E. coli* cells. Using suspensions with non-viable cells, treated or untreated with propidium monoazide, the threshold values (C_t) for non-viable were closer; therefore, the delta $C_{tNON\text{-}VIABLE\text{-}PMA}$

in this case is 1.025. This result suggests that the viability dye did not efficiently penetrate the outer membrane of *E. coli* and cell wall, and most of the DNA from the dead cells was amplified after DNA isolation. Although the PMA treatment has no adverse effect on the detection of viable *E. coli*, in the case of non-viable bacteria, the high dose of PMA (50 µM) had a low penetration effect. It is considered that a signal reduction higher than 4 for dead cells indicates that 94% of the DNA from these bacteria was removed, and the percentage increases to 99.6% for a value of 8 [36].

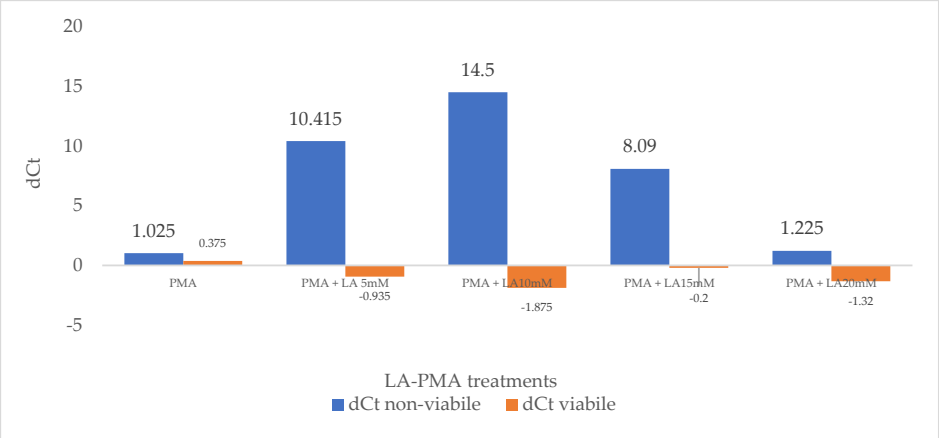


Figure 1. The effect of PMA treatment and LA-PMA treatments on delta dC_t for viable and non-viable cell suspensions.

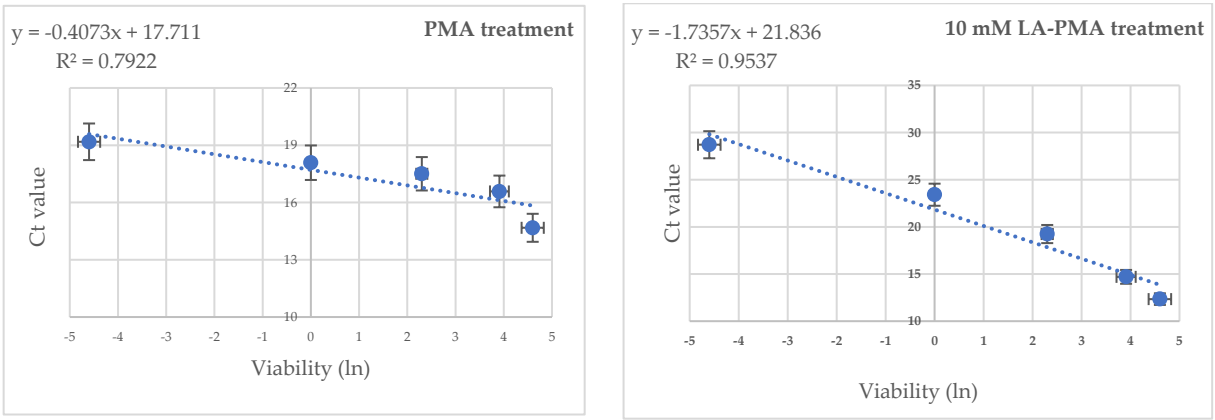
Second, the sublethal effect of low concentrations of LA (5–30 mM) on *E. coli* ATCC 8739 cells was evaluated. The permeabilizer function of lactic acid has been known for a long time and explains its antimicrobial effect while supporting its use in decontamination procedures [34]. The viability of *E. coli* cultures that were grown in media with lactic acid up to 20 mM was negligibly affected, while a ~30% decrease in viability was noted in media with 30 mM of LA.

Following previous results relating to the sublethal effect of lactic acid in concentrations up to 20 mM, suspensions of live and heat-killed *E. coli* ATCC 8739 were exposed to different concentrations of LA (5 to 20 mM) and PMA. Control probes were only treated with PMA, followed by photolysis and DNA amplification. Moreover, control probes only treated with LA proved that this compound does not influence DNA amplification. The delta Ct (dC_t) was defined as the difference in the mean C_t values of LA-treated and non-treated probes and reflected the signal reduction level. Increasing concentrations of LA do not significantly influence the delta Ct for viable cells ($dC_{tVIABLE}$), and values lower than 2 were obtained (Figure 1). This proves that viable cells efficiently excluded PMA with or without LA pre-treatment. However, in the case of non-viable 10 mM LA pre-treated cells, the $C_{tNON\text{-}VIABLE,\ LA\text{-}PMA}$ signal was 14.5 times higher than the $C_{tNON\text{-}VIABLE,\ PMA}$ obtained without lactic acid incubation. The signal reduction reached the greatest value ($dC_{tNON\text{-}VIABLE}$ = 14.5) at a lactic acid concentration of 10 mM before gradually decreasing at higher LA concentrations. Additionally, cells exposed for 30 min to 5 mM and 15 mM lactic acid increased propidium monoazide penetration, and the values for $dC_{tNON\text{-}VIABLE}$ were 10.415 and 8.090, respectively (Figure 1).

3.2. Limit of Detection for LA-PMA qPCR Assay in a Background of Dead Bacteria

Different works have proved that large quantities of free DNA or DNA from dead cells might interfere with DNA extraction and subsequent qPCR detection from viable cells [8,10,37,38]. Therefore, *E. coli* suspensions with defined ratios of viable (100%, 50%, 10%, 1%, and 0.1%) and non-viable cells were prepared. Half of these suspensions were treated with 10 mM LA and PMA, while the other half were only with PMA, and then the DNA was amplified via real-time PCR with specific primers for the uivA gene. As expected,

the highest Ct value was noted for LA-PMA-treated samples with 100% heat-killed cells. A plot of the natural logarithm of DNA percentage versus C_t values showed a linear correlation with a coefficient of regression (R^2) of 0.9537 (Figure 2b) for LA-PMA treated samples and 0.7922 (Figure 2a) for samples exposed to PMA. The improved LA-PMA qPCR assay yielded a good quantification prediction of the viable fraction in dead–alive cell mixtures with higher than 1% viable cells. However, a significant deviation from linearity was noticed in samples with less than 0.1% viable cells.



(**a**) (**b**)

Figure 2. The effect of PMA (**a**) and 10 mM LA-PMA (**b**) treatments on the detection of viable cells in defined ratios of viable (100%, 50%, 10%, and 1%) and dead cells. The average value of the linear coefficients of regression (R^2) obtained from two independent experiments in triplicate are indicated.

3.3. Detection of E. coli ATCC 8739 via LA-PMA-qPCR Assay in Artificially Inoculated Milk

To evaluate *E. coli* ATCC 8793 detection using LA-PMA-qPCR in food samples, milk samples were artificially spiked with log 2 CFU/mL alive cells, dead cells, and a mix of culturable and heat-killed cells. The standard curve in milk was prepared using 10-fold dilutions of genomic DNA from log 7 CFU/mL *E. coli* culture. The artificially spiked samples and samples for standard curve preparation were pre-treated with LA, followed by PMA and subsequent DNA isolation and qPCR amplification. The correlation coefficient (R^2 = 0.9792) and the slope (−2.95) of the standard curve were automatically generated, and they showed a good linear relationship between the values of each sample (Figure 3).

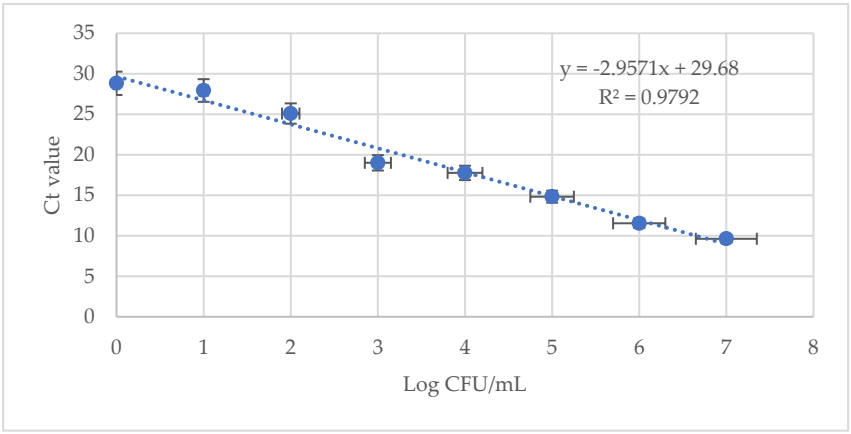


Figure 3. Standard curve for quantification of *E. coli* ATCC 8794 in artificially spiked milk using 10 mM LA-PMA assay. The average value of the linear coefficient of regression (R^2) obtained in two independent experiments in triplicate is indicated.

The average C_t values for alive and mixed alive–dead samples were 25.32 ± 0.30 and 25.69 ± 0.07, respectively, proving that these samples contained log 2 CFU/mL viable bacterial cells. In milk samples inoculated with non-alive *E. coli* cells, the average C_t was 28.52 ± 0.55.

4. Discussion

Viable qPCR represents a valuable tool in the field of pathogen detection, offering rapid, sensitive, and specific detection of viable pathogens in diverse sample types. Its applications span various fields, including clinical diagnostics, food safety, environmental monitoring, and biodefense, contributing significantly to efforts aimed at controlling infectious diseases and ensuring public health. In recent years, propidium monoazide has been widely used in combination with qPCR assays to limit false positive results in the detection of *Escherichia coli* from different food (e.g., milk, vegetables, and ground meat), environmental, and biological samples [7,15,39–42]. However, some practical limitations were found, and different strategies were proposed for overcoming some of these problems, including improving the dye penetration, extensive optimization procedures for complex samples, and changing the intercalation dye PMA with a palladium compound [18,19,43]. The above-named limitations led us to seek improved methods to detect and quantify microbes and reduce the false positive/negative results.

To remedy the shortcoming of v-qPCR regarding the ability of PMA dye to penetrate the cells with impaired membrane integrity, a new practical approach based on using lactic acid as an enhancer was proposed. Lactic acid is known as a potent outer membrane-disintegrating agent that causes the release of lipopolysaccharides in Gram-negative bacteria [33,34]. The ability of LA to effectively penetrate the outer membrane of Gram-negative bacteria and act in synergy with antimicrobials has been reported [44]. Recently, the lactic acid cell-penetrating effect has been exploited in various biomedical applications, including the development of lactic acid-based drug delivery systems, gene delivery vectors, and diagnostic probes [45]. Although the mechanisms are still poorly understood, the ability of lactic acid to enhance cellular uptake will improve the targeted delivery of therapeutic agents to specific cells and enhance the efficacy of treatments while minimizing off-target effects [44].

First, we evaluated the ability of LA to improve PMA penetration and, thus, the efficiency of the v-qPCR assay to detect an alive fraction of Gram-negative *E. coli* ATCC 8739. Cell suspensions containing 100% viable bacteria and 100% heat-killed cells were treated or not with different concentrations of LA (5–20 mM), followed by PMA photolysis and DNA amplification. To quantify the signal reduction level, we calculated delta C_t (dC_t) as a difference in the mean C_t values of LA-treated and non-treated probes. The mean $C_{tVIABLE}$ values for the DNA extracted from viable cells treated or not treated with LA were not significantly different ($p > 0.05$); thus, the delta $C_{tVIABLE}$ values varied between −0.935 and −1.875. This proved that PMA was not able to enter the viable cells and reduce the C_t signal in both cases, with and without LA pre-treatment. However, the mean $C_{tNON\text{-}VIABLE}$ values for DNA extracted from killed cells were significantly different ($p < 0.05$), except for bacterial suspensions treated with 20 mM LA and PMA. Based on the $dC_{tNON\text{-}VIABLE}$ values, the 10 mM LA pre-treatment had the best effect and improved PMA penetration into heat-killed cells that suppressed the detection of dead cells. Both 5 mM LA and 15 mM LA pre-treatment improved *E. coli* ATCC 8739 PMA-qPCR detection, but the values were lower compared to $dC_{tNON\text{-}VIABLE}$ = 14.5 obtained for the 10 mM LA pre-treatment. Similar data were observed by Nkuipou-Kenfack et al., 2013, for the deoxycholate effect on *Salmonella typhimurium* PMA-qPCR detection when PMA penetration decreased at higher concentrations of DOC [22]. In another study concerning *E. coli* detection using SDS-PMA-qPCR, the authors tested different SDS concentrations (0–1000 µg/mL) and reported that the optimal concentration of SDS for enhancing PMA (40 µM) penetration into dead cells was 100 µg/mL [27].

Previous works have shown that the accuracy of PMA-qPCR detection could be affected by the high background of dead cells [8,10,37,38]. To analyze the limit of detection of the LA-PMA-qPCR assay's defined ratios, culturable viable (100%, 50%, 10%, 1%, and 0.1%) and non-viable cells were prepared. When used for the detection of *E. coli* ATCC 8739 under culture conditions, LA-PMA-qPCR assay gave a good quantitative prediction for viable cells in mixtures of alive–dead cells only when the fraction of viable bacteria was equal to or higher than 1%. Other studies reported that the prediction of *E. coli* and *Listeria innocua* viable cells detected using PMA-qPCR in alive–dead mixtures with less than 1% viable cells was not feasible [21,22]. Testing the effect of PMA treatment on defined ratios of viable (0–100%) and non-viable *E. coli* cells, Nocker et al., 2006, noted a linear correlation $R^2 = 0.9742$ after plotting the natural logarithm of normalized DNA concentrations and the corresponding C_t values obtained from *stx1* gene amplification [6]. In this case, the PMA-qPCR assay was optimized, and cells were treated with PMA (50 µM) and exposed to light for a longer period of time (120 s), which could explain the differences with other reported data. However, in our experiments, the qPCR assay with the primer pair that targeted the *uivA* gene (amplicon length 83 bp) generated a standard curve with $R^2 = 0.9912$ and a slope of −3.45, suggesting robust DNA amplification with these primers. Additionally, it is possible that a longer amplicon of up to 200 bp could increase v-qPCR efficiency, as was recently suggested by Van Holm, 2021 [19].

Finally, LA-PMA-qPCR was successfully applied to detect viable *E. coli* cells in artificially spiked milk. For UHT milk, the standard plating methods require different enrichment media plus long detection/confirmation times (2 days) to target aerobic or anaerobic viable contaminant bacteria. When it comes to analyzing food samples using qPCR, the complexity of food matrices poses several challenges: the presence of PCR inhibitors, variability in food composition, texture, and structure, the presence of enzymes that degrade DNA, and so on [46–50]. In our experiments, the lowest detection limit was log 2 CFU/mL for viable cells or mixtures with log 2 CFU/mL alive and log 2 CFU/mL heat-killed bacteria. Similarly, Dong et al., 2019, reported a detection limit in spiked milk of 3×10^2 CFU/mL viable *E. coli* ATCC 25922 using DOC-PMA-qPCR [27]. Still, other studies have suggested that fat and other components of opaque fluids, such as milk, could protect cells from the effects of DOC and PMA [21,51].

Incorporating lactic acid into viability qPCR assays is relatively straightforward and can be easily integrated into existing laboratory protocols. Moreover, lactic acid is readily available, cost-effective, and compatible with standard laboratory equipment and reagents, making it a practical choice for enhancing the performance of v-qPCR assays. However, more studies are required to evaluate the ability of LA to improve PMA penetration using different species of Gram-positive and Gram-negative bacteria.

5. Conclusions

Overall, the results of this study proved that the use of lactic acid in conjunction with viability qPCR offers a powerful strategy for the accurate, sensitive, and specific detection of viable Gram-negative pathogens in a culture and food matrix. Lactic acid (10 mM) greatly enhanced membrane permeability to PMA for heat-killed *E. coli* cells compared to a conventional PMA-qPCR assay ($dC_{tNON\text{-}VIABLE} = 14.5$), thus improving v-qPCR efficiency and the limit of detection in a mixture of dead and alive cells. Lactic acid has low toxicity and is cheap; therefore, it is economically interesting for use in larger-scale applications concerning pathogen detection in food matrices.

By leveraging lactic acid's ability to enhance and facilitate DNA extraction from viable cells, this approach contributes to advancing pathogen detection methodologies and supporting efforts to safeguard public health and food safety.

Author Contributions: Conceptualization, L.-D.D.; methodology, L.-D.D., Q.J.A.-Z. and A.G.M.; software, L.-D.D.; investigation, L.-D.D., Q.J.A.-Z. and A.G.M.; writing—original draft preparation, L.-D.D.; writing—review and editing, L.-D.D. and F.M.; supervision, F.M.; funding acquisition, F.M. All authors have read and agreed to the published version of the manuscript.

Funding: The APC was funded by the University of Agricultural Sciences and Veterinary Medicine-Bucharest.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Acknowledgments: The technical assistance provided by Florentina Badea is gratefully acknowledged.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Pires, S.M.; Desta, B.N.; Mughini-Gras, L.; Mmbaga, B.T.; Fayemi, O.E.; Salvador, E.M.; Gobena, T.; Majowicz, S.E.; Hald, T.; Hoejskov, P.S.; et al. Burden of foodborne diseases: Think global, act local. *Curr. Opin. Food Sci.* **2021**, *39*, 152–159. [CrossRef]
2. Gourama, H. Foodborne pathogens. In *Food Safety Engineering*; Springer: Berlin/Heidelberg, Germany, 2020; pp. 25–49.
3. Denamur, E.; Clermont, O.; Bonacorsi, S.; Gordon, D. The population genetics of pathogenic *Escherichia coli*. *Nat. Rev. Microbiol.* **2021**, *19*, 37–54. [CrossRef] [PubMed]
4. Rani, A.; Ravindran, V.B.; Surapaneni, A.; Mantri, N.; Ball, A.S. Trends in point-of-care diagnosis for *Escherichia coli O157: H7* in food and water. *Int. J. Food Microbiol.* **2021**, *349*, 109233. [CrossRef] [PubMed]
5. Singha, S.; Thomas, R.; Viswakarma, J.N.; Gupta, V.K. Foodborne illnesses of *Escherichia coli* O157origin and its control measures. *J. Food Sci. Technol.* **2023**, *60*, 1274–1283. [CrossRef]
6. Nocker, A.; Cheung, C.Y.; Camper, A.K. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *J. Microbiol. Methods* **2006**, *67*, 310–320. [CrossRef] [PubMed]
7. Nocker, A.; Sossa-Fernandez, P.; Burr, M.D.; Camper, A.K. Use of propidium monoazide for live/dead distinction in microbial ecology. *Appl. Environ. Microbiol.* **2007**, *73*, 5111–5117. [CrossRef] [PubMed]
8. Dinu, L.D.; Bach, S. Detection of viable but non-culturable *Escherichia coli* O157: H7 from vegetable samples using quantitative PCR with propidium monoazide and immunological assays. *Food Control* **2013**, *31*, 268–273. [CrossRef]
9. Rudi, K.; Moen, B.; Drømtorp, S.M.; Holck, A.L. Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. *Appl. Environ. Microbiol.* **2005**, *71*, 1018–1024. [CrossRef] [PubMed]
10. Elizaquível, P.; Sánchez, G.; Aznar, R. Application of propidium monoazide quantitative PCR for selective detection of live *Escherichia coli O157: H7* in vegetables after inactivation by essential oils. *Int. J. Food Microbiol.* **2012**, *159*, 115–121. [CrossRef] [PubMed]
11. Shi, Z.; Li, X.; Fan, X.; Xu, J.; Liu, Q.; Wu, Z.; Pan, D. PMA-qPCR method for the selective quantitation of viable lactic acid bacteria in fermented milk. *Front. Microbiol.* **2022**, *13*, 984506. [CrossRef] [PubMed]
12. Guo, J.; Wang, W.; Zhao, H.; Luo, Y.; Wan, M.; Li, Y. A new PMA-qPCR method for rapid and accurate detection of viable bacteria and spores of marine-derived *Bacillus velezensis* B-9987. *J. Microbiol. Methods* **2022**, *199*, 106537. [CrossRef] [PubMed]
13. Okada, A.; Tsuchida, M.; Rahman, M.M.; Inoshima, Y. Two-Round Treatment with Propidium Monoazide Completely Inhibits the Detection of Dead *Campylobacter Spp.* Cells by Quantitative PCR. *Front. Microbiol.* **2022**, *13*, 801961. [CrossRef] [PubMed]
14. Infante, N.; Rodríguez, R.; Bartolo, Y.; Sánchez, O.; Sanz, I.; Bermeo, L.; Morató, J. Biofunctionalization of Cork with Moringa oleifera Seeds and Use of PMA Staining and qPCR to Detect Viability of *Escherichia coli*. *Water* **2021**, *13*, 2731. [CrossRef]
15. Rey, M.D.; Cap, M.; Favre, L.C.; Rodríguez Racca, A.; Dus Santos, M.J.; Vaudagna, S.R.; Mozgovoj, M. Evaluation of PMA-qPCR methodology to detect and quantify viable Shiga toxin-producing *Escherichia coli* in beef burgers. *J. Food Process. Preserv.* **2021**, *45*, e15338. [CrossRef]
16. Do, T.N.; Lao, T.D.; Le, T.A. Establishment of PMA Real-Time PCR Method to Detect Viable Cells of *Listeria monocytogenes* and *Salmonella spp.* in Milk and Dairy Products. *Asian J. Pharm. Res. Health Care* **2021**, *20*, 146–156.
17. Truchado, P.; Gil, M.I.; Kostic, T.; Allende, A. Optimization and validation of a PMA qPCR method for *Escherichia coli* quantification in primary production. *Food Control* **2016**, *62*, 150–156. [CrossRef]
18. Zeng, D.; Chen, Z.; Jiang, Y.; Xue, F.; Li, B. Advances and challenges in viability detection of foodborne pathogens. *Front. Microbiol.* **2016**, *7*, 1833. [CrossRef] [PubMed]
19. Van Holm, W.; Ghesquière, J.; Boon, N.; Verspecht, T.; Bernaerts, K.; Zayed, N.; Chatzigiannidou, I.; Teughels, W. A viability quantitative PCR dilemma: Are longer amplicons better? *Appl. Environ. Microbiol.* **2021**, *87*, e02653-20. [CrossRef] [PubMed]
20. Lee, J.L.; Levin, R.E. Discrimination of viable and dead Vibrio vulnificus after refrigerated and frozen storage using EMA, sodium deoxycholate and real-time PCR. *J. Microbiol. Methods* **2009**, *79*, 184–188. [CrossRef]
21. Yang, X.; Badoni, M.; Gill, C.O. Use of propidium monoazide and quantitative PCR for differentiation of viable *Escherichia coli* from *E. coli* killed by mild or pasteurizing heat treatments. *Food Microbiol.* **2011**, *28*, 1478–1482. [CrossRef]

22. Nkuipou-Kenfack, E.; Engel, H.; Fakih, S.; Nocker, A. Improving efficiency of viability-PCR for selective detection of live cells. *J. Microbiol. Methods* **2013**, *93*, 20–24. [CrossRef] [PubMed]
23. Zhou, B.; Liang, T.; Zhan, Z.; Liu, R.; Li, F.; Xu, H. Rapid and simultaneous quantification of viable *Escherichia coli O157: H7* and *Salmonella spp*. in milk through multiplex real-time PCR. *J. Dairy Sci.* **2017**, *100*, 8804–8813. [CrossRef] [PubMed]
24. Canh, V.D.; Kasuga, I.; Furumai, H.; Katayama, H. Viability RT-qPCR combined with sodium deoxycholate pre-treatment for selective quantification of infectious viruses in drinking water samples. *Food Environ. Virol.* **2019**, *11*, 40–51. [CrossRef] [PubMed]
25. Wang, L.; Ye, C.; Xu, H.; Aguilar, Z.P.; Xiong, Y.; Lai, W.; Wei, H. Development of an SD-PMA-mPCR assay with internal amplification control for rapid and sensitive detection of viable *Salmonella* spp., *Shigella* spp. and *Staphylococcus aureus* in food products. *Food Control* **2015**, *57*, 314–320. [CrossRef]
26. Dong, L.; Liu, H.; Meng, L.; Xing, M.; Wang, J.; Wang, C.; Chen, H.; Zheng, N. Quantitative PCR coupled with sodium dodecyl sulfate and propidium monoazide for detection of viable *Staphylococcus aureus* in milk. *J. Dairy Sci.* **2018**, *101*, 4936–4943. [CrossRef] [PubMed]
27. Dong, L.; Liu, H.; Meng, L.; Xing, M.; Lan, T.; Gu, M.; Zheng, N.; Wang, C.; Chen, H.; Wang, J. Quantitative PCR coupled with sodium dodecyl sulfate and propidium monoazide for detection of culturable *Escherichia coli* in milk. *J. Dairy Sci.* **2019**, *102*, 6914–6919. [CrossRef] [PubMed]
28. Takahashi, H.; Gao, Y.; Miya, S.; Kuda, T.; Kimura, B. Discrimination of live and dead cells of *Escherichia coli* using propidium monoazide after sodium dodecyl sulfate treatment. *Food Control* **2017**, *71*, 79–82. [CrossRef]
29. Wang, H.; Gill, C.O.; Yang, X. Use of sodium lauroyl sarcosinate (sarkosyl) in viable real-time PCR for enumeration of *Escherichia coli*. *J. Microbiol. Methods* **2014**, *98*, 89–93. [CrossRef] [PubMed]
30. Hong, W.; Xiong, J.; Nyaruaba, R.; Li, J.; Muturi, E.; Liu, H.; Yu, J.; Yang, H.; Wei, H. Rapid determination of infectious *SARS-CoV-2* in PCR-positive samples by SDS-PMA assisted RT-qPCR. *Sci. Total Environ.* **2021**, *797*, 149085. [CrossRef] [PubMed]
31. Zi, C.; Zeng, D.; Ling, N.; Dai, J.; Xue, F.; Jiang, Y.; Li, B. An improved assay for rapid detection of viable *Staphylococcus aureus* cells by incorporating surfactant and PMA treatments in qPCR. *BMC Microbiol.* **2018**, *18*, 132. [CrossRef] [PubMed]
32. Li, H.; Xin, H.; Li, S.F. Multiplex PMA–qPCR Assay with internal amplification control for simultaneous detection of viable *Legionella pneumophila*, *Salmonella typhimurium*, and *Staphylococcus aureus* in environmental waters. *Environ. Sci. Technol.* **2015**, *49*, 14249–14256. [CrossRef] [PubMed]
33. Alakomi, H.L.; Paananen, A.; Suihko, M.L.; Helander, I.M.; Saarela, M. Weakening effect of cell permeabilizers on gram-negative bacteria causing biodeterioration. *Appl. Environ. Microbiol.* **2006**, *72*, 4695–4703. [CrossRef] [PubMed]
34. Alakomi, H.L.; Skytta, E.; Saarela, M.; Mattila-Sandholm, T.; Latva-Kala, K.; Helander, I.M. Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. *Appl. Environ. Microbiol.* **2000**, *66*, 2001–2005. [CrossRef]
35. Barbau-Piednoir, E.; Denayer, S.; Botteldoorn, N.; Dierick, K.; De Keersmaecker, S.C.; Roosens, N.H. Detection and discrimination of five *E. coli* pathotypes using a combinatory SYBR® Green qPCR screening system. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 3267–3285. [CrossRef] [PubMed]
36. Biotium Site. Available online: https://biotium.com/free-sample/ (accessed on 15 January 2024).
37. Wang, L.; Li, Y.; Mustapha, A. Detection of viable *Escherichia coli* O157:H7 by ethidium monoazide real-time PCR. *J. Appl. Microbiol.* **2009**, *107*, 1719–1728. [CrossRef]
38. Pan, Y.O.; Breidt, F., Jr. Enumeration of viable *Listeria monocytogenes* cells by real-time PCR with propidium monoazide and ethidium monoazide in the presence of dead cells. *Appl. Environ. Microbiol.* **2007**, *73*, 8028–8031. [CrossRef]
39. Lee, A.S.; Lamanna, O.K.; Ishida, K.; Hill, E.; Nguyen, A.; Hsieh, M.H. A novel propidium monoazide-based PCR assay can measure viable uropathogenic *E. coli* in vitro and in vivo. *Front. Cell. Infect. Microbiol.* **2022**, *12*, 794323. [CrossRef]
40. Ouf, J.M.; Yuan, Y.; Singh, P.; Mustapha, A. Detection of viable but nonculturable *Escherichia coli* O157:H7 in ground beef by propidium monoazide real-time PCR. Detection of Viable but Nonculturable *Escherichia coli* O157:H7 in Ground Beef by Propidium Monoazide real-time PCR. *Int. J. Agric. Sci. Food Technol.* **2017**, *3*, 26–31.
41. Taskin, B.; Gozen, A.G.; Duran, M. Selective quantification of viable *Escherichia coli* bacteria in biosolids by quantitative PCR with propidium monoazide modification. *Appl. Environ. Microbiol.* **2011**, *77*, 4329–4335. [CrossRef]
42. Golpayegani, A.; Douraghi, M.; Rezaei, F.; Alimohammadi, M.; Nodehi, R.N. Propidium monoazide–quantitative polymerase chain reaction (PMA-qPCR) assay for rapid detection of viable and viable but non-culturable (VBNC) *Pseudomonas aeruginosa* in swimming pools. *J. Environ. Health Sci. Eng.* **2019**, *17*, 407–416. [CrossRef]
43. Cechova, M.; Beinhauerova, M.; Babak, V.; Kralik, P. A viability assay combining palladium compound treatment with quantitative PCR to detect viable *Mycobacterium avium* subsp. *paratuberculosis* cells. *Sci. Rep.* **2022**, *12*, 4769. [CrossRef] [PubMed]
44. Wang, Y.; Wei, Y.; Shang, N.; Li, P. Synergistic inhibition of plantaricin E/F and lactic acid against Aeromonas hydrophila LPL-1 reveals the novel potential of class IIb bacteriocin. *Front. Microbiol.* **2022**, *13*, 774184. [CrossRef] [PubMed]
45. Shen, X.; Li, T.; Xie, X.; Feng, Y.; Chen, Z.; Yang, H.; Wu, C.; Deng, S.; Liu, Y. PLGA-Based Drug Delivery Systems for Remotely Triggered Cancer Therapeutic and Diagnostic Applications. *Front. Bioeng. Biotechnol.* **2020**, *8*, 381. [CrossRef]
46. Labrador, M.; Giménez-Rota, C.; Rota, C. Real-time PCR method combined with a matrix lysis procedure for the quantification of *Listeria monocytogenes* in meat products. *Foods* **2021**, *10*, 735. [CrossRef] [PubMed]
47. Vamanu, E.; Pop, M.I.; Pop, I.O.; Mishra, S.K.; Singh, K.S. Therapeutic potential of functional product based on wild edible mushrooms. *AgroLife Sci. J.* **2023**, *12*, 229–235. [CrossRef]

48. Hodzic, E.; Glavinic, A.; Wademan, C. A novel approach for simultaneous detection of the most common food-borne pathogens by multiplex qPCR. *Biomol. Biomed.* **2023**, *23*, 640. [CrossRef]
49. Suther, C.; Moore, M.D. Quantification and discovery of PCR inhibitors found in food matrices commonly associated with foodborne viruses. *Food Sci. Hum. Wellness* **2019**, *8*, 351–355. [CrossRef]
50. Siala, M.; Barbana, A.; Smaoui, S.; Hachicha, S.; Marouane, C.; Kammoun, S.; Gdoura, R.; Messadi-Akrout, F. Screening and detecting *Salmonella* in different food matrices in Southern Tunisia using a combined enrichment/real-time PCR method: Correlation with conventional culture method. *Front. Microbiol.* **2017**, *8*, 2416. [CrossRef] [PubMed]
51. Løvdal, T.; Hovda, M.B.; Björkblom, B.; Møller, S.G. Propidium monoazide combined with real-time quantitative PCR underestimates heat-killed *Listeria innocua*. *J. Microbiol. Methods* **2011**, *85*, 164–169. [CrossRef] [PubMed]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

AgroLife Scientific Journal - Volume 13, Number 1, 2024
ISSN 2285-5718; ISSN CD-ROM 2285-5726; ISSN ONLINE 2286-0126; ISSN-L 2285-5718

DEVELOPMENT OF THE LACTIC ACID PERMEABILIZER v-qPCR TECHNIQUE FOR *Salmonella* spp. DETECTION AND QUANTIFICATION

Quthama AL-ZAIDI[1], Adelina Georgiana MATACHE[1], Laura-Dorina DINU[1], Eleonora CALZONI[2], Florentina MATEI[1, 3]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Biotechnology, 59 Marasti Blvd, District 1, Bucharest, Romania
[2]University of Perugia, Department of Chemistry, Biology and Biotechnology, 06100, Perugia, Italy
[3]Transilvania University of Brașov, Faculty of Food Industry and Tourism, 148 Castelului Street, 500014, Brașov, Romania

Corresponding author email: laura.dinu@biotehnologii.usamv.ro

Abstract

In the food industry, one of the biggest challenges is microbial contamination. Viability real-time PCR (v-qPCR) technology has made the detection and quantification of pathogens easier, and some issues remain with the accuracy of the results, especially in samples containing viable and non-viable cells. V-qPCR with propidium monoazide (PMA) and pre-treatment with permeability agents is one promising method to circumvent this issue. Therefore, the aim of the study was to improve the Salmonella spp. detection using a method with lactic acid (LA 5-10 mM) pre-treatment that permeabilized Gram-negative outer membrane. The signal reduction (dC_t = 13.97) after 10 mM LA pre-treatment was similar with the reduction in C_t signal (dC_t = 13.62) when only PMA treatment was applied to a suspension of non-viable cells. In the case of viable cells, the LA pre-treatment improved the v-qPCR detection. These preliminary studies provide useful information on the use of lactic acid, which has proven to be an effective, cheap and low-toxic permeabilizing agent.

Key words: *lactic acid, PMA, v-qPCR, pathogen detection, food safety application.*

INTRODUCTION

The pathogen *Salmonella* spp. can cause severe foodborne illness, so it is important to develop and improve detection technologies for this bacterium. The traditional detection approach based on cell enrichment (at least 10^4 cells per mL of *Salmonella* concentration) involves time-consuming steps to detect viable cells (Lee et al., 2015). In order to improve the *Salmonella* monitoring in food, this pathogenic microorganism must be detected quickly, accurately, quantitatively, and more efficiently, given short shelf life of food matrixes (Vichaibun & Kanchanaphum, 2020). Molecular techniques are increasingly acknowledged as beneficial substitutes of traditional microbiological methods based on their speed, sensitivity, and specificity. But DNA from both dead and living bacteria can be amplified using real-time PCR (qPCR) or polymerase chain reaction (PCR) (Masters et al., 1994; Wolffs et al., 2005; Li et al., 2013; Barbau-Piednoir et al., 2014). Therefore, viability qPCR (v-qPCR) is a promising method based on pre-treatment with photochromic dyes that bind to the DNA of dead or damaged cells, making it possible to specifically identify DNA only from living cells (Qin et al., 2020). The basic premise of the v-qPCR assay is that all alive microorganisms must have an intact cell membrane or be resistant to biological reagents, regardless of their metabolic state. Otherwise, these reagents enter the cells and will interact with DNA that can no longer be used as a template in qPCR experiments due to the photochemical reaction resulting from exposure to high-intensity light beam (Codony et al., 2023). Since these dyes only enter damaged or dead cells, the process depends on the integrity of the bacterial cells (Nocker et al., 2006). Ethidium monoazide (EMA) was initially shown to be selective in detecting living cells (Nogva et al., 2003; Rudi et al., 2005; Rudi et al., 2005). Later, propidium monoazide (PMA) has been proved to be more selective than ethidium monoazide (EMA) for

alive bacteria detection and quantification, due to the molecule's higher charge (Nocker et al., 2006; ElizaquÃvel et al., 2012; Thilakarathna et al., 2022). Several studies have demonstrated the effectiveness of v-qPCR in detecting and quantifying different microbial species, including foodborne pathogens. It has been noted that the v-qPCR method may have some practical limitations, even with improvements made to the assay, such as changes in the qPCR protocol, working time, or dye concentration used (Fittipaldi et al., 2012; Nkuipou-Kenfack et al., 2013). The complexity of the matrix (food, medical or environmental) which can block the signal from dead cells, leading to an overestimation of the number of live cells in samples, is one of the biggest obstacles. Another issue is related to the ability of some bacterial strains to avoid dye uptake. Therefore, *Salmonella enterica* serovar *typhimurium ATCC 53648* and *Listeria monocytogenes ATCC 49594* strains were identified by Nkuipou-Kenfack et al. (2013) using sodium deoxycholate permeabilizer (DOC) 0.01-0.3% (w/v) as pre-treatment, then treating cells with 10 μM PMA, for 5-30 minutes, at different temperature values (0°C, 20°C and 40°C). It was observed that only the detection of the *Salmonella* strain successfully benefited from DOC, as there was a signal drop more than 10. Furthermore, the investigation showed a strong correlation between the length and temperature of dye incubation and the effectiveness of PMA treatment (Nkuipou-Kenfack et al., 2013). Organic solvents such as acetone, methanol, and detergents have been proved to permeabilize bacterial cells (Jamur & Oliver, 2010). In another experiment, a multiplex PMA-qPCR assay with sarcosyl pre-treatment was successfully applied to detect *Legionella pneumophila*, *S. typhimurium*, and *S. aureus* from water samples (Li et al., 2015). The aim of the study was to test a new type of cell permeabilizer, which would allow the photoactive dye PMA to enter the *Salmonella* spp. cells more easily and thus improve the accuracy of the v-qPCR assay. Lactic acid (LA), the material suggested in this study, has the primary benefit of having far less toxicity when compared to the other permeabilizers used. It is also less expensive and simpler to handle.

MATERIALS AND METHODS

Bacterial strain and culture conditions

Salmonella typhimurium ATCC 14028 strain from the Microorganisms Collection of the Laboratory of Applied Microbiology - Faculty of Biotechnology, was cultured for 24 h, at 37°C, on 20-25 mL Tryptone Soy Broth (TSB) medium (Neogen, USA). To measure the optical density at 600 nm (OD 600 nm) and to calculate the microbial load of the inoculum, an UV-1800 spectrophotometer (ChromTech, USA) was used. At the same time, decimal dilutions in sterile distilled water (10^{-1}-10^{-8}) were prepared to determine the number of colonies plated onto Tryptone Soy Agar (TSA) growth medium (Scharlau, Spain), expressed as CFU/mL.

Inactivation of bacterial cells by heat treatment

An amount of 10 mL of inoculum containing 10^8 bacterial cells/mL was incubated in glass tubes for 45 min at 80°C in a water bath (Memmert, Germany) to inactivate the cells. To test the loss of viability one milliliter of suspension was plated onto TSA medium for 24 h at 37°C.

Testing the effect of lactic acid (10-20 mM) on microbial vitality

A 30% (v/v) L-(+)-lactic acid stock solution (Sigma, USA) was used to prepare 10 to 20 mM LA solutions, pH=5-5.5. To test the sublethal effect of lactic acid on bacteria, two replicates of *Salmonella* spp. inoculum (3 mL) were combined with 3 mL of lactic acid solution at various concentrations between 10 to 20 mM. The control was represented by the mixture of 3 mL inoculum and 3 mL sterile distilled water. After 24 h at 37°C, the optical density was checked.

Testing the synergistic effect lactic acid - antibiotic CIP1

The Kirby-Bauer method was used to test the synergistic effect produced by the permeabilizer and antibiotic - ciprofloxacin 1 mcg - CIP1 (Sigma, USA). The antibiotic disks were soaked into permeabilizer solutions (LA 5-20 mM, EDTA 10 mM) and placed on the surface of the TSA medium inoculated with bacterial strain. After 24 h at 37°C incubation, the diameter of lysis zone was measured.

Pre-treatment with lactic acid (5 and 10 mM)

Aliquots (400 µL/each) of heat-killed cells and others with living cells (400 µL/each) were created in order to examine the qPCR signal reduction. They were subjected to bacterial outer membrane permeabilization treatment using 5 mM and 10 mM lactic acid and incubated under stirring conditions (150 rpm), for 30 min. at room temperature, before PMA treatment.

PMA treatment, photolysis and cell washing step

To prepare a stock solution of 20 mM PMA, 1 mg of PMA (Biotium, Hayward, CA, USA) was dissolved in 98 µL of sterile distilled water. The stock solution was kept at -20ºC, in the dark. PMA was used according to the manufacturer's instructions, in a 50 µM working concentration. Therefore, 1 µL of PMA stock was added into 400 µL aliquots for 10 minutes, and tubes were incubated at room temperature, 150 rpm, in the dark. A 1000-W halogen light source (Omnilux, Germany) was used to expose the sample tubes to light. It was positioned 20 cm away. The tubes were kept on ice during the photolysis process to prevent overheating and sporadic mixing. Following five minutes in the light, the cells were harvested using centrifugation at 12,000 rpm for two minutes. To ensure that any remaining traces of PMA solution would not impede the extraction of free DNA, the cells were twice washed with sterile distilled water. To isolate the DNA, the pellet was again suspended in 200 µL of distilled water. Control sample sets (400 µL aliquots with alive and others with heat-killed cells) were not treated with LA for set I, not treated with PMA for set II and without both LA and PMA treatment for set III.

DNA extraction and qPCR amplification

In this step, the Quick-DNA Fungal/Bacterial Miniprep kit (Zymo Research, Germany) was used according to the manufacturer's instructions. Primer set Sc-8-F (5'–ATCGTGATACAGAACGCCG-3') and Sc-8-R (5'-TCTTCGTCATCCACCCAGA-3') was previously reported that target *Salmonella* spp. strains and the amplicon size is 83 bp (Yu et al., 2016). The qPCR assay was performed using a final volume of 25 µL/sample consisting of 12.5 µL Maxima SYBR Green/ROX qPCR Master mix (ThermoFisher, USA), 1 µL target DNA (up to 500 ng/reaction) 0.5 µL each forward and reverse primer (final concentration 0.3 µM/each) and 10.5 µL PCR-grade water. In order to quantify the microbes, quantitative PCR was carried out with the Rotor-Gene 6000 5plex HRM (Qiagen-Corbett Life Science, Australia). The three stages of the melting program were, depending on the product size (83 bp), denaturation at 95ºC for 10 min., followed by 40 cycles of 15 s denaturation at 95ºC, 30 s annealing at 60ºC, and 20 s elongation at 72ºC. Each time, a PCR-grade water solution (1 µL) was used in place of a DNA template as a negative control for the amplification process.

For signal reduction analysis after PMA and LA-PMA treatments, delta Ct (dCtPMA) for live and dead cells was calculated as shown:

$dC_{tALIVE\text{-}PMA} = C_{tALIVE,\ PMA} - C_{tALIVE,\ nonPMA}$

$dC_{tNON\text{-}VIABLE\text{-}PMA} = C_{tNON\text{-}VIABLE,\ PMA} - C_{tNON\text{-}VIABLE,\ nonPMA}$

$dC_{tALIVE\text{-}LA\text{-}PMA} = C_{tALIVE,\ LA\text{-}PMA} - C_{tALIVE,\ PMA}$

$dC_{tNON\text{-}VIABLE\text{-}LA\text{-}PMA} = C_{tNON\text{-}VIABLE,\ LA\text{-}PMA} - C_{tNON\text{-}VIABLE,\ PMA}$

Statistical analysis

The software IBM SPSS Statistics 23 was used for the statistical analysis. ANOVA was used for the analysis of variance at 95% significance (P = 0.05). For the qPCR analysis the average findings from two distinct tests with duplicates were analyzed. After plating, the counts were log converted, and the average and standard deviation were computed. Every experiment was run twice, with duplicates or triplicates.

RESULTS AND DISCUSSIONS

The sublethal effect of lactic acid on the strain *Salmonella typhimurium* ATCC 14028

The experiment was performed to determine the highest concentration of lactic acid at which the bacterial strains grow normally without being inhibited. Alakomi et al. (2000) have proved the direct correlation between the permeabilization potential of the lactic acid and its concentration (Alakomi et al., 2000). However, if the acidity of the substance ends

up damaging cell viability, then pre-treatment can create false negative results, which is not desirable. The results are shown in Figure 1 and proved that lactic acid concentration up to 10 mM do not significantly decrease the microbial viability.

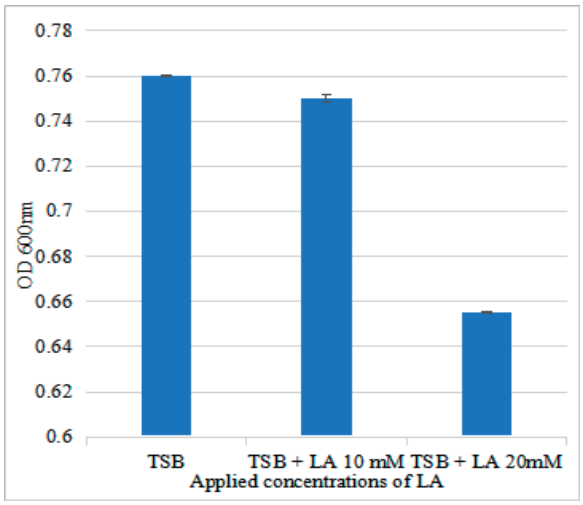


Figure 1. The sublethal effect of lactic acid (LA) on the growth of *Salmonella typhimurium* ATCC 14028 in tryptic soy broth (TSB)

The synergistic effect lactic acid - antibiotic on the strain *Salmonella typhimurium* ATCC 14028

It is well known that Gram-negative bacteria are inherently harder to kill with antibiotics than Gram-positive bacteria as Gram-negative microbes have an outer membrane that is difficult to be penetrated by antibiotic molecules. Therefore, we tested the outer membrane-disrupting and synergistic effect of different concentrations of LA (5-20 mM) and antibiotic ciprofloxacin (CIP1). The results about additive effect antibiotic - permeabilizer, tested by Kirby-Bauer method are shown in Figure 2. Combination antibiotic and different concentrations of lactic acid and EDTA up to 10 mM were more effective and produced a higher lysis zone compared to ciprofloxacin, where the diameter of the lysis zone was 22±2.82 mm. The most effective was combination between CIP1 and LA 5 mM that produced a clear zone of 28±2.83 mm (P>0.05), proving the potential of lactic acid to be used as an antibiotic synergist in therapy. The chelating agent EDTA is a well known described synergist with a reported ability to potentiate antibiotics, therefore was used as a positive control in our experiments (Wesseling & Martin, 2022).

The effect of PMA treatment on qPCR amplification of *Salmonella typhimurium* ATCC 14028

First, we compared the qPCR results obtained with aliquots of 100% alive and others with heat-killed cells, without LA and PMA treatment (Table 1).

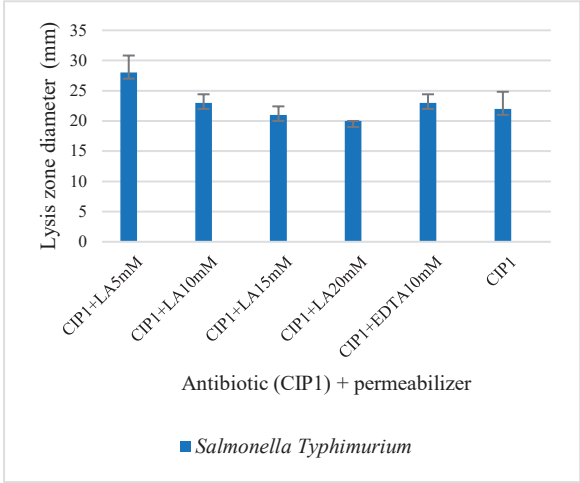


Figure 2. The synergistic effect antibiotic (CIP1) and permeabilizer (acid lactic LA 5-20 mM, EDTA 10 mM) on *Salmonella typhimurium* ATCC 14028

Table 1. Mean threshold values after different treatments with lactic acid (LA) and propidium monoazide (PMA) applied to *Salmonella* spp. suspensions of viable and non-viable cells

Treatment	C_t *viable*	C_t *non-viable*
-	9.99±0.10	**11.41±0.24**
LA 5 mM	ND	10.78±0.09
LA 10 mM	10.48±0.34	10.34±0.02
PMA 50 µM	13.41±0.28	**25.04±0.26**
	dC_t *viable*	dC_t *non-viable*
	3.42	13.62

ND- not determined

The mean threshold values (C_t) for non-viable and viable control samples were closer, 11.41±0.24 and 9.99±0.10, respectively. The results were not significantly different (P>0.05), proving that DNA from heat-killed cells were amplified (false positive result). Similar, control samples with 100% non-viable bacteria treated only with LA (5 mM, 10 mM) had mean C_t values of 10.78±0.09 and 10.34±0.02, respectively. At the same time, LA do not influence the DNA amplification from viable cells, mean threshold value for 10 mM

LA-treated cells being 10.48±0.34 compared to 9.99±0.10 for non-LA treated samples (Table 1). Another set of control samples were treated only with PMA, followed by photolysis and DNA amplification. Mean C_t values were significantly different (P>0.05), 25.04±0.26 for heat-killed and 13.41±0.28 for viable cells. These results proved that PMA efficiently penetrated the dead cells of *Salmonella* spp. and most of the DNA was not amplified after DNA isolation. The difference in the mean C_t values of PMA-treated and non-treated probes ($dC_{tNON\text{-}VIABLE\text{-}PMA}$) with non-viable cells is 13.62 suggesting a high qPCR signal reduction. It is considered that a signal reduction higher than 8 for non-viable cells indicates that 99.6% of DNA from these bacteria was removed (https://biotium.com/free-sample/). However, the DNA amplification from living cells was slightly influenced by the PMA treatment, as C_t values of PMA treated and untreated samples were similar and $dC_{tVIABLE\text{-}PMA}$ = 3.42 (Table 1). In another study with *Salmonella enterica* serovar *typhimurium* ATCC 53648 the signal reduction after 10 µM PMA treatment was related to the exposure time (5-30 min). The longest exposure time tested (30 minutes) produced the strongest signal reduction, with a difference of approximately 11 cycles between C_t values obtained from PMA-treated viable and non-viable samples (Nkuipou-Kenfack et al., 2013). In our experiment the difference between mean values obtained from PMA-treated viable and non-viable samples was 11.63 cycles, proving that PMA enter to all non-viable cells and inhibited the DNA amplification.

The effect of LA and PMA treatment on qPCR amplification of *Salmonella typhimurium* ATCC 14028

To assess the impact of LA and PMA treatment on qPCR assay results, the "viable dCt" and "nonviable dCt" indices were calculated. The qPCR signal reductions after 5mM and 10 mM LA pre-treatment, followed by PMA treatment were analyzed (Figure 3). Delta C_t values are the difference between LA-PMA treated and LA-treated samples, for both viable and non-viable cells. Both PMA and LA-PMA treatments had non-viable dC_t values greater than 8, demonstrating that PMA efficiently penetrated the dead cells of *Salmonella* spp. and inhibited DNA amplification. No significant differences between dC_t non-viable for different treatments with 5 mM and 10 mM LA-PMA and PMA (P>0.05) were noted. Moreover, viable dCt value after 10 mM LA-PMA treatment was lower (2.325) than dCt viable after propidium monoazide application (3.425), suggesting that LA might improve the PMA-qPCR assay. For living cells threshold values in samples without PMA and samples treated with PMA should be similar and $dC_{tVIABLE\text{-}PMA}$ less than 3. Nkuipou-Kenfack et al. (2013) showed that co-incubation of cells with PMA and deoxycholate improved the qPCR detection for non-viable gram-negative bacteria *Salmonella enterica* serovar *typhimurium* ATCC 53648. Using 0.1% DOC the PMA-qPCR detection of non-viable cells improved with ~ 2 cycles compared to PMA-qPCR. DOC concentrations up to 0.3% do not influence the qPCR signal from viable cells (Nkuipou-Kenfack et al., 2013). The difference between this result and our work might be related to the strain used and experiment design. It has to be pointed out that in our study cell suspensions were not co-incubated with permeabilizer and PMA as in the previous study. The specificity of the qPCR amplification with *Sc-8-F/R* primer set was verified. Based on the dissociation curves result, non-specific products were not produced.

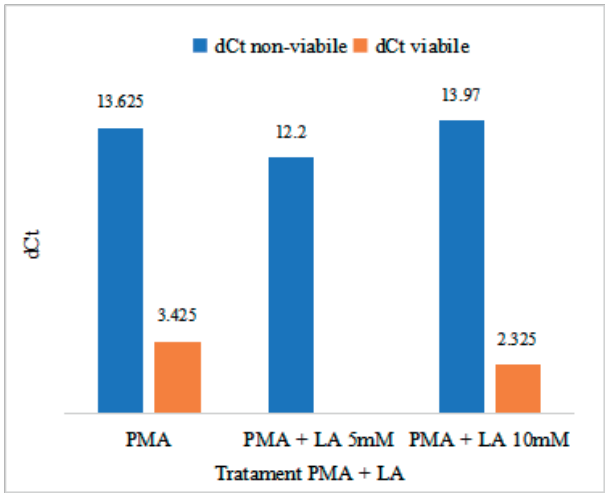


Figure 3. The signal reduction detected by qPCR after LA and PMA treatments applied to *Salmonella* spp. suspensions of viable and non-viable cells

CONCLUSIONS

This study aimed to contribute to the efforts to improve v-qPCR efficiency testing lactic acid, a new type of permeabilizing agent. The sublethal concentration of lactic acid enhanced the detection of viable Gram-negative bacteria ($dC_{tVIABLE\text{-}LA\text{-}PMA}$ = 2.32). However, the signal reduction ($dC_{tNON\text{-}VIABLE\text{-}LA\text{-}PMA}$ = 13.97) after 10 mM LA pre-treatment was similar with the reduction in C_t signal ($dC_{tNON\text{-}VIABLE\text{-}PMA}$ = 13.62) when only PMA treatment was applied to a suspension of non-viable cells. These preliminary studies provide useful information on the use of lactic acid, which has proven to be an effective, cheap and low-toxic enhancer for v-qPCR assay.

REFERENCES

Alakomi, H. L., Skytta, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., Helander, I. M. (2000). Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. *Applied and environmental microbiology*, *66*(5), 2001-2005.

Barbau-Piednoir, E., Mahillon, J., Pillyser, J., Coucke, W., Roosens, N. H., & Botteldoorn, N. (2014). Evaluation of viability-qPCR detection system on viable and dead *Salmonella* serovar Enteritidis. *Journal of microbiological methods*, *103*, 131-137.

Codony, F., Barreto, L., Agustí, G., & Asensio, D. (2023). Understanding the reaction balances behind the viability PCR protocols based on photoreactive dyes. *Journal of Microbiological Methods*, 106737.

ElizaquÃvel, P., Gloria, S. Ã., & Aznar, R. (2012). Quantitative detection of viable foodborne E. *coli O157*:*H7*, *Listeria monocytogenes* and *Salmonella* in fresh-cut vegetables combining propidium monoazide and real-time PCR. *Journal of Food Control 25*(2):704–708.

Fittipaldi, M., Nocker, A., & Codony, F. (2012). Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. *Journal of microbiological methods*, *91*(2), 276-289.

Jamur, M. C., & Oliver, C. (2010). Permeabilization of cell membranes. *Immunocytochemical methods and protocols*, 63-66.

Lee, K. M., Runyon, M., Herrman, T. J., Phillips, R., & Hsieh, J. (2015). Review of *Salmonella* detection and identification methods: Aspects of rapid emergency response and food safety. *Food control*, *47*, 264-276.

Li, M., Zhao, G., Liu, J., Gao, X., & Zhang, Q. (2013). Effect of Different Heat Treatments on the Degradation of *Salmonella* Nucleic Acid. *Journal of Food Safety*, *33*(4), 536-544.

Li, H., Xin, H., & Li, S. F. Y. (2015). Multiplex PMA–qPCR Assay with internal amplification control for simultaneous detection of viable *Legionella pneumophila, Salmonella typhimurium*, and *Staphylococcus aureus* in environmental waters. *Environmental science & technology*, *49*(24), 14249-14256.

Masters, C. I., Shallcross, J. A., & Mackey, B. M. (1994). Effect of stress treatments on the detection of *Listeria monocytogenes* and *enterotoxigenic Escherichia coli* by the polymerase chain reaction. *Journal of Applied Microbiology*, *77*(1), 73-79.

Nkuipou-Kenfack, E., Engel, H., Fakih, S., & Nocker, A. (2013). Improving efficiency of viability-PCR for selective detection of live cells. *Journal of microbiological methods*, *93*(1), 20-24.

Nocker, A., Cheung, C. Y., & Camper, A. K. (2006). Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *Journal of microbiological methods*, *67*(2), 310-320.

Nogva, H. K., Drømtorp, S. M., Nissen, H., & Rudi, K. (2003). Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5′-nuclease PCR. *Biotechniques*, *34*(4), 804-813.

Qin, H., Shi, X., Yu, L., Li, K., Wang, J., Chen, J., Yang, F., Xu, H. & Xu, H. (2020). Multiplex real-time PCR coupled with sodium dodecyl sulphate and propidium monoazide for the simultaneous detection of viable *Listeria monocytogenes, Cronobacter sakazakii, Staphylococcus aureus* and *Salmonella* spp. in milk. *International Dairy Journal*, *108*, 104739.

Rudi, K., Moen, B., Drømtorp, S. M., & Holck, A. L. (2005). Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. *Applied and environmental microbiology*, *71*(2), 1018-1024.

Rudi, K., Naterstad, K., Drømtorp, S. M., & Holo, H. (2005). Detection of viable and dead *Listeria monocytogenes* on gouda-like cheeses by real-time PCR. *Letters in Applied Microbiology*, *40*(4), 301-306.

Thilakarathna, S. H., Stokowski, T., & Chui, L. (2022). An Improved Real-Time Viability PCR Assay to Detect *Salmonella* in a Culture-Independent Era. *International Journal of Molecular Sciences*, *23*(23), 14708.

Vichaibun, V., & Kanchanaphum, P. (2020). Quantitative LAMP and PCR detection of *Salmonella* in chicken samples collected from local markets around Pathum Thani province, Thailand. *International journal of food science*, *2020*(3), 1–6.

Wolffs, P., Norling, B., & Rådström, P. (2005). Risk assessment of false-positive quantitative real-time PCR results in food, due to detection of DNA originating from dead cells. *Journal of Microbiological Methods*, *60*(3), 315-323.

Wesseling, C. M. J, Martin, N. I. (2022). Synergy by Perturbing the Gram-Negative Outer Membrane: Opening the Door for Gram-Positive Specific Antibiotics. *ACS Infection Disseases, 8*(9), 1731-1757.

Yu, Q., Zhai, L., Bie, X., Lu, Z., Zhang, C., Tao, T., Li, J., Lv, F., and Zhao, H. (2016). Survey of five food-borne pathogens in commercial cold food dishes and their detection by multiplex PCR. *Food Control*, *59*, 862-869.

BMC Microbiology

RESEARCH Open Access

Functional properties of lactic acid bacteria isolated from Tilapia (*Oreochromis niloticus*) in Ivory Coast

Wahauwouélé Hermann Coulibaly[1,3], N'goran Richard Kouadio[2], Fatoumata Camara[2], Camelia Diguță[3*] and Florentina Matei[3]

Abstract

Background Probiotics have recently been applied in aquaculture as eco-friendly alternatives to antibiotics to improve fish health, simultaneously with the increase of production parameters. The present study aimed to investigate the functional potential of lactic acid bacteria (LAB) isolated from the gut of Tilapia (*Oreochromis niloticus*) originating from the aquaculture farm of Oceanologic Research Center in Ivory Coast.

Results Twelve LAB strains were identified by 16 S rDNA gene sequence homology analysis belonging to two genera *Pediococcus* (*P. acidilactici* and *P. pentosaceus*) and *Lactobacillus* (*L. plantarum*) with a predominance of *P. acidilactici*. Several aspects including functional, storage, and safety characteristics were taken into consideration in the selection process of the native LAB isolates as potential probiotics. All LAB isolates showed high antagonistic activity against bacterial pathogens like *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus mirabilis*, and *Staphylococcus aureus*. In addition, the LAB isolates exhibited different degrees of cell surface hydrophobicity in the presence of hexane, xylene, and chloroform as solvents and a good ability to form biofilm. The strong antioxidant activity expressed through the DPPH scavenging capacity of LAB intact cells and their cell-free supernatants was detected. LAB strains survived between 34.18% and 49.9% when exposed to low pH (1.5) and pepsin for 3 h. In presence of 0.3% bile salts, the growth rate ranged from 0.92 to 21.46%. Antibiotic susceptibility pattern of LAB isolates showed sensitivity or intermediate resistance to amoxicillin, cephalothin, chloramphenicol, imipenem, kanamycin, penicillin, rifampicin, streptomycin, tetracycline and resistance to oxacillin, gentamicin, and ciprofloxacin. No significant difference in antibiotic susceptibility pattern was observed between *P. acidilactici* and *P. pentosaceus* strains. The non-hemolytic activity was detected. Following the analysis of the enzyme profile, the ability of LAB isolates to produce either lipase or β-galactosidase or both enzymes was highlighted. Furthermore, the efficacy of cryoprotective agents was proved to be isolate-dependent, with LAB isolates having a high affinity for D-sorbitol and sucrose.

Conclusion The explored LAB strains inhibited the growth of pathogens and survived after exposure to simulated gastrointestinal tract conditions. The safety and preservative properties are desirable attributes of these new probiotic strains hence recommended for future food and feed applications.

*Correspondence:
Camelia Diguță
camifilo@yahoo.com

Full list of author information is available at the end of the article

© The Author(s) 2023. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated in a credit line to the data.

Keywords Tilapia fish, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Lactobacillus plantarum*, Probiotics, Aquaculture

Background

In West Africa, intensive and semi-intensive systems of aquaculture farming remain the most common among fish farmers [1, 2]. Bamba et al. [3]; Gabriel et al. [4] and Crentsil and Ukpong [5] reported a massive use of agro-industrial by-products of plant origin (wheat bran, corn bran, rice bran, low rice flour) at a lower cost as feed for fish farming on most fish farms in sub-Saharan Africa. However, these agro-industrial by-products have a low protein, nutritional and immune contribution [6]. Furthermore, an inadequate application of antibiotics to boost fish production could lead to adverse disorders such as an imbalance in the gut microbiota, poisoning, immunity reduction as well as predisposition to the development of diseases [7–9]. Moreover, using antibiotics could be a potential risk to the health of consumers since the vast majority of antibiotics used are the same used to treat human infections [9]. Currently, probiotics are intensively promoted as healthy alternatives for sustainable aquaculture [10–14].

According to the World Health Organization (WHO) and the Food and Agriculture Organization (2014), probiotics were defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [15, 16]. Lactic acid bacteria (mainly *Lactobacillus sp., Bifidobacterium* sp. and *Pediococcus* sp.) [17–20], *Bacillus* sp. [20, 21] and a few yeasts (mainly *Saccharomyces boulardii* and *S. cerevisiae*) [22–24] are intensively studied as probiotics to improve aquatic life health and performance of fish. However, it was found that the probiotic bacteria isolated from other hosts used in aquaculture do not colonize efficiently the fish gut as the native (indigenous) probiotics [25–27]. In their study, Boutin et al. [26] reported that native probiotic strains are a better choice than exogenous probiotics which could cause the homeostatic disturbance of the fish microbiota. Recently, research has focused more on host-associated microorganisms as a source of probiotics, due to the fact that the health beneficial effects could be species-specific, as well as that they adapt much more easily to the aquatic environment (e.g. salinity, temperature) [28–34]. Microorganisms with potential use as probiotics have been isolated from the gastrointestinal tract of Atlantic cod (*Gadus morhua*) [31], common carp [32], giant freshwater prawn (*Macrobrachium rosenbergii*) [33], rainbow trout (*Oncorhynchus mykiss*) [34], Nile Tilapia (*Oreochromis niloticus*) [29, 30, 35], Mediterranean trout (*Salmo macrostigma*) [36]. The choice of inappropriate microbes could have been the cause of the negative results observed in probiotic research [27, 37–39]. Different functionality, safety, and storage criteria have been established to investigate the microbial strains with probiotic potential, thus allowing the screening of the most promising strains [27, 37–39]. Generally, the criteria for the selection of probiotics are highlighted as antibacterial activity, antibiotic susceptibility, simulation of gastrointestinal conditions [40–46] biofilm-forming ability [47–50], hemolytic activity [51], hydrophobicity [52], antioxidant activity [53, 54], and enzymes production [36, 55, 56].

Currently, many commercial probiotics which contain one or more live microorganisms are introduced in fish farming industries mainly to improve the growth performance and boost the health of fish [57–60]. According to the study of Nimrat and Vuthiphandchai [57], none of the 12 commercial probiotics used in marine shrimp culture in Thailand did offer correct informations about the composition or number of micro-organisms or qualitative extracellular enzymes described on the labels. Furthermore, none of the commercial probiotics could inhibit the growth of the shrimp pathogen *V. harveyi* [57].

Several studies have proven some beneficial effects linked to the administration of native probiotics on fish species, including high feed conversion efficiency, supply of nutrients and enzymatic input to digestion, increased growth performance and stimulation of the immune system [61–72]. overall suggesting that native probiotics could be relevant alternatives to antibiotics to control emerging fish diseases, increase stress resistance and improve water quality [61–72]. Fish production may therefore be improved by using indigenous probiotics for the sustainable development of African aquaculture [73].

Thus, the current study aimed to investigate the lactic acid bacterial (LAB) strains isolated from the gut of Tilapia (*Oreochromis niloticus*) as potential probiotics, by addressing their functional properties (antibacterial activity, biofilm-forming ability, simulation of gastrointestinal conditions, hydrophobicity, antioxidant, and enzymatic activities), safety (antibiotic sensibility and hemolytic activity) and storage (freeze-drying survival).

Results

Molecular identification of LAB isolates

The LAB strains included in the study (Table 1) were isolated from the intestine of Tilapia (*Oreochromis niloticus*) originating from the aquaculture farm of the Oceanologic Research Center in Ivory Coast. The full-length 16 S rDNA genes of all the LAB isolates were sequenced to identify them at the species level. A BLAST search of the 16 S rDNA gene sequences obtained was

Table 1 LAB strains accession number

Strains	Accession number
Pediococcus acidilactici LB100	ON141894
P. acidilactici LB98	ON141895
P. acidilactici LB187	ON141896
P. acidilactici LB194	ON141897
P. acidilactici LB166	ON141898
P. acidilactici LB156	ON141899
P. acidilactici LB143	ON141900
P. acidilactici LB45	ON141901
P. acidilactici LB137	ON141902
P. pentosaceus LB195	ON141903
P. pentosaceus LB82	ON141904
Lactobacillus plantarum LB96	ON141905

performed at NCBI and revealed high similarity values to many bacterial 16 S rDNA sequences deposited in the NCBI database. LAB strains identified belonged to two genera *Pediococcus* and *Lactobacillus*. The partial 16 S rDNA gene sequences of nine LAB strains (LB45, LB98, LB100, LB137, LB143, LB156, LB166, LB187, and LB194) were identified as *P. acidilactici* (showed 96.38 −98.21% homology to the GenBank sequences). The other two LAB strains (LB82 and LB195) were identified as *P. pentosaceus* (97.43-99% homology to GenBank sequences). The LB96 strain had 97.66% homology with the known *Lactobacillus plantarum* sequences. The partial 16 S rDNA sequences of LAB strains were deposited in the NCBI database (the accession numbers are listed in Table 1).

The phylogenetic tree revealed the existence of several groups of LAB species (Fig. 1). Thus, the *L. plantarum* LB96 (ON141905) was related to *Lactobacillus plantarum* TMPC 3M613 strain (OM757925), supporting a bootstrap value of 88%. *P. pentosaceus* LB82 (ON141904) and LB195 (ON141903) were related to *P. pentosaceus* FB 145 strain (MF945626) with a bootstrap score of 40%. *P. acidilactici* strains (LB45, LB98, LB100, LB137, LB143, LB156, LB166, LB187, LB194) were related to different *P. acidilactici* strains.

Functional properties of LAB isolates

Antibacterial activity of the cell-free supernatants (CFS) from the LAB strains

Antibacterial activity against pathogenic bacteria was considered an important criterion for the selection of probiotics. In this research, the antibacterial activity of cell-free supernatants (CFS) from the LAB isolates was assessed against five pathogens including *E. coli, K. pneumoniae, P. mirabilis, P. aeruginosa*, and *S. aureus* using agar well diffusion method. The value of the inhibition zone diameter expressed in mm is summarized in Table 2. The LAB isolates showed statistically significant ($p<0.05$) inhibition rates regardless of the pathogen. Our results showed that the CFSs obtained from all LAB strains exhibited good antibacterial activity against tested pathogenic bacteria. Moreover, CFS from the LB143 strain seems to exert the highest inhibition effect against *S. aureus*, *P. mirabilis*, and *K. pneumoniae.* A similar inhibitory activity against *P. aeruginosa* and *E. coli* was observed for CFS from LB195.

Ability to form a biofilm

All LAB isolates had a high capacity to form a biofilm. The absorbance values were higher than 0.5, ranging from 0.928±00 (LB96) to 3.211±0.01 (LB143). The statistical analyses showed a significant difference ($p<0.05$) between the isolates (Table 3).

Hydrophobicity

The hydrophobicity test was carried out in the presence of hexane, xylene, and chloroform as solvents. The results revealed that hydrophobicity rates were 1.53±0.1 and 16.30±0.4% in the presence of hexane for isolates LB195 and LB156, respectively. In the presence of xylene as the solvent, isolate LB137 manifested the highest hydrophobicity (51.10±0.8%), while the lowest was observed at isolate LB82 (1.17±0.8%). The use of chloroform as solvent showed hydrophobicity values that ranged from 9.4±0.14% (LB96) to 87.2±0.14% (LB166). Furthermore, statistical analyses showed a significant difference ($p<0.05$) between isolates for the same solvent and between different solvents (Table 3).

Antioxidant activity

In general, antioxidant activity levels were statistically significant ($p<0.05$) for both supernatants and LAB intact cells. Overall, the antioxidant activity levels observed in the supernatants were higher than those in the intact cells (Table 3). However, for isolates LB96, LB137, and LB195 antioxidant activity values were highest in intact cells than in the supernatant with 87.28±0.40, 70.40±0.57%, and 57.19±0.27%, respectively (Table 3). For each sample (supernatant and intact cells), statistical analyses showed a significant ($p<0.05$) difference between the isolates.

Tolerance to bile salts and resistance to pepsin and acid pH of LAB isolates

A critical step toward the selection of probiotic strains was to survive conditions that mimic the gastrointestinal tract. The LAB isolates bile salts tolerance, as well as the resistance in the presence of pepsin and acid pH evolution tests are shown in Fig. 2. Generally, an adaptation of all LAB isolates was observed after 4 h of exposure to bile salts characterized by cell growth, while exposition after 3 h to 0.3% pepsin and acid pH (1.5) was marked with a decrease in bacterial load. Based on growth

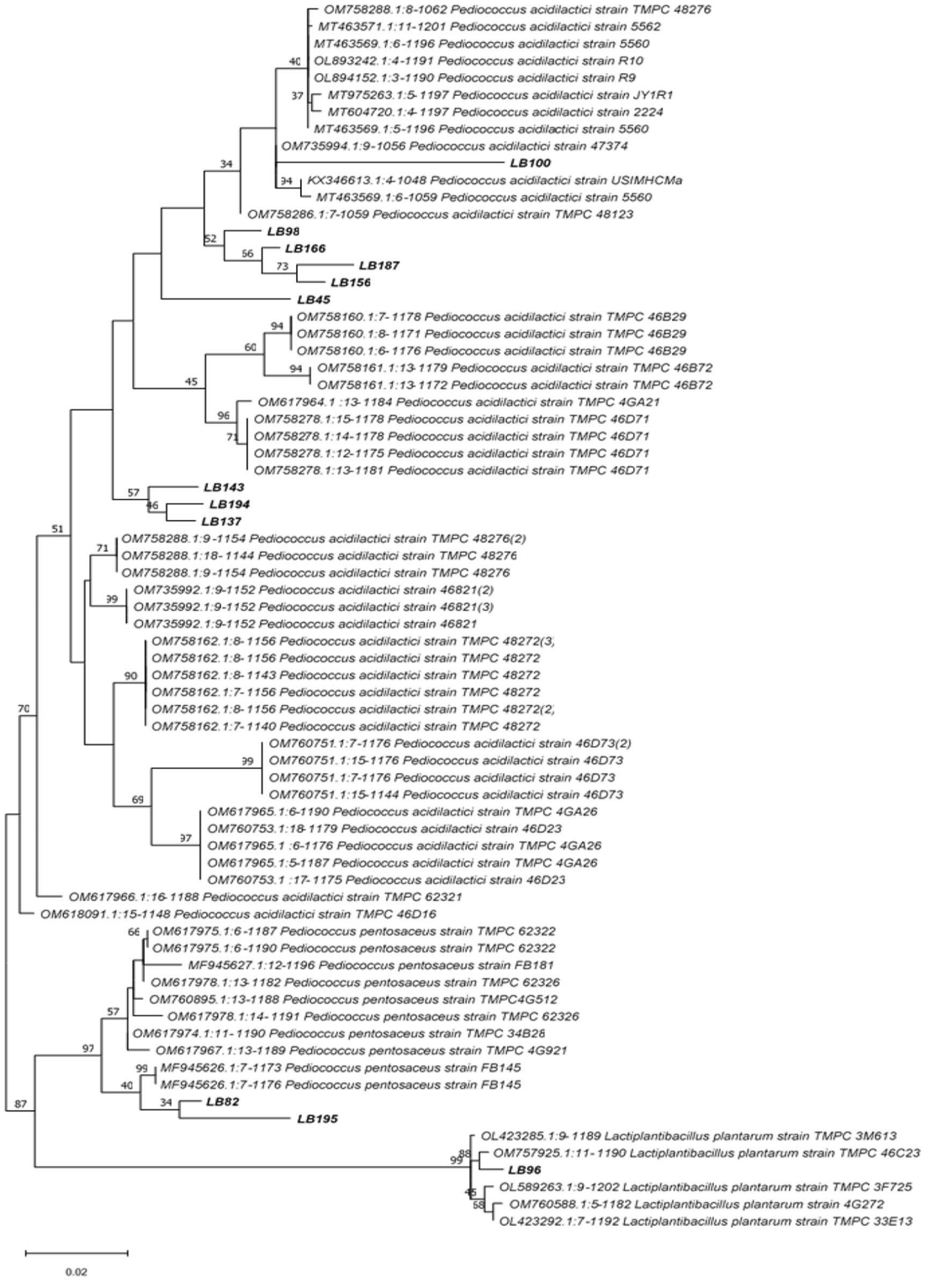


Fig. 1 The phylogenetic tree showing the relative position of LAB isolates as inferred by the neighbour-joining method with 16 S rDNA gene sequences

rates, three profiles were observed. The most significant growth ($p<0.05$) was observed for isolate LB 194, with a growth rate of 27.33%, followed by isolates LB45, LB82, LB98, LB100, and LB166 (growth rate between 15.36 and 16.88%). The least growth rates were observed in isolates LB 143 and LB 195 (0.92 and 1.06%). The growth rates showed a significant difference ($p<0.05$) between the three profiles.

Table 2 Antibacterial activity of LAB strains against pathogenic microorganisms

Isolates	*P. aeruginosa* ATCC 27853	*E. coli* ATCC 25922	*S. aureus* ATCC 25913	P. *mirabilis* JCM 1669	*K. pneumoniae* ATCC 43816
LB45	+ +	+ +	+ +	+ +	+ +
LB82	+ + +	+ +	+ +	+ +	+ +
LB96	+ +	+ +	+ +	+ +	+ +
LB98	+ +	+ + +	+ +	+ +	+ +
LB100	+ +	+ +	+ +	+ +	+ +
LB137	+ +	+ + +	+ +	+ +	+ +
LB143	+ +	+ +	+ + +	+ + +	+ + +
LB156	+ +	+ + +	+ +	+ +	+ +
LB166	+ +	+ +	+ +	+ +	+ +
LB187	+ +	+ +	+ +	+ +	+ + +
LB194	+ +	+ +	+ +	+ +	+ +
LB195	+ + +	+ + +	+ +	+ +	+ +

(+): 1–5 mm ; (+ +): 6–17 mm ; (+ + +): 18–29 mm

Despite the overall decrease in bacterial load, the level of resistance to pepsin and acid pH (1.5) was reflected by a survival rate ranging from 34.18 to 49.9%. The highest survival rate was obtained for the LB166 isolate which was significantly different ($p<0.05$), while the lowest rate was observed in the LB96 isolate.

Each value represents the mean value ± standard deviation (SD) (n = 3). Bars with different lower-case letters denoted significantly different ($p<0.05$).

In vitro **investigation of enzymatic activities of LAB isolates**.

To detect amylase, protease, lipase, and β-galactosidase activities the LAB isolates were inoculated into selective media for each enzyme. Our results revealed that the nine LAB strains tested were positive for lipases (pink-orange colony under UV 352 nm) and β-galactosidase (green colony) (Table 4). LB 143 did not exhibit lipase activity, while LB 137 and LB 156 did not exhibit β-galactosidase activity. No activity was detected for the amylases, cellulases, and proteases, respectively (Table 4).

Safety properties of LAB isolates

Antibiotic susceptibility

The antibiotic susceptibility of the LAB isolates was tested using the antibiotic disc diffusion method on MRS agar plates. A total of 12 antibiotics were included in the assay: gentamicin, chloramphenicol, kanamycin, streptomycin, tetracycline as inhibitors of protein synthesis, amoxicillin, cephalothin, oxacillin, penicillin, imipenem as inhibitors of cell wall synthesis, ciprofloxacin as inhibitors of DNA replication and rifampicin as inhibitors of nucleic acids synthesis. All LAB isolates showed variations in antibiotic susceptibility to 9 out of the 12 antibiotics and also showed multidrug resistance for oxacillin, gentamicin, and ciprofloxacin (Table 5). No significant difference in antibiotic susceptibility profile was observed between *P. acidilactici* and *P. pentosaceus* strains.

Hemolytic activity

Probiotic strains must be risk-free (γ-hemolysis), which makes them safe for consumption [15, 37]. In our study, all LAB strains showed γ-hemolysis activity (without clearing zones around the colonies on blood agar plates) (Fig. 3), thus ensuring the safety to be used as potential probiotics.

Table 3 Ability to form a biofilm (AFB), antioxidant activity (AA), and hydrophobicity (H) of LAB isolates

Isolates	AFB	Antioxidant activity (AA) (%)		Hydrophobicity (H) (%)		
		Supernatants	Intact cells	Hexane	Xylene	Chloroform
LB 45	1.425 ± 0.04^{a}	90.23 ± 0.32^{a}	36.74 ± 0.05^{b}	10.94 ± 0.01^{a}	7.23 ± 0.1^{b}	15.85 ± 0.32^{c}
LB 82	2.886 ± 0.002^{b}	51.92 ± 0.03^{a}	49.95 ± 1.34^{a}	9.96 ± 0.6^{a}	1.17 ± 0.8^{b}	51.9 ± 0^{c}
LB 96	0.928 ± 0^{c}	61.18 ± 0.25^{b}	87.28 ± 0.40^{a}	12.92 ± 1^{a}	10.83 ± 0.3^{ab}	9.4 ± 0.14^{b}
LB 98	2.843 ± 0.06^{b}	74.29 ± 0.12^{a}	50.34 ± 0.03^{b}	9.40 ± 0.2^{a}	3.48 ± 0.4^{b}	11.05 ± 0.17^{ac}
LB 100	2.722 ± 0.03^{b}	84.06 ± 0.08^{a}	39.52 ± 0.73^{b}	11.82 ± 2.1^{a}	8.87 ± 0.6^{b}	20.58 ± 0.29^{c}
LB 137	1.616 ± 0.05^{d}	67.86 ± 0.08^{b}	70.40 ± 0.57^{a}	7.38 ± 1.3^{a}	51.10 ± 0.8^{b}	10.49 ± 0.06^{c}
LB 143	3.211 ± 0.01^{e}	75.57 ± 0.81^{a}	73.28 ± 0.38^{a}	9.05 ± 0.4^{a}	4.97 ± 0.2^{b}	22.78 ± 0.07^{b}
LB 156	1.489 ± 0^{d}	64.52 ± 0.03^{a}	44.19 ± 0.26^{b}	16.30 ± 0.4^{a}	8.31 ± 0.02^{b}	20.47 ± 0^{c}
LB 166	3.048 ± 0^{e}	63.85 ± 5.4^{a}	54.96 ± 1.36^{a}	15.55 ± 0.01^{a}	6.99 ± 0.03^{b}	87.2 ± 0.14^{c}
LB 187	1.362 ± 0.06^{d}	57.84 ± 1.18^{a}	34.06 ± 0.08^{b}	11.52 ± 0.8^{a}	7.62 ± 0.5^{b}	23.18 ± 0.25^{c}
LB 194	2.710 ± 0.007^{b}	76.60 ± 0.85^{a}	54.02 ± 0.02^{b}	11.13 ± 0.3^{a}	20.84 ± 1.2^{b}	$22.68 \pm 0.9b^{c}$
LB 195	3.046 ± 0.01^{e}	50.64 ± 0.05^{b}	57.19 ± 0.27^{a}	1.53 ± 0.1^{a}	4.21 ± 0.6^{b}	73.54 ± 0.85^{c}

(-) Negative to hemolytic activity; Values expressed as mean ± standard deviation for three independent measurements.

AFB: Mean values with the same letter in a column were not significantly different ($p>0.05$)

AA: Mean values with the same letter in a line were not significantly different ($p>0.05$)

H: Mean values with the same letter in a line were not significantly different ($p>0.05$)

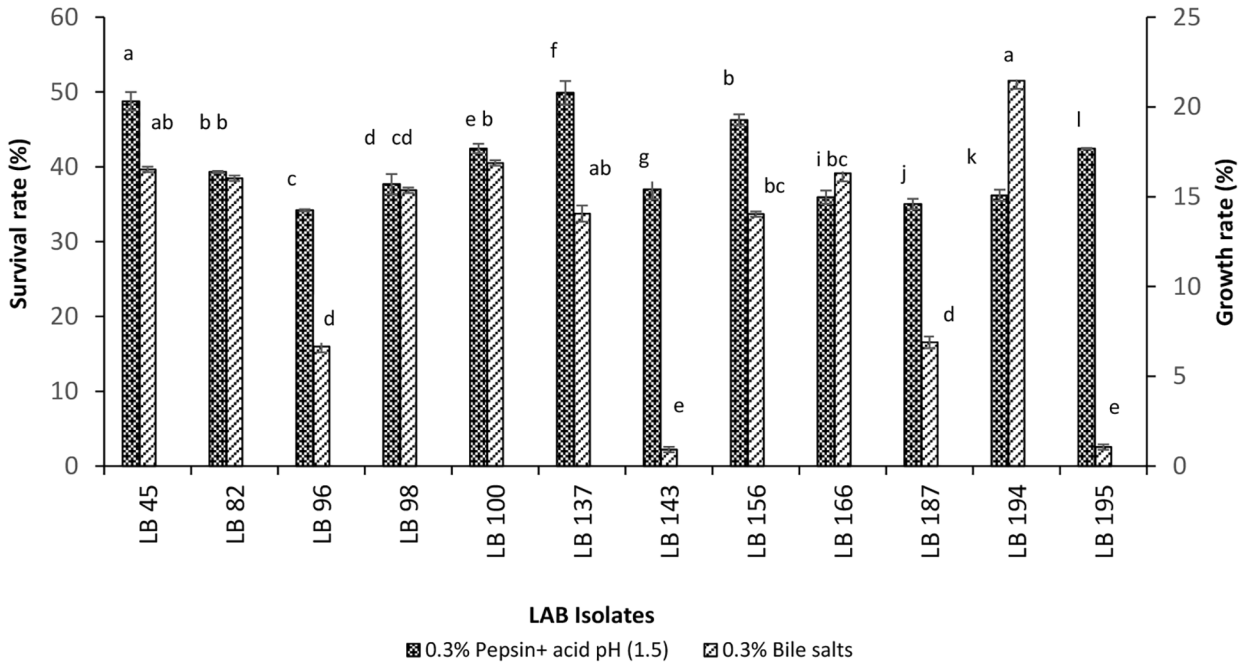


Fig. 2 Growth rate and survival rate of LAB isolates in the presence of 0.3% bile salts and 0.3% pepsin and pH 1.5 respectively

Table 4 Hydrolytic enzyme profile of the LAB isolates

Isolates	Enzymatic activities				
	Amylolytic	Cellulolytic	Lipolytic	Proteolytic	β-galactosidase
LB 45	-	-	+	-	+
LB 82	-	-	+	-	+
LB 96	-	-	+	-	+
LB 98	-	-	+	-	+
LB 100	-	-	+	-	+
LB 137	-	-	+	-	-
LB 143	-	-	-	-	+
LB 156	-	-	+	-	-
LB 166	-	-	+	-	+
LB 187	-	-	+	-	+
LB 194	-	-	+	-	+
LB 195	-	-	+	-	+

(+) and (-) indicate the presence and absence of the enzymatic activities

Storage and preservation of LAB isolates: freeze-drying procedures

Preservation of the viability of LAB strains during freeze-drying is a critical challenge. Three cryoprotectants (D-sorbitol, D-glucose, and sucrose) were tested for their ability to protect the LAB cells during freeze drying, while sterile deionized water served as the negative control. The survival rate of freeze-dried LAB isolates is presented in Fig. 4. A strain-dependent relationship existed between the cryoprotectant's effectiveness. The isolates LB143 and LB98 had over 62% survival rate with all tested cryoprotectants, with maximum of 83.77±0.44% and 87.97±5.13% when sucrose was used as a cryoprotectant. On the contrary, the LB187 isolate had less than 10% survival rate with all cryoprotectants. LB143, LB96, LB137, and LB195 isolates demonstrated a high survival rate, from 53.50±0.33% to 74.52±9.64 with D-sorbitol (2%), whereas in the case of the LB82 and LB143 strains, glucose 2% (w/v) led to survival of 56.60±0.18%, and 66.44±0.14% respectively. Statistical analyses revealed a significant difference ($p<0.05$) in the survival rates of different cryoprotectants between isolates. The boxplot presented in Fig. 5 shows that the D-sorbitol (41.48%) offered better protection of the LAB isolates during freeze-drying compared with sucrose (34.17%) and glucose (21.78%).

Hierarchical ascending classification (HAC)

The HAC carried out showed that all the LAB isolates had the same characteristics (Fig. 6). Three groups were generated, the first group included isolates LB 82, LB166, and LB 195. The second group included isolates LB194; LB187; LB156 and LB100. The last group consisted of isolates LB143; LB137; LB98; LB96, and LB45.

Discussion

Today, aquaculture in the Ivory Coast has not yet reached a viable economic dimension, despite immense physical, hydrological (150 000 ha of lagoons, 350,000 ha of lakes, numerous shallows, etc.), climatic, and human resources [1]. The development of Ivorian aquaculture is hampered by several factors, the most significant of which are the

Table 5 Antibiotic susceptibility pattern of LAB isolates

Isolates	AML	OX	C	PEN	CN	TE	KAN	CIP	STR	RAM	IPM	GM
LB45	25 S	0R	28 S	27 S	11R	26 S	21 S	14R	30 S	35 S	26 S	22 S
LB82	28 S	0R	30 S	30 S	7R	24 S	25 S	10R	20 S	37 S	37 S	30 S
LB96	30 S	0R	29 S	30 S	14R	25 S	20 S	0R	25 S	32 S	25 S	27 S
LB98	23 S	0R	30 S	25 S	10R	19I	23 S	0R	19IR	38 S	40 S	30 S
LB100	23 S	0R	26 S	20 S	10R	23 S	19IR	13R	17IR	24 S	30 S	20 S
LB137	21 S	0R	24 S	22 S	10R	22 S	20 S	15I	28 S	23 S	32 S	18I
LB143	27 S	0R	30 S	30 S	12R	22 S	23 S	0R	25 S	40 S	35 S	30 S
LB166	23 S	0R	25 S	25 S	0R	20 S	20 S	12R	23 S	30 S	25 S	23 S
LB187	18IR	10	30 S	32 S	12R	20 S	20 S	0R	22 S	32 S	30 S	25 S
LB194	25 S	0R	30 S	27 S	12R	20 S	22 S	0R	22 S	35 S	28 S	24 S
LB195	23 S	0R	29 S	27 S	12R	21 S	24 S	0R	20 S	36 S	38 S	30 S

Legend: Resistant (R) ≤ 14 mm; intermediate resistant (IR) 15–19 mm; susceptible (S) > 19 mm; AML (Amoxicillin); OX (Oxacillin); C (Chloramphenicol); PEN (Penicillin); CN (Cephalothin); TE (Tetracycline); KAN (Kanamycin); CIP (Ciprofloxacin); STR (Streptomycin); RAM (Rifampicin); IPM (Imipenem); GM (Gentamicin)

Fig. 3 Hemolysis activity of LAB strains and *S. aureus* ATCC 25,913 (positive control)

availability of high-quality feed at exorbitant prices, a lack of technical skills, and the poor quality and quantity of fish [1]. Thus, the use of functional food (food enriched with probiotics) seems to be an ecological, economical, and sustainable solution.

Several aspects, including functional characteristics (antibacterial activity, biofilm-forming ability, simulation of gastrointestinal conditions, hydrophobicity, antioxidant activity), safety characteristics (molecular identification, antibiotic sensibility, and hemolytic activity), and storage (freeze-drying survival), have been taken into consideration in the selection process of LAB isolates as potential native probiotics.

Careful selection remains the main tool to obtain high quality probiotics. Proper strain identification at the species level is one key criterion to classify a microbial isolate as probiotics, especially for the microorganisms to be used in the food chain [74–76]. In this context, the well-known amplification and sequencing of the 16 S ribosomal DNA region are reliable tools to identify species at the expense of classical methods [74, 75]. In the present study, 12 LAB strains isolated from the intestine of Tilapia (*Oreochromis niloticus*) were identified by 16 S rDNA gene sequence homology analysis and belonged to *P. acidilactici* (9 strains), *P. pentosaceus* (2 strains), and *L. plantarum* (1 strain). The list of probiotics includes mainly members of the genera *Lactobacillus* and *Bifidobacterium* [35, 61, 71], but species belonging to the genus *Pediococcus*, particularly *P. acidilactici* and *P. pentosaceus*, have been investigated many times, indicating that newly isolated strains may play a key role in the new generation of functional ingredients [19, 77–80].

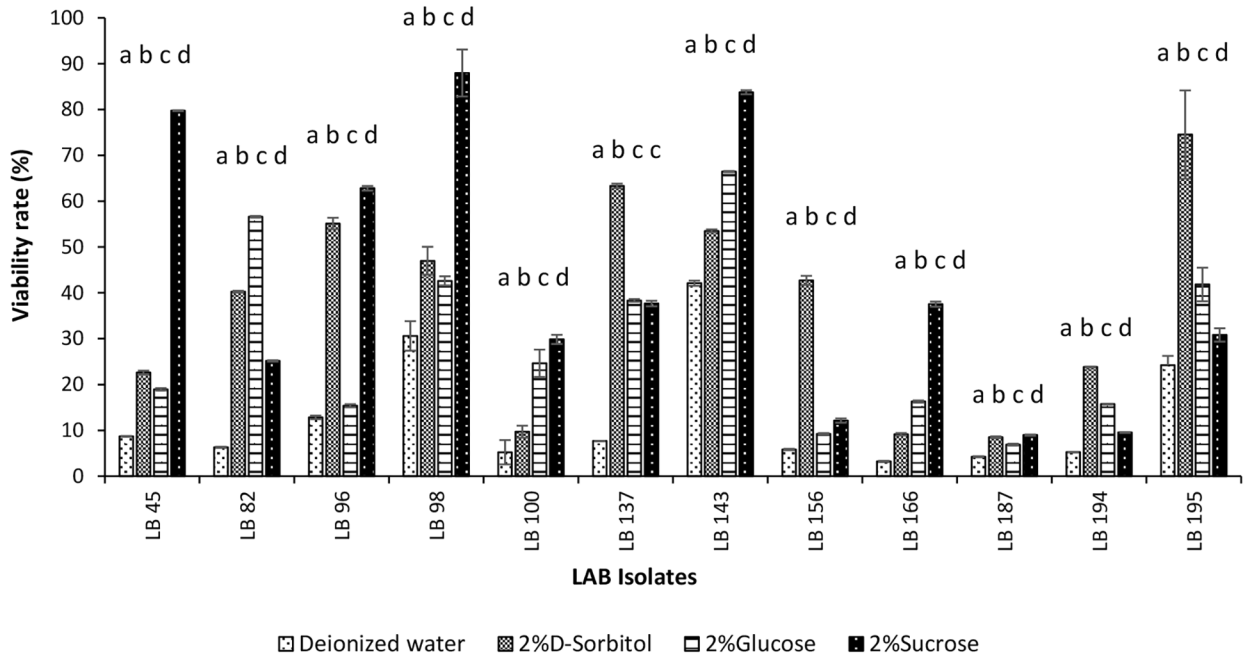


Fig. 4 Viability rate of LAB isolates in the presence of different protective agents at end of freeze-drying Each value represented the mean value ± standard deviation (SD) (n = 3). Bars with different lower-case letters denoted significantly different ($p < 0.05$)

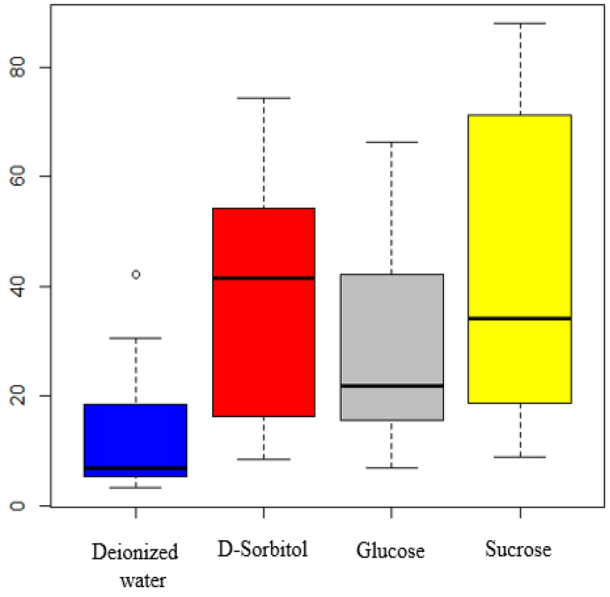


Fig. 5 Affinity of LAB isolates for protective agents

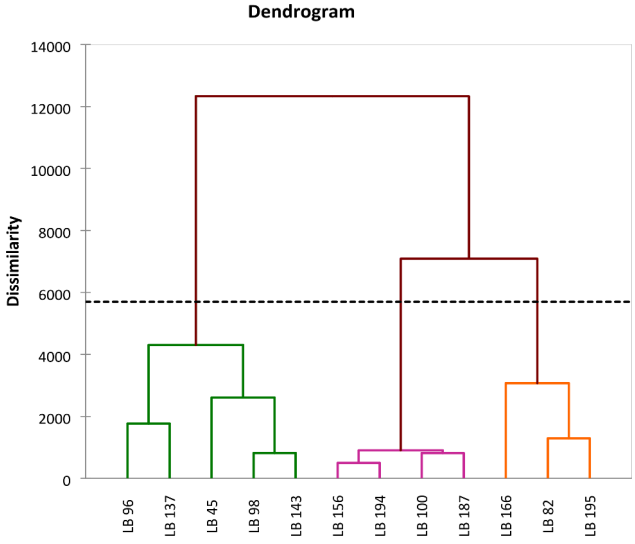


Fig. 6 Cluster dendrogram of LAB species

Some bacterial pathogens, such as *Escherichia sp., Klebsiella* sp., *Staphylococcus* sp., *Proteus* sp., and *Pseudomonas* sp., were isolated from fish and can indicate multiple sources of contamination [81–83]. All LAB isolates showed strong growth inhibition of all reference pathogens: *P. aeruginosa*, *E. coli*, *S. aureus*, *P. mirabilis*, and *K. pneumoniae*. These results are in agreement with previous studies where *Lactobacillus* and *Pediococcus* strains exhibited a broad spectrum of antagonistic activity against fish pathogens [19, 77, 84, 85].

In terms of probiotics, biofilm formation offered a more rapid capacity for metabolite production and resistance to hostile environments [47–50]. In our study, all the isolates showed a good ability to form biofilm. Lamari et al. [86] selected several LAB strains with the ability to form biofilm and high adherence to polystyrene microplates and hydrocarbon. This characteristic was important for

bacteria's ability to adhere to an abiotic surface, which could be a potential indicator for LAB to colonize the gut and further antagonize the pathogens [86].

Cell surface hydrophobicity is another property considered important for the probiotics' overall adhesion capacity to various types of surfaces. The hydrophobicity of LAB isolates selected in the presence of three different solvents revealed that values depended on the solvent used. Overall, the hydrophobicity values of LAB isolates with chloroform were higher than those with other solvents. The values vary between 1.53% (LB 195) and 16.30% (LB 166) for hexane, 3.48% (LB 98) and 51.10% (LB 137) for xylene, and 9.4% (LB 96) and 87.2% (LB 166) for chloroform. Yasmin et al. [51] reported high hydrophobicity values for *Bifidobacterium* strains for xylene; in this work, the highest hydrophobicity values were obtained in the case of chloroform. The results are confirmed in another study on *L. fermentum* URLP18 and *L. lactis* URLA2 strains, which showed high aggregation capacities and high affinity towards xylene, followed by chloroform [84].

Free radical-scavenging ability, as a criterion for probiotics, had been studied by several authors [53, 54]. These authors reported that extracellular liquid, intracellular liquid, and intact cells had free radical-scavenging properties. In our study, CFS and intact cells were investigated. Remarkably, the CFS of the nine LAB strains exhibited higher DPPH scavenging activities than the intact cells, as follows: LB45 (90.23%); LB82 (51.92%); LB98 (74.29%); LB100 (84.06%); LB143 (75.57%); LB156 (64.52%); LB166 (63.85%); LB187 (57.84%); and LB194 (76.60%). Yasmin et al. [51] reported that the *Bifidobacterium* exhibited strong antioxidant activity in cell-free supernatant, whose values varied between 80.72% and 87.72%.

Several studies reported the ability of probiotic microorganisms to produce extracellular enzymes improving the nutrient digestibility, growth performances, and health status of fish [13, 36, 55, 56]. For instance, Iorizzo et al. [36] showed that the LABs isolated from the intestinal tract of the Mediterranean trout (*Salmo macrostigma*) are producers of extracellular enzymes that help absorb the nutrients in the fish intestine. Our results showed the ability of 9 out of 12 LAB isolates to synthesize both β-galactosidase and lipases. No other enzymatic activity (amylases, cellulases, and proteases) was detected. This finding was in contrast with the results reported by Muñoz-Atienza et al. [42], in which the majority of the LAB strains did not shown lipolytic activity (with few exceptions). On the other hand, the LAB strains isolated from the intestine of freshwater fish species exhibited amylase, lipase, and protease activities [84]. Similarly, Marchwiska and Gwiazdowska [87] reported that different *Lactobacillus* and *Pediococcus* isolated from swine faeces for feed additive composition had protease and amylase activities, but no lipase activity. *Bacillus* species have already been known as enzyme producers, being one of the reasons for their use in aquaculture as probiotics enhancing feed digestibility, digestive enzyme activities, and growth performance [21].

Furthermore, antibiotic susceptibility is a prerequisite from a safety standpoint because probiotic bacteria might transfer antibiotic-resistance genes either directly or indirectly to pathogenic bacteria. This approach requires evidence that the LAB strain does not show resistance to antibiotics used in human and veterinary medicine. In our study, all LAB isolates were susceptible or intermediately resistant to 9 antibiotics and resistant to 3 antibiotics out of the 12 antibiotics used. LAB isolates showed resistance to oxacillin, gentamycin, and ciprofloxacin. Our result was important because LAB isolates showed sensitivity to penicillin, ampicillin, and chloramphenicol, among the most commonly used antibiotics in aquaculture. The results obtained were not consistent with those documented by Diguță et al. [78], which indicated that two *Pediococcus* strains (L3 and L5) isolated from the Kombucha consortium were found to be resistant to amoxicillin, streptomycin, kanamycin, and tetracycline. Furthermore, antibiotic sensitivity is a variable and strain-dependent property. In the context of probiotic LAB selection, drug-resistant probiotic bacteria are serious health threats. Increasing and abusive use of antibiotics has given rise to resistant bacteria through the transfer of resistance plasmids between bacteria [7–9, 88–90]. The resistance to an antibiotic may be accompanied by resistance to one or more other antibiotics, even if the bacteria have no contact with these antibiotics. Bacterial strains with transferable antibiotic resistance genes should not be used in animal feeds, fermentation, or probiotic foods for human consumption, according to the European Food Safety Authority (EFSA) [91]. Given the increase of drug-resistant probiotics has been recently developed the online ProbResist database, which centralizes reports of probiotic bacteria that have been demonstrated experimentally to be resistant to antibiotics [92].

The examination of hemolytic activity is strongly recommended by the EFSA as long as the isolated bacteria are intended for use in food products, even if they have "generally recognized as safe" (GRAS) or "quality presumption of safety" (QPS) status. In this study, all the isolates exhibited no hemolytic activity (γ-hemolysis), indicating that they are non-pathogenic and considered safe for animal or human probiotic applications. Similar results were previously obtained with two *Pediococcus* strains isolated from Kombucha (L3 and L5) [78]. Yasmin et al. [51] shown that eight *Bifidobacterium* strains isolated from raw camel milk did not exhibit hemolytic

activity. Lack of hemolytic activity is significant during the selection of probiotic strains when it comes to probiotic safety because such strains are non-virulent, and the lack of hemolysin assures that virulence will not arise among bacterial strains [15].

The ability of LAB strains to effectively function in the gastrointestinal tract (including bile salts tolerance and low gastric pH resistance) is the most important criterion for their selection as probiotics. Probiotic bacteria must first make it through the stomach, where the pH can be as low as 1.5 to 2 before reaching the intestinal tract [93, 94]. In our study, the resistance tests of these bacteria at low pH levels (ranging from 1 to 3) revealed that all strains are resistant at pH 1.5 for 3 h, while most strains lose viability in 1 h at pH 1.5. The resistance at 0.3% pepsin and low pH (1.5) was characterized by a high level of cell viability. The survival rates of the 12 LAB isolates ranged from 34.8 to 49.9%. Chemlal-Kerrhaz et al. [39] showed that two isolates of LAB from the Nile Tilapia (*Oreochromis niloticus*) tolerated a concentration of 0.3% bile salts for 4 h and pH 2 for 3 h.

In addition, all strains tolerated the concentration of 0.3% bile salts for 4 h. The growth rate of the 12 LAB isolates varied between 0.92 and 21.46%. The results obtained were lower than in previous studies which reported high survival abilities of different *Pediococcus* strains [78] and *L. fermentum* URLP18 isolated from *C. mrigala* [84] in the presence of high bile salts concentration (until 2%). Our study showed a similar outcome; all isolates tolerated a concentration of 0.3% bile salts for 4 h and exhibited resistance at pH 1.5, however with different intensities.

Freeze-drying, as a LAB conditioning technique, is recognized to cause severe damage to organisms, particularly at the membrane level as well as to their proteins, but the addition of cryoprotective agents may mitigate injury or inactivation by increasing cell survival during freeze-drying [78, 95]. In this study, three cryoprotective agents were assessed for their influence on the LAB isolates' viability rate at the end of the freeze-drying process. Our results indicate that the LAB isolates showed an important affinity for D-sorbitol and sucrose. This trend has been observed by Diguță et al. [78], where sucrose was responsible for the best viability rate of *P. acidilactici* and *P. pentosaceus* at the end of freeze-drying. Considering the strain-dependent variation in response to the stress conditions during the freeze-drying process, cryoprotectant agents must be investigated to choose them for conditioning of LAB strains with high efficiency on cell viability and economically feasible, before being included in functional foods or feeds.

Conclusion

Based on 16 S rDNA gene sequencing, 12 LAB strains isolated from the intestine of Tilapia (*Oreochromis niloticus*) were identified as belonging to *P. acidilactici, P. pentosaceus*, and *L. plantarum* species with a predominance of *P. acidilactici*. All LAB isolates showed a high antibacterial activity as well as a strong antioxidant activity. Additionally, they showed no hemolytic activity, a typical pattern of antibiotic susceptibility, and a good ability to form a biofilm, respectively. All LAB isolates exhibited either lipase or β-galactosidase or both enzymes production. Some LAB strains showed good survival rates in the simulated gastrointestinal conditions. Conditioning of LAB strains by freeze-drying using D-sorbitol or sucrose as cryoprotectant agents could be used to formulate probiotic products in powdered form. According to these results, *P. acidilactici* LB137 and *P. pentosaceus* LB195 present promising probiotic properties and could be applied as health promoters for fish. Further in vivo studies might use these strains in monoculture or co-culture to obtain enriched/supplemented food for fish farming, ultimately ensuring that healthier fish will be part of a healthier human diet.

Materials and methods

Bacterial strains and culture conditions

Twelve LAB strains were isolated from the intestine of Tilapia (*Oreochromis niloticus*) originating from the aquaculture farm of Oceanologic Research Center in Ivory Coast. Five pathogenic bacteria including *P. mirabilis* JCM1669 (University Nangui Abrogoua of Ivory Coast), *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *K. pneumoniae* ATCC 43816, and *S. aureus* ATCC 25913 (American Type Culture Collection (ATCC), Manassas, VA, USA) were used as indicator strains. LAB strains were routinely grown in MRS (De Man, Rogosa, and Sharpe) broth or agar (Oxoid Limited, Hampshire, United Kingdom) for 24–48 h at 37 °C under microaerophilic conditions (5% CO_2). The reference pathogenic bacteria were grown in tryptic soy broth (TSB) or agar (TSA) (Scharlab S.L., Barcelona, Spain) for 18–24 h at 37 °C under aerobic conditions. All strains were stored at −20 °C in an adequate culture medium containing 30% (*v*/*v*) glycerol (Scharlab S.L., Barcelona, Spain) and subcultured twice before being used in assays.

Identification of LAB strains

LAB strains were grown in MRS broth for 48 h at 37 °C. Cells were harvested by centrifugation at 5000 x g for 10 min. Genomic DNA extraction was performed using a ZR Fungal/Bacterial DNA kit (Zymo Research, Irvine, CA, USA), according to the manufacturer's instructions. The DNA concentration and purity were verified with a SpectraMax® QuickDrop™ (Molecular Devices, San

Jose, CA, USA). LAB strains were identified by analysis of 16 S rDNA amplified with the universal primers 27 F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) (Biolegio B.V. Nijmegen, The Netherlands). The reaction mixture consisted of 50 µl of 10X DreamTaq Green Buffer (contains 20 mM $MgCl_2$), 0.5 µM of each primer, 0.2 mM dNTPs, 0.025 U of DreamTaq DNA Polymerase (Thermo Fisher Scientific, Baltics, UAB, Vilnius, Lithuania), and 10 ng of bacterial DNA. The amplification program cycles started with an initial denaturation at 95 °C for 3 min, followed by 35 cycles (94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min), and a final extension step at 72 °C for 7 min. The amplification reactions of the 16 S rDNA region were performed using a thermal cycler (MultiGene Thermal Cycler Labnet International, Inc., Edison, NJ, USA). The PCR products were detected by agarose gel electrophoresis (2% (w/v) agarose, 90 V, 60 min), and visualized using a GelDoc-It Imaging System (Analytik Jena, Upland, CA, USA). Sequencing was performed in both directions with the universal primers 27 F and 1492R by Cellular and Molecular Immunological Application, Holland (CEMIA, Greece). The partially obtained nucleotide sequences were aligned with multiple available homologous sequences in the National Center for Biotechnology Information (NCBI) GenBank databases (http://www.ncbi.nlm.nih.gov, accessed on 29 November 2021) to identify at the species level based on high similarity. The phylogenetic tree was constructed via the neighbour-joining method [96] using MEGA (Molecular Evolution Genetic Analysis) software, version X [97].

Functional characterization LAB isolates

Antibacterial activity of LAB isolates

Antagonistic activities of the LAB isolates were recorded against five pathogens indicators (*E. coli* ATCC 25922, *K. pneumoniae* ATCC 43816, *P. aeruginosa* ATCC 27853, *P. mirabilis* JCM1669, and *S. aureus* ATCC 25913) by the agar well diffusion method described by Balouiri et al. [98] with some modifications. The LAB isolates were cultured in MRS broth at 37 °C for 48 h. and centrifuged at 10,000 x g at 4 °C, for 5 min. Cell-free supernatants (CFS) were obtained by filtration using sterile 0.22 µm Millipore filters (VWR International, Rosny-sous-Bois, France). 1mL of the overnight pathogen culture (adjusted $OD_{600\ nm}$ to 0.2±0.05, representing approximately 10^7– 10^8 cfu/ml) was added to a sterile Petri dish (90 mm), overlaid with approximately 20 mL of TSA cooled to 45 °C, and gently homogenized until solidification. Wells with a diameter of 6 mm have been punched aseptically with a sterile tip, filled with 100 µl of CFS tested, and incubated at 37 °C for 24 h. A clear zone of 1 mm or more around each well was considered positive inhibition, which demonstrated the antibacterial activity of the CFS.

Ability to form a biofilm

The LAB isolates were grown in MRS broth at 37 °C for 48 h and bacterial load was adjusted to the same optical density $OD_{600\ nm}$ of 0.2±0.05. Bacterial cells were centrifuged at 4000 rpm for 10 min and the pellets were washed three times with NaCl solution (0.9%) and dried at 50 °C for 30 min. The bacterial biofilms were stained with 1 ml of 0.1% crystal violet (Sigma Aldrich, Saint Louis, MO, USA) for 20 min and washed with the NaCl solution until the liquid was clear. The dye was eluted with ethanol (96%). The quantification was performed by measuring absorbance value (OD) at the 595 nm wavelength spectrophotometer (BioBase, Jinan, Shandong, China). The ability to form a biofilm was considered positive for OD≥0.5.

Tolerance of LAB isolates to bile salts

The bile salts tolerance test of the LAB isolates was performed according to the method described by Diguță et al. [78] with some modifications. Test tubes containing MRS broth were supplemented with 0.3% bile salts (Oxoid Limited, Hampshire, United Kingdom), inoculated with each LAB isolate (adjusted to the $OD_{600\ nm}$ at 0.2±0.05), and incubated at 37 °C, for 4 h. The cell viability was tested by the plate count method at 0 h and after 4 h of incubation. Tolerance toward bile salts was estimated by $\mathrm{growthrate} = \left(\frac{\mathrm{logCFUNi}}{\mathrm{logCFUNt}}\right) \times 100$, where Ni and Nt mean the viable cells (CFU/ml) at 0 h and after 4 h of incubation.

Resistance of LAB isolates to pepsin and acid pH

The ability of LAB isolates to survive the presence of pepsin and acid pH was done using the method described by Diguță et al. [78]. After overnight culture, LAB cells were centrifuged at 2000 x g for 10 min and pellets were suspended and washed twice with sterile physiological saline (0.9% NaCl). The pellets were suspended in phosphate-buffered saline (PBS) solution (VWR International, Rosny-sous-Bois, France) previously supplemented with 0.3% pepsin (Sigma-Aldrich, Saint Louis, MO, USA) and pH was adjusted to 1.5 with 1 N HCl. The cell viability was tested by the plate count method at 0 h and after 3 h of incubation. The percentage (%) survival of LAB isolates was calculated by the following formula: $\%\mathrm{viability} = \left(\frac{\mathrm{logUFCNt}}{\mathrm{logUFCNi}}\right) \times 100$, where Ni and Nt mean the viable cells (CFU/ml) at 0 h and after 3 h of incubation.

Evaluation of LAB isolates hydrophobicity

Overnight LAB cultures were centrifuged at 12000 x g for 5 min at 4 °C. The cell pellets were washed twice using PBS solution (pH 7.2) and adjusted to the optical density of 1±0.05 at 650 nm wavelength (H_0). To determine the cell surface hydrophobicity, three solvents

were used: hexane (VWR International, Rosny-sous-Bois, France), xylene (Bernd Kraft GmbH, Duisburg, Germany), and chloroform (Bernd Kraft GmbH, Duisburg, Germany). The mixture of 2.4 ml of cell suspension with 0.4 mL of solvent was vigorously vortexed for 2 min. After the phase stabilization and separation period of 30 min at room temperature, the aqueous phase was carefully recovered and the optical density was measured at 650 nm wavelength (H_1). The hydrophobicity values were calculated according to the following formula: $H\% = \left(\frac{H0-H1}{H0}\right) \times 100$.

DPPH Free Radical Scavenging ability

After the LAB cells were incubated at 37 °C in MRS broth overnight, the cells were harvested by centrifugation at 12000 x g for 5 min at 4 °C. The supernatant samples were collected and cell pellets were washed twice with PBS solution and suspended in the same solution to adjust to $OD_{600\ nm}$ 0.2±0.05 and served as intact cells. The 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity was determined by the method described by Brand-Williams et al. [99]. A volume of 2 ml of DPPH (Alfa Aesar, Kandel, Germany) (100 µM in methanol) was added to 1 ml of the cell suspension or 1 ml of the supernatant, the mixtures were mixed vigorously and incubated at laboratory temperature in the dark for 30 min. In the case of the intact cell, the absorbance of the resulting solution was measured in triplicate at 517 nm wavelength after centrifugation at 12,000 x g, for 5 min. The deionized water was used as the negative control. The presence of antioxidant activity is shown by the change in color of the mixture from purple to yellow. The scavenging ability was defined as: $\%AA = \left(\frac{ODDPPH-ODsample}{ODDPPH}\right) \times 100$.

Plate screening of enzymes producing LAB isolates

LAB isolates were inoculated in spots on the surface of culture media distributed in Petri dishes. Amylase activity was evaluated on MRS Agar medium supplemented with 1% of soluble starch (VWR International, Rosny-sous-Bois, France). After incubation at 37 °C for 4 days, the positive reaction was indicated by a clear zone surrounding LAB isolates by adding Iodine-potassium iodide solution (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Cellulase activity was tested on MRS Agar supplemented with 1% carboxymethylcellulose (Sigma-Aldrich, Merck, Darmstadt, Germany). The zone of clearance was visualized after staining with 0.1% Congo red solution (Sigma-Aldrich, Merck, Darmstadt, Germany) and washing the plate with 1 M NaCl. Lipase activity was determined on MRS Agar medium supplemented with 0.25 mL olive oil, 0.01% $CaCl_2xH_2O$, and 0.0001% (w/v) rhodamine B (Alfa Aesar, Kandel, Germany). Positive reactions were observed by pink-orange colony under UV 350 nm. Protease activity was detected on skim milk (1%) agar medium (PanReac AppliChem, Darmstadt, Germany). After the incubation period, the LAB isolates showing a clear zone of the degradation of casein were read as positive for protease production. The β-galactosidase activity was determined on MRS agar containing 20 µl of X-Gal (20 mg/ml in DMSO) (PanReac AppliChem, Darmstadt, Germany). The green color colonies were regarded as bacteria producing β-galactosidase enzyme.

Safety characterization of the LAB isolates

Antibiotic susceptibility

The LAB strains were tested for antibiotic susceptibilities by the disc diffusion method described by CLSI [100]. Twelve (12) antibiotics belonging to 8 classes of antibiotics were used, namely Beta-lactams (Penicillin: PEN 6 µg; Amoxicillin: AML 10 µg; Oxacillin: OX 5 µg), Cephalosporins (Cephalothin: CN 30 µg), Aminoglycosides (Gentamicin: GM 10 µg; Kanamycin: KAN 1 mg; Streptomycin: STR 500 µg), Quinolones (Ciprofloxacin: CIP 5 µg), Cyclines (Tetracycline: TE 30 µg), Rifampicin (Rifampicin: RAM 30 µg), Carbapenems (Imipenem: IPM 10 µg), Phenicols (Chloramphenicol: C 30 µg). All antibiotics were provided by Oxoid Limited (Hampshire, United Kingdom). A volume of 100 µl of LAB fresh cultures (adjusted to the $OD_{600\ nm}$ at 0.2±0.05) was inoculated into MRS agar plates and dried. Antibiotic discs were placed on the MRS plates agar and incubated at 37 °C, for 48 h. The diameter of the zone of inhibition was measured and classified as sensitive (S); intermediate resistant (IR); or resistant (R) in agreement with the Clinical and Laboratory Standards Institute CLSI [100].

Hemolytic activity of LABs

An aliquot of each LAB culture (5µL) was applied onto Columbia Agar plates containing 5% (w/v) sheep blood (Oxoid Limited, Hampshire, United Kingdom) and cultured at 37 °C, for 48 h. The hemolytic activity was assessed by β-hemolysis (the clear halo around colonies), α-hemolysis (the green halo around colonies), or γ-hemolysis (no halo around colonies). Here, *S. aureus* ATCC 25913 (β-hemolytic) was used as a positive control strain.

Storage and preservation of LAB isolates: freeze-drying procedures

The LAB cultures left in MRS broth overnight were centrifuged at 4000 x g for 10 min at 4 °C. The pellets were washed twice using saline solution (0.9% NaCl) and suspended in 2 ml of cryoprotectant solutions. Three cryoprotectants (at a final concentration of 2%) were tested: D-sorbitol (Sigma-Aldrich, Saint Louis, MO, USA), D-glucose, and sucrose (PanReac AppliChem, Darmstadt, Germany). After freezing at -20 °C overnight, cell suspensions (prepared as described above) were freeze-dried in

a chamber-type freeze-dryer (FreeZone6, LABCONCO, 6 L Benchtop Freeze Dry System, Kansas, MO, USA) at −55 °C and 0.3 mbar, for 4 h. The cell viability was tested before and after the freeze-drying procedure by the plate count method. Distilled water was used as a control. The survival rate of LAB strains was calculated as:

$\%\text{viability} = \left(\frac{\log\text{CFUN}}{\log\text{CFUN0}}\right) \times 100$, where N_0 and N mean the viable cells (CFU/ml) before and after freeze drying, respectively.

Statistical analysis

All the experiments were carried out in triplicate and the results were expressed as the mean ± standard deviation. The calculations, figures, and boxplots were performed using Excel 2016. For the comparison of the means of the studied parameters, one-way analysis of variance (ANOVA) and Tukey's test were performed with the XLStat software (Version 2016). For $p<0.05$, the means were considered significant. XLSTAT software was used to create a dendrogram to group LAB species with similar characteristics.

Acknowledgements

Thanks to the Romanian government and AUF: Agence Universitaire de la Francophonie for a post-doctoral grant by "Eugen Ionescu" 2021 fellowship.

Author Contribution

W.H.C: conceptualization, investigation, methodology, validation, writing—original draft. N.R.K: investigation, methodology. C.D: investigation, methodology, writing—original draft, validation. F.C and F.Mi: conceptualization, supervision, writing—review and editing, validation. All authors read and approved the final manuscript.

Funding

This work was supported by grants from the Romanian government and AUF: Agence Universitaire de la Francophonie for a post-doctoral "Eugen Ionescu" 2021 fellowship.

Data Availability

All the datasets generated in the present study are included in the manuscript. The partial 16 S rDNA sequences of LAB strains were deposited in the NCBI GenBank. Accession numbers:
https://www.ncbi.nlm.nih.gov/nuccore/ON141894.1/.
https://www.ncbi.nlm.nih.gov/nuccore/ON141895.1/.
https://www.ncbi.nlm.nih.gov/nuccore/ON141896.1/.
https://www.ncbi.nlm.nih.gov/nuccore/ON141897.1/.
https://www.ncbi.nlm.nih.gov/nuccore/ON141898.1/.
https://www.ncbi.nlm.nih.gov/nuccore/ON141899.1/.
https://www.ncbi.nlm.nih.gov/nuccore/ON141900.1/.
https://www.ncbi.nlm.nih.gov/nuccore/ON141901.1/.
https://www.ncbi.nlm.nih.gov/nuccore/ON141902.1/.
https://www.ncbi.nlm.nih.gov/nuccore/ON141903.1/.
https://www.ncbi.nlm.nih.gov/nuccore/ON141904.1/.
https://www.ncbi.nlm.nih.gov/nuccore/ON141905.1/.

Declarations

Ethics approval

The work research complies with the current animal welfare laws in Ivory Coast. The experimental animals' Tilapia (*Oreochromis niloticus*) is not an endangered fish; the provisions of the Govt. of Ivorian's Wildlife Protection Act of 1965 are not applicable for experiments on this Tilapia. All experimental protocols were approved by the University Nangui Abrogoua ethics committee. All methods are reported following ARRIVE guidelines.

Conflict of interest

The authors declare no conflict of interest.

Competing interests

The authors declare no competing interests.

Author details

[1]Biotechnology and Food Microbiology Laboratory, Food Science and Technology, Formation and Research Unit, University Nangui Abrogoua, 02 BP 801 Abidjan 02, Abidjan, Côte d'Ivoire
[2]Nutrition and Food Safety Laboratory, Food Science and Technology, Formation and Research Unit, University Nangui Abrogoua, 02 BP 801 Abidjan 02, Abidjan, Côte d'Ivoire
[3]Applied Microbiology Laboratory, Faculty of Biotechnologies, University of Agronomic Sciences and Veterinary Medicine Bucharest, 59 Mărăsti Blvd, Bucharest 011464, Romania

Received: 6 April 2022 / Accepted: 17 May 2023

References

1. FAO. Fishing profil by country, the Republic of Côte d'Ivoire. Rome (Italie): FAO. 2008; http://ftp.fao.org/FI/document/fcp/fr/FI_CP_CI.pdf (30/10/2014).
2. Hecht T. Review of feeds and fertilizers for sustainable aquaculture development in sub-saharan Africa. In: Hasan MR, Hecht T, DeSilva SS, Tacon AGJ, editors. Study and analysis of feeds and fertilizers for sustainable aquaculture development. Rome (Italie): FAO; 2007.
3. Bamba Y, Ouattara A, Kouassi SDC, Gourène G. Production of *Oreochromis niloticus* with feed made from agricultural by-products. Sci and Nat. 2008;5(1):89–99.
4. Gabriel UU, Akinrotimi OA, Bekibele DO, Onunkwo DN, Anyanwu PE. Locally produced fish feed: potentials for aquaculture development in sub-saharan Africa. Afr J Agric Res. 2007;2:287–95.
5. Crentsil C, Ukpong IG. Economics of fish production in Amansie-West District of Ghana: implication for Food Security in West Africa. Asian J Agric Ext Econ Soc. 2014;3:179–88.
6. Médale F, Le Boucher R, Dupont-Nivet M, Quillet E, Aubin J, Panserat S. Des aliments à base de végétaux pour les poissons d'élevage. INRA Productions Animales. 2013;26:303–16.
7. O'Neill J. Antimicrobials in Agriculture and the Environment: Reducing Unnecessary Use and Waste. The Review on Antimicrobial Resistance. Available online: http://amr-review.org/sites/default/files/Antimicrobials%20in%20agriculture%20and%20the%20environment%20-%20Reducing%20unnecessary%20use%20and%20waste.pdf ((accessed on 25 March 2023).)
8. Chuah L, Effarizah ME, Goni AM, Rusul G. Antibiotic application and emergence of multiple antibiotic resistance (MAR) in global catfish aquaculture. Curr Environ Health Rep. 2016;3:118–27.
9. Watts JEM, Schreier HJ, Lanska L, Hale MS. The Rising Tide of antimicrobial resistance in aquaculture: sources, sinks and solutions. Mar Drugs. 2017;15(6):158.
10. Irianto A, Austin B. Probiotics in aquaculture. J Fish Dis. 2002;25:633–42.
11. Banerjee G, Ray AK. The advancement of probiotics research and its application in fish farming industries. Res Vet Sci. 2017;115:66–77.
12. Hasan KN, Banerjee G. Recent studies on probiotics as beneficial mediator in aquaculture: a review. JoBAZ. 2020;81:53.
13. El-Saadony MT, Alagawany M, Patra AK, Kar I, Tiwari R, Dawood MAO, Dhama K, Abdel-Latif HMR. The functionality of probiotics in aquaculture: an overview. Fish Shellfish Immunol. 2021;117:36–52.
14. Hancz C. Application of probiotics for environmentally friendly and sustainable aquaculture: a review. Sustainability. 2022;14(22):15479.
15. FAO/WHO. Guidelines for the evaluation of probiotics in food. London, Ontario, Canada: Food and Agriculture Organization of the United Nations and World Health Organization Working Group Report; 2002.
16. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. Expert consensus document: the International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol. 2014;11:506–14.

17. Jahangiri L, Esteban M. Administration of Probiotics in the water in finfish aquaculture systems: a review. Fishes. 2018;3:33.
18. Chauhan A, Singh R. Probiotics in aquaculture: a promising emerging alternative approach. Symbiosis. 2019;77:99–113.
19. Gong L, He H, Li D, Cao L, Khan TA, Li Y, et al. A new isolate of *Pediococcus pentosaceus* (SL001) with antibacterial activity against fish pathogens and potency in facilitating the immunity and growth performance of grass carps. Front Microbiol. 2019;10:1384.
20. Ringø E, Van Doan H, Lee SH, Soltani M, Hoseinifar SH, Harikrishnan R, et al. Probiotics, lactic acid bacteria and bacilli: interesting supplementation for aquaculture. J Appl Microbiol. 2020;129(1):116–36.
21. Kuebutornye FKA, Abarike ED, Lu Y. A review on the application of *Bacillus* as probiotics in aquaculture. Fish Shellfish Immunol. 2019;87:820–8.
22. Navarrete P, Tovar-Ramírez D. Use of yeasts as probiotics in fish aquaculture. Sustain Aquac Tech. 2014;1:57196.
23. Dumitrache C, Mihai C, Frîncu M. Yeast - sustainable nutrient source for fish feed - review. Sci Papers Ser E Land Reclam Earth Observation Surveying Environ Eng. 2022;XI:464–9.
24. Diguță CF, Mihai C, Toma RC, Cîmpeanu C, Matei F. *In vitro* assessment of yeasts strains with probiotic attributes for aquaculture use. Foods. 2023;12:124.
25. Buntin N, Chanthachum S, Hongpattarakere T. Screening of lactic acid bacteria from gastrointestinal tracts of marine fish for their potential use as probiotics. Songklanakarin J Sci Technol. 2008;30(s1):141–8.
26. Boutin S, Audet C, Derome N. Probiotic treatment by indigenous bacteria decreases mortality without disturbing the natural microbiota of *Salvelinus fontinalis*. Can J Microbiol. 2013;59:662–70.
27. Wanka KM, Damerau T, Costas B, Krueger A, Schulz C, Wuertz S. Isolation and characterization of native probiotics for fish farming. BMC Microbiol. 2018;18:119.
28. Mladineo I, Bušelić I, Hrabar J, Radonić I, Vrbatović A, Jozić S, Trumbić Ž. Autochthonous bacterial isolates successfully stimulate *in vitro* peripheral blood leukocytes of the European Sea bass (*Dicentrarchus labrax*). Front Microbiol. 2016;7:1244.
29. Ridha MT, Azad IS. Effect of autochthonous and commercial probiotic bacteria on growth, persistence, immunity and disease resistance in juvenile and adult Nile tilapia *Oreochromis niloticus*. Aquac Res. 2016;47:2757–67.
30. Van Doan H, Hoseinifar SH, Khanongnuch C, Kanpiengjai A, Unban K, Van Kim V, Srichaiyo S. Host-associated probiotics boosted mucosal and serum immunity, disease resistance and growth performance of Nile tilapia (*Oreochromis niloticus*). Aquaculture. 2018;491:94–100.
31. Dhanasiri AKS, Brunvold L, Brinchmann MF, Korsnes K, Bergh Ø, Kiron V. Changes in the intestinal microbiota of wild Atlantic cod *Gadus morhua* L. upon captive rearing. Microb Ecol. 2011;61:20–30.
32. Chi C, Jiang B, Yu X-B, Liu T-Q, Xia L, Wang G-X. Effects of three strains of intestinal autochthonous bacteria and their extracellular products on the immune response and disease resistance of common carp. Fish Shellfish Immunol. 2014;36(1):9–18.
33. Mohamad N, Manan H, Sallehhuddin M, Musa N, Ikhwanuddin M. Screening of lactic acid Bacteria isolated from giant freshwater prawn (*Macrobrachium rosenbergii*) as potential probiotics. Aquac Rep. 2020;18:100523.
34. Araújo C, Muñoz-Atienza E, Nahuelquín Y, Poeta P, Igrejas G, Hernández PE, et al. Inhibition of fish pathogens by the microbiota from rainbow trout (*Oncorhynchus mykiss, Walbaum*) and rearing environment. Anaerobe. 2015;32:7–14.
35. Chemlal-Kherraz D, Sahnouni F, Matallah-Boutiba A, Boutiba Z. The probiotic potential of lactobacilli isolated from Nile tilapia (*Oreochromis niloticus*)'s intestine. Afr J Biotechnol. 2012;11(68):13220–7.
36. Iorizzo M, Albanese G, Testa B, Ianiro M, Letizia F, Succi M, Tremonte P, D'Andrea M, Iaffaldano N, Coppola R. Presence of lactic acid bacteria in the intestinal tract of the Mediterranean trout (*Salmo macrostigma*) in its natural environment. Life. 2021;11(7):667.
37. Shewale RN, Sawale PD, Khedkar CD, Singh A. Selection criteria for probiotics: a review. Int J Probiotics Prebiotics. 2014;9:17–22.
38. de Melo Pereira GV, de Oliveira Coelho B, Magalhães Júnior AI, Thomaz-Soccol V, Soccol CR. How to select a probiotic? A review and update of methods and criteria. Biotechnol. Adv. 2018;36:2060–76.
39. Byakika S, Mukisa I, Byenkya Y, Muyanja C, Byenkya Byaruhanga Y. A review of criteria and methods for evaluating the probiotic potential of microorganisms. Food Rev Int. 2019;35:427–66.
40. Verschuere L, Rombaut G, Sorgeloos P, Verstraete W. Probiotic bacteria as biological control agents in aquaculture. Microbiol Mol Biol Rev. 2000;64:655–71.
41. Defoirdt T, Sorgeloos P, Bossier P. Alternatives to antibiotics for the control of bacterial disease in aquaculture. Curr Opin Microbiol. 2011;14:251–8.
42. Muñoz-Atienza E, Gómez-Sala B, Araújo C, Campanero C, del Campo R, Hernández PE, Herranz C, Cintas LM. Antimicrobial activity, antibiotic susceptibility and virulence factors of lactic acid bacteria of aquatic origin intended for use as probiotics in aquaculture. BMC Microbiol. 2013;13:15.
43. Sharifuzzaman S, Austin B. Probiotics for disease control in aquaculture. in Diagnosis and control of Diseases of Fish and Shellfish, Austin B, Newaj-Fyzul A, editors (Hoboken, NJ: John Wiley & Sons Ltd), 189–222. https://doi.org/10.1002/9781119152125.ch8.
44. Ringø E, Hoseinifar SH, Ghosh K, Doan HV, Beck BR, Song SK. Lactic acid bacteria in finfish—An update. Front Microbiol. 2018;9:1818.
45. Li X, Ringø E, Hoseinifar SH, Lauzon HL, Birkbeck H, Yang D. The adherence and colonization of microorganisms in fish gastrointestinal tract. Rev Aquac. 2019;11:603–18.
46. Danilova T, Adzhieva A, Danilina G, Polyakov N, Soloviev A, Zhukhovitsky V. Antimicrobial activity of supernatant of *Lactobacillus plantarum* against pathogenic microorganisms. Bull Exp Biol Med. 2019;167:751–4.
47. Salas-Jara MJ, Ilabaca A, Vega M, García A. Biofilm forming *Lactobacillus*: new challenges for the development of probiotics. Microorganisms. 2016;4(3):35.
48. Todhanakasem T, Triwattana K, Pom J, Havanapan P, Koombhongse P, Thitisak P. Physiological studies of the *Pediococcus pentosaceus* biofilm. Lett Appl Microbiol. 2021;72(2):178–86.
49. Chamignon C, Guéneau V, Medina S, Deschamps J, Gil-Izquierdo A, Briandet R, Mousset PY, Langella P, Lafay S, Bermúdez-Humarán LG. Evaluation of the probiotic properties and the capacity to form biofilms of various *Lactobacillus* strains. Microorganisms. 2020;8(7):1053.
50. Kim JH, Lee ES, Song KJ, Kim BM, Ham JS, Oh MH. Development of desiccation-tolerant probiotic biofilms inhibitory for growth of foodborne pathogens on stainless steel surfaces. Foods. 2022;11(6):831.
51. Yasmin I, Saeed M, Khan WA, Khaliq A, Chughtai MFJ, Iqbal R, Tehseen S, Naz S, Liaqat A, Mehmood T, Ahsan S, Tanweer S. *Vitro* probiotic potential and safety evaluation (hemolytic, cytotoxic activity) of *Bifidobacterium* strains isolated from raw Camel milk. Microorganisms. 2020;8:354.
52. Krausova G, Hyrslova I, Hynstova I. *In Vitro* evaluation of adhesion capacity, hydrophobicity, and auto-aggregation of newly isolated potential probiotic strains. Fermentation. 2019;5:100.
53. Mishra V, Shah C, Mokashe N, Chavan R, Yadav H, Prajapati J. Probiotics as potential antioxidants: a systematic review. J Agric Food Chem. 2015;63:3615–26.
54. Feng T, Wang J. Oxidative stress tolerance and antioxidant capacity of lactic acid bacteria as probiotic: a systematic review. Gut Microbes. 2020;12:1801944.
55. Bairagi A, Sarkar Ghosh K, Sen SK, Ray AK. Enzyme producing bacterial flora isolated from fish digestive tracts. Aquacult Int. 2002;10:109–21.
56. Ray AK, Ghosh K, Ringo E. Enzyme-producing bacteria isolated from fish gut: a review. Aquac Nutr. 2012;18(5):465–92.
57. Nimrat S, Vuthiphandchai V. *Vitro* evaluation of commercial probiotic products used for marine shrimp cultivation in Thailand. Afr J Biotechnol. 2011;10:4643–50.
58. Abomughaid MM. Isolation and identification of some probiotic bacteria and their potential role in improving immune response and resistance of Nile Tilapia (*Oreochromis niloticus*) in comparison with a commercial product. International J Microbiol. 2020; 2020: 8865456.
59. Melo-Bolívar JF, Pardo RYR, Hume ME, Díaz LMV. Multistrain probiotics use in main commercially cultured freshwater fish: a systematic review of evidence. Rev Aquac. 2021;13:1758–80.
60. El-Kady AA, Magouz FI, Mahmoud SA, Abdel-Rahim MM. The effects of some commercial probiotics as water additive on water quality, fish performance, blood biochemical parameters, expression of growth and immune-related genes, and histology of Nile tilapia (*Oreochromis niloticus*). Aquaculture. 2022;546:737249.
61. Al-Dohail MA, Hashim R, Aliyu-Paiko M. Effects of the probiotic, *Lactobacillus acidophilus*, on the growth performance, haematology parameters and immunoglobulin concentration in African Catfish (*Clarias gariepinus*, Burchell 1822) fingerling. Aquac Res. 2009;40(14):1642–52.
62. Nayak SK. Probiotics and immunity: a fish perspective. Fish Shellfish Immunol. 2010;29:2–14.
63. Lakshmi B, Viswanath B, Sai Gopal DVR. Probiotics as antiviral agents in shrimp aquaculture. J Pathog. 2013;8:424123.

64. De B, Meena DK, Behera BK, Das P, Das Mohapatra PK, Sharma AP. Probiotics in fish and shellfish culture: Immunomodulatory and ecophysiological responses. Fish Physiol Biochem. 2014;40:921–71.
65. Allameh S, Noaman V, Nahavandi R. Effects of probiotic bacteria on fish performance. Adv Tech Clin Microbiol. 2017;1(2):11.
66. Yi C-C, Liu C-H, Chuang K-P, Chang Y-T, Hu S-Y. A potential probiotic *Chromobacterium aquaticum* with bacteriocin-like activity enhances the expression of indicator genes associated with nutrient metabolism, growth performance and innate immunity against pathogen infections in zebrafish (*Danio rerio*). Fish Shellfish Immunol. 2019;93:124–34.
67. Zabidi A, Yusoff FM, Amin N, Yaminudin NJM, Puvanasundram P, Karim MMA. Effects of probiotics on growth, survival, water quality and disease resistance of red hybrid Tilapia (*Oreochromis* spp.) fingerlings in a biofloc system. Anim (Basel). 2021;11(12):3514.
68. Iorizzo M, Albanese G, Letizia F, Testa B, Tremonte P, Vergalito F, Lombardi SJ, Succi M, Coppola R, Sorrentino E. Probiotic potentiality from versatile *lactiplantibacillus plantarum* strains as resource to enhance freshwater fish health. Microorganisms. 2022;10(2):463.
69. Nathanailides C, Kolygas M, Choremi K, Mavraganis T, Gouva E, Vidalis K, Athanassopoulou F. Probiotics have the potential to significantly mitigate the environmental impact of freshwater fish farms. Fishes. 2021;6:76.
70. Silva VV, Salomão RAS, Mareco EA, Pai MD, Santos VB. Probiotic additive affects muscle growth of Nile tilapia (*Oreochromis niloticus*). Aquac Res. 2021;52:2061–9.
71. Sahandi J, Jafaryan H, Soltani M, Ebrahimi P. The use of two *Bifidobacterium* strains enhanced growth performance and nutrient utilization of rainbow trout (*Oncorhynchus mykiss*) Fry. Probiotics Antimicrob Proteins. 2019;11(3):966–72.
72. Sîrbu E, Dima MF, Tenciu M, Cretu M, Coadă MT, Ţoţoiu A, Cristea V, Patriche N. Effects of dietary supplementation with probiotics and prebiotics on growth, physiological condition, and resistance to pathogens challenge in Nile Tilapia (*Oreochromis niloticus*). Fishes. 2022;7:273.
73. Kouhoundé S, Coulibaly WH, Adeoti K, Somda M, Dadié AT, Savadogo A, Fatiou Toukourou F, Baba-Moussa F, Probiotics. A sustainable option for food safety and preservation in Africa In: Studium Press LLC, editor Trends in probiotics applications, edn 1st. Houston 2018; pp. 112–134.
74. Ben Amor K, Vaughan EE, de Vos WM. Advanced molecular tools for the identification of lactic acid bacteria. J Nutr. 2007;137:741–7.
75. Mohania D, Nagpal R, Kumar M, Bhardwaj A, Yadav M, Jain S, Marotta F, Singh V, Parkash O, Yadav H. Molecular approaches for identification and characterization of lactic acid bacteria. J Dig Dis. 2008;9:190–8.
76. Sharma A, Lee S, Park YS. Molecular typing tools for identifying and characterizing lactic acid bacteria: a review. Food Sci Biotechnol. 2020;29:1301–18.
77. Bajpai VK, Han J-H, Rather IA, Park C, Lim J, Paek WK, Lee JS, Yoon J-I, Park Y-H. Characterization and antibacterial potential of lactic acid bacterium *Pediococcus pentosaceus* 4l1 isolated from freshwater fish *Zacco koreanus*. Front Microbiol. 2016;7:2037.
78. Diguță FC, Nițoi GD, Matei F, Luță G, Cornea CP. The biotechnological potential of *Pediococcus* spp. isolated from Kombucha microbial consortium. Foods. 2020;9:1780.
79. Jiang S, Cai L, Lv L, Li L. *Pediococcus pentosaceus*, a future additive or probiotic candidate. Microb Cell Fact. 2021;20:45.
80. Matei B, Salzat J, Diguță CF, Cornea CP, Luta G, Utoiu ER, Matei F. Lactic acid bacteria strains isolated from Kombucha with potential probiotic effect. Rom Biotech Lett. 2018;23(3):13592–8.
81. Ringø E, Løvmo L, Kristiansen M, Bakken Y, Salinas I, Myklebust R, Olsen RE, Mayhew TM. Lactic acid bacteria vs. pathogens in the gastrointestinal tract of fish: a review. Aquacult Res. 2010;41:451–67.
82. Gufe C, Canaan Hodobo T, Mbonjani B, Majonga O, Marumure J, Musari S, Jongi G, Makaya PV, Machakwa J. Antimicrobial profiling of bacteria isolated from fish sold at informal market in Mufakose, Zimbabwe. Int J Microbiol. 2019;8(7):59–63.
83. Acharjee M, Hoque R, Shreya SS, Tabassum N, Acharjee MR, Rezanujjaman M, Rahman M, Amin A, Mahmud MR. Antibiotic susceptibility pattern of fish pathogens: a new approach of emerging the bacterial resistance through biofilm formation in *in-vitro* condition. Saudi J Biol Sci. 2021;28(12):6933–8.
84. Govindaraj K, Samayanpaulraj V, Narayanadoss V, Uthandakalaipandian R. Isolation of lactic acid bacteria from intestine of freshwater fishes and elucidation of probiotic potential for aquaculture application. Volume 13. Prot: Probiotics & Antimicro; 2021. pp. 1598–610.
85. Allameh SK, Yusof FM, Daud HM, Ringø E, Ideris A, Saad CR. Characterization of a probiotic *Lactobacillus fermentum* isolated from snakehead, *Channa striatus* stomach. J World Aquac Soc. 2013;44(6):835–44.
86. Lamari F, Sadok K, Bakhrouf A, Gatesoupe F-J. Selection of lactic acid bacteria as candidate probiotics and *in vivo* test on *Artemia nauplii*. Aquac Int. 2014;22:699–709.
87. Marchwińska K, Gwiazdowska D. Isolation and probiotic potential of lactic acid bacteria from swine feces for feed additive composition. Arch Microbiol. 2022;204:61.
88. Michael CA, Dominey-Howes D, Labbate M. The antibiotic resistance crisis: causes, consequences, and management. Front Public Health. 2014;2:145.
89. Viswanathan VK. Off-label abuse of antibiotics by bacteria. Gut Microbes. 2014;5(1):3–4.
90. Daniali M, Nikfar S, Abdollahi M. Antibiotic resistance propagation through probiotics. Expert Opin Drug Metab Toxicol. 2020;16:1207–15.
91. EFSA. Guidance on the characterization of microorganisms used as feed additives or as production organisms. EFSA J. 2018;16:5206.
92. Dou W, Abdalla HB, Chen X, Sun C, Chen X, Tian Q, Wang J, Zhou W, Chi W, Zhou X, Ye H, Bi C, Tian X, Yang Y, Wong A. ProbResist: a database for drug-resistant probiotic bacteria. Database (Oxford). 2022: baac064. https://doi.org/10.1093/database/baac064.
93. Binda S, Hill C, Johansen E, Obis D, Pot B, Sanders ME, Tremblay A, Ouwehand AC. Criteria to qualify microorganisms as "Probiotic" in foods and dietary supplements. Front Microbiol. 2020;11:1662.
94. Dunne C, O'Mahony L, Murphy L, Thornton G, Morrissey D, O'Halloran S, Feeney M, Flynn S, Fitzgerald G, Daly C, Kiely B, CO'Sullivan G, Shanahan F, Collins JK. *In vitro* selection criteria for probiotic bacteria of human origin: correlation with *in vivo* findings. Am J Clin Nutr. 2001;73:386S–92.
95. Hubalek Z. Protectants used in the cryopreservation of microorganisms. Cryobiology. 2003;46:205–29.
96. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987;4:406–25.
97. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Mol Biol Evol. 2018;35:1547–9.
98. Balouiri M, Sadiki M, Ibnsouda SK. Methods for *in vitro* evaluating antimicrobial activity: a review. J Pharm Anal. 2016;6(2):71–9.
99. Brand-Williams W, Cuvelier ME, Berset C. Use of free radical method to evaluate antioxidant activity. Lebensm WissTechnol. 1995;28:25–30.
100. CLSI. CLSI Supplement M100S. Performance standards for antimicrobial susceptibility testing, 26th ed.; Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2016.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

fermentation

MDPI

Review

Achievements of Autochthonous Wine Yeast Isolation and Selection in Romania—A Review

Raluca-Ștefania Rădoi-Encea [1,†], Vasile Pădureanu [2,*,†], Camelia Filofteia Diguță [1], Marian Ion [3], Elena Brînduşe [3] and Florentina Matei [1,2,*]

[1] Faculty of Biotechnologies, University of Agronomic Sciences and Veterinary Medicine Bucharest, 59 Mărăști Blvd., District 1, 011464 Bucharest, Romania
[2] Faculty of Food Industry and Tourism, Transilvania University of Brașov, 148 Castelului St., 500014 Brașov, Romania
[3] Research and Development Institute for Viticulture and Oenology, Valea Călugărească, 107620 Prahova, Romania
* Correspondence: padu@unitbv.ro (V.P.); florentina.matei@biotehnologii.usamv.ro (F.M.)
† These authors contributed equally to this work.

Abstract: Winemaking in Romania has a long-lasting history and traditions and its viticulture dates back centuries. The present work is focused on the development of wine yeast isolation and selection performed in different Romanian winemaking regions during past decades, presenting the advanement of the methods and techniques employed, correlated with the impact on wine quality improvement. Apart from the historical side of such work, the findings will reveal how scientific advancement in the country was correlated with worldwide research in the topic and influenced local wines' typicity. To create an overall picture of the local specificities, the work refers to local grape varieties and the characteristics of the obtained wines by the use of local yeasts as compared to commercial ones. Numerous autochthonous strains of *Saccharomyces* were isolated from Romanian vineyards, of which several demonstrated strong oenological characteristics. Meanwhile, different non-*Saccharomyces* yeast strains were also isolated and are nowadays receiving the attention of researchers seeking to develop new wines according to wine market tendencies and to support wine's national identity.

Keywords: Romania; winemaking; autochthonous yeasts; non-*Saccharomyces* yeast; terroir

check for updates

Citation: Rădoi-Encea, R.-Ș.; Pădureanu, V.; Diguță, C.F.; Ion, M.; Brînduşe, E.; Matei, F. Achievements of Autochthonous Wine Yeast Isolation and Selection in Romania—A Review. *Fermentation* **2023**, *9*, 407. https://doi.org/10.3390/fermentation9050407

Academic Editors: Agustín Aranda, Roberta Comunian and Luigi Chessa

Received: 15 March 2023
Revised: 13 April 2023
Accepted: 19 April 2023
Published: 23 April 2023

Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Winemaking in Romania has a long-lasting history and traditions and its viticulture dates back centuries [1]. With the EU accession in 2007, Romania started a journey with the final goal of putting Romania on the international high-quality wines map. Access to pre- and post-accession funds increased investment in wine making technology, the replacement of low-quality vines, and the replanting vineyards with improved genetic sources [2].

According to OIV (International Organization of Vine and Wine) 2022 statistics [3], Romania is nowadays the sixth largest wine producer in Europe and the thirteenth largest wine producer in the world ranking. The total wine production was estimated at around 4.45 million hl in 2021, increasing from around 3.63 million hl in 2015.

Meanwhile, the total area cultivated with vines decreased from 253.203 ha (1995) to 191.459 ha (2015). Since 2015, when Romania legally declared that wine is considered a food product [4], the area cultivated with vines has still shown some fluctuation, but it remained relatively balanced until 2021, when the number reached 188.891 ha [5].

The delimitation of Romanian viticultural areas was established by the National Office of Vine and Wine Products and is based on the climatic conditions determining the qualitative potential of the grapes and wines, the relief conditions, the applied technologies,

the level of the obtained productions, and the qualitative characteristics of the resulting products [6]. Therefore, the Romanian viticultural space consists of 37 vineyards which comprise, in total, 120 viticultural centers and 46 independent viticultural centers, grouped in 8 regions and 3 viticultural areas, as presented in Table 1 and shown in Figure 1.

Table 1. The Romanian viticultural space.

Viticultural Area	Viticultural Region	Vineyards Denominations
Central area, inside the Carpathian arch	The Transylvanian plateau	Târnave, Alba, Sebeș-Apold, Lechința, Aiud
Peri-Carpathian hills	The hills of Moldova	Cotnari, Huși, Iași, Dealu Bujorului, Ivești, Nicorești, Panciu, Odobești, Cotești, Zeletin, Covurlui, Colinele Tutovei
	The hills of Muntenia and Oltenia	Dealu Mare, Sâmburești, Ștefănești, Drăgășani, Dealurile Craiovei, Dealurile Buzăului, Podgoria Severinului, Plaiurile Drancei
	Banat	6 independent centers
	Crișana and Maramureș	Diosig, Miniș-Măderat, Valea lui Mihai, Podgoria Silvaniei
Danube Pontic area	The Dobrogea hills	Murfatlar, Sarica-Niculitel, Istria-Babadag
	The Danube terraces	Ostrov, Greaca
	Region of sands and other favorable lands in the South of the country	Calafat, Sadova-Corabia, Podgoria Dacilor

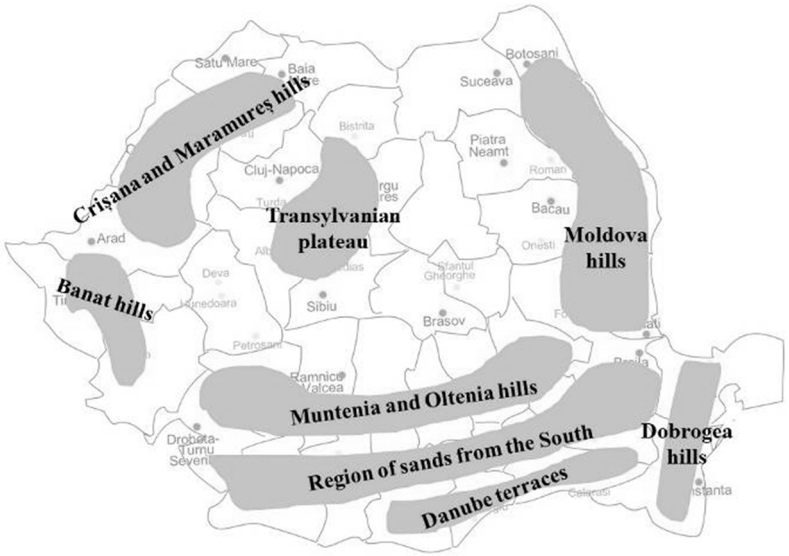


Figure 1. The Romanian viticultural regions and their geographical location.

The trend in Romanian winemaking is to maintain an uprising path in terms of total wine production volume, while also increasing the diversity of local wine types. These goals can be achieved starting from the use of local grape varieties, as well as via the isolation, selection, and then the use of autochthonous yeasts in the production of Romanian wines.

In recent decades, winemakers could choose from a wide variety of commercial yeasts provided by several well-known companies; these are yeasts that display a wide range of special characteristics, adapted to specific needs [7]. In line with the evolution of consumers' preferences and even with climate changes that bring about a higher-than-previous sugar concentration, finding yeasts with special traits was and is a continuous project [8].

Vineyard yeast biodiversity characterization and wine yeast selection are not new entries in wine-making research, but considering the history of wine, these approach

can be considerred as young. In the history of winemaking, the use of selected starter cultures did not become widespread practice until the 1970s, and the vast majority of the industrial yeasts belong to *Saccharomyces cerevisiae*; however, currently, it is recongnized that non-*Saccharomyces* species may also be relevant for alcoholic fermentation [9]. It is generally recognized that the current set of the commercial *S. cerevisiae* strains or derived hybrids is not sufficient to provide new technological or organoleptic properties in wine; therefore, new strains are desired, if not essential [10]. Hybrid genomes of *Saccharomyces cerevisiae*/*Saccharomyces kudriavzevii* yeast strains used for wine making in France (Alsace), Germany, and Hungary have been characterized by the use of microsatellite markers [11]. Autochthonous strains represent alternative genetic resources by which the industry can overcome current challenges. The preservation of spontaneous microflora is essential to obtain the typical flavor and aroma of wines deriving from different grape varieties [12]. Meanwhile, the last two decades, practices of organic vine growing influenced fungi (yeast and molds) biodiversity. This was clearly proven in France, in the Bourgogne region, with respect to the Chardonnay variety [13]. In recent years, on the European level, researchers from different groups and countries have focused on yeast selection and biodiversity issues. Ecological and geographic studies have highlighted that unique strains are associated with particular grape varieties in specific geographical locations [14]. An example of such initiatives was provided by the European project, WILDWINE Project (EU contract 315065), focused on the selection of wild microorganism in five worldwide- recognized wine regions: Nemea and Crete (Greece), Piedmont (Italy), Bordeaux (France), and Priorat (Spain) [15]. In Italy, a wide range of vineyards were examined, covering most of the wine's Italian regions: in the northwest, in the Piedmont region and Monferrato vineyards concerning Barbera grapes [16]; the Barbera variety was also studied in the "Nizza" Barbera d'Asti DOC zone [17]. In Sicily, a wide study was conducted on hundreds of isolates and the superiority of the local strains over the commercial strains was proved [18]. Another focus was on Montepulciano d'Abruzzo "Colline Teramane" premium wine DOCG, produced in Teramo province; the presence of atypical *S. cerevisiae* strains only in a particular vineyard in a restricted area suggests the role of local selective pressure in the origin of distinctive *Saccharomyces* yeast populations [19]. In Spain, several groups conducted similar work, and screening results were reported for wine regions such as Douro, Extremadura, Galicia, La Mancha and Uclés, Ribera del Duero, Rioja, Sherry area, and Valencia [20]. Moreover, in the DOQ Priorat region, isolation was performed on varieties such as Grenache and Carignan [21]; in the northwest, in the Galicia region, biodiversity was studied, comparing organic and conventional culture [22]. Relatively recently, isolates from three appellations of Spanish origin were checked for fingerprinting of interdelta polymorphism; ancient vineyards managed with organic practices showed intermediate to low levels of strain diversity, indicating the existence of stable populations of *S. cerevisiae* strains [23]. In another European area, in the Greek island of Kefalonia, in the Mavrodafni wine region, at the end of the alcoholic fermentation, indigenous yeasts were isolated; selected strains are already in industrial use [24]. In the European eastern neighborhood, in Georgia, a traditional winemaking country, long-term biodiversity studies were conducted in the Dagestan region using various isolation techniques and various substrates [25].

The present work is focused on the development of wine yeast isolation and selection performed in different Romanian winemaking regions during recent decades, presenting the advance of the employed methods and techniques, correlated with the impact on wine quality improvement. Apart from the historical side of the importance of such work, our findings will reveal how scientific advancement in the country is correlated to worldwide research in the topic.

2. Materials and Methods

The current review is based on the available scientific articles that record research regarding the isolation and selection of local wine yeasts from different Romanian vineyards. Most of the sources approached are indexed in different international databases, such as

Google Scholar, ScienceDirect, Web of Knowledge-Clarivate, and CABI. However, the available records in the international databases start from 2005, while records in some native language (Romanian), available in different national libraries, go as far as the beginning of the century, in 1915 [26]. In addition, to create an overall picture of the local specificities, scientific reports were also used in relation to local grape varieties (Fetească regală, Fetească albă, Crâmpoșie, Băbească neagră, Fetească neagră, Grasă de Cotnari, Cadarcă, Tămâioasă românească, etc.) and the characteristics of the wines obtained by the use of local yeasts compared to the commercial ones.

3. Results and Discussion

This review took into account the reported work on wine yeast isolation and selection activities performed in the wine-growing areas of Romania from 1915 to the present.

From the chronological point of view, according to Brînduşe et al. [26], the first report, from 1915, comes from the doctoral thesis of Nițescu M.A. [27]. He made an ample physiological characterization of yeast isolated from different regions and local grape varieties, such as Cotnari (Grasă, Fetească albă), Iași (Fetească neagră), Pietroasele (Grasă), Drăgășani (Tămâioasă românească, Negru moale, Negru vârtos, Crâmpoșie), and Odobești (Tămâioasă românească). This study, conducted in Paris, was positively appreciated by Ribéreau Gayon and Peynaud in 1960, according to the same source [26]. Following this study, in the 1920s–1930s, Dr. Russ and his team (Dr. Moldovan and Dr. Mavromati) founded the national school of wine microbiology and the first Romanian wine yeast collection. In the years 1945–1965, different researchers focused on local wine yeast selection [28–34]. Beginning in the 1970s, isolation and selection work has increased, and the results are detailed below.

In terms of the vineyard region, yeast isolation and selection work was reported in several areas, covering most of the Romanian winemaking regions. For instance, in the Transylvanian plateau, Dănoaie [35] and Stamate et al. [36] focused on the yeast biodiversity in Târnave vineyard, while Oprean [37] studied several Sibiu wine-growing areas. In Moldova, such experiments were conducted by Sandu-Ville et al. [38,39], followed by Viziteu et al. [40] in Cotnari vineyard, by Vasile et al. [41] and by Nechita et al. [42] in the Iași-Copou vineyard, as well as by Găgeanu et al. [43] in Dealurile Bujorului vineyard. In the hills of Muntenia, the research started in Valea Călugărească center by Kontek and Kontek [44,45], followed by Matei Rădoi et al. [46] and Brînduşe et al. [47,48], and in the Buzău vineyard by Bărbulescu et al. [49]. In the Oltenia hills in Tamburești, Banu Mărăcine, Drăgășani, and Târgu Jiu, studies were conducted by Dragomir Tutulescu and Popa [50], while Beleniuc [51] isolated wine yeast from the Murfatlar vineyard in the Dobrogea hills.

3.1. Employed Techniques of Yeast Isolation, Identification and Selection

Different approaches were taken into account during the isolation work, starting from grape washing water [42,43,45,47,52], continuing with the juice from fresh crushed grapes [40,46,52] or must in different fermenting stages: respectively, at the beginning, middle, and end of fermentation [42,48,53]. The employed microbiological media were the classical ones, meaning Sabouraud medium or Yeast Extract Peptone Dextrose (YEPD) supplemented with chloramphenicol. Bărbulescu et al. also made use of a specific medium for yeast isolation (malt extract–peptone yeast extract agar), then another specific medium (yeast extract–malt extract sucrose agar) for the maintenance of the culture [49].

The selection work followed typical steps, i.e., respectively, by monitoring the parameters of the fermentations and the characteristics of the obtained wines. Classically, there were employed tests such as ethanol tolerance [7,42] or the refermentation capacity of the strains [42]. Of the yeasts tested by Nechita et al. from Iași-Copou, five strains proved to be tolerant of high concentrations of ethanol of about 14–15% [42]. Regarding their capacity to restart the stagnated fermentation at 11.5% ethanol and 70 g/L sugars, the strains managed to bring the fermentation to an end and produce dry wines. Dragomir, Tutulescu, and Popa used the standard methods accepted by OIV to isolate, identify, and described their strains'

biological, physical, and oenological characteristics from the Oltenia area [50,54]. In the end, most of the authors reported the results of the physicochemical and organoleptical characteristics of the obtained wines after using the selected strains. Following this path, Vasile et al. isolated 86 local yeast strains from the Iași-Copou vineyard, followed by a final selection for the best fermentative characteristics and wine profiles [41,55]. In terms of the killer profile of the isolated yeast, only one report was identified in the databases, in which Matei and Găgeanu reported a killer positive strain isolated in Dealurile Bujorului county [56].

Less conventional methods were used in the characterization and wine yeast selection. For instance, Antoce and Nămoloșanu employed a calorimetric method using a multiplex batch micro-calorimeter (isothermal, conduction type) for the rapid yeast testing for ethanol tolerance in order to select strains that were useful for winemaking [57]. They demonstrated that the method could eliminate labor-intensive cell counting, as well as its high sensitivity and the possibility of measuring cultures grown in intense-colored or high-turbidity media, such as red wine. In addition, this method offers the benefit of simultaneously monitoring a large number of samples in a 48–72-h experiment.

The identification work, hand in hand with yeast biodiversity studies, had a slow evolution in terms of the employed techniques in past decades. Such work rquires know-how and specific tools, and the predominant methods were based on classical morpho-physiological tests, according to Barnett et al. [58,59], Krieger-van Rij [60], and Delfini [61]. Most authors reported studies on the macroscopic features of the colonies, pseudo-mycelium formation, and sporulation on a specific medium [43,44,46,47,52]. Tests such as fermentation and assimilation of different carbohydrates, nitrogen utilization, the use of ethanol as the sole carbon source, and arbutin split were taken into account [37,47,52]. Several authors were using rapid biochemical tests; that is, API galleries [40,46].

Some teams made use of MALDI-TOF mass spectrometry, especially that of Bărbulescu et al., wherein the isolated strains were prepared for the analysis after the extraction of peptides with formic acid, ethanol, and acetonitrile [49]. A similar approach was taken by Corbu and Csutak when studying yeast biodiversity in different traditional fermented foods, including wine [62]. For a more accurate physiological identification of the tested strains, phenotypic phylogeny analyses were also performed using Biolog Microbial ID System according to the manufacturers' specifications [63].

The molecular approach came later on in the country, when PCR-ITS RFLP techniques were employed by Gaspar et al. [64] in Sebeș vineyard (Apold-Blaj centre), followed by Găgeanu et al. [43] in Dealurile Bujorului vineyard, and Dumitrache et al. [53] in Pietroasa center (Dealu Mare vineyard); these results were also coupled with sequencing data. These teams performed conventional DNA extraction, followed by PCR amplification with ITS 1 and ITS 4 primers, continuing with *Hinf*I, *Hae*III, and *Hha*I digestion [43], or *Alu*I and *Taq*I [64], and comparing the obtained profiles with the existent databases.

The first PCR-RAPD approach was taken by Oprean, when different *Saccharomyces* and non-*Saccharomyces* strains, isolated from Sebeș-Apold vineyard, were identified [65]. Relatively recently, apart from using the ITS-RFLP technique of the ITS1-5.8S rDNA-ITS2 region, taking advantage of the restriction enzymes such as *Hinf*I, *Hae*III, *Cfo*I, and *Msp*I, Corbu and Csutak have also employed the RAPD method for the identification of yeast involved in wine spontaneous fermentation [62,63]. In their case, the intraspecific biodiversity (genetic relatedness) of the isolates was detected by analyzing the RAPD profile obtained for each strain and by calculating the similarity index using the Jaccard coefficient (Sij). Similarly, the interspecific biodiversity of the microbial communities from spontaneous fermented products was determined by comparing their profile to the RAPD profile of their co-fermenters; in the end, the dendograms were generated by PyElph, using the UPGAMA (unweighted pair group method with arithmetic mean) method.

3.2. Yeast Biodiversity and Identification Results

The wine yeast studies in Romania followed two different patterns. Most of the authors have isolated and selected different strains, followed by identification only for the strains proving special and/or demonstrating specific winemaking profiles and characteristics. Systematic studies were started only in later 1970s by Kontek et al. (1975–1977). Later on, a few studies took into account the study of the vineyard or fermented grape must yeast biodiversity as a whole [46,62].

A first ample biodiversity report study was performed by Kontek in 1977 [66], in Dealu Mare vineyard (Valea Călugărească centre), adopting the classification proposed by Lodder and Kreger-van Rij [67]. Among 244 isolates, the predominant genus was *Saccharomyces*, with the following species and var.: *S. ellipsoideus* (dominant), *S. bayanus*, *S. carlsbergensis*, *S. cerevisiae*, *S. exiguous*, *S. heterogenicus*, *S. florentinus*, *S. fructuum*, *S. italicus*, *S. oviformis*, *S. rosei*, *S. steinerii*, *S. uvarum*, and *S. logos*. In terms of non-*Saccharomyces* (NS) species, they reported *Candida mycoderma*, *Candida peliculosa*, *Kloeckera apicullata*, *Kloeckera africana*, *Torulopsis stellata*, *Pichia membranaefaciens*, and *Rhodotorula mucilaginosa*.

Later on, Matei Rădoi et al. performed a similar study in the Valea Călugărească center, Dealu Mare vineyard, comparing the data obtained by Kontek team in the 1970s in a double approach: classical morphophysiological study; and by API 20C AUX—Biomerieux [46]. The isolation was performed during 2007–2009 on Cabernet Sauvignon, Merlot, Fetească Neagră, and Pinot Noir varieties. A change in the yeast species profiles was noticed throughout the decades; specifically, the 1970s as compared to the 2000s. Among 262 isolates, the dominant species isolated in the vineyard belonged to the NS species, such as *C. famata*, *K. apiculata*, and *Debaryomyces hansenii*. One year later, a similar study was published in the same area [47], in which the dominant NS species were *C. utilis*, *K. apiculata*, *R. mucilaginosa*, and *D. hansenii*, with the employed method and the results being very close among the two teams. Other reported isolates belonged to *Candida lusitaniae*, *C. stellata*, *C. utilis*, *C. magnoliae*, *C. pelliculosa*, *Pichia anomala*, *P. jadinii*, *Torulaspora delbrueckii*, and *Hanseniaspora uvarum* (Table 2).

Multiple NS species were identified from the Cotnari vineyard by Viziteu et al., namely, *C. mycoderma*, *Hansenula anomala*, *H. uvarum*, *Kluyveromyces* spp., *P. membranafaciens*, and *T. stellata* [40].

Vasile et al. selected three *S. ellipsoideus* strains and determined their influence on the must of three grape varieties from Iași-Copou, namely, Fetească albă, Sauvignon blanc, and Chardonnay [41,55]. Other *Sacharomyces* spp. were reported by Găgeanu et al. in Dealurile Bujorului county (Table 3), such as *S. bayanus*, for instance [43].

The strains isolated and tested in Oltenia county by Dragomir Tutulescu and Popa in 2009–2010 were identified as *K. apiculata*, *P. membranafaciens*, *Rhodotorula glutinis*, *S. ellipsoideus* (the most abundant during must fermentation), and *S. oviformis*, but they also found few representatives of *S. rosei*, *Candida vinaria*, and *Metschnikowia reukaufii* [50,54].

In 2014, Oprean identified in Sebeș-Apold county, by molecular tools, *S. ellipsoideus* and *S. oviformis*, as well as NS yeasts such as *Candida vini* and *K. apiculata* [65]. Similarly, in Blaj centre, Stamate et al. reported as dominant, among 139 isolates, the species of *S. cerevisiae* var. *ellipsoideus*, *K. apiculata*, *S. oviformis*, and *S. bayanus* during must fermentation, while *K. apiculata*, *C. mycoderma*, and *T. stellata* were abundant on the grapes [36].

A general image on the *Saccharomyces* spp. isolated and selected in Romania is presented in Table 3. The main identified *Saccharomyces* species and varieties belong to *S. bayanus*, *S. cerevisiae*, *S. chevalieri*, *S. ellipsoideus*, *S. florentinus*, *S. oviformis* (synonym *S. cerevisiae*), or *S. uvarum*.

Table 2. The non-*Saccharomyces* (NS) yeasts isolated from various winemaking areas in Romania.

Genus	Species	Centre/Vineyard	References
Candida	*C. colliculosa*	Valea Călugărească, Dealu Mare	[46,47]
		Recaș	[68]
	C. famata	Valea Călugărească, Dealu Mare	[46,47]
	C. lusitaniae	Valea Călugărească, Dealu Mare	[46,47]
	C. magnoliae	Valea Călugărească, Dealu Mare	[46,47]
		Recaș	[68]
	C. mycoderma	Cernavodă, Murfatlar	[52]
		Cotnari vineyard	[40]
	C. pelliculosa	Valea Călugărească, Dealu Mare	[46,47]
	C. sphaerica	Valea Călugărească, Dealu Mare	[46,47]
	C. tropicalis	Recaș	[68]
	C. utilis	Valea Călugărească, Dealu Mare	[46,47]
	C. vini	Drăgășani, Tamburești	[50]
Clavispora	*C. lusitaniae*	Valea Călugărească, Dealu Mare	[47]
Debaryomyces	*D. hansenii*	Valea Călugărească, Dealu Mare	[46,47]
Dekkera	*D. anomala*	Pietroasa vineyard	[53]
Geotrichum	*G. penicillatum*	Valea Călugărească, Dealu Mare	[47]
Hanseniaspora	*H. uvarum*	Recaș	[68]
Hansenula	*H. anomala*	Cernavodă, Murfatlar	[52]
		Cotnari vineyard	[40]
Kloeckera	*K. apiculata*	Cernavodă, Murfatlar	[52]
		Valea Călugărească, Dealu Mare	[46,47]
		Drăgășani	[33]
		Recaș	[68]
Lachancea	*L. kluyveri*	Cotnari vineyard	[40]
Metschnikowia	*M. pulcherrima*	Drăgășani	[50]
		Pietroasa vineyard	[53]
Pichia	*P. angusta*	Recaș	[68]
	P. anomala	Valea Călugărească, Dealu Mare	[46,47]
	P. fermentans	Cernavodă, Murfatlar	[52]
		Recaș	[68]
	P. jadinii	Valea Călugărească, Dealu Mare	[46,47]
	P. kudriavzevii	Ilfov area	[63]
	P. membranaefaciens	Drăgășani, Tamburești	[50]
		Cotnari vineyard	[40]
	P. ohmeri	Valea Călugărească, Dealu Mare	[47]
Rhodotorula	*R. glutinis*	Valea Călugărească, Dealu Mare	[46,47]
		Recaș	[68]
	R. minuta	Valea Călugărească, Dealu Mare	[46,47]
	R. mucilaginosa	Cernavodă, Murfatlar	[52]
		Valea Călugărească, Dealu Mare	[47]
		Recaș	[68]
Torulaspora	*T. delbrueckii*	Valea Călugărească, Dealu Mare	[46,47]

Table 2. *Cont.*

Genus	Species	Centre/Vineyard	References
Torulopsis	*T. stellata*	Cernavodă, Murfatlar	[52]
		Cotnari vineyard	[40]
Zygosaccharomyces	*Z. bailii*	Cotnari vineyard	[40]
	Z. rouxii	Cotnari vineyard	[40]

Table 3. The *Saccharomyces* species and varieties isolated from various winemaking areas in Romania.

Species	Centre/Vineyard	References
S. bayanus	Cernavodă, Murfatlar	[52]
	Dealurile Bujorului	[43]
	Cotnari vineyard	[40]
S. cerevisiae	Buzău vineyard	[49]
	Pietroasa vineyard	[53]
	Recaș	[68]
	Valea Călugărească, Dealu Mare	[46]
	Cotnari vineyard	[40]
S. chevalieri	Cotnari vineyard	[40]
S. ellipsoideus	Cernavodă, Murfatlar	[52]
	Dealurile Bujorului	[43]
	Iași-Copou vineyard	[41]
	Cotnari vineyard	[40]
S. florentinus	Cotnari vineyard	[40]
S. oviformis (synonym *S. cerevisiae*)	Cernavodă, Murfatlar	[52]
	Dealurile Bujorului	[43]
	Cotnari vineyard	[40]
S. uvarum	Cotnari vineyard	[40]

3.3. Selected Yeast Properties and the Final Characteristics of Local Wines

From the available records, a wide range of grape varieties were tested, of which nine are registered as local varieties (Table 4), while the wines' characteristics (Table 5) were assessed for both red wines and white wines, though more attention have been given to the white wines.

In the case of white wines, the local selected yeasts were tested on local varieties (Fetească albă, Fetească regală, Tămâioasă românească), as well as on international varieties (Aligoté, Chardonnay, Sauvignon blanc, Pinot gris, Muscat ottonel).

Regarding Feteasca albă, this type of wine was obtained and tested in Dealu Bujorului, with 13.5% alcohol (*v*/*v*) and without residual sugar detected [69], and in Iași, with 11.6% alcohol (*v*/*v*) and 0.2 g/L sugars [55]. Colibaba et al. [70], Dobrei et al. [71], and Bora et al. [69] obtained Fetească regală wine from Iași, Miniș-Măderat, and Dealu Bujorului, with an average alcohol content of 13.7% (*v*/*v*). The residual sugar content was very different—from 1.9 g/L (Dealu Bujorului) and 3.9 g/L (Miniș-Măderat) to 6.63 g/L (Iași).

Aligoté wines showed some differences in terms of ethanol content from one location to another, but also within the same location. Thus, the Aligoté obtained in Dealu Bujorului had a content of 13.1% ethanol (*v*/*v*) with no residual sugars detected [69], while those obtained in Iași had, respectively, 10.08% ethanol (*v*/*v*) with 0.72 g/L sugars [70], and 11.33% ethanol without a mention of the residual sugars [72].

Colibaba et al. [73] and Bora et al. [69] also obtained Italian Riesling wines with around 11% ethanol, but the first author obtained a dry wine with 0.77 g/L residual sugar, while the second author obtained a sweet wine with 72 g/L residual sugar.

Table 4. Wine grape varieties from Romanian vineyards fermented with selected autochthonous yeast.

Grape Varieties	Vine Regions	References
Aligoté	Iași	[72]
	Iași	[70]
	Dealu Bujorului	[69]
Băbească gri	Dealu Bujorului	[69]
Cabernet sauvignon	Dealu Mare	[73]
	Miniș-Măderat	[71]
	Dobra (Satu Mare)	[74]
Cadarcă	Miniș-Măderat	[74]
Chardonnay	Iași	[55]
Fetească albă	Dealu Bujorului	[69]
	Iași	[55]
Fetească neagră	Miniș-Măderat	[74]
	Panciu	[75]
	Rateşti (Satu Mare) and Aliman (Constanța)	[74]
Fetească regală	Dealu Bujorului	[69]
	Iași	[70]
	Miniș-Măderat	[71]
Frâncușă	Iași	[70]
Grasa de Cotnari	Iași	[70]
Italian riesling	Dealu Bujorului	[69]
	Iași	[70]
Merlot	Aliman (Constanța)	[74]
Muscat ottonel	Dealu Bujorului	[69]
	Iași	[70,76,77]
Neuburger	Iași	[70]
Pinot gris	Iași	[70]
	Miniș-Măderat	[71]
Pinot noir	Rateşti (Satu Mare)	[74]
Rose traminer	Iași	[70]
Sarba	Dealu Bujorului	[69]
Sauvignon	Dealu Mare	[78]
Sauvignon blanc	Dealu Bujorului	[69]
	Iași	[55,70]
Tamaioasă românească	Iași	[70]
Traminer	Miniș-Măderat	[71]

Muscat Ottonel wines were obtained in two Moldova areas, one from Dealu Bujorului and three from Iași. The wine obtained in Dealu Bujorului was a sweet wine, with 11% ethanol and 30.7 g/L residual sugar [69]. Colibaba et al. [70] and Vararu et al. [76] obtained dry wines from Iași, with less than 2 g/L sugar and 12.2%, respectively, and 13.6% ethanol. The glycerol content of Vararu et al. wine was almost 13 g/L. Focea et al. obtained a sparkling wine with 10.3% ethanol, but without mentioning the sugar content [77].

As for Pinot gris, two wines with an increased ethanol content of about 14% were obtained in Iași [70] and in Miniș-Măderat [71]. Vișan et al. [78] obtained three Sauvignon semi-dry wines from Dealu Mare, with an average of 12.5% ethanol, 11 g/L sugar, and about 8–10 g/L glycerol. Vasile et al. [55] and Colibaba et al. [70] each made a dry Sauvignon blanc from Iași, with 11.2–11.9% ethanol and approx. 1 g/L sugar; wine from 2010 had a content of 7.4 g/L glycerol. The Sauvignon blanc obtained from Dealu Bujorului [69] was semi-dry, with 12 g/L sugar and higher ethanol content of 14.4%.

On red wines' side, Cabernet sauvignon was tested in Dealu Mare [73], Miniș-Măderat [71], and in Dobra, Dealurile Silvaniei [74]. This type of wine had an alcohol content between 12% and 15% (*v*/*v*); the highest value was obtained in Miniș-Măderat. The residual sugar content was 3.8 g/L in the 2012 study, 10.05 g/L in the 2015 study, and not specified in the 2018 study. Vișan et al. also emphasized that the glycerol content was 9 g/L [73], which contributes to the wine's texture and body [79]. Manolache et al. [74,75] and Dobrei et al. [71] obtained and tested Feteasca neagră wine, with an average of 13.49% ethanol (*v*/*v*) and 3.48–3.9 g/L residual sugar.

Table 5. Wines obtained in Romanian winemaking areas after fermentation with local yeast and their physicochemical properties.

Grape Varieties	Vine Region	Alcohol Vol. (%)	Residual Sugars (g/L)	Total Acidity (g/L)	Volatile Acidity (g/L)	References
Aligoté	Iași	11.33	*	6.72	0.35	[72]
	Dealu Bujorului	13.1	nd	5.5	0.37	[69]
	Iași	10.08	0.72	9.14	0.33	[70]
Băbească gri	Dealu Bujorului	13.2	12.7	5.9	0.38	[69]
Cabernet Sauvignon	Dealu Mare	13.1	3.8	4.3	0.7	[73]
Cadarcă	Miniș-Măderat	15	10.05	5.5	0.43	[71]
	Dobra (Satu Mare)	12	*	5.42	0.47	[74]
	Miniș-Măderat	13.25	2.44	5.55	0.32	[71]
Chardonnay	Iași	12.4	nd	5.9	0.29	[55]
Fetească albă	Dealu Bujorului	13.5	nd	4	0.39	[69]
	Iași	11.6	0.2	5.6	0.28	[55]
Fetească neagră	Miniș-Măderat	13.97	3.48	5.93	0.42	[71]
	Panciu	13.5	3.9	5.32	0.88	[75]
	Ratești (Satu Mare)	13.06	*	5.98	0.57	[74]
	Aliman (Constanța)	13.43	*	5.41	0.73	[74]
Fetească regală	Dealu Bujorului	13.8	1.9	5.3	0.42	[69]
	Iași	13.94	6.63	6.92	0.43	[70]
	Miniș-Măderat	13.39	3.9	5.7	0.53	[71]
Frâncușă	Iași	11.87	0.63	8.54	0.41	[70]
Grasa de Cotnari	Iași	11.6	1.7	8.55	0.25	[70]
Italian Riesling	Dealu Bujorului	11	72	4.9	0.61	[69]
	Iași	11.83	0.77	7.07	0.29	[70]
Merlot	Aliman (Ostrov)	14.14	*	5.25	0.65	[57]
Muscat ottonel	Dealu Bujorului	11	30.7	4.4	0.54	[69]
	Iași	12.2	1.34	6.43	0.33	[70]
	Iași	13.6	1.67	6.4	0.35	[76]
Sparkling Muscat ottonel	Iași	10.3	*	6.2	0.33	[77]
Neuburger	Iași	12.44	10.63	7.71	0.45	[70]

Table 5. *Cont.*

Grape Varieties	Vine Region	Alcohol Vol. (%)	Residual Sugars (g/L)	Total Acidity (g/L)	Volatile Acidity (g/L)	References
Pinot gris	Iași	14.49	4.81	6.68	0.33	[70]
	Miniș-Măderat	13.39	2.04	5.93	0.47	[71]
Pinot noir	Ratești (Dealurile Silvaniei)	13.47	*	6.01	0.53	[74]
Rose Traminer	Iași	14.1	1.67	6.73	0.25	[70]
Șarba	Dealu Bujorului	14.1	23	5.8	0.54	[69]
Sauvignon	Dealu Mare	12.2	10	5.8	0.3	[78]
		13	12	5.4	0.4	
		12.5	12	5.2	0.4	
Sauvignon blanc	Dealu Bujorului	14.35	12	5.2	0.57	[69]
	Iași	11.24	1.1	5.94	0.29	[70]
	Iași	11.9	0.9	5.95	0.2	[55]
Tămâioasă românească	Iași	11.63	15.47	6.93	0.31	[70]
Traminer	Miniș-Măderat	12.3	50	5.9	0.47	[71]

*: the authors did not mention the residual sugar content in the respective wines; nd: not detected.

Special wines were also obtained in Dealu Mare, Valea Călugărească center by Kontek and Kontek (1976); specifically, Jerez type wines, made of pellicular autochthonous yeast isolates belonging to *S. bayanus* species. These wines reached 15–16% alcohol, a maximum of 4 g H_2SO_4/L acidity, and the most appreciated were the ones with residual sugar of 16–17 g/L. The same authors also reported a cryophilic yeast, identified by classical tools as *S. carslbergensis*, initially isolated from must fermenting at 5 °C; this strain led to rapid wine clarification and produced low volatile content and high glycerol content. Similarly, for the cryophilic property, Tudose et al. selected a *S. ellipsoideus* strain in Iași-Copou centre, which was also resistant to high sulphur hydrogen content [80].

For high-quality sparkling wines, isolates of *S. oviformis* and *S. carlsbergensis* were selected in Blaj county during the 1980s [35]; they were capable of complete sugar consumption, while not stimulating the malolactic fermentation and not producing high volatility.

In the 1980s–1990s, generally, special attention was given to high-alcohol, low-foaming, and high-glycerol wine yeast strains, e.g., in Valea Călugărească center [81] and Iași county [82].

Starting with the 2000s, attention was more focused on the aromatic profile of wines made of local grape varieties and local yeast, while less attention was given to the high alcoholic strength. For instance, Liță et al. reported different local strains of *S. cerevisiae* var. *ellipsoideus* as appropriate candidates for dry white wines made of local varieties, such as Fetească albă and Fetească regală [83]. Moreover, in 2017, Lengyel and Panaitescu reported a local yeast isolated from Gârbova area (Sebeș-Apold vineyard), which was capable of improving the terpene flavor compounds content in Muscat ottonel wines [84]. A deeper study and methodology was reported by Vararu et al. after analyzing the aromatic profile of Muscat ottonel variety fermented with commercial and local yeast from Copou Iași centre [76]; a visual and easy to understand foot-printing was also performed, based on a multiple variable analysis, which established differences in the fermentative volatilome.

3.4. New Selection Directions in the Terroir Concept Context

The conventional practice of producing wines on an industrial scale with the use of *Saccharomyces* species involves controlled fermentation from all points of view. The wines thus obtained can be denominated according to the geographical indication (GI) if certain legislative requirements are followed. However, for an even greater specificity, a possible direction might be the use of local yeasts from each geographical region, in addition to using grapes harvested from those areas.

On another note, one way to obtain local wines is the spontaneous fermentation of grapes, but there are multiple disadvantages. The obtained wines may have different characteristics from one vintage to another, depending on many environmental variables, such as climate (temperature, precipitation, sunlight, wind), biology (microbiota, flora, and fauna), relief (topographic coordinates, geomorphology), and geology (soil types, irrigation, fertilization), as well as human implications, namely, traditions, culture, applied technology, agronomic practices, and legislation [8,85,86]. All these are involved in the concept of *terroir*.

Knight et al. consider the possibility of the existence of the concept of "microbial *terroir*", which implies that the microbial consortia in a certain wine-growing area are specific to that certain area and are producing flavors typical of the area [85]. Their experiments showed that the organoleptic properties of wine are given by *S. cerevisiae* indigenous strains and their origin, which may sustain the microbial aspect of *terroir*; in addition, the biodiversity of the yeast in the vineyards is affected by the micro and macroclimatic conditions of the vine varieties and the geographical location of the vineyard, a fact that would explain why the yeast consortia are different between two different wine-growing regions [87].

A research direction that emerges from the above-mentioned data is the use of autochthonous yeast in the wine industry in order to produce specific wines for certain wine-growing areas. Spontaneous fermentation is an uncontrolled and complex biotechnological process, in which the alteration microorganisms could rapidly multiply and reach too-high levels quickly, which may negatively impact the quality of the finished products [88]; this, even if spontaneous fermentation is correlated with greater complexity, greater wine body, and uncommon flavors [89–91], and it could improve the qualities of the wine by creating unique regional fingerprints [92], it is a process to be avoided. Therefore, one could combine spontaneous fermentation with indigenous yeasts with the safety of controlled processes from the industrial environment [86]. This would imply the use of selected local yeasts as new starter cultures in the winemaking industry, which would be reflected in the specific fingerprint of the finished product [86,93].

The new selection directions regarding the local wine yeasts tend to follow different paths, i.e., obtaining new wines with predetermined properties (high glycerol content, low ethanol content, reduced acidity); creating new and specific technological flows for obtaining certain types of wines, especially in order to avoid the production of certain compounds (biogenic amines, volatile sulfur compounds) in the finished wines; obtaining new wines of controlled origin and with a geographical indication; and completing the oenological practices in the legal specifications.

Thus, the research could be divided into two different directions, namely, that with the use of *Saccharomyces* yeasts, and that with the use of non-conventional (non-*Saccharomyces*) yeasts, in different variations, such as simple cultures, co-fermentation, or in sequential fermentation with *Saccharomyces* yeasts in different proportions. As described above, already, several non-*Saccharomyces* (NS) local yeast were detected during the isolation work and are stored in the owners' collections. In this regard, the usefulness of unconventional yeasts and the need to isolate and select such wine yeasts is further emphasized.

Considering the existence of numerous studies [94–98] which confirm that NS wine yeasts are beneficial a very large proportion, and even essential to obtaining wines with extraordinary organoleptic and sensory properties (Table 6), the selection of these yeast species is desirable in the near future. Among the NS species, only *Dekkera* spp. was reported as having only spoilage impact on wines. In Europe, numerous studies have been registered that argue in favor of non-conventional yeasts for the fermentation of the grape must. It is well-known that numerous NS yeast genera, including, but not limited to, the ones mentioned in Table 6, possess desirable oenological properties, such as the production of glycerol and other higher alcohols [99–101], the decreased ethanol content in the finished wine [102], and also the production of extracellular enzymes [103–105], esters [101,106], or polysaccharides [107].

Table 6. Biotechnological role of some non-*Saccharomyces* yeasts.

Genus	Relevant Species	Initial Technological Significance	Real Biotechnological Role	References
Hanseniaspora/ *Kloeckera*	*H. uvarum*/ *H. apiculata*	Contamination /Spoilage	Higher alcohols, acetate, and ethyl esters production	[90,108]
Candida	*C. stellata*	Contamination	Glycerol production, fructophily	[109]
	C. zemplinina/ Starmerella bacillaris	Contamination	Glycerol, succinic acid production; decrease of alcohol content	[94,98]
Metschnikowia	*M. pulcherrima*	Contamination	Esters, terpenes, and thiols production, increase in aroma complexity	[91,98,107]
Pichia	*P. anomala*	Contamination /Spoilage	Increased production of volatile compounds, killer against *Dekkera*/*Brettanomyces*	[110]
	P. kluyveri		3-mercaptohexan-1-ol and 3-mercaptohexan-1-ol acetate production	[111]
Lachancea/ *Kluyveromyces*	*L. thermotolerans*	Contamination	Glycerol overproduction, reduction of volatile acidity	[112]
Torulaspora	*T. delbrueckii*	Spoilage	Succinic acid, polysaccharides production	[113]
Dekkera/ *Brettanomyces*	*D. bruxellensis*	Spoilage	Spoilage	[8,100]
Schizosaccharomyces	*S. pombe*	Spoilage	Malolactic deacidification; propanol and pyruvic acid production	[98,114]

Taking into account all the properties and real biotechnological roles of these NS yeasts in the production of wine, a new path for their use in grape must fermentation is open, which will avoid the production of certain chemical compounds in the final wines instead of desirable compounds such as esters and glycerol. However, due to the fact that NS yeasts are not able to finish the alcoholic fermentation (they are less efficient in the production of ethanol), the technology should be accmpanied by a sequential inoculation of the grape must [91]. Thus, the NS yeast may be inoculated at the beginning of the fermentation, and, after the fermented must reaches a content of approximatively 10% ethanol, a *Saccharomyces* yeast will be added. In this way, the fermentation will be concluded by the *Saccharomyces* species, while the NS species will produce the necessary metabolites to positively influence the aroma of the wine. A similar alternative involves the simultaneous inoculation of the two types of yeast. Finally, mixed or sequential fermentations with *Saccharomyces* and NS allow the developtment of local wines with a low alcohol content [91].

4. Conclusions

From a historical point of view, the first wine yeast selection work in Romania started in 1915 as part of the international research process started by French teams at the time, and the first local wine yeasts collection was delivered in years 1920s. After the 1970s and until the 1990s, the selection work reached almost all Romanian winemaking regions. The use of novel molecular identification and characterization tools followed the international trend, reaching the country later on (after 2010). The advancement in the past ten years was highly depentend on such techniques, and special selected yeast are nowadays in several local collections. However, their inclusion in international collection was not found in any report, and this is an aspect which should be taken into account in the near future.

Several autochthonous strains of *Saccharomyces* were isolated from Romanian vineyards, grapes, and musts, a part of which demonstrated oenological qualities that are desirable for Romanian local wines.

Moreover, numerous NS yeast strains, belonging to a multitude of different genera, have been isolated and identified from vineyards and wine research stations in Romania, but few Romanian authors have studied and published the use of local NS yeasts in winemaking.

The selection of local yeasts is of great interest for Romanian wine production due to the fact that there is the possibility of expanding the diversity of wines on the market, but also due to the high demand for local, unique products. Actually, it was reported recently [115] that a large majority of Romanian people prefer to consume only local wines. It is also worth mentioning the fact that a larger range of local yeasts used leads to developing a wider range of local wines, which supports Romanian gastronomic identity, culture, and tradition.

Author Contributions: Conceptualization, F.M. and R.-Ș.R.-E.; methodology, R.-Ș.R.-E. and C.F.D.; formal analysis, R.-Ș.R.-E.; investigation, R.-Ș.R.-E., E.B., M.I. and V.P.; resources, R.-Ș.R.-E., V.P., E.B., M.I. and F.M.; writing—original draft preparation, R.-Ș.R.-E. and F.M.; writing—review and editing, R.-Ș.R.-E., V.P., C.F.D. and F.M.; visualization, E.B. and M.I.; supervision, F.M. and V.P.; project administration, F.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

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

References

1. Mărăcineanu, L.; Giugea, N.; Perț, C.; Căpruciu, R. Tradition and Quality of Romanian Viticulture. *Ann. Univ. Craiova* **2018**, *23*, 139–143.
2. Mart, M. Everything You Need to Know about Romanian Wine. *Vincarta* **2017**.
3. International Organisation of Vine and Wine. Country Reports of Romania. 2022. Available online: https://www.oiv.int/what-we-do/country-report?oiv; https://www.oiv.int/what-we-do/data-discovery-report?oiv (accessed on 18 November 2022).
4. Law 164 of 24 June 2015 of Vines and Wine under the System of the Common Organization of the Wine Market. Article 17, Published in Official Journal of Romania no. 472 of 30 June 2015. Available online: https://legislatie.just.ro/Public/DetaliiDocument/169280 (accessed on 4 December 2022).
5. National Institute of Statistics. *Romanian Statistical Yearbook—Time Series (CD-ROM)*; NIS: Bucharest, Romania, 2021.
6. Giugea, N.; Muntean, C.; Mărăcineanu, C.; Călugăru, V. *Resurse Oenoturistice*; Alma: Craiova, Romania, 2020; ISBN 978-606-567-398-4.
7. Antoce, A.O.; Nămoloșanu, I.C.; Matei Rădoi, F. Comparative Study Regarding the Ethanol Resistance of Some Yeast Strains Isolated from Romanian Vineyards. *Rom. Biotechnol. Lett.* **2011**, *16*, 5981–5988.
8. Pretorius, I.S. Tasting the Terroir of Wine Yeast Innovation. *FEMS Yeast Res.* **2020**, *20*, foz084. [CrossRef]
9. Gonzalez, R.; Morales, P. Truth in wine yeast. *Microb. Biotechnol.* **2022**, *15*, 1339–1356. [CrossRef] [PubMed]
10. Molinet, J.; Cubillos, F.A. Wild Yeast for the Future: Exploring the Use of Wild Strains for Wine and Beer Fermentation. *Front. Genet.* **2020**, *11*, 589350. [CrossRef]
11. Erny, C.; Raoult, P.; Alais, A.; Butterlin, G.; Delobel, P.; Matei-Radoi, F.; Casaregola, S.; Legras, J.L. Ecological success of a group of *Saccharomyces cerevisiae/Saccharomyces kudriavzevii* hybrids in the northern european wine-making environment. *Appl. Environ. Microbiol.* **2012**, *78*, 3256–3265. [CrossRef]
12. Pretorius, I.S. Tailoring wine yeast for the new millennium: Novel approaches to the ancient art of winemaking. *Yeast* **2000**, *16*, 675–729. [CrossRef]
13. Grangeteau, C.; Roullier-Gall, C.; Rousseaux, S.; Gougeon, R.D.; Schmitt-Kopplin, P.; Alexandre, H.; Guilloux-Benatier, M. Wine microbiology is driven by vineyard and winery anthropogenic factors. *Microb Biotechnol.* **2017**, *10*, 354–370. [CrossRef]
14. Tofalo, R.; Perpetuini, G.; Schirone, M.; Fasoli, G.; Aguzzi, I.; Corsetti, A.; Suzzi, G. Biogeographical characterisation of *Saccharomyces cerevisiae* wine yeast by molecular methods. *Front. Microbiol.* **2013**, *4*, 166. [CrossRef]
15. Mas, A.; Padilla, B.; Esteve-Zarzoso, B.; Beltran, G.; Reguant, C.; Bordons, A. Taking Advantage of Natural Biodiversity for Wine Making: The WILDWINE Project. *Agric. Agric. Sci. Procedia* **2016**, *8*, 4–9. [CrossRef]
16. Alessandria, V.; Marengo, F.; Englezos, V.; Gerbi, V.; Rantsiou, K.; Cocolin, L. Mycobiota of Barbera grapes from the Piedmont region from a single vintage year. *Am. J. Enol. Vitic.* **2015**, *66*, 244–250. [CrossRef]
17. Costantini, A.; Vaudano, E.; Pulcini, L.; Boatti, L.; Gamalero, E.; Garcia-Moruno, E. Yeast Biodiversity in Vineyard during Grape Ripening: Comparison between Culture Dependent and NGS Analysis. *Processes* **2022**, *10*, 901. [CrossRef]
18. Di Maio, S.; Polizzotto, G.; Di Gangi, E.; Foresta, G.; Genna, G.; Verzera, A.; Scacco, A.; Amore, G.; Oliva, D. Biodiversity of Indigenous Saccharomyces Populations from Old Wineries of South-Eastern Sicily (Italy): Preservation and Economic Potential. *PLoS ONE* **2012**, *7*, e30428. [CrossRef]

19. Tofalo, R.; Perpetuini, G.; Fasoli, G.; Schirone, M.; Corsetti, A.; Suzzi, G. Biodiversity study of wine yeasts belonging to the "terroir" of Montepulciano d'Abruzzo "Colline Teramane" revealed *Saccharomyces cerevisiae* strains exhibiting atypical and unique 5.8S-ITS restriction patterns. *Food Microbiol.* **2014**, *39*, 7–12. [CrossRef] [PubMed]
20. Morata, A.; Arroyob, T.; Bañuelosa, M.A.; Blancoc, P.; Brionesd, A.; Cantorale, J.M.; Castrilloc, D.; Cordero-Buesoe, G.; del Fresnoa, J.M.; Escott, C.; et al. Wine yeast selection in the Iberian Peninsula: *Saccharomyces* and non-*Saccharomyces* as drivers of innovation in Spanish and Portuguese wine industries. *Crit. Rev. Food Sci. Nutr.* **2022**, *10*, 1–29. [CrossRef]
21. Padilla, B.; Garcia- Fernández, D.; González, B.; Izidoro, I.; Esteve-Zarzoso, B.; Beltran, G.; Mas, A. Yeast Biodiversity from DOQ Priorat Uninoculated Fermentations. *Front. Microbiol.* **2016**, *7*, 930. [CrossRef]
22. Castrillo, D.; Rabuñal, E.; Neira, N.; Blanco, P. Yeast diversity on grapes from Galicia, NW Spain: Biogeographical Patterns and the Influence of the Farming System. *OENO One* **2019**, *53*, 573–587. [CrossRef]
23. De Celis, M.; Ruiz, J.; Martín-Santamaría, A.; Alonso, M.; Marquina, D.; Navascués, E.; Gómez-Flechoso, M.Á.; Belda, I.; Santos, A. Diversity of *Saccharomyces cerevisiae* yeasts associated to spontaneous and inoculated fermenting grapes from Spanish vineyards. *Lett. Appl. Microbiol.* **2019**, *68*, 580–588. [CrossRef]
24. Koulougliotis, D.; Eriotou, E. Isolation and identification of endogenous yeast strains in grapes and must solids of Mavrodafni kefalonias and antioxidant activity of the produced red wine. *Ferment Technol.* **2016**, *5*, 1. [CrossRef]
25. Kachalkin, A.V.; Abdullabekova, D.A.; Magomedova, E.S.; Magomedov, G.G.; Chernov, I.Y. Yeasts of the vineyards in Dagestan and other regions. *Microbiology* **2015**, *84*, 425–432. [CrossRef]
26. Brînduşe, E.; Nechita, A.; Ion, M.; Paşa, R.; Fîciu, L.; Ciubucă, A. *Valorificarea Biodiversităţii Drojdiilor Autohtone de Vinificaţie În Scopul Obţinerii Vinurilor Cu Tipicitate de Areal Viticol*; Editura PIM: Iaşi, Romania, 2022; ISBN 978-606-13-7088-7.
27. Nițescu, M.A. Contribution de l'étude Des Levures Roumaines. Ph.D. Thesis, Faculte de Sciences, Universite Paris Cite, Paris, France, 1915.
28. Ringhianu, J.; Septilici, G. Folosirea Drojdiilor Selecționate La I.A.S. Și Importanța Lor În Obținerea Vinurilor de Calitate. *Rev. I.A.S.* **1958**, *1*, 4–7.
29. Septilici, G. *Contribuții La Studiul Comparativ al Câtorva Specii de Drojdii*; Institutul de Cercetări Horti-Viticole, Lucrări Științifice: Paris, France, 1961; Volume 4, pp. 39–47.
30. Septilici, G.; Gherman, M. Elemente noi în pregătirea, livrarea și folosirea culturilor de drojdii în vinificație. *Rev. Grădina Via Și Livada* **1963**, *3*, 12–19.
31. Septilici, G.; Gherman, M. Rolul drojdiilor specializate în obținerea vinurilor de calitate. *Rev. Grădina Via Și Livada* **1963**, *7*, 47–52.
32. Septilici, G.; Sandu-Ville, G. Conducerea fermentației musturilor în toamne cu temperaturi scăzute prin folosirea drojdiilor criofile. *Rev. Grădina Via Și Livada* **1965**, *11*, 89–94.
33. Gherman, M. Influența culturilor de drojdii în amestec asupra calității vinurilor. *Rev. Grădina Via Și Livada* **1963**, *39*, 58–67.
34. Gherman, M. Drojdii de genuri și specii diferite pentru reglarea acidității. *Rev. Grădina Via Și Livada* **1965**, *38*, 72–75.
35. Dănoaie, F. Cercetări Privind Fiziologia Drojdiilor Izolate Din Podgoria Târnave. Ph.D. Thesis, Babeș-Bolyai University, Cluj-Napoca, Romania, 1989.
36. Stamate, C.; Țârdea, C.; Burnete, C. Isolation and Identification of Microflora on Grapes for the Varieties in Viticultural Centres Blaj, Târnave Vineyard. *Horticultura* **2006**, *49*, 537–542.
37. Oprean, L. Biotechnological Characteristics of Some *Saccharomyces* Species Isolated from Wine Yeast Culture. *Food Sci. Biotechnol.* **2005**, *14*, 722–726.
38. Sandu-Ville, G. Contribuții La Studiul Și Clasificarea Drojdiilor de Vin Din Microflora Vinicolă a Podgoriei Copou-Iași. Ph.D. Thesis, Institutul Agronomic Iași, Iași, Romania, 1974.
39. Sandu-Ville, G.; Savin, C. Conducerea Fermentației Alcoolice a Musturilor Cu Ajutorul Levurilor Criofile. *Cercet. Agron. În Mold.* **1987**, *2*, 68–74.
40. Viziteu, G.A.; Manoliu, A.; Andor, I. Data Concerning the Yeasts Microbiota from Cotnari Vineyard. *Rom. Biotechnol. Lett.* **2008**, *13*, 3673–3680.
41. Vasile, A.; Cotea, V.V.; Măntăluță, A.; Paşa, R.; Savin, C. The Preliminary Selection of Isolated Yeast Strains from the Indigenous Flora of Iași Vineyard. *Sci. Artic. UASVM Iași—Hortic. Ser.* **2009**, *52*, 811–816.
42. Nechita, A.; Savin, C.; Paşa, R.; Zamfir, C.I.; Codreanu, M. Isolation of New Types of Yeasts Strains from Indigenous Flora of Iași Vineyards. *Sci. Artic. UASVM Iași—Hortic. Ser.* **2014**, *57*, 177–182.
43. Găgeanu, A.; Câmpeanu, G.; Diguță, C.; Matei, F. Isolation and Identification of Local Wine Yeast Strains from Dealurile Bujorului Vineyard. *Sci. Bulletin. Ser. F. Biotechnol.* **2012**, *XVI*, 22–25.
44. Kontek, A.; Kontek, A. Caracteristicile Morfofiziologice Și Tehnologice Ale Unei Tulpini de *Saccharomyces carlsbergensis* Izolată Din Vin. *An. ICVV* **1975**, *VI*, 543–552.
45. Kontek, A.; Kontek, A. Contribuții La Studiul Taxonomic al Drojdiilor Din Podgoria Dealu Mare. *An. ICVV* **1976**, *VII*, 597–609.
46. Matei Rădoi, F.; Brînduşe, E.; Nicolae, G.; Tudorache, A.; Teodorescu, R.I. Yeast Biodiversity Evolution over Decades in Dealu Mare—Valea Călugărească Vineyard. *Rom. Biotechnol. Lett.* **2011**, *16*, 113–120.
47. Brînduşe, E.; Tudorache, A.; Fotescu, L. Influence of Ecological Culture System on the Dynamics and Biodiversity of Non-*Saccharomyces* Autochthonous Wine Yeasts. *Sci. Artic. UASVM Iași—Hortic. Ser.* **2012**, *55*, 331–336.
48. Brînduşe, E.; Tudorache, A.; Fotescu, L. Selected Autochthonous Yeast Strains with Influence on Wine Quality. *Sci. Artic. UASVM Iași—Hortic. Ser.* **2012**, *55*, 365–370.

49. Bărbulescu, I.D.; Begea, M.; Frîncu, M.; Tamaian, R. Identification of Yeasts Tested for Fermentation on Different Carbon Sources. *Prog. Cryog. Isot. Sep.* **2015**, *18*, 51–58.
50. Dragomir Tutulescu, F.; Popa, A. Wine-Growing Areas in Oltenia (Romania) Major Natural Sources for the Isolation, Identification and Selection of Oenological Microorganisms. *Not. Bot. Horti Agrobot. Cluj-Napoca* **2009**, *37*, 139–144.
51. Beleniuc, G. Contribuții La Studiul Drojdiilor Izolate Din Podgoria Murfatlar. Ph.D. Thesis, Al. I. Cuza University: Iași, Romania, 1996.
52. Beleniuc, G. Researches Regarding the Taxonomic Identification of Spontaneous Microflora from Viti-Vinicol Cernavodă Center from Murfatlar Vineyard. *Res. J. Agric. Sci.* **2013**, *45*, 93–99.
53. Dumitrache, C.; Frîncu, M.; Rădoi, T.A.; Bărbulescu, I.D.; Mihai, C.; Matei, F.; Tudor, V.; Teodorescu, R.I. Identification by PCR ITS-RFLP Technique of New Yeast Isolates from Pietroasa Vineyard. *Sci. Bull. Ser. B Hortic.* **2020**, *LXIV*, 287–293.
54. Dragomir Tutulescu, F.; Popa, A. Viticultural Areas from Oltenia—Romania Which Have at Disposal a Rich and Performant Microflora of Oenological Interest. *Agricultura* **2010**, *75*, 33–39.
55. Vasile, A.; Pașa, R.; Savin, C. The Influence of New Yeast Strains from the Indigenous Flora of Iași Vineyard on the Alcoholic Fermentation Process. *Sci. Artic. UASVM Iași—Hortic. Ser.* **2010**, *53*, 459–464.
56. Matei, F.; Găgeanu, A. Killer Profile of Wine Yeast Strains Isolated in Dealurile Bujorului Vineyard. *Rom. Biotechnol. Lett.* **2011**, *16*, 144–147.
57. Antoce, A.O.; Nămoloșanu, I.C. A Rapid Method for Testing Yeast Resistance to Ethanol for the Selection of Strains Suitable for Winemaking. *Rom. Biotechnol. Lett.* **2011**, *16*, 5953–5962.
58. Barnett, J.A.; Payne, R.W.; Yarrow, D. *Yeasts: Characteristics and Identification*, 1st ed.; Cambridge University Press: Cambridge, UK, 1983; ISBN 978-0-521-25296-6.
59. Barnett, J.A. A History of Research on Yeasts 2: Louis Pasteur and His Contemporaries, 1850–1880. *Yeast* **2000**, *16*, 755–771. [CrossRef]
60. Kreger-van Rij, N.J.W. (Ed.) *The Yeasts: A Taxonomic Study*, 3rd ed.; Elsevier Science: Groningen, The Netherlands, 1984; ISBN 978-0-444-80421-1.
61. Delfini, C. Innovative Trends in Oenology and in the Selection of Yeasts and Malolactic Bacteria for Wine Industry. *Riv. Vitic. E Enol.* **1992**, *45*, 17–30.
62. Corbu, V.; Csutak, O. Molecular and Physiological Diversity of Indigenous Yeasts Isolated from Spontaneously Fermented Wine Wort from Ilfov County, Romania. *Microorganisms* **2023**, *11*, 37. [CrossRef]
63. Corbu, V.; Csutak, O. Biodiversity Studies on *Pichia kudriavzevii* from Romanian Spontaneous Fermented Products. *AgroLife Sci. J.* **2020**, *9*, 104–113.
64. Gaspar, E.; Gyöngyvér, M.; Oprean, L.; Iancu, R. Identification and Isolation of Yeast Strains *Saccharomyces bayanus* from Native Sources Using Mollecular Methods. *Ann. Rom. Soc. Cell Biol.* **2011**, *16*, 286–291.
65. Oprean, L. Molecular Identification of Selected Yeast Strains Isolated from Sebeș-Apold Vineyard, Apoldu de Jos Viticultural Area. *Ann. Rom. Soc. Cell Biol.* **2014**, *19*, 45–48.
66. Kontek, A. Studiul Factorilor Care Condiționează Formarea Acidității Volatile a Vinurilor La Vinificarea Primară. Ph.D. Thesis, University of Craiova, Craiova, Romania, 1977.
67. Lodder, J.; Kreger-van Rij, N.J.W. *The Yeasts. A Taxonomic Study*, 2nd ed.; North Holland Publishing Company: Amsterdam, The Netherlands, 1967.
68. Lengyel, E.; Oprean, L.; Tița, O.; Păcală, M.L.; Iancu, R.; Stegăruș, D.; Ketney, O. Isolation and Molecular Identifications of Wine Yeast Strains from Autochthonous Aromatic and Semi Aromatic Varieties. *Ann. Rom. Soc. Cell Biol.* **2012**, *17*, 134–138.
69. Bora, F.D.; Donici, A.; Oșlobanu, A.; Fițiu, A.; Babeș, A.C.; Bunea, C.I. Qualitative Assessment of the White Wine Varieties Grown in Dealu Bujorului Vineyard, Romania. *Not. Bot. Horti Agrobot.* **2016**, *44*, 593–602. [CrossRef]
70. Colibaba, L.C.; Cotea, V.V.; Lacureanu, F.G.; Tudose-Sandu-Ville, S.; Rotaru, L.; Niculaua, M.; Luchian, C.E. Studies of Phenolic and Aromatic Profile of Busuioacă de Bohotin Wines. *BIO Web Conf.* **2015**, *5*, 02008. [CrossRef]
71. Dobrei, A.G.; Dobrei, A.; Iordănescu, O.; Nistor, E.; Balla, G.; Mălăescu, M.; Drăgunescu, A. Research Concerning the Correlation of Soil with Wines Quality in Some Varieties of Wine Grapes in Miniș-Măderat Vineyards. *J. Hortic. For. Biotechnol.* **2015**, *19*, 98–105.
72. Vararu, F.; Moreno-Garcia, J.; Moreno, J.; Niculaua, M.; Nechita, B.; Zamfir, C.; Colibaba, C.; Dumitru, G.D.; Cotea, V.V. Minor Volatile Compounds Profiles of "Aligoté" Wines Fermented with Different Yeast Strains. *Not. Sci. Biol.* **2015**, *7*, 123–128. [CrossRef]
73. Vișan, L.; Dobrinoiu, R.; Dumbravă, M. The Chemical and Aromatic Composition in a Romanian Wine Cabernet Sauvignon. *Rom. Biotechnol. Lett.* **2012**, *17*, 6855–6861.
74. Manolache, M.; Pop, T.I.; Babeș, A.C.; Farcaș, I.A.; Muncaciu, M.L.; Călugăr, A.; Gal, E. Volatile Composition of Some Red Wines from Romania Assessed by GC-MS. *Stud. Univ. Babeș-Bolyai Chem.* **2018**, *63*, 125–142. [CrossRef]
75. Manolache, M.; Pop, T.I.; Babeș, A.C.; Farcaș, I.A.; Godoroja, M.; Călugăr, A.; Gal, E. Assessment of Volatile Compounds of Some Red Wine Samples from Republic of Moldova and Romania Using GC-MS Analysis. *Agricultura* **2018**, *105*, 40–47.
76. Vararu, F.; Moreno-Garcia, J.; Niculaua, M.; Cotea, V.V.; Mayen, M.; Moreno, J. Fermentative Volatilome Modulation of Muscat Ottonel Wines by Using Yeast Starter Cultures. *LWT—Food Sci. Technol.* **2020**, *129*, 109575. [CrossRef]
77. Focea, M.C.; Luchian, C.; Zamfir, C.; Niculaua, M.; Moroșanu, A.M.; Nistor, A.; Andrieș, T.; Lacureanu, G.; Cotea, V.V. Organoleptic Characteristics of Experimental Sparkling Wines. *Sci. Artic. UASVM Iași—Hortic. Ser.* **2017**, *60*, 221–226.

78. Vișan, L.; Dobrinoiu, R. Studies on the Aroma of Sauvignon Wine. *Sci. Bulletin. Ser. F. Biotechnol.* **2013**, *17*, 127–131.
79. Scanes, K.T.; Hohmann, S.; Prior, B.A. Glycerol Production by the Yeast *Saccharomyces cerevisiae* and Its Relevance to Wine: A Review. *S. Afr. J. Enol. Vitic.* **1998**, *19*, 17–24. [CrossRef]
80. Tudose, I.; Savin, C.; Stoica, C. Selecția Și Testarea Potențialului Criofil al Unor Tulpini de *Saccharomyces ellipsoideus* Izolate Din Podgoria Iași, Centrul Viticol Copou. *An. ICVV* **2002**, *17*, 256–261.
81. Kontek, A.; Kontek, A.; Rusea, V.; Sandu-Ville, G. Selecția Și Caracterizarea a Două Suse de Levuri Cu Capacitate Alcooligenă Ridicată. *An. ICVV* **1991**, *13*, 279–292.
82. Sandu-Ville, G.; Popescu, C. Conducerea Fermentației Alcoolice a Musturilor Prin Utilizarea de Drojdii Cu Caracter Nespumant. *Cercet. Agron. În Mold.* **1980**, *1*, 89–92.
83. Liță (Mihai), C.; Antoce, A.O.; Nămoloșanu, I.C. Studiul Influenței Unor Tulpini de Drojdii Selecționate Asupra Fermentației Alcoolice. *Lucr. Științifice USAMV Iași Ser. Hortic.* **2006**, *49*, 701–706.
84. Lengyel, E.; Panaitescu, M. The Management of Selected Yeast Strains in Qualifying Terpene Flavours in Wine. *Manag. Sustain. Dev.* **2017**, *9*, 27–30. [CrossRef]
85. Knight, S.; Klaere, S.; Fedrizzi, B.; Goddard, M.R. Regional Microbial Signatures Positively Correlate with Differential Wine Phenotypes: Evidence for a Microbial Aspect to Terroir. *Sci. Rep.* **2015**, *5*, 14233. [CrossRef]
86. Belda, I.; Zarraonaindia, I.; Perisin, M.; Palacios, A.; Acedo, A. From Vineyard Soil to Wine Fermentation: Microbiome Approximations to Explain the "*terroir*" Concept. *Front. Microbiol.* **2017**, *8*, 821. [CrossRef] [PubMed]
87. Alexandre, H. Wine Yeast Terroir: Separating the Wheat from the Chaff—For an Open Debate. *Microorganisms* **2020**, *8*, 787. [CrossRef]
88. Francesca, N.; Gaglio, R.; Alfonzo, A.; Settani, L.; Corona, O.; Mazzei, P.; Romano, R.; Piccolo, A.; Moschetti, G. The Wine: Typicality or Mere Diversity? The Effect of Spontaneous Fermentations and Biotic Factors on the Characteristics of Wine. *Agric. Agric. Sci. Procedia* **2016**, *8*, 769–773. [CrossRef]
89. Jackson, R.S. *Wine Tasting: A Professional Handbook (Food Science and Technology)*, 2nd ed.; Academic Press: Cambridge, MA, USA, 2009; ISBN 978-0-12-374181-3.
90. Jolly, N.P.; Varela, C.; Pretorius, I.S. Not Your Ordinary Yeast: Non-*Saccharomyces* Yeasts in Wine Production Uncovered. *FEMS Yeast Res.* **2014**, *14*, 215–237. [CrossRef] [PubMed]
91. Carrau, F.; Boido, E.; Ramey, D. Chapter Three—Yeasts for Low Input Winemaking: Microbial Terroir and Flavor Differentiation. *Adv. Appl. Microbiol.* **2020**, *111*, 89–121. [CrossRef]
92. Fleet, G.H. Wine Yeasts for the Future. *FEMS Yeast Res.* **2008**, *8*, 979–995. [CrossRef]
93. Comitini, F.; Capece, A.; Ciani, M.; Romano, P. New Insights on the Use of Wine Yeasts. *Curr. Opin. Food Sci.* **2017**, *13*, 44–49. [CrossRef]
94. Garcia, M.; Esteve-Zarzoso, B.; Arroyo, T. Non-Saccharomyces Yeasts: Biotechnological Role for Wine Production. In *Grape and wine Biotechnology*; IntechOpen: London, UK, 2016; pp. 249–271. ISBN 978-953-51-2693-5.
95. Manzanares, P.; Valles, S.; Viana, F. Chapter 4—Non-*Saccharomyces* Yeasts in the Winemaking Process. In *Molecular Wine Microbiology*; Academic Press: Cambridge, MA, USA, 2011; pp. 85–109. ISBN 978-0-12-375021-1.
96. Englezos, V.; Rantsiou, K.; Torchio, F.; Rolle, L.; Gerbi, V.; Cocolin, L. Exploitation of the Non-*Saccharomyces* Yeast *Starmerella bacillaris* (Synonym *Candida zemplinina*) in Wine Fermentation: Physiological and Molecular Characterization. *Int. J. Food Microbiol.* **2015**, *199*, 33–40. [CrossRef]
97. Sgouros, G.; Chalvantzi, I.; Mallouchos, A.; Paraskevopoulos, Y.; Banilas, G.; Nisiotou, A. Biodiversity and Enological Potential of Non-*Saccharomyces* Yeasts from Nimean Vineyards. *Fermentation* **2018**, *4*, 32. [CrossRef]
98. Benito, A.; Calderon, F.; Benito, S. The Influence of Non-*Saccharomyces* Species on Wine Fermentation Quality Parameters. *Fermentation* **2019**, *5*, 54. [CrossRef]
99. Ciani, M.; Maccarelli, F. Oenological Properties of Non-*Saccharomyces* Yeasts Associated with Wine-Making. *J. Microbiol. Biotechnol.* **1998**, *14*, 199–203. [CrossRef]
100. Belda, I.; Ruiz, J.; Esteban-Fernandez, A.; Navascues, E.; Marquina, D.; Santos, A.; Moreno-Arribas, M.V. Microbial Contribution to Wine Aroma and Its Intended Use for Wine Quality Improvement. *Molecules* **2017**, *22*, 189. [CrossRef] [PubMed]
101. Lee, S.B.; Banda, C.; Park, H.D. Effect of Inoculation Strategy of Non-*Saccharomyces* Yeasts on Fermentation Characteristics and Volatile Higher Alcohol and Esters in Campbell Early Wines. *Aust. J. Grape Wine Res.* **2019**, *25*, 384–395. [CrossRef]
102. Rocker, J.; Strub, S.; Ebert, K.; Grossmann, M. Usage of Different Aerobic Non-*Saccharomyces* Yeasts and Experimental Condition as a Tool for Reducing the Ethanol Content in Wines. *Eur. Food Res. Technol.* **2016**, *242*, 2051–2070. [CrossRef]
103. Maturano, Y.P.; Rodriguez Assaf, L.A.; Toro, M.E.; Nally, M.C.; Vallejo, M.; Castellanos de Figueroa, L.I.; Combina, M.; Vazquez, F. Multi-Enzyme Production by Pure and Mixed Cultures of *Saccharomyces* and Non-*Saccharomyces* Yeasts during Wine Fermentation. *Int. J. Food Microbiol.* **2012**, *155*, 43–50. [CrossRef]
104. Capozzi, V.; Garofalo, C.; Chiriatti, M.A.; Grieco, F.; Spano, G. Microbial Terroir and Food Innovation: The Case of Yeast Biodiversity in Wine. *Microbiol. Res.* **2015**, *181*, 75–83. [CrossRef]
105. Nechita, A.; Filimon, V.R.; Pașa, R.; Damian, D.; Zaldea, G.; Filimon, R.; Zaiț, M. Oenological Characterization of Some Yeast Strains Isolated from the Iași Vineyard Romania. *Rom. J. Hortic.* **2020**, *I*, 141–148. [CrossRef]

106. Lai, Y.T.; Hsieh, C.W.; Lo, Y.C.; Liou, B.K.; Lin, H.W.; Hou, C.Y.; Cheng, K.C. Isolation and Identification of Aroma-Producing Non-*Saccharomyces* Yeast Strains and the Enological Characteristic Comparison in Wine Making. *LWT—Food Sci. Technol.* **2022**, *154*, 112653. [CrossRef]
107. Domizio, P.; Liu, Y.; Bisson, L.F.; Barile, D. Use of Non-*Saccharomyces* Wine Yeasts as Novel Sources of Mannoproteins in Wine. *Food Microbiol.* **2014**, *43*, 5–15. [CrossRef]
108. Andorra, I.; Berradre, M.; Rozes, N.; Mas, A.; Guillamon, J.M.; Esteve-Zarzoso, B. Effect of Pure and Mixed Cultures of the Main Wine Yeast Species on Grape Must Fermentations. *Eur. Food Res. Technol.* **2010**, *231*, 215–224. [CrossRef]
109. Magyar, I.; Toth, T. Comparative Evaluation of Some Oenological Properties in Wine Strains of *Candida stellata*, *Candida zemplinina*, *Saccharomyces uvarum* and *Saccharomyces cerevisiae*. *Food Microbiol.* **2011**, *28*, 94–100. [CrossRef]
110. Domizio, P.; Romani, C.; Lencioni, L.; Comitini, F.; Gobbi, M.; Mannazzu, I.; Ciani, M. Outlining a Future for Non-*Saccharomyces* Yeasts: Selection of Putative Spoilage Wine Strains to Be Used in Association with *Saccharomyces cerevisiae* for Grape Juice Fermentation. *Int. J. Food Microbiol.* **2011**, *147*, 170–180. [CrossRef] [PubMed]
111. Anfang, N.; Brajkovich, M.; Goddard, M.R. Co-Fermentation with *Pichia kluyveri* Increases Varietal Thiol Concentrations in Sauvignon Blanc. *Aust. J. Grape Wine Res.* **2009**, *15*, 1–8. [CrossRef]
112. Comitini, F.; Gobbi, M.; Domizio, P.; Romani, C.; Lencioni, L.; Mannazzu, I.; Ciani, M. Selected Non-*Saccharomyces* Wine Yeasts in Controlled Multistarter Fermentations with *Saccharomyces cerevisiae*. *Food Microbiol.* **2011**, *28*, 873–882. [CrossRef] [PubMed]
113. Sadoudi, M.; Tourdot-Marechal, R.; Rousseaux, S.; Steyer, D.; Gallardo-Chacon, J.J.; Ballester, J.; Vichi, S.; Guerin-Schneider, R.; Caixach, J.; Alexandre, H. Yeast-Yeast Interactions Revealed by Aromatic Profile Analysis of Sauvignon Blanc Wine Fermented by Single of Co-Culture of Non-*Saccharomyces* and *Saccharomyces* Yeasts. *Food Microbiol.* **2012**, *32*, 243–253. [CrossRef]
114. Benito, S.; Palomero, F.; Morata, A.; Calderon, F.; Palomero, D.; Suarez-Lepe, J.A. Selection of Appropriate *Schizosaccharomyces* Strains for Winemaking. *Food Microbiol.* **2014**, *42*, 218–224. [CrossRef]
115. Dumitrescu, R. Romanians Prefer to Drink Local Wines. Available online: https://www.romania-insider.com/romanians-prefer-drink-local-wine-2023 (accessed on 3 February 2023).

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

microorganisms

MDPI

Article

Promising Probiotic Properties of the Yeasts Isolated from *Rabilé*, a Traditionally Fermented Beer Produced in Burkina Faso

Iliassou Mogmenga [1,2,3], Marius Kounbèsiounè Somda [1], Cheik Amadou Tidiane Ouattara [1], Ibrahim Keita [1], Yérobessor Dabiré [2,4], Camelia Filofteia Diguță [5,*], Radu Cristian Toma [4,*], Lewis I. Ezeogu [2], Jerry O. Ugwuanyi [2], Aboubakar S. Ouattara [1] and Florentina Matei [5]

1 Laboratoire de Microbiologie et de Biotechnologies Microbiennes, Université Joseph KI-ZERBO, Ouagadougou 03 BP 7021, Burkina Faso
2 Department of Microbiology, Faculty of Biological Sciences, University of Nigeria, Nsukka 410001, Enugu State, Nigeria
3 Centre Universitaire de Banfora, Université Nazi BONI, Bobo-Dioulasso 01 BP 1091, Burkina Faso
4 Laboratoire de Biochimie, Biotechnologie, Technologie Alimentaire et Nutrition (LABIOTAN), Département de Biochimie Microbiologie, Université Joseph KI-ZERBO, Ouagadougou 03 BP 7021, Burkina Faso
5 Faculty of Biotechnologies, University of Agronomic Sciences and Veterinary Medicine of Bucharest, 011464 Bucharest, Romania
* Correspondence: camelia.diguta@biotehnologii.usamv.ro (C.F.D.); radu.toma@biotehnologii.usamv.ro (R.C.T.)

Abstract: In recent years, research on yeasts as probiotics has gained more and more interest, which will allow the development of "new" products in the probiotics market. In this context, seventeen yeast strains isolated from *Rabilé*, a traditional beer produced in Burkina Faso, were assessed for their probiotic attributes. The yeast identification was performed by molecular methods, including PCR-RFLP and 5.8S-ITS region sequencing. *Saccharomyces cerevisiae* (14 strains) was the predominantly identified species, followed by *Pichia kudriavzevii* (2 strains) and *Rhodotorula mucilaginosa* (1 strain). Except for *R. mucilaginosa*, all yeast strains grew well at human temperature. The yeast strains showed high resistance when they were exposed to simulated gastrointestinal conditions. Auto-aggregation ability was between 70.20 ± 10.53% and 91.82 ± 1.96%, while co-aggregation with *E. coli* ranged from 24.92 ± 3.96% to 80.68 ± 9.53% and with *S. enterica serovar* Typhimurium from 40.89 ± 8.18% to 74.06 ± 7.94%. Furthermore, the hydrophobicity of isolated strains toward n-hexane was in the range from 43.17 ± 5.07% to 70.73 ± 2.42%. All yeast strains displayed high antioxidant capabilities, and the strains did not show hemolysis halos, such that they can be considered safe. Additionally, *S. cerevisiae* strains strongly inhibited the growth of foodborne pathogens. This is the first preliminary study to identify and characterize the yeast strains isolated from *Rabilé* with interesting probiotic properties.

Keywords: yeasts; *Rabilé*; Burkina Faso; *Saccharomyces*; non-*Saccharomyces*; probiotic properties

check for updates

Citation: Mogmenga, I.; Somda, M.K.; Ouattara, C.A.T.; Keita, I.; Dabiré, Y.; Diguță, C.F.; Toma, R.C.; Ezeogu, L.I.; Ugwuanyi, J.O.; Ouattara, A.S.; et al. Promising Probiotic Properties of the Yeasts Isolated from *Rabilé*, a Traditionally Fermented Beer Produced in Burkina Faso. *Microorganisms* **2023**, *11*, 802. https://doi.org/10.3390/microorganisms11030802

Academic Editors: Djamel Drider and Françoise Coucheney

Received: 20 February 2023
Revised: 12 March 2023
Accepted: 16 March 2023
Published: 21 March 2023

Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

The current sustainability of food systems must be rethought in order to reduce hunger and prevent food and disease vulnerabilities in some parts of the world, mostly in developing countries [1–3]. For this reason, local fermented foods have been put back in the spotlight in Africa [3–8]. Several traditional fermented foods in Africa are obtained by the mediation of microorganisms (mainly lactic acid bacteria (LAB) and yeasts), such as *Rabilé* and *Ben-saalga* (Burkina Faso); Mawè (Benin); Gari, Fufu, and Kunu-zaki (Nigeria); kule naoto and Amabere amaruranu (Kenya); and Amasi and Mahewu (South Africa) (reviewed by Obafemi et al. [6]). According to FAO/WHO [9] and updates by Hill et al. [10], probiotics are defined as live microorganisms that confer beneficial effects on the host when administered in the proper amounts. Although several LAB species belonging to *Lactobacillus* and

Bifidobacterium genera are the most studied and commercialized probiotics [11–16], yeast biotechnology is well-known and is used in the manufacturing of fermented foods and in starter/co-starter cultures in the development of new functional foods with high-value nutraceuticals [17–23]. In the last few years, research on yeasts as potential probiotics with valuable properties, which had previously been relatively neglected, has intensified to discover new "wild" yeast strains isolated from traditional fermented foods; such approaches may revolutionize the probiotics market, which has been dominated mainly by lactic acid bacteria [24–30]. *Saccharomyces cerevisiae* var. *boulardii* has been approved for commercial use as a probiotic yeast [31,32]. Additionally, *S. cerevisiae*, the best-known species, has been extensively studied for its valuable probiotic characteristics [33–35]. Currently, non-*Saccharomyces* species have been studied for their potential probiotic attributes [33–39]. Ogunremi et al. [36] have isolated new yeast strains from some traditional cereal-based fermented products from Nigeria, such as *Candida tropicalis*, *Issatchenkia orientalis*, *Pichia kudriavzevii*, and *Pichia kluyveri*, and characterized them as starter cultures with multifunctional potentials to produce cereal-based probiotic products. The probiotic properties of yeasts isolated from whole-grain millet sourdoughs have been evaluated [39]. According to the reviewed articles, the exploration of the probiotic potential of yeasts is based on the usual in vitro testing of the main functional characteristics, namely, species identification, safety requirements, the ability to survive the transition through the gastrointestinal tract (body temperature, stomach pH, various digestive enzymes, and bile salts), the ability to adhere to cell surfaces (hydrophobicity and self- and co-aggregation capacities), and antimicrobial activity [40–43]. Different *Saccharomyces* and non-*Saccharomyces* strains other than the commercially available *Saccharomyces boulardii* have been demonstrated to have valuable probiotic properties that could improve both human and animal health (modulating metabolism and immunity and antimicrobial activity), enhance livestock feed digestion and growth performance, and obtain functional foods/feeds, in addition to other novel applications [21,24–29,34,44–46].

LAB and yeasts have been reported as the main microorganisms isolated from traditional beers [6,47–49]. *Rabilé*, a traditional fermented beer from Burkina Faso, is also used as a condiment to supply protein in cereal-based foods [49]. To our knowledge, research on the probiotic properties of yeasts isolated from *Rabilé* has not been carried out.

Hence, the goal of this research was to investigate in vitro the functional and probiotic properties of yeast strains isolated from *Rabilé* as a basis for establishing the nutraceutical value of *Rabilé*.

2. Materials and Methods

2.1. Microorganisms and Growth Conditions

For this study, 17 yeast strains isolated from *Rabilé* beer [49] were selected to evaluate their probiotic properties. Strains were maintained by cultivation in yeast extract dextrose-peptone broth (YPD) (containing 2% *w/v* dextrose, 2% *w/v* peptone, and 1% *w/v* yeast extract) at 30 °C for 24 h and stored in the presence of 20% (*v*/*v*) glycerol at −20 °C.

Escherichia coli ATCC 8739, *Listeria monocytogenes* ATCC 7644, *Salmonella enterica* serovar Typhimurium ATCC 14028, *S. enteritidis* ATCC 13076, and *Staphylococcus aureus* ATCC 33592 (provided by the American Type Culture Collection (ATCC) (Manassas, VA, USA) were used as the reference pathogenic bacteria for the antimicrobial tests, while *E. coli* ATCC 8739 and *S. enterica* serovar Typhimurium ATCC 14028 were used in the co-aggregation assays. Pathogenic bacteria were maintained by cultivation in tryptic soy broth (TSB) or tryptic soy agar (TSA) (Scharlab, Barcelona, Spain) at 37 °C for 24 h.

2.2. Molecular Identification of Yeast Isolates

2.2.1. Amplification of the 5.8S-ITS Region and RFLP Analysis

The DNA extraction was performed with fresh yeast cultures using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, CA, USA), according to the supplier's instructions, and the extracts were stored at −20 °C until use. The universal primers

ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [50] were used to amplify the 5.8S-ITS region by PCR. All PCRs were carried out in 50 µL of solution containing 5 µL of 10X DreamTaq Green Buffer supplemented with $MgCl_2$, 2.5 µL of each 10 µM primer, 1 µL of 10 mmol dNTP, 0.25 µL of 1U DreamTaq polymerase (Thermo Fisher Scientific, Baltics, UAB, Vilnius, Lithuania), and 10 ng·µL^{-1} fungal DNA. PCR amplifications were performed in a MultiGene PCR System (MyCycler thermal cycler, BIO-RAD, Hercules, USA) under the conditions described by Esteve-Zarzoso et al. [51]. Then, without further purification, each PCR product was digested separately with three restriction enzymes—*Hae*III, *Hinf*I, and *Hha*I (Thermo Fisher Scientific, Baltics, UAB, Vilnius, Lithuania)—at 37 °C for a minimum of 2 h, according to the supplier's instructions. PCR products and their restriction fragments were separated on 2% (*w*/*v*) agarose gel, and their lengths were approximated by comparison with the known DNA size standards (GeneRuler 100 bp Plus DNA Ladder; Thermo Fisher Scientific, Baltics, UAB, Vilnius, Lithuania). The electrophoretic patterns were captured using the GelDoc-It Imaging System (Analytik Jena, Upland, CA, USA). The RFLP patterns were compared with restriction analyses performed by Esteve-Zarzoso et al. [51] and with those of the reference yeast species freely available in the Yeast-id database (http://www.yeast-id.com, accessed on 29 November 2021). The similarity of restriction patterns was analyzed using Pearson's correlation coefficient r. The yeast strains were grouped using the unweighted pair group method with arithmetic averages (UPGMA) [52].

2.2.2. Yeast Strain Identification by Sequencing

The 5.8-ITS sequences of some yeast isolates belonging to the different detected yeast groups were sequenced by the Cellular and Molecular Immunological Application (CEMIA, Greece) to confirm the identifications at the species level by ITS-RFLP. The sequenced sequences were submitted using BLASTN tools (http://www.ncbi.nlm.nih.gov/BLAST/, accessed on 14 February 2022) for alignment with different sequences available in the NCBI database based on similarity percentages.

2.3. Growth Capacity at 37 °C

Quantities of 10 mL of YPD Broth in sterile tubes were inoculated with fresh yeast cultures (adjusted to OD600 at 0.3 ± 0.05). After incubation at 37 °C for 24 h, the growth rates of the yeast strains were recorded by measuring optical densities at 600 nm.

2.4. Survival of Simulated Gastrointestinal (GI) Digestion

2.4.1. Tolerance of Pepsin Presence and Acidic pH

The pepsin and pH 2.5 tolerance tests of the yeast strains were performed according to Burns et al. [53]. Briefly, overnight yeast cultures grown in YPD broth were centrifuged (4000× *g*, 5 min). Cell pellets were washed twice with sterile phosphate buffer (PB) (pH 7.0) (0.5382% (*w*/*v*) NaH_2PO_4 and 1.6363% (*w*/*v*) $Na_2HPO_4.7H_2O$) and resuspended in 10 mL of sterile PB with low pH (2.5) and containing pepsin (0.3%). Then, the samples thus prepared were incubated at 37 °C for 3 h in static conditions. Aliquots were taken at 0 h (initial) and after 3 h (final) and diluted in sterile saline 0.85% solution. Survival rates were assessed by the drop plate method on YPD Agar and were calculated using the following formula:

$$SR\% = \frac{\text{Log CFU/mL (final)}}{\text{Log CFU/mL (initial)}} \times 100$$

2.4.2. Tolerance of Bile Salts

Bile salt tolerance of the yeast strains was determined following the method reported by Pedersen et al. [54], with a few modifications. The YPD broth supplemented with bile salts (0.3%) (HiMedia Laboratories, Pvt. Ltd., Maharashtra, India) was inoculated with fresh yeast cultures and then incubated at 37 °C for 4 h. Aliquots were taken at 0 h (initial) and after 4 h (final) and diluted in a sterile saline 0.85% solution. Survival rates were

assessed by the drop plate method on YPD Agar and were calculated using the following formula:

$$SR\% = \frac{\text{Log CFU/mL (final)}}{\text{Log CFU/mL (initial)}} \times 100$$

2.5. Auto-Aggregation Ability

Auto-aggregation assays were performed according to Binetti et al. [55]. Briefly, the fresh yeast cells were collected by centrifugation (4000× *g*, for 10 min) and washed twice with 0.2 mol·L^{-1} PB solution (pH 7.2). The OD600 was adjusted to 1.0 ± 0.05. The cell pellets were resuspended in 2 mL sterile PB by vortexing for 30 s, then incubated at 37 °C for 24 h. Aliquots of these suspensions (1 mL) were carefully removed from the upper zone to measure OD at 600 nm. Auto-aggregation ability was calculated as:

$$\text{Auto} - \text{aggregation } (\%) = 1 - \frac{DO_0}{DO_t} \times 100$$

where OD_0 and OD_t are the optical densities at 0 h and after 24 h, respectively.

2.6. Co-Aggregation Activity

The co-aggregation assays were assessed using the method described by Kos et al. [56], with some modifications. *E. coli* ATCC 8739 and *S. enterica* serovar Typhimurium ATCC 14028 were used as target bacteria. The cell suspensions were prepared as above. A quantity of 2 mL of bacterial suspension was mixed with an equal volume of yeast suspension by vortexing for 10 s and then incubated at 37 °C for 24 h. Unmixed yeast and bacterial suspensions were used as controls, under the same conditions. After incubation, the absorbances (ODs) of all suspensions were measured at 600 nm. The percentage of co-aggregation was expressed as:

$$\text{Co} - \text{aggregation } (\%) = \frac{(OD_{\text{yeast}} + OD_{\text{pathogen}})/2) - OD(\text{yeast} + \text{pathogen})}{(OD_{\text{yeast}} + OD_{\text{pathogen}})/2} \times 100$$

The percentages of auto-aggregation and co-aggregation were considered low (below 30%), intermediary (between 30% and 60%), or high (greater than 60%) [41].

2.7. Hydrophobicity

Cell surface hydrophobicity was determined according to the method of Alkalbani et al. [42]. Overnight yeast cultures were centrifuged at 4000× *g* for 10 min. Cell pellets were washed twice with 0.1 mol·L^{-1} PB solution (pH 7.0) and adjusted to the OD600 at 1.0 ± 0.05 (ODi). Quantities of 3 mL of cell suspensions were mixed well with 0.6 mL of n-hexane (VWR International, Rosny-sous-Bois, France) by vortexing (10 s). After incubation at 37 °C for 3 h, the aqueous phases were carefully recovered and OD600 nm values were measured (ODf). Cell surface hydrophobicity (H%) was determined using the following formula:

$$H\% = \frac{OD_i - OD_f}{OD_i} \times 100$$

2.8. Hemolytic Activity

Hemolytic activity was conducted by spot-inoculating the yeast strains on Columbia Agar with Sheep Blood Plus plates (Oxoid, UK). After inoculation, the plates were incubated at 37 °C for 48 h according to the methodology used by Menezes et al. [57]. The occurrence of a clear area (denoted as β-hemolysis) or a green-hued zone surrounding the yeast colonies (denoted as α-hemolysis) was considered a positive result, which meant disqualification as a probiotic [9]. Only the yeast strains that showed neither hemolysis halos nor green-hued zones after growth on blood agar plates (denoted as γ-hemolysis) were considered safe and used for the next studies [9].

2.9. Antioxidant Activity

The antioxidant activities of the yeast strains were determined using the methodology described by Chen et al. [58], with slight modifications. Briefly, the yeast cell suspensions were prepared as above. Quantities of 500 µ L of cell suspension were mixed with 1 mL of 1,1 diphenyl-2-picrylhydrazyl (DPPH) solution (0.2 mM in methanol) and vigorously vortexed for 2 min. After incubation at room temperature in darkness for 30 min, the supernatants were recovered by centrifugation (10,000× *g*, 5 min), and their absorbances were measured at 517 nm. Deionized water was used in the control solution. The percentage of DPPH radical scavenging was calculated as:

$$\text{Ac } (\%) = \frac{1 - \text{OD(sample)}}{\text{OD(control)}} \times 100$$

2.10. Antibacterial Activity

The antibacterial activities of the yeast strains were assessed by employing the cross-streaking method described by Diguță et al. [33] against *E. coli* ATCC 8739, *L. monocytogenes* ATCC 7644, *S. enteritidis* ATCC 13076, *S. enterica* serovar, Typhimurium ATCC 14028, and *S. aureus* ATCC 33592. Fresh yeast cultures were first inoculated as 90 mm long streaks in the middle of Petri dishes on YPD agar and grown at 37 °C for 48 h. Subsequently, each indicator bacterium (overnight culture) was deposited by drawing a streak perpendicular to the previously seeded yeast strains, very close to but without touching them, and again incubated at 37 C for 24 h. The antibacterial activities of the yeast strains were recorded by measuring the bacterial growth inhibition sizes in millimeters (mm) using a ruler.

2.11. Statistical Analyses

All assays were repeated in triplicate, and the results were recorded as the means ± standard deviations (SDs). Analysis of variance (ANOVA) was used to compare the means of different variables, with the significance level at $p = 0.05$. The differences between the means of the tests were evaluated by the Fisher HDS test. Additionally, principal component analysis was performed to identify the correlations between the different variables and to select the yeasts with valuable probiotic attributes.

3. Results

3.1. Molecular Identification of Yeast Strains

A total of 17 yeast strains isolated from *Rabilé*, a traditional fermented beer, were investigated in this study. The morphological, cultural, and physiological characteristics of these yeast strains were determined, and the results were published elsewhere [49]. The yeast identifications at the species level were conducted by molecular methods, including PCR-RFLP and 5.8S-ITS region sequencing. According to the sizes of the PCR products, three groups were identified: group I (850 bp), group II (620 bp), and group III (510 bp) (Table 1). ITS-RFLP analysis confirmed the presence of three different groups (species). Fourteen yeast isolates displayed RFLP patterns corresponding to *Saccharomyces cerevisiae*, two isolates belonged to *Pichia kudriavzevii*, and one isolate belonged to *Rhodotorula mucilaginosa* (Table 1).

The dendrogram obtained by the comparison and clustering of RFLP patterns of yeast strains with the restriction enzymes *Hinf*I, *Hha*I, and *Hae*III and the amplicon profiles revealed the existence of the family relationships between the strains examined in this study (Figure 1).

Table 1. Molecular identification of yeast isolates by RFLP analysis of the 5.8S-ITS regions.

Yeast Isolates	PCR ProductSize (bp)	Restriction Fragments (bp)			Identification
		***Hinf*I**	***Hha*I**	***Hae*III**	
BB 1-2; BB 2-3; BB 3-2; BB 3-5; Bf 2-4; Bf 2-6; Bf 2-7; Dr 1-2; Dr 1-5; Dd 3-4; Fd 2-6; Fd 2-8; Ga 2-10; Ga 3-3	850	370 + 110	370 + 320 + 150	320 + 230 + 170 + 130	*Saccharomyces cerevisiae*
BB 3-7; Dr 1-8	510	220 + 150	210 +180 + 70	380 + 90	*Pichia kudriavzevii*
BB 3-3	620	340 + 210 + 70	300 + 220 + 90	410 + 210	*Rhodotorula mucilaginosa*

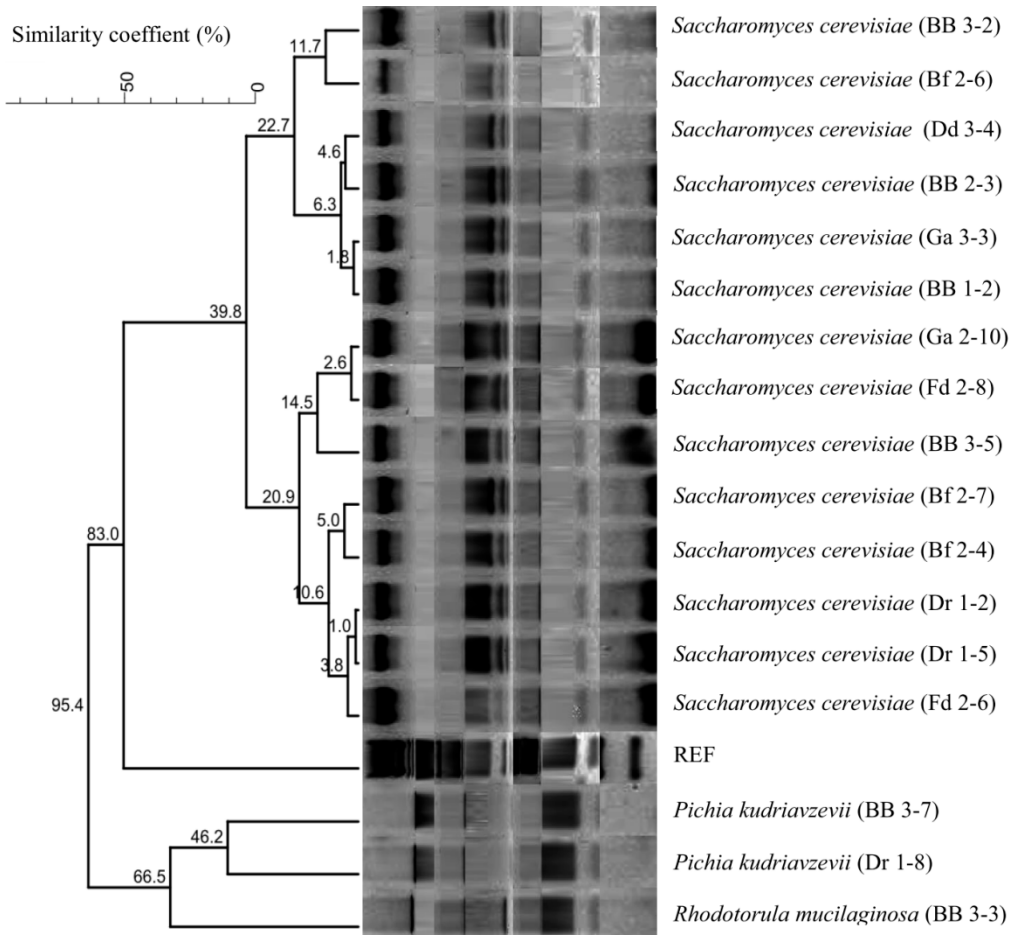


Figure 1. Dendrogram produced by comparing the RFLP-PCR profiles of yeast strains with the restriction enzymes *Hinf*I, *Hha*I, and *Hae*III with the PCR product profiles using Gel Compar Software, Applied Maths, Sint-Martens-Latem, Belgium, and clustering the data using the UPGMA method.

The identifications at the species level obtained by ITS-RFLP analysis were confirmed by sequencing of the 5.8-ITS sequence of one yeast strain from each group and validated by high similarity percentages (from 99.35% to 100%) with respect to different ITS sequences available in the NCBI databases. In addition, the sequences stored in the NCBI database can be downloaded using the accession numbers OQ179952 (*S. cerevisiae* Ga 2-10), OQ179953 (*R. mucilaginosa* BB 3-3), and OQ179954 (*P. kudriavzevii* BB 3-7).

3.2. Tolerance at 37 °C (Body Temperature)

The yeast strains were able to grow at 37 °C (body temperature), with OD600 nm values ranging between 1.68 ± 0.11 (*P. kudriavzevii* Dr 1-8) and 4.27 ± 0.05 (*S. cerevisiae* BB 3-5) (Table 2). However, the *R. mucilaginosa* BB 3-3 strain did not grow very well at 37 °C, which means that this strain may not be suitable for human probiotics (Table 2).

Table 2. Assessment of the capacities of yeast strains to grow at 37 °C and resist the simulated gastrointestinal conditions.

Strains	At 37 °C	Survival Rate (%)	
	DO600 nm	0.3% Pepsin and pH 2.5	0.3% Bile Salts
BB 3-7	2.58 ± 0.25 ^{f}	96.96 ± 1.27 ^{abc}	100.00 ± 0.00 ^{a}
Dr 1-8	1.68 ± 0.11 ^{g}	96.30 ± 1.42 ^{abc}	100.00 ± 00 ^{a}
BB 3-3	0.36 ± 0.01 ^{h}	91.55 ± 4.97 ^{cdef}	97.79 ± 1.07 ^{b}
BB 1-2	2.73 ± 0.17 ^{f}	99.98 ± 0.00 ^{a}	98.65 ± 1.29 ^{ab}
BB 2-3	3.32 ± 0.11 ^{cd}	95.41 ± 6.97 ^{abcde}	95.41 ± 2.74 ^{c}
BB 3-2	3.30 ± 0.05 ^{d}	88.46 ± 3.34 ^{ef}	99.25 ± 0.65 ^{ab}
BB 3-5	4.03 ± 0.10 ^{ab}	91.22 ± 1.92 ^{cdef}	99.36 ± 0.33 ^{ab}
Bf 2-4	3.69 ± 0.16 ^{bc}	86.01 ± 1.98 ^{f}	100.00 ± 0.00 ^{a}
Bf 2-6	2.87 ± 0.05 ^{ef}	93.24 ± 5.41 ^{abcde}	99.55 ± 0.56 ^{ab}
Bf 2-7	2.90 ± 0.04 ^{ef}	95.60 ± 3.41 ^{abcd}	95.56 ± 0.43 ^{c}
Dr 1-2	2.81 ± 0.12 ^{f}	96.77 ± 1.94 ^{abc}	98.73 ± 1.16 ^{ab}
Dr 1-5	2.25 ± 0.00 ^{f}	89.03 ± 9.45 ^{def}	99.53 ± 0.80 ^{ab}
Dd 3-4	3.19 ± 0.17 ^{de}	92.36 ± 7.08 ^{bcdef}	99.80 ± 0.33 ^{ab}
Fd 2-6	2.68 ± 0.03 ^{f}	97.82 ± 1.54 ^{abc}	99.51 ± 0.60 ^{ab}
Fd 2-8	3.36 ± 0.13 ^{cd}	98.69 ± 1.37 ^{ab}	100.00 ± 00 ^{a}
Ga 2-10	4.27 ± 0.05 ^{a}	96.59 ± 1.75 ^{abc}	100.00 ± 00 ^{a}
Ga 3-3	3.46 ± 0.08 ^{cd}	96.95 ± 3.75 ^{abc}	99.12 ± 1.51 ^{ab}

Means and standard deviations (SDs) of three determinations. Values with the same letters are not significantly different at $p = 0.05$.

3.3. Survival of Simulated Gastrointestinal Conditions

3.3.1. Tolerance of Pepsin 0.3% and pH 2.5 at 37 °C

The effects of the simulated gastric conditions (0.3% pepsin and pH 2.5) on cell viability are presented in Table 2. The survival rates of the yeast strains varied from 86.01 ± 1.98% to 99.98 ± 0.00%. The *S. cerevisiae* strain (Bf 2-4) showed the lowest rate, while the *S. cerevisiae* strain (BB 1-2) showed the highest survival rate.

3.3.2. Bile Salt Tolerance

All of the yeast isolates tested were able to tolerate 0.3% bile salts. The *S. cerevisiae* BB 2-3 strain showed the lowest percentage (95.41 ± 2.74%). The *P. kudriavzevii* strains (BB 3-7 and Dr 1-8) and the *S. cerevisiae* strains (Bf 2-4, Fd 2-8, and Ga 2-10) showed the highest survival percentages (100.00 ± 0.00%) (Table 2).

3.4. Auto-Aggregation, Co-Aggregation, and Hydrophobicity of Yeast Isolates

The results obtained for the auto-aggregation, co-aggregation, and hydrophobicity activities of the 17 yeasts strains are displayed in Table 3. The cell surface hydrophobicity and auto-aggregation capacities of probiotic candidates are considered important for the overall ability to adhere to a hydrocarbon solvent and epithelial cells and intestinal mucosa, these factors being relevant to the ability to colonize the GI tract. As shown in Table 3, the auto-aggregation rate varied between 70.20 ± 10.53% and 91.82 ± 1.96% ($p < 0.05$) after 24 h of incubation.

Table 3. Auto-aggregation (%) and co-aggregation with pathogens (%) and hydrophobicity (%) results for the yeast strains.

Strains	Auto-Aggregation (%)	Co-Aggregation (%)		Hydrophobicity (%)
		E. coli	*S. enterica serovar* Typhimurium	
BB 3-7	81.25 ± 1.83 ^{ab}	63.27 ±3.60 ^{abc}	74.06 ± 7.94 ^{a}	60.97 ± 2.20 ^{abc}
Dr 1-8	86.53 ± 4.82 ^{a}	65.04 ± 7.56 ^{abc}	67.44 ± 5.03 ^{ab}	68.84 ± 5.23 ^{a}
BB 3-3	89.40 ± 2.30 ^{a}	80.68 ± 9.53 ^{a}	70.83 ± 5.05 ^{a}	68.99 ± 5.37 ^{a}
BB 3-2	87.17 ± 2.86 ^{a}	65.37 ± 4.35 ^{abc}	69.38 ± 3.05 ^{a}	51.99 ± 4.61 ^{bcde}
BB 1-2	70.20 ± 10.53 ^{b}	50.15 ± 4.16 ^{bcd}	66.38 ± 5.50 ^{abc}	43.17 ± 5.07 ^{e}
BB 2-3	79.89 ± 7.08 ^{ab}	56.16 ± 5.56 ^{bcd}	57.9 ± 6.08 ^{abcd}	63.09 ± 5.18 ^{ab}
BB 3-5	85.43 ± 5.25 ^{a}	36.06 ± 3.45 ^{de}	45.71 ± 4.35 ^{bcd}	59.17 ± 4.88 ^{abcd}
Bf 2-4	84.38 ± 4.96 ^{a}	24.92 ± 3.96 ^{e}	40.89 ± 8.18 ^{d}	60.11 ± 3.06 ^{abc}
Bf 2-6	89.11 ± 2.40 ^{a}	49.91 ± 4.58 ^{bcd}	58.82 ± 6.70 ^{abcd}	67.97 ± 2.12 ^{a}
Bf 2-7	87.91 ± 3.47 ^{a}	54.39 ± 4.90 ^{bcd}	61.97 ± 2.71 ^{abcd}	67.00 ± 2.45 ^{a}
Dr 1-2	89.13 ± 3.55 ^{a}	72.74 ± 8.02 ^{ab}	68.76 ± 5.00 ^{a}	60.06 ± 2.52 ^{abcd}
Dr 1-5	81.48 ± 5.77 ^{ab}	38.82 ± 10.8 ^{de}	44.55 ± 7.09 ^{cd}	64.53 ± 4.90 ^{a}
Dd 3-4	87.55 ± 2.28 ^{a}	66.33 ± 5.56 ^{abc}	61.72 ± 5.19 ^{abcd}	48.23 ± 0.94 ^{de}
Fd 2-6	88.97 ± 1.54 ^{a}	43.83 ± 7.03 ^{cde}	59.74 ± 5.03 ^{abcd}	70.73 ± 2.42 ^{a}
Fd 2-8	88.93 ± 1.74 ^{a}	45.43 ± 13.15 ^{cde}	61.21 ± 9.99 ^{abcd}	65.11 ± 4.25 ^{a}
Ga 2-10	91.82 ± 1.96 ^{a}	66.58 ± 7.02 ^{abc}	72.78 ± 11.53 ^{a}	63.04 ± 3.47 ^{ab}
Ga 3-3	84.34 ± 2.27 ^{a}	65.49 ±4.6 ^{abc}	66.16 ± 5.29 ^{abc}	49.89 ± 3.41 ^{cde}

Means and standard deviations (SDs) of three determinations. Values with the same letters are not significantly different at $p = 0.05$.

Furthermore, co-aggregation activities were evaluated to investigate the abilities of the yeast strains to prevent the colonization of the intestine by pathogens. In our study, the co-aggregation percentages of the yeast strains with *E. coli* ATCC 8739 ranged from 24.92 ± 3.96% to 80.68 ± 9.53% and with *S. enterica serovar* Typhimurium ATCC 14028 from 40.89 ± 8.18% to 74.06 ± 7.94%, with significant differences ($p < 0.05$) among the isolates (Table 3).

The hydrophobicities of the yeast strains toward n-hexane varied considerably (Table 3), with values in the range from 43.17 ± 5.07% to 70.73 ± 2.42% ($p = 0.05$). The highest hydrophobicity values were determined for the *S. cerevisiae* strain Fd 2-6 (70.73 ± 2.42%), *R. mucilaginosa* BB 3-3 (68.99 ± 5.37%), and *P. kudriavzevii* Dr 1-8 (68.84 ± 5.23%), respectively.

3.5. Hemolytic Activity

According to FAO/WHO [9], the safety aspects of any probiotic candidate should be considered, including specifications and lack of harmful activities. Probiotic organisms should be GRAS-compliant. In our study, all yeast strains demonstrated γ-hemolysis activity, which demonstrated their safety for use.

3.6. Antioxidant Activity

DPPH free radical scavenging is one of the methods most often used to evaluate the antioxidant potential of probiotic candidates. The antioxidant activities of intact yeast cells were in the range of 45% to 78% (Figure 2). Among all 17 isolates studied, *P. kudriavzevii* BB 3-7 exhibited the highest percentage of DPPH reduction, and *S. cerevisiae* strains Dr 1-5 and Fd 2-6 exhibited the lowest values.

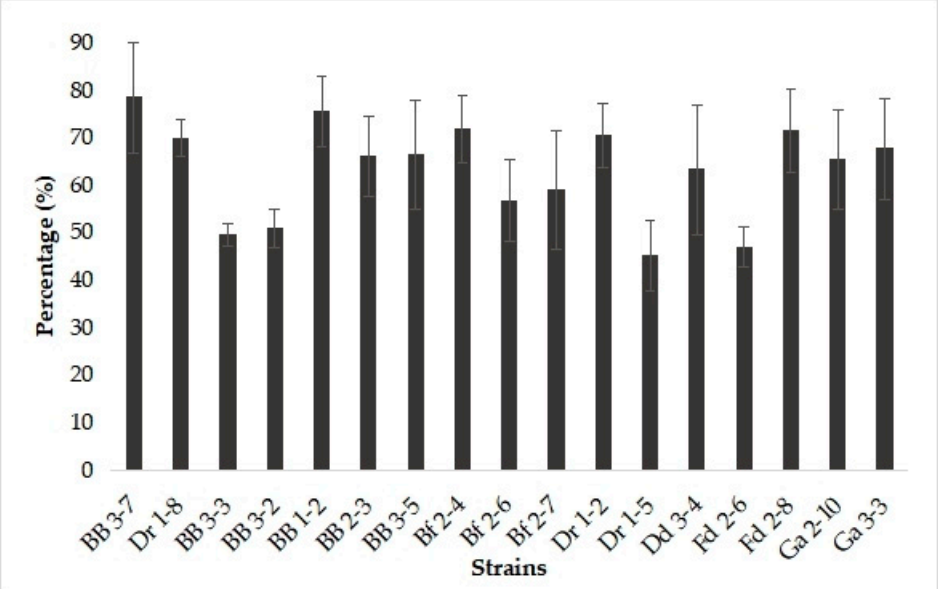


Figure 2. Antioxidant activities (%) of the yeast isolates evaluated by DPPH reduction.

3.7. Antibacterial Activities of the Yeast Strains

The antibacterial activities of the yeast strains were assessed against foodborne bacteria, including Gram-positive bacteria (*L. monocytogenes* and *S. aureus*) and Gram-negative bacteria (*E. coli*, *S. enteritidis*, and *S. typhimurium*). As stated in Table 4, *S. cerevisiae* strains were the most effective in inhibiting the growth of all tested target pathogenic bacteria. Among *Saccharomyces* strains, the Fd 2-6, Dd 3-4, Ga 2-10, BB 3-2, and Bf 2-6 strains showed high antibacterial activity with large clear zones of inhibition ranging between 20.00 ± 0.00 mm and 30.00 ± 0.00 mm in size (Table 4). *P. kudriavzevii* strains (BB 3-7 and Dr 1-8) showed low antibacterial activity, and the *R. muciloginosa* strain (BB 3-3) showed no activity against pathogenic bacteria (Table 4).

Table 4. Antibacterial activities of the yeast strains (inhibitory zones in mm).

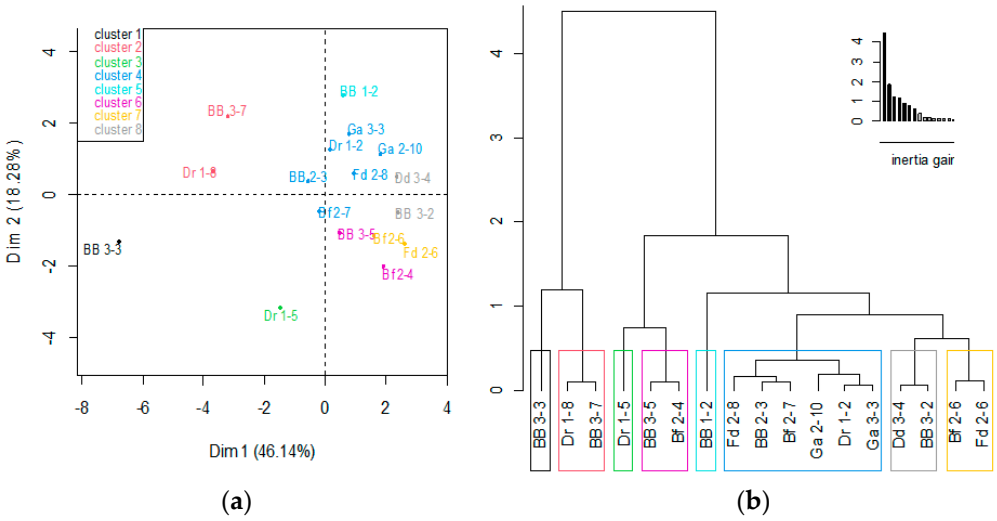
Strains	*E. coli* ATCC 8739	*S. aureus* ATCC 33592	*L. monocytogenes* ATCC 13932	*S. enteritidis* ATCC 13076	*S. typhimurium* ATCC 14028
Fd 2-6	23.66 ± 1.15 ^{a}	24.33 ± 0.57 ^{a}	26.66 ± 2.88 ^{a}	27.00 ± 2.64 ^{a}	24.66 ± 0.57 ^{a}
Dd 3-4	23.33 ± 2.88 ^{a}	21.33 ± 2.30 ^{ab}	20.33 ± 0.57 ^{bc}	26.00 ± 1.00 ^{a}	25.00 ± 1.00 ^{a}
Ga 2-10	21.66 ± 2.88 ^{ab}	21.66 ± 0.57 ^{ab}	20.00 ± 0.00 ^{bc}	24.33 ± 1.15 ^{ab}	20.33 ± 0.57 ^{bc}
BB 3-2	20.00 ± 0.00 ^{ab}	20.00 ± 0.00 ^{abc}	30.00 ± 0.00 ^{a}	25.00 ± 0.00 ^{ab}	25.00 ± 0.00 ^{a}
Bf 2-6	20.00 ± 0.00 ^{ab}	20.00 ± 0.00 ^{abc}	21.33 ± 1.15 ^{b}	24.66 ± 0.57 ^{ab}	21.33 ± 1.15 ^{ab}
Fd 2-8	19.33 ± 1.15 ^{abc}	20.00 ± 0.00 ^{abc}	15.00 ± 0.00 ^{de}	20.00 ± 0.00 ^{cd}	16.33 ± 1.52 ^{def}
Ga 3-3	19.33 ± 1.15 ^{abc}	19.33 ± 0.57 ^{bcd}	15.00 ± 0.00 ^{de}	18.66 ± 1.15 ^{cd}	16.33 ± 0.57 ^{def}
Bf 2-4	17.33 ± 2.30 ^{bcd}	20.00 ± 0.00 ^{abc}	20.00 ± 0.00 ^{bc}	21.33 ± 1.15 ^{bc}	17.66 ± 2.08 ^{bcde}
BB 1-2	15.00 ± 0.00 ^{cd}	16.66 ± 2.88 ^{cde}	17.33 ± 2.51 ^{cd}	20.00 ± 2.00 ^{cd}	17.33 ± 2.51 ^{cde}
Dr 1-2	15.00 ± 0.00 ^{cd}	16.66 ± 2.88 ^{cde}	20.00 ± 0.00 ^{bc}	17.33 ± 0.57 ^{de}	17.66 ± 0.57 ^{bcde}
BB 3-5	13.66 ± 1.52 ^{de}	14.33 ± 0.57 ^{ef}	16.00 ± 1.73 ^{d}	16.33 ± 0.57 ^{de}	14.33 ± 0.57 ^{efg}
BB 2-3	13.33 ± 2.08 ^{de}	14.33 ± 0.57 ^{ef}	12.00 ± 1.73 ^{e}	17.33 ± 1.15 ^{de}	13.33 ± 1.15 ^{fg}
Bf 2-7	13.00 ± 1.00 ^{de}	15.00 ± 0.00 ^{def}	16.66 ± 0.57 ^{cd}	17.66 ± 2.51 ^{cd}	18.33 ± 0.57 ^{bcd}
Dr 1-5	10.00 ± 0.00 ^{e}	11.00 ± 3.60 ^{f}	14.33 ± 1.15 ^{de}	13.66 ± 1.52 ^{e}	10.66 ± 1.15 ^{g}
BB 3-7	5.33 ± 0.57 ^{f}	6.33 ± 0.57 ^{g}	5.33 ± 0.57 ^{f}	5.33 ± 0.57 ^{f}	4.33 ± 1.52 ^{h}
Dr 1-8	4.00 ± 1.73 ^{fg}	5.00 ± 0.00 ^{g}	6.00 ± 1.00 ^{f}	5.00 ± 0.00 ^{f}	4.00 ± 1.73 ^{h}
BB 3-3	0.00 ± 0.00 ^{g}	0.00 ± 0.00 ^{h}	0.00 ± 0.00 ^{g}	0.00 ± 0.00 ^{g}	0.00 ± 0.00 ^{i}

Means and standard deviations (SDs) of three determinations. Values with the same letters are not significantly different at $p = 0.05$.

3.8. Selection of Yeasts with the Highest Probiotic Potential

A PCA was carried out on 17 yeast isolates and the variables of probiotic properties were determined; this allowed the clustering of yeast strains according to their potential probiotic properties.

The classification of strains according to their probiotic properties is presented in Figure 3a. The hierarchical tree presented in Figure 3b shows eight clusters. Cluster 1 (BB 3-3 strain) was characterized by low values for the variable tests, including 0.3% bile salts, tolerance at 37 °C, and no antibacterial activity. Cluster 2 (Dr 1-8 and BB 3-7 strains) and cluster 3 (Dr 1-5) were characterized by low values of antibacterial activity and low tolerance at 37 °C. Next, cluster 4 (Ga 2-10, Dr 1-2, Ga 3-3 Bf 2-7, Bf 2-6, BB 2-3, and Fd 2-8 strains), cluster 5 (BB 1-2 strain), and cluster 6 (BB 3-5 and Bf 2-4 strains) were characterized by variables whose high values did not differ significantly from the means. Cluster 7 (Fd 2-6 strain) and cluster 8 (Dd 3-4 and BB 3-2 strains) were distinguished by high values for the variable tests.



(**a**) (**b**)

Figure 3. Ascending hierarchical classification of the individuals (**a**) and the hierarchical tree (**b**).

4. Discussion

Yeasts have been intensively studied and exploited as starter cultures to produce fermented foods [2–7,18–23]. Recently, *Saccharomyces* and non-*Saccharomyces* strains isolated from African fermented foods have been studied and promoted as promising probiotics with health benefits [4,8,35,36,47–49,54,59]. In our study, the molecular identification of yeast strains isolated from *Rabilé* beer revealed the presence of three species—*S. cerevisiae*, *P. kudriavzevii*, and *R. mucilaginosa*—and the predominance of *S. cerevisiae*. *S. cerevisiae* has been reported as the predominant yeast isolated from fermented foods and beverages, being known to enhance nutritional and organoleptic properties, as well as probiotic properties [8,33–35,60,61]. Recently, *P. kudriavzevii* strains have been proposed as probiotic candidates and have been found to enhance fermented African foods [36,59].

Further, the yeast strains were investigated to see whether they qualified as probiotics based on several criteria mentioned in review papers [40–43]. To exert probiotic effects, yeast strains should tolerate low pH levels and bile salts and also be adaptable to corporeal temperature. All strains were subjected to acidic conditions (pH 2.5) and 0.3% pepsin at a temperature of 37 °C—the relevant conditions for the stomach. Our yeast strains were isolated from low-pH environments (*Rabilé*) [49] where they co-existed with LAB, which could explain their tolerance of acidic conditions. Diguță et al. [33] reported the abilities of *M. pulcherrima* OBT05, *S. cerevisiae* BB06, and *T. delbrueckii* MT07 to grow well in large pH ranges (1.5–7.5). Similar studies confirmed the tolerance of low pH of yeast strains isolated from different indigenous fermented foods [35,36,62]. Most natural or traditional fermented foods, including African traditional alcoholic beverages, such as palm wines,

and partially fermented, grain-based gruels and beverages are produced by yeasts or LAB, as well as multi-strain mixtures [3,7,8,18,36].

In addition to low-pH tolerance, tolerance of bile salts is considered to be an essential criterion for the evaluation of potential probiotics to exert influence in the GI tract beyond the small intestine [42,53]. These bile salts have antimicrobial activities, and to reach the intestinal tract in a viable form any ingested microorganism has to resist them, otherwise it will be unable to withstand the presence of bile in the duodenum [40,42]. Our yeast strains showed high survival rates in artificial gastric juice conditions (between 86.01 ± 1.98% and 99.98 ± 0.00%) and stronger tolerances at the 0.3% bile salt concentration (between 95.41 ± 2.74% and 100.00 ± 0.00%), which are supported by previously reported results. In the study by Ogunremi et al. [36], *Issatchenkia orientalis*, *P. kluyveri*, and *P. kudriavzevii* strains showed tolerance of 2% bile salts. Additionally, Adisa et al. [39] reported that *Kluveromyces lactis* and *S. cerevisiae* tolerated up to 2% bile salt concentrations.

A prerequisite for the persistence of yeast probiotics in the GI tract is their capacity to adhere to the intestinal mucosa. In the current study, our yeast strains revealed high percentages of auto-aggregation (>70%) after 24 h, confirming the results reported in other research. Variable results were found by Fernandez-Pacheco et al. [62] after 30 min of incubation (3.85–64.43%), with the best percentages determined for *Hanseniaspora osmophila* and *Candida pararugosa*. Indeed, auto-aggregation increases the time yeast strains can spend in the intestine and allows them to have a positive impact on health. Menezes et al. [57] found variable results (66.0 ± 2.1% and 99.3 ± 0.6%), with the best auto-aggregation percentages shown by the *S. cerevisiae* strain CCMA 0716. In their study, Fernandez-Pacheco et al. [62] reported that percentages of auto-aggregation varied between 17% and 62% after 30 min of incubation, with *R. mucilaginosa* 32 showing the highest value. Diguță et al. [33] reported the strongest auto-aggregation capacity of *S. cerevisiae* BB06 (92.08 ± 1.49%) after 24 h. According to these results, some researchers have reported that the auto-aggregation of yeasts seems to be a strain-dependent property [36,57]. The high percentages of auto-aggregation observed in yeasts may derive from the fact that yeast cells are relatively large and heavy and precipitate relatively quickly [36,40–42]. Trunk et al. [63] reported that the mechanism of bacterial auto-aggregation can involve simple surface electrostatic effects due to charges on bacterial surfaces. Furthermore, the co-aggregation abilities of yeasts can be considered as strategies to prevent the attachment and subsequent colonization of pathogens. Our yeast strains were able to co-aggregate with the tested bacterial pathogens, with the highest percentage obtained for *R. mucilaginosa* BB 3-3 with *E. coli* and for *P. kudriavzevi*i BB 3-7 with *S. enterica serovar* Typhimurium. Ogunremi et al. [36] reported a high co-aggregation ability of *P. kudriavzevii* OG32 with *E. coli* (71.57%). From the point of view of probiotic activity, the high hydrophobicity of cell surfaces could be the reason why some strains have distinct health benefits and slower elimination kinetics in the GI tract [42]. However, the varying degrees of adherence of our yeast strains to n-hexane were observed in a range between 43% and 71%. Binetti et al. [55] reported hydrophobicity values that ranged from 45.3% to 85.5% for yeasts isolated from autochthone cheese. Ogunremi et al. [36] evaluated the hydrophobicities of *I. orientalis*, *P. kluyveri*, and *P. kudriavzevii* strains isolated from cereal-based, traditional fermented food products of Nigeria with respect to toluene and octane and found good hydrophobic affinity to n-hexadecane (33.61–42.30%) in *P. kudriavzevii* strains. With xylene as a hydrocarbon, Fernandez-Pacheco et al. [62] found hydrophobicity rates that ranged between 2.6% and 34.6%, with the highest value obtained for *Meyerozyma caribbica* 35. In general, the capability of microorganisms to adhere to surfaces is a complex, multistep process that includes hydrophobic forces, electrostatic interactions, and interactions between the physical and chemical properties of the microbial surface and intestinal mucosa [29,42]. Cell surface hydrophobicity and auto-aggregation capability are the main parameters of probiotic candidates relevant to adherence to the intestinal epithelium of the host and the formation of biofilms [40–42].

The natural antioxidant capacities of yeast cells have been reported in several studies [29,33,58]. Chen et al. [58] reported a higher antioxidant capacity of intact yeast cells than cell extracts. One explanation would be the high content of (1/3)-β-D- glucan and other β-glucans found in the yeast cell wall and, additionally, antioxidant enzymes, such as catalase, superoxide dismutase, and glutathione peroxidase. According to their percentages of antioxidant activity, Gil-Rodríguez et al. [29] classified yeasts into five groups: very low (< 20%), low (20–30%), good (30–40%), very good (40–50%), and excellent (> 50%). Based on this grouping, the yeast isolates in the present study were classified into two levels: three isolates (BB 3-3, Fd 2-6, and Dr 1-5) showed very good activity, and fourteen (Ga 2-10, Ga 3-3, Bf 2-6, Dd 3-4, Fd 2-8, Dr 1-2, BB 3-2, BB 3-7, BB 3-5, BB 1-2, Bf 2-7, Bf 2-4, Dr 1-8, and BB 2-3) showed excellent activity.

From a biotechnological point of view, antimicrobial activity is an essential criterion, since yeast strains could be used in biological controls and food preservation and as promising probiotic candidates with health benefits. Our research revealed that only *S.cerevisiae* strains showed high antibacterial effects against bacterial pathogens, such as *E. coli*, *L. monocytogenes*, *S. aureus*, *S. enteritidis*, and *S. typhimurium.* In another work, Diguță et al. [33] demonstrated the high antibacterial activity of *S. cerevisiae* BB06 against nine foodborne pathogenic bacteria, namely, *Bacillus cereus*, *Enterococcus faecalis*, *E. coli*, *L.monocytogenes*, *L. ivanovii*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, and *S. aureus*. The antimicrobial activities of yeast against pathogens could be due to the competition for nutrients and, simultaneously, the production of organic acids, hydrogen peroxide, and diacetyl. In another study, Adisa et al. [39] reported the antimicrobial activities of *S. cerevisiae* against *Klebsiella* spp. and of *K. lactis* against *Pseudomonas* spp. and *Staphylococcus* spp. Fernandez-Pacheco et al. [62] also reported, among 20 yeast isolates, 1 yeast (*Diutina rugosa* 12) that presented antimicrobial activity against *Dekkera bruxellensis* and *Zygosaccharomyces* spp.

According to the PCA analysis, *S. cerevisiae* strains (especially Fd 2-6, Dd 3-4, and BB 3-2 strains) were selected as having numerous valuable probiotic properties and could be potential candidates for establishing the high nutraceutical value of *Rabilé* beer.

5. Conclusions

In this study, 17 yeast strains isolated from traditional *Rabilé* beer produced in Burkina Faso were identified by molecular methods as *S. cerevisiae* (14 strains), *P. kudriavzevii* (2 strains), and *R. mucilaginosa* (1 strain). Selecting a yeast strain with all the desired probiotic attributes tested proved to be difficult. However, the strains evaluated in this study have many of the essential and critical probiotic characteristics that recommend them for probiotic use. These strains were able to grow at human body temperature (except *R. mucilaginosa*) and survive in the gastrointestinal tract. Meanwhile, *S. cerevisiae* strains showed strong antibacterial activities against the pathogens that were used. Additionally, these strains exhibited high antioxidant properties. Furthermore, they showed high percentages of hydrophobicity and strong auto-aggregation abilities, as well as various degrees of co-aggregation with *E. coli* and with *S. enterica serovar* Typhimurium. The tested yeast strains revealed no hemolytic activities and can therefore be considered safe. However, the probiotic potentials of *R. mucilaginosa* and *P. kudriavzevii* were lower than that of *S. cerevisiae*. So, taking into account these in vitro probiotic qualities, yeast strains isolated from *Rabilé* are promising strains and have the potential to be used as probiotic supplements. Further investigation will be performed on molecular aspects, functional attributes (sensibility of antibiotics, enzymatic profiling, and valuable metabolite profiling), and technological properties (preservation for extending the shelf life of final products). Additionally, our probiotic yeast strains with proven multifunctional properties could be useful in the development of functional foods which exhibit various health benefits.

Author Contributions: Conceptualization, F.M., C.F.D., A.S.O. and J.O.U.; methodology, I.M., M.K.S., C.F.D. and R.C.T.; software, I.M., C.F.D. and R.C.T.; validation, F.M., C.F.D., A.S.O. and C.A.T.O.; formal analysis, I.M., I.K., J.O.U., C.F.D. and R.C.T.; investigation, I.M., M.K.S., Y.D. and L.I.E.; resources, M.K.S., I.K, Y.D. and L.I.E.; data curation, M.K.S., Y.D. and L.I.E.; writing—original draft

preparation, I.M., C.F.D. and R.C.T.; writing—review and editing, F.M., C.F.D., A.S.O., J.O.U. and C.A.T.O.; visualization, I.K., J.O.U., A.S.O. and C.A.T.O.; supervision, F.M., C.F.D., A.S.O., J.O.U. and C.A.T.O.; project administration, F.M. and C.F.D.; funding acquisition, F.M. and C.F.D. All authors have read and agreed to the published version of the manuscript.

Funding: The authors are grateful to AUF (Agence Universitaire de la Francophonie) for supporting Iliassou MOGMENGA through the programme de bourses « Eugen Ionescu » 2020–2021 at the University of Agronomic Sciences and Veterinary Medicine of Bucharest, Romania. They also acknowledge the German DAAD service for their financial support through its In-Country/In-Region Scholarship Program 2017 (91682081), the University of Nigeria Nsukka (UNN), Enugu State, Nigeria.

Data Availability Statement: All data are available in this manuscript.

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

References

1. FAO. *The Future of Food and Agriculture—Alternative Pathways to 2050*; FAO: Rome, Italy, 2018.
2. Choi, K.R.; Yu, H.E.; Lee, S.Y. Microbial food: Microorganisms repurposed for our food. *Microb. Biotechnol.* **2022**, *15*, 18–25. [CrossRef]
3. Setta, M.C.; Matemu, A.; Mbega, E.R. Potential of probiotics from fermented cereal-based beverages in improving health of poor people in Africa. *J. Food Sci. Technol.* **2020**, *57*, 3935–3946. [CrossRef] [PubMed]
4. Franz, C.M.; Huch, M.; Mathara, J.M.; Abriouel, H.; Benomar, N.; Reid, G.; Galvez, A.; Holzapfel, W.H. African fermented foods and probiotics. *Int. J. Food Microbiol.* **2014**, *190*, 84–96. [CrossRef] [PubMed]
5. Diaz, M.; Kellingray, L.; Akinyemi, N.; Adefiranye, O.O.; Olaonipekun, A.B.; Bayili, G.R.; Ibezim, J.; Du Plessis, A.S.; Houngbédji, M.; Kamya, D.; et al. Comparison of the microbial composition of African fermented foods using amplicon sequencing. *Sci. Rep.* **2019**, *9*, 13863. [CrossRef]
6. Obafemi, Y.D.; Oranusi, S.U.; Ajanaku, K.O.; Akinduti, P.A.; Leech, J.; Cotter, P.D. African fermented foods: Overview, emerging benefits, and novel approaches to microbiome profiling. *Npj Sci. Food* **2022**, *6*, 15. [CrossRef]
7. Kayitesi, E.; Onojakpor, O.; Moyo, S.M. Highlighting the impact of lactic-acid-bacteria-derived flavours or aromas on sensory perception of African fermented cereals. *Fermentation* **2023**, *9*, 111. [CrossRef]
8. Mehlomakulu, N.N.; Moyo, S.M. Yeast derived metabolites and their impact on nutritional and bioactive properties of African fermented maize products. *Food Biosci.* **2023**, *51*, 102300. [CrossRef]
9. FAO; WHO. *Guidelines for the Evaluation of Probiotics in Food*; Food and Agriculture Organization of the United Nations and World Health Organization Working Group Report; FAO: London, ON, Canada, 2002.
10. Hill, C.; Guarner, F.; Reid, G.; Gibson, G.R.; Merenstein, D.J.; Pot, B.; Morelli, L.; Canani, R.B.; Flint, H.J.; Salminen, S.; et al. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* **2014**, *11*, 506–514. [CrossRef]
11. Picard, C.; Fioramonti, J.; Francois, A.; Robinson, T.; Neant, F.; Matuchansky, C. Review article: Bifidobacteria as probiotic agents–physiological effects and clinical benefits. *Aliment. Pharmacol. Ther.* **2005**, *22*, 495–512. [CrossRef]
12. Kumar, H.; Collado, M.C.; Wopereis, H.; Salminen, S.; Knol, J.; Roeselers, G. The Bifidogenic Effect Revisited—Ecology and Health Perspectives of Bifidobacterial Colonization in Early Life. *Microorganisms* **2020**, *8*, 1855. [CrossRef]
13. Chen, J.; Chen, X.; Ho, C.L. Recent development of probiotic bifidobacteria for treating human diseases. *Front. Bioeng. Biotechnol.* **2021**, *9*, 770248. [CrossRef] [PubMed]
14. Jeong, J.-J.; Park, H.J.; Cha, M.G.; Park, E.; Won, S.-M.; Ganesan, R.; Gupta, H.; Gebru, Y.A.; Sharma, S.P.; Lee, S.B.; et al. The Lactobacillus as a probiotic: Focusing on liver diseases. *Microorganisms* **2022**, *10*, 288. [CrossRef] [PubMed]
15. Torres-Maravilla, E.; Reyes-Pavón, D.; Benítez-Cabello, A.; González-Vázquez, R.; Ramírez-Chamorro, L.M.; Langella, P.; Bermúdez-Humarán, L.G. Strategies for the identification and assessment of bacterial strains with specific probiotic traits. *Microorganisms* **2022**, *10*, 1389. [CrossRef]
16. Chou, L.-S.; Weimer, B. Isolation and characterization of acid- and bile-tolerant isolates from strains of *Lactobacillus acidophilus*. *J. Dairy Sci.* **1999**, *82*, 23–31. [CrossRef]
17. Csutak, O.; Sabău, E.; Pelinescu, D.; Corbu, V.; Cîrpici, I.; Vassu, T. Molecular identification and metabolic screening of some yeast strains from foods. *AgroLife Sci. J.* **2016**, *5*, 51–58.
18. Johansen, P.G.; Owusu-Kwarteng, J.; Parkouda, C.; Padonou, S.W.; Jespersen, L. Occurrence and importance of yeasts in indigenous fermented food and beverages produced in Sub-Saharan Africa. *Front. Microbiol.* **2019**, *10*, 1789. [CrossRef] [PubMed]
19. Rai, A.K.; Pandey, A.; Sahoo, D. Biotechnological potential of yeasts in functional food industry. *Trends Food Sci. Technol.* **2019**, *83*, 129–137. [CrossRef]
20. Tullio, V. Yeast genomics and its applications in biotechnological processes: What is our present and near future? *J. Fungi* **2022**, *8*, 752. [CrossRef]

21. Zhimo, V.Y.; Biasi, A.; Kumar, A.; Feygenberg, O.; Salim, S.; Vero, S.; Wisniewski, M.; Droby, S. Yeasts and bacterial consortia from kefir grains are effective biocontrol agents of postharvest diseases of Fruits. *Microorganisms* **2020**, *8*, 428. [CrossRef]
22. Barbulescu, I.D.; Dumitrache, C.; Diguta, C.F.; Matei, P.M.; Frincu, M.; Marculescu, S.I.; Ciric, A.I.; Tudor, V.; Boroiu, E.M.; Teodorescu, R. Evolution at the microfermenter level of the growth dynamics of *Saccharomyces cerevisiae* and *Starmella bacillaris* yeasts with potential for use in winemaking at the pietroasa winery. *AgroLife Sci. J.* **2022**, *11*, 9–16. [CrossRef]
23. Chen, P.; Zhang, Q.; Dang, H.; Liu, X.; Tian, F.; Zhao, J.; Zhang, H.; Chen, W. Screening for potential new probiotic based on probiotic properties and α-glucosidase inhibitory activity. *Food Control.* **2014**, *35*, 65–72. [CrossRef]
24. Abid, R.; Waseem, H.; Ali, J.; Ghazanfar, S.; Muhammad Ali, G.; Elasbali, A.M.; Alharethi, S.H. Probiotic yeast *Saccharomyces*: Back to nature to improve human health. *J. Fungi* **2022**, *8*, 444. [CrossRef] [PubMed]
25. Vysoka, M.; Szotkowski, M.; Slaninova, E.; Dzuricka, L.; Strecanska, P.; Blazkova, J.; Marova, I. Oleaginous Yeast Extracts and Their Possible Effects on Human Health. *Microorganisms* **2023**, *11*, 492. [CrossRef] [PubMed]
26. Kunyeit, L.; Rao, R.P.; Anu-Appaiah, K.A. Yeasts originating from fermented foods, their potential as probiotics and therapeutic implication for human health and disease. *Crit. Rev. Food Sci. Nutr.* **2023**. [CrossRef]
27. Staniszewski, A.; Kordowska-Wiater, M. Probiotic and potentially probiotic yeasts—Characteristics and food application. *Foods* **2021**, *10*, 1306. [CrossRef]
28. Tamang, J.P.; Lama, S. Probiotic properties of yeasts in traditional fermented foods and beverages. *J. Appl. Microbiol.* **2022**, *132*, 3533–3542. [CrossRef]
29. Gil-Rodríguez, A.M.; Carrascosa, A.V.; Requena, T. Yeasts in foods and beverages: In vitro characterisation of probiotic traits. *LWT-Food Sci. Technol.* **2015**, *64*, 1156–1162. [CrossRef]
30. Pihurov, M.; Păcularu-Burada, B.; Cotârleț, M.; Vasile, M.A.; Bahrim, G.E. Novel insights for metabiotics production by using artisanal probiotic cultures. *Microorganisms* **2021**, *9*, 2184. [CrossRef]
31. Pais, P.; Almeida, V.; Yılmaz, M.; Teixeira, M.C. *Saccharomyces boulardii*: What makes it tick as successful probiotic? *J. Fungi* **2020**, *6*, 78. [CrossRef]
32. Fu, J.; Liu, J.; Wen, X.; Zhang, G.; Cai, J.; Qiao, Z.; An, Z.; Zheng, J.; Li, L. Unique probiotic properties and bioactive metabolites of *Saccharomyces boulardii*. *Probiotics Antimicrob. Proteins* **2022**, 1–16. [CrossRef]
33. Diguță, C.F.; Mihai, C.; Toma, R.C.; Cîmpeanu, C.; Matei, F. In vitro assessment of yeasts strains with probiotic attributes for aquaculture use. *Foods* **2023**, *12*, 124. [CrossRef]
34. Palma, M.L.; Zamith-Miranda, D.; Martins, F.S.; Bozza, F.A.; Nimrichter, L.; Montero-Lomeli, M.; Marques, E.T.A.; Douradinha, B. Probiotic *Saccharomyces cerevisiae* strains as biotherapeutic tools: Is there room for improvement? *Appl. Microbiol. Biotechnol.* **2015**, *99*, 6563–6570. [CrossRef] [PubMed]
35. Motey, G.A.; Johansen, P.G.; Owusu-Kwarteng, J.; Ofori, L.A.; Obiri-Danso, K.; Siegumfeldt, H.; Larsen, N.; Jespersen, L. Probiotic potential of *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* isolated from West African spontaneously fermented cereal and milk products. *Yeast* **2020**, *37*, 403–412. [CrossRef] [PubMed]
36. Ogunremi, O.R.; Sanni, A.I.; Agrawal, R. Probiotic potentials of yeasts isolated from some cereal-based Nigerian traditional fermented food products. *J. Appl. Microbiol.* **2015**, *119*, 797–808. [CrossRef] [PubMed]
37. Corbu, V.; Csutak, O. Biodiversity studies on *Pichia kudriavzevii* from Romanian spontaneous fermented products. *AgroLife Sci. J.* **2020**, *9*, 104–114.
38. Corbu, V.M.; Csutak, O. Molecular and physiological diversity of indigenous yeasts isolated from spontaneously fermented wine wort from Ilfov county, Romania. *Microorganisms* **2023**, *11*, 37. [CrossRef]
39. Adisa, A.M.; Ifesan, O.B.T.; Enujiugha, V.N.; Adepeju, A.B. Microbiological and probiotic assessment of yeast isolated from wholegrain millet sourdoughs. *J. Adv. Microbiol.* **2020**, *20*, 1–10. [CrossRef]
40. Shruthi, B.; Deepa, N.; Somashekaraiah, R.; Adithi, G.; Divyashree, S.; Sreenivasa, M.Y. Exploring biotechnological and functional characteristics of probiotic yeasts: A review. *Biotechnol. Rep.* **2022**, *34*, e00716. [CrossRef]
41. De Melo Pereira, G.V.; de Oliveira Coelho, B.; Magalhães Júnior, A.I.; Thomaz-Soccol, V.; Soccol, C.R. How to select a probiotic? A review and update of methods and criteria. *Biotechnol. Adv.* **2018**, *36*, 2060–2076. [CrossRef]
42. Alkalbani, N.S.; Osaili, T.M.; Al-Nabulsi, A.A.; Olaimat, A.N.; Liu, S.-Q.; Shah, N.P.; Apostolopoulos, V.; Ayyash, M.M. Assessment of yeasts as potential probiotics: A review of gastrointestinal tract conditions and investigation methods. *J. Fungi* **2022**, *8*, 365. [CrossRef]
43. Fernández-Pacheco, P.; Pintado, C.; Briones Pérez, A.; Arévalo-Villena, M. Potential probiotic strains of *Saccharomyces* and non-*Saccharomyces*: Functional and Biotechnological Characteristics. *J. Fungi* **2021**, *7*, 177. [CrossRef]
44. Elghandour, M.M.Y.; Tan, Z.L.; Abu Hafsa, S.H.; Adegbeye, M.J.; Greiner, R.; Ugbogu, E.A.; Cedillo Monroy, J.; Salem, A.Z.M. *Saccharomyces cerevisiae* as a probiotic feed additive to non and pseudo-ruminant feeding: A review. *J. Appl. Microbiol.* **2019**, *28*, 658–674.
45. Pang, Y.; Zhang, H.; Wen, H.; Wan, H.; Wu, H.; Chen, Y.; Li, S.; Zhang, L.; Sun, X.; Li, B.; et al. Yeast probiotic and yeast products in enhancing livestock feeds utilization and performance: An overview. *J. Fungi* **2022**, *8*, 1191. [CrossRef] [PubMed]
46. Burdick Sanchez, N.C.; Broadway, P.R.; Carroll, J.A. Influence of yeast products on modulating metabolism and immunity in cattle and Swine. *Animals* **2021**, *11*, 371. [CrossRef] [PubMed]
47. Keita, I.; Somda, M.K.; Savadogo, A.; Mogmenga, I.; Koita, O.; Traore, A.S. Isolation and molecular identification of yeast strains from "*Rabilé*" a starter of local fermented drink. *Afr. J. Biotechnol.* **2016**, *15*, 823–829.

48. Keita, I.; Mogmenga, I.; Somda, M.K.; Doumbia, L.; Ouattara, A.S.; Koita, O.A.; Traore, A.S. Yeasts from a traditional ferment *Rabile*: Molecular and physiological characterization. *Int. J. Biotechnol. Mol. Biol. Res.* **2022**, *12*, 1–8.
49. Mogmenga, I.; Dadiré, Y.; Somda, M.K.; Keita, I.; Ezeogu, L.I.; Ugwuanyi, J.; Traoré, A.S. Isolation and identification of indigenous yeasts from "*Rabilé*", a starter culture used for production of traditional beer "*dolo*", a condiment in Burkina Faso. *Adv. Microbiol.* **2019**, *9*, 646. [CrossRef]
50. White, T.J.; Bruns, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*; Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., Eds.; Academic Press: San Diego, CA, USA, 1990; pp. 315–322.
51. Esteve-Zarzoso, B.; Belloch, C.; Uruburu, F.; Querol, A. Identification of yeasts by RFLP analysis of the 5.85 rRNA gene and the two ribosomal internal transcribed spacers. *Int. J. Syst. Bacteriol.* **1999**, *49*, 329–337. [CrossRef] [PubMed]
52. Vauterin, L.; Vauterin, P. Computer-aided objective comparison of electrophoresis patterns for grouping and identification of microorganisms. *Eur. Microbiol.* **1992**, *1*, 37–41.
53. Burns, P.; Vinderola, G.; Binetti, A.; Quiberoni, A.; de los Gavilán-Reyes, C.G.; Reinheimer, J. Bile-resistant derivatives obtained from non-intestinal dairy lactobacilli. *Int. Dairy J.* **2008**, *18*, 377–385. [CrossRef]
54. Pedersen, L.L.; Owusu-Kwarteng, J.; Thorsen, L.; Jespersen, L. Biodiversity and probiotic potential of yeasts isolated from fura, a West African spontaneously fermented cereal. *Int. J. Food Microbiol.* **2012**, *159*, 144–151. [CrossRef] [PubMed]
55. Binetti, A.; Carrasco, M.; Reinheimer, J.; Suárez, V. Yeasts from autochthonous cheese starters: Technological and functional properties. *J. Appl. Microbiol.* **2013**, *115*, 434–444. [CrossRef]
56. Kos, B.; Šušković, J.; Vuković, S.; Šimpraga, M.; Frece, J.; Matošić, S. Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophillus* M92. *J. Appl. Microbiol.* **2003**, *94*, 981–987. [CrossRef] [PubMed]
57. Menezes, A.G.T.; Ramos, C.L.; Cenzi, G.; Melo, D.S.; Dias, D.R.; Schwan, R.F. Probiotic potential, antioxidant activity, and phytase production of indigenous yeasts isolated from indigenous fermented foods. *Probiotics Antimicrob. Proteins* **2020**, *12*, 280–288. [CrossRef] [PubMed]
58. Chen, L.-S.; Ma, Y.; Maubois, J.-L.; Chen, L.-J.; Liu, Q.-H.; Guo, J.-P. Identifcation of yeasts from raw milk and selection for some specific antioxidant properties. *Int. J. Dairy Technol.* **2010**, *63*, 47–54. [CrossRef]
59. Greppi, A.; Saubade, F.; Botta, C.; Humblot, C.; Guyot, J.-P.; Cocolin, L. Potential probiotic *Pichia kudriavzevii* strains and their ability to enhance folate content of traditional cereal-based African fermented food. *Food Microbiol.* **2017**, *62*, 169–177. [CrossRef]
60. Heitmann, M.; Zannini, E.; Arendt, E. Impact of *Saccharomyces cerevisiae* metabolites produced during fermentation on bread quality parameters: A review. *Crit. Rev. Food Sci. Nutr.* **2018**, *58*, 1152–1164. [CrossRef]
61. Fernandez-Pacheco, P.; Arévalo-Villena, M.; Bevilacqua, A.; Corbo, M.R.; Briones Pérez, A. Probiotic characteristics in *Saccharomyces cerevisiae* strains: Properties for application in food industries. *LWT* **2018**, *97*, 332–340. [CrossRef]
62. Fernández-Pacheco, P.; Rosa, I.Z.; Arévalo-Villena, M.; Gomes, E.; Pérez, A.B. Study of potential probiotic and biotechnological properties of non-*Saccharomyces* yeasts from fruit Brazilian ecosystems. *Braz. J. Microbiol.* **2021**, *52*, 2129–2144. [CrossRef]
63. Trunk, T.; Khalil, H.S.; Leo, J.C. Bacterial autoaggregation. *AIMS Microbiol.* **2018**, *4*, 140–164. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

foods

MDPI

Article

Chitosan-Based Edible Coatings Containing Essential Oils to Preserve the Shelf Life and Postharvest Quality Parameters of Organic Strawberries and Apples during Cold Storage

Paul-Alexandru Popescu [1], Laurentiu Mihai Palade [1,2,*], Ioana-Cătălina Nicolae [3], Elisabeta Elena Popa [1], Amalia Carmen Miteluț [1], Mihaela Cristina Drăghici [1], Florentina Matei [1] and Mona Elena Popa [1]

[1] Faculty of Biotechnology, University of Agronomic Sciences and Veterinary Medicine of Bucharest, 011464 Bucharest, Romania
[2] National Research & Development Institute for Food Bioresources-IBA Bucharest, 6 Dinu Vintilă Street, District 2, 021102 Bucharest, Romania
[3] Research Center for Studies of Food Quality and Agricultural Products, University of Agronomic Sciences and Veterinary Medicine of Bucharest, 011464 Bucharest, Romania
* Correspondence: palade_laurentiu_mihai@yahoo.com

Abstract: Edible coatings and films have been researched for more than three decades due to their ability to be incorporated with different functional ingredients or compounds as an option to maintain the postharvest quality of fruits and vegetables. The aim of this study was to evaluate the effect of three types of chitosan-based (CH) edible coatings obtained from medium and high molecular weight chitosan, containing ascorbic or acetic acid and sea buckthorn or grape seed essential oils on the physical–chemical and microbiological properties of organic strawberries and apple slices during cold storage at 4 °C and 8 °C. Scanning electron microscope images showed both a smooth structure and a fracture and pore structure on strawberry coatings and a dense and smooth structure on the apple slices coatings. Further, the edible coatings managed to reduce the microbial load of yeasts and molds of the coated strawberries during the storage period. Overall, the treatments preserved the ascorbic acid, total polyphenol content, and antioxidant activity for all the tested samples compared to the control sample, throughout the storage period. In addition, the water activity (a_w) of the coated samples presented lower values (0.96–0.98) than the control samples. The obtained results indicate that the developed chitosan-based edible coatings could maintain the postharvest parameters of the tested samples, also leading to their shelf-life prolongation.

Keywords: edible coatings; chitosan; shelf life; strawberries; apples

check for updates

Citation: Popescu, P.-A.; Palade, L.M.; Nicolae, I.-C.; Popa, E.E.; Miteluț, A.C.; Drăghici, M.C.; Matei, F.; Popa, M.E. Chitosan-Based Edible Coatings Containing Essential Oils to Preserve the Shelf Life and Postharvest Quality Parameters of Organic Strawberries and Apples during Cold Storage. *Foods* **2022**, *11*, 3317. https://doi.org/10.3390/foods11213317

Academic Editor: Hiléia Karla Silva Souza

Received: 27 September 2022
Accepted: 21 October 2022
Published: 23 October 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Food products are vital for the survival of human beings and, over time, consumers have become more demanding in terms of their quality. However, food products are naturally perishable and can be degraded by environmental factors, such as fungal or bacterial contamination [1,2]. Therefore, it is essential to protect them from spoilage microorganisms in order to increase their shelf life and satisfy consumer demands [3].

Conventional food packaging materials are made from petroleum-based polymers and have been used to protect food products and maintain their quality and sensorial attributes [4,5]. Due to their flexibility, lightness, and transparency, these films have been massively used, rendering the achievement of modified atmosphere packaging (MAP) for commodities that are highly perishable [6,7]. Innovative packaging methods, such as edible coatings, polymeric films, or modified atmosphere packaging, are used more and more in order to maintain the quality of fresh-cut produce during the transport and distribution phases [8]. When gas is flushed into the pack or the produce receives no alteration of the atmosphere, along with the permeability properties of the employed

packaging, this allows equilibrium MAP (EMAP) to be reached [6,9]. However, this has led to a serious ecological problem because of their nonbiodegradability [10,11]. Their complete replacement by biodegradable and sustainable packaging materials is currently being studied and is desired by both the industry as well as the final consumers [12]. Edible coatings and films are among the newly developed biodegradable packaging materials. EU regulations EC 1935/2004 and EC 10/2011 comprise specific recommendations with regard to the declaration of conformity that must accompany food commodities; these provide guidelines on the use of packaging materials (active and intelligent), release of substances considered food additives thereof (must be accredited), as well as materials used during processing (i.e., direct or indirect contact with food along the production chain) [13,14]. The main advantage of edible films and coatings compared to traditional synthetic packaging is that they are safe for the environment and the consumer [15–17]. They consist of a thin layer of a nontoxic, edible surface applied to the food product, with or without subsequent removal, with the objective of extending the shelf life and quality of the product [7,18].

Edible coatings and films are produced from polymers derived exclusively from renewable resources and applied as a liquid form directly on the surface of food [19,20]. At the compositional level, a material is used as the structural matrix of the edible coating and bioactive compounds, nano-composites or natural extracts are added to improve the functional properties of the coating [21]. Arguably, edible coatings pertaining a certain thickness along with selective permeability may alter the inner atmosphere composition of the package without gas filling/flushing, rendering them EMAP coatings and may avoid further packaging [6,22]. Most recently, Jalali et al., 2020 developed an integrated mathematical modelling as a guide for strawberry shelf-life prediction under realistic conditions by taking into consideration the transpiration and respiration rate. The model simulated the strawberries shelf life under open tray conditions and MAP conditions, taking into consideration the temperature and humidity as test parameters and allowing the optimization of the supply chain [23]. Each material is chosen according to the characteristics of the food to be coated [24,25]. For example, studies show that fried food products can be coated with cellulose-based edible films because they prevent oil absorption [26], meat products can be treated with alginate edible films because of the delays of the lipid oxidation process [27] or chitosan base edible films in order to preserve the color and prevent lipid oxidation [28]. Pectin-based edible films can be used on fruits and vegetables in order to maintain the color, prevent weight loss, and reduce microbial growth [29]. Edible films and coatings are defined as thin layers applied to foods to protect them, to extend their shelf life and to improve certain qualitative properties such as the appearance. They are created from edible and natural renewable sources (proteins, polysaccharides, lipids) and contain antioxidants, anti-browning agents and colorants. Sometimes additives are added to improve the efficiency of the coating; by themselves or in combination with additives, they can help prevent drying out, immobilize microbes, or reduce access to oxygen [30–32]. Different methods of application of coatings are used: spraying, brushing or electro-spraying [4]. Several polysaccharides, such as starch, alginate, chitosan, pullulan, pectin and carrageenan are currently being used by the researchers to produce edible coatings [33].

Chitosan is a well-known biopolymer used for several years in different sectors of the industry and is known for its antibacterial [34,35] and antifungal properties [36–38]. The main advantages when using chitosan as matrix for a packaging material, beside its antimicrobial capabilities, is that it is biodegradable, thus being a sustainable choice for the food industry [39,40]. Chitosan, a deacetylated derivative of chitin, is nowadays used as a biodegradable food packaging material because of its great film-forming and good antimicrobial properties [41]. Other properties that make chitosan a good packaging material are its ability to be cross-linked in order to avoid dissolution in acidic solutions, high porosity, good hydrophilicity and big adhesion area [24]. In addition to these properties, chitosan is mainly produced from see-food industrial waste, thus being a sustainable material and that has excellent functional properties when combined with other materials. Chitosan can be used as packaging film or as coating applied directly on the food product [41].

For several years now, there has been a growing demand for fresh fruits and vegetables, especially for minimally processed ones due to consumer needs for fresh, healthy snacks for their busy lives. However, these foods are more vulnerable to damage and loss of market quality [31,32]. For this reason, many methods are used to address these advantages and allow freshly-cut products to have a longer shelf life than the 2–9 days assuming proper temperatures are assured (cold storage) [42]. The use of edible coatings is one of them. These consist of using a material for coating and adding natural antimicrobials to preserve the quality and improve the shelf life of the food product. This method has already been studied on different fruits and vegetables [3].

With more than thirty years of scientific insight gathered, currently, various studies are conducted worldwide to address the need to improve the properties of edible coatings and films to develop an alternative to synthetic polymers and hazardous chemicals [19,24,25]. Moreover, by knowing the composition of the food coating, the organoleptic properties and the shelf life of the food can be improved. Edible coatings/films have become the subject of many research topics in the food industry, especially on fruits, such as strawberries [43–45], apples [46,47], cherries [48,49], kiwis [50], melons [51], and pears [52].

Strawberries are fruits that possess a very short shelf life because of their high metabolic activity and water content and the susceptibility of being contaminated with fungal strains, especially grey mold (*Botrytis cinerea*). Physical injuries can also occur in different stages of the traceability chain because of their soft texture and lack of protective rind [53]. The main postharvest technique used in the industry to extend the shelf life of strawberries is refrigeration at temperatures of 0 °C [54]. With superior barrier attributes, edible coatings are intended to prolong the shelf life and exhibit a significant effect as postharvest treatment on fresh-cut fruit; by maintaining the physiological and physicochemical properties of the produce, they are considered to attain efficiency and functionality towards the retention of postharvest quality [7]. In recent years, edible coatings have been used to extend the shelf life of strawberries by delaying the textural changes, reducing the respiration rate, and providing a gas permeability barrier, thus maintaining the sensorial and nutritional values at higher levels [55]. Edible coatings made from soy or wheat gluten with incorporated thymol and calcium chloride were applied on fresh strawberries in order to analyze the improvements of physical–chemical and microbiological properties of the coated samples. The tests were carried out over a 9-day period, and the appearance of the samples remained unchanged. The firmness, ascorbic acid content, total soluble solids, and total sugars were maintained over the storage period in comparison to the control sample. The coated samples with thymol and soy protein or white gluten recorded higher chroma hue angle values and the lowest values of anthocyanin content [53].

Four types of edible coatings made from low methoxyl pectin (LMP), carboxymethyl cellulose (CMC), Persian gum (PG), and tragacanth gum (TG) were developed and applied on fresh strawberries in order to test their shelf life improvement capacity. A physical–chemical analysis, such as weight loss, ascorbic acid, total phenolics, and anthocyanins content were evaluated over a period of 16 days of storage at 4 °C. The results show that the coated samples presented better sensory attributes compared to the control samples. The strawberry samples coated with the CMC solution had reduced decay and weight loss, as well as better values of the physicochemical analysis [56].

The antifungal properties of chitosan/glycerol films were tested over *E. coli*, *S. aureus*, and *B. cereus* using the disc-diffusion technique by Salvia-Trujillo et al. (2015) [47]. When coated with a chitosan/glycerol 30% solution [55], the strawberry samples had an improved shelf life, with excellent antibacterial and antifungal activity (1 week). Moreover, it maintained the sensorial properties of the strawberries, with no alteration in appearance, texture, and flavor [55].

Fresh-cut apples are also vulnerable to microbial and enzymatic spoilage because their natural protective barrier is removed, and the internal tissue, which is an excellent source of nutrients, is exposed to external factors [57]. Apples are usually resistant to microbial decay because of the presence of phenolic and flavonoid compounds, such as catechins,

phloridzins, tannins, and chlorogenic acid, on the apple peel [58]. There are several studies that researched the effect of different chitosan edible films on apples or fresh-cut apples, the results highlighting that the edible films managed to reduce the respiration rate [59,60], the microbial load [57,59,61], and the weight loss [57,60,62].

The scope of this research paper was to evaluate the impact on the shelf life and nutritional values of three newly developed chitosan-based edible coatings on strawberries and apple slices. The chitosan-based edible coatings were obtained as follows: 2% medium molecular weight chitosan and 1% acetic acid; 2% medium molecular weight chitosan and 1% acetic acid; and 1% high molecular weight chitosan and 2% ascorbic acid. These formulations were enriched with grape seed or sea buckthorn essential oils; these were chosen over a previous study performed by the same authors in which the EOs were analyzed for their antifungal properties [63].

2. Materials and Methods

2.1. Biological Material—Strawberries and Apples

Organic strawberries (*Fragaria* × *ananassa*) and apples (*Malus domestica cv Florina*) were purchased from a local market in Bucharest (Romania) and transferred to the laboratory within 2 h after purchase. The biological material was kept overnight at refrigerated temperatures (4 °C) before the coatings were applied. The next day, the fruits were visually inspected for decay or damage. Fruits free of physical injuries were selected for the further treatment taking into account the similarity in shape, color, and size.

2.2. Materials

Medium molecular weight chitosan with a deacetylation degree of 75–85% (448877) and high molecular weight chitosan with a >75% deacetylation degree (419419) were purchased from Sigma-Aldrich (Burlington, MA, USA)and used as received. Ascorbic acid (AC05150) and acetic acid (AC0344) were purchased from Scharlau (Spain), and Tween® 20 (Polysorbate) (97062-332) was purchased from VWR (Radnor, PA, USA) and used as received. Grape seed essential oil was purchased from Herbavit (Bucharest, Romania), and sea buckthorn essential oil was purchased from Hofigal (Bucharest, Romania) and used as received. Folin–Ciocalteu reagent (F9252), xylene (214736), sodium bicarbonate (S6014), sodium acetate (S8750), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (247642), and potassium chloride (P3911) were purchased from Merck (Darmstadt, Germany).

2.3. Preparation and Characterization of Chitosan Coatings

Edible coating chitosan-based solutions were prepared by dissolving medium molecular weight chitosan 2% w/v, high molecular weight chitosan 1% w/v in acetic acid or ascorbic acid solutions, under constant stirring on a stirring plate (Fisherbrand, Isotemp, China) for approximately 1 h at a constant temperature of 80 °C. Subsequently, the obtained solutions were left to cool at room temperature for 1 h. In order to ensure the mixing of the essential oils with the chitosan solutions, 2 mL of Tween 20 was used as emulsifying agent and then stirred for 5 min. Grape seed (7.5%) or sea buckthorn (7.5%) essential oils were added to each coating solution and then stirred again for 15 min in order to completely emulsify. Composition and coding of the obtained edible coating materials are described in Table 1. The surface morphology of the coatings was investigated using a scanning electron microscope (SEM), FEI Inspect S50 Electron Microscope (FEI, Hillsborough, OG, USA).

Table 1. Composition of the obtained chitosan-based edible coatings.

Coating	Code	Composition
A	MMC-AcA-GSEO	2% Medium molecular weight chitosan, 1% acetic acid, grape seed EO
B	MMC-AcA-SBEO	2% Medium molecular weight chitosan, 1% acetic acid, sea buckthorn EO
C	HMC-AsA-GSEO	1% High molecular weight chitosan, 2% ascorbic acid, grape seed EO

2.4. Coating Treatment

Before applying the coating treatment, the chosen strawberries and apples were rinsed for 1 min with distilled water and then stored for 1 h at room temperature in order to ensure full water evaporation. The strawberries were coated as such (whole fruit), whereas the apples were cut into slices of the same dimensions. The control samples consisted of fruits without any coating applied.

The edible coatings were used to treat the strawberries and apple slices through dipping method. The fruits were dipped into the coating solutions for 20 s and then dried at room temperature for 1 h to remove excess solution from fruit surface (Figure 1). After the coatings were fully dried, the strawberries and apple slices were packaged in perforated polyethylene terephthalate (PET) containers (each container had approximately 80 g of fruit sample) and stored at two distinct temperatures (4 °C and 8 °C). The physicochemical and microbiological properties were evaluated for a period of 7 days.

(**a**) (**b**)

Figure 1. (**a**) Visual aspect of the coated strawberries during the drying process; (**b**) Visual aspect of the coated apple slices during the drying process.

2.5. Scanning Electron Microscopy (SEM) Assay

Apple and strawberry fruit samples coated with chitosan layer were cut into small pieces, approximately 5 × 5 mm in dimension, and fixed on the support stub using carbon adhesive tape. Samples were scanned using FEI Inspect S50 Electron Microscope, in low vacuum mode. Images were acquired at a pressure of 220 Pa and magnification of 200× and 400×.

2.6. Dry Matter

The dry matter and humidity were determined by using a thermobalance (RADAWAG MAC 50, Poland). Briefly, five (5) grams of sample were distributed homogeneously in a thin layer on the weighing plate, to obtain reproducible results. Following treatment at 105 °C, the amount of dry matter was determined. All measurements were performed in triplicate.

2.7. Water Activity (a_w)

Strawberries and apple slices (approximatively 80 g per treatment) were homogenized using a stomacher (Interscience, BagMixer 400, Saint Nom, France). The water activity content of the samples was determined using a water activity meter (NOVASINA, LabMaster-aw, Lachen, Switzerland) with an accuracy of ±0.0030 aw within the adjustment range (https://www.novasina.ch/produkt/labmaster-aw-neo/, accessed on 15 October 2022). The samples were placed in containers and allowed to reach the device's temperature in the thermostatic chamber before reading.

2.8. Determination of Total Polyphenol Content (TPC)

An ethanolic (75%) extraction of polyphenols in a sample:solvent ratio of 1:5 was employed by macerating samples for 48 h in the dark at room temperature. After filtration, the resulting ethanolic extracts were collected and stored at −20 °C until further analysis. TPC was determined using the Folin–Ciocalteu method [64] adapted to microscale [65]. Briefly, 20 µL ethanolic extracts was mixed with 1580 µL of distilled water, plus 100 µL of Folin–Ciocalteu reagent and vigorously stirred. After 1 min, 300 µL of aqueous sodium carbonate 20% was added, and the mixture was vigorously stirred again and allowed to stand at room temperature in the dark, for 2 h. Absorbance was measured at 765 nm (on a Thermo Helios Alpha UV/Vis Spectrophotometer, Waltham, MA, USA); TPC was calculated from a calibration curve, using gallic acid as standard. The results were expressed as mg gallic acid equivalents (mg GAE)/L.

2.9. Evaluation of Antioxidant Activity (AA) using the DPPH Method

Antioxidant activity was determined by evaluating the free radical scavenging effect on the 1,1-dipheny l-2-picrylhydrazyl radical (DPPH) as described by Villaño et al. (2007) with slight modifications [66]. Briefly, an aliquot of 0.5 mL ethanolic extracts was added to 1.95 mL of DPPH solution (60 µM in ethanol), vortexed, and the absorbance was read at t = 0 (A 515(0)) and t = 30 (A 515(30)) min using a Thermo Helios Alpha UV/Vis Spectrophotometer. The AA was calculated from a calibration curve, by plotting %ΔA515 against known quercetin concentrations (3–50 µM), where

$$\%\Delta A515 = [(A\ 515(0) - A\ 515(30))/A\ 515(0)] \times 100.$$

The results were expressed as µM quercetin equivalents (µM QE).

2.10. Determination of Ascorbic Acid Content

The ascorbic acid content was evaluated using the indophenol–xylene extraction method [67]. Briefly, 10 g of sample was mixed with 20 mL of 2% (*w*/*v*) oxalic acid, ground in a mortar, and brought to 100 mL (final volume) with 2% (*w*/*v*) oxalic acid. After resting for 10 min at room temperature and filtration, 2 mL of the content was mixed with 1 mL of 2% (*w*/*v*) oxalic acid, 5 mL of sodium acetate solution, 2 mL of indophenol colorant, and 20 mL of xylene, followed by centrifugation for 20 min at 4 °C and 9000 rpm. The absorbance of the resulting sample extracts was measured at 500 nm, and the results were expressed as mg ascorbic acid/100 g sample (fresh weight).

2.11. Microbiological Assay—Molds and Yeasts

Sample preparation for mold and yeast counts was achieved in aseptic conditions (laminar flow, gloves, and scalpels), for each replicate, based on the method described by Jafari et al. (2021) [68] and Eshghi et al. (2022) [69]. Briefly, 10 g of sample was mixed with 90 mL of sterilized distilled water in 100 mL Erlenmeyer flask. One (1) mL of each of the appropriate serial dilution was plated by standard microbiological pour plate technique on malt extract agar (MEA) medium (Scharlau, Spain). All plates were incubated for 5 days at 25 °C, and results were expressed as total number of colonies per dilution. All microbiological determinations were performed in duplicate.

2.12. Statistical Analysis

The study assessed strawberry and apple fruit samples selected from 3 treatments. The DM%/a_w/TPC/AA/Vit C levels registered by each fruit type were measured over time. All data are expressed as mean ± standard error of the mean (SEM). Results were submitted to JMP 11 Statistical Discovery™ from SAS. Repeated measurement analysis of variance (ANOVA) was performed to investigate the statistical differences among groups for all analyzed parameters employing the Standard Least Squares method. The model effects were treatment (Control, MMC-AcA-GSEO, MMC-AcA-SBEO, or HMC-AsA-GSEO),

time (day 0, day 3, day 5, day 7, and day 9), and their interaction (Treatment × Time), in order to evaluate the overall treatment effect, measured at 4 °C and 8 °C. Values of $p < 0.05$ were considered significant.

3. Results and Discussion

3.1. Scanning Electron Microscopy (SEM) Assay

Figure 2 shows the morphology of the applied coatings on whole strawberries and apple slices. When analyzing strawberry coatings, fractures and pores were observed in the coatings of fruits dipped in MMC-AcA-SBEO (Figure 2b) and HMC-AsA-GSEO (Figure 2c), while the MMC-AcA-GSEO (Figure 2a) coating showed a continuous and smooth structure. However, the coatings applied on strawberries appeared to be very thin, and consequently, the thickness could not be measured on the coated fruit. The presence of porosity in a sponge-like structure may have occurred due to the structure-elevated roughness associated with the evaporation of the oil; this could be attributed to the disruption of the crosslinking of the chitosan film (C–H bonds) due to the hydrophobic nature of the essential oil components [45]. Similar effects were observed also by Oberlintner et al. (2021) in a biodegradability study of active chitosan biopolymer films enriched with *Quercus* polyphenol extract where a reduced thickness of the chitosan matrix occurred, but no notable difference in morphology was observed [40]. Further, the structure of the chitosan-based coatings appeared dense and smooth on apple slices, the thickness varying from 4.43 ± 0.5 µm on the tissue of the sliced sample to 13.61 ± 0.5 µm on the apple peal. This difference may result from the high humidity of the slice tissue.

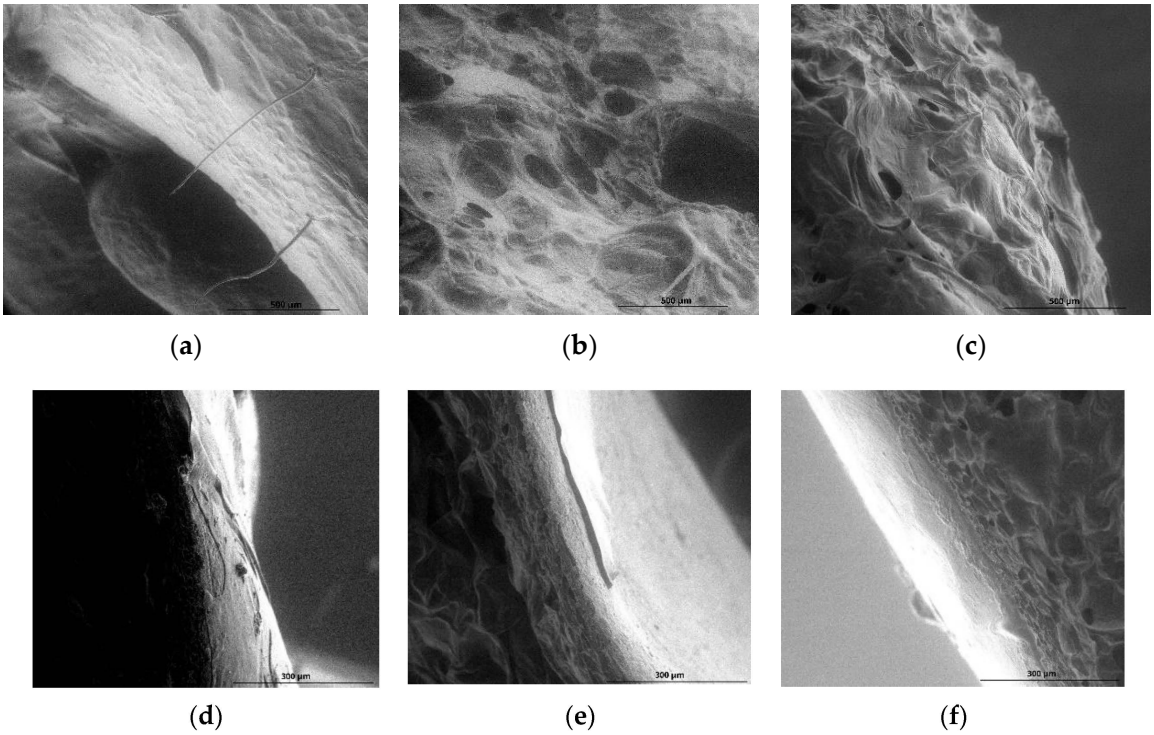


Figure 2. Scanning electron microscopy (SEM) images of (**a**) strawberry coated with medium molecular weight chitosan, acetic acid, and grape seed EO (magnification 500×); (**b**) strawberry coated with medium molecular weight chitosan, acetic acid, and sea buckthorn EO; (**c**) strawberry coated with high molecular weight chitosan, ascorbic acid, and grape seed EO; (**d**) apple slices coated with medium molecular weight chitosan, acetic acid, and grape seed EO (magnification 500×); (**e**) apple slices coated with medium molecular weight chitosan, acetic acid, and sea buckthorn EO; and (**f**) apple slices coated with high molecular weight chitosan, ascorbic acid, and grape seed EO.

3.2. Effect of Edible Coating on Fruit Quality Parameters in Strawberries

3.2.1. Dry Matter and Water Activity

The main effects of treatment, time, and their interaction were assayed for the dry matter (DM%) and water activity (a_w) at 4 °C and 8 °C in strawberries during a storage period of 7 days. DM% measured in coated fruit stored at 4 °C did not vary significantly until day 5 (10.1–11.3%) compared with the control, except for MMC-AcA-SBEO, which showed a significant drop at day 7 (Figure 3a). By contrast, repeated measurement analysis revealed different patterns for coated strawberries stored at 8 °C when compared to 4 °C. The improvement in DM% for both MMC coatings over the control was displayed by the significant effects of Treatment ($p < 0.0001$*) and Time × Treatment ($p < 0.0001$*). The HMC-AsA-GSEO revealed a drop at day 3 (7.09%), followed by a significant increase toward day 7 (11.01%) compared with the control.

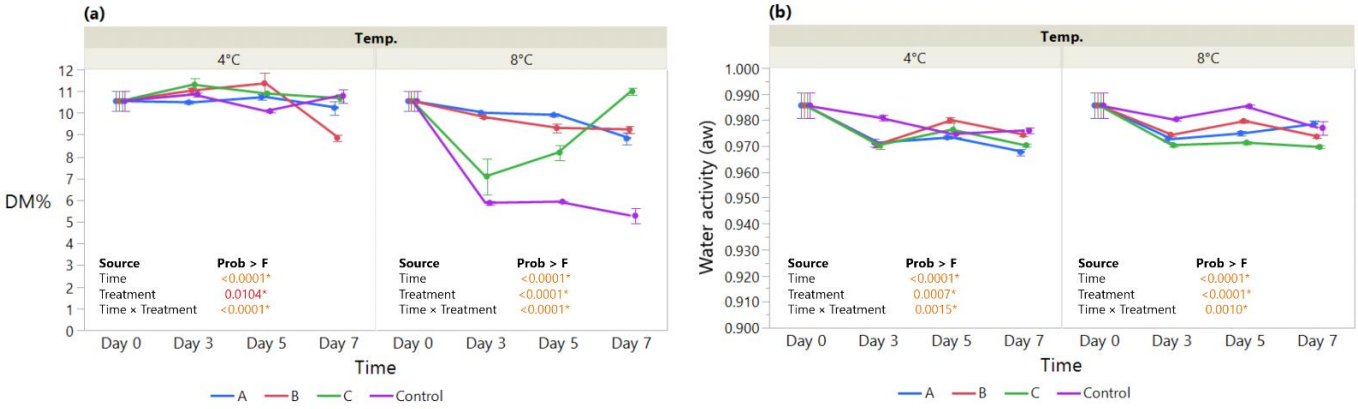


Figure 3. The effect of coating treatment on strawberry (**a**) Dry matter (DM%) and (**b**) Water activity (a_w) during a 7-day storage period at 4 °C and 8 °C. The *p*-values for the effects of Treatment (coating), Time and Interaction Treatment × Time are shown. Data are means ± SEM ($n = 3$). Asterisk (*) indicates significance ($p < 0.05$*).

In terms of water activity (a_w) measured at 4 °C and 8 °C, the improvement for coated fruit was displayed by the significant Treatment effect ($p = 0.0007$* at 4 °C and $p < 0.0001$* at 8 °C) (Figure 3b). Over time, the coated fruit maintained overall a_w levels below the control, as displayed by the Time × Treatment interaction effect ($p = 0.0015$* at 4 °C and $p < 0.0010$* at 8 °C), except for MMC-AcA-SBEO (treatment B) at 4 °C on days 5 (0.98) and 7 (0.97).

Provided that moisture transfer occurs between the fruit produce and the surrounding environment, bound water become a function of time throughout cost–storage [70]. Water activity values of strawberries (Figure 3b) are maintained between 1.00 and 0.95, a range that favors microbial spoilage of fresh fruit [71,72]. Our results show definitive overall improvement at both 4 °C and 8 °C for coatings over the control and are in line with previous findings [72–74].

In conjunction with the DM% evolution, the weight loss of strawberries, resulting from the loss of water content, are consistent in terms of the improvement due to coating application.

Given that water activity regulates moisture migration, differences in DM% were drastic for HMC-AsA-GSEO, whereas the MMC exhibited steady trends throughout storage. This might be attributed to the difference in coating permeability, which in turn accounts for the amount of water available for chemical and physical reactions [72]. For example, Choi et al. (2016) observed that the excessive addition of EO altered the structure of an HPMC (Hydroxy Propyl Methyl Cellulose) coating, resulting in a loosened structure, more prone to water migration [75].

3.2.2. Total Polyphenol Content, Antioxidant Activity, and Vitamin C Content

The effect of an edible coating on total polyphenol content, antioxidant activity, and vitamin C content was assayed in strawberries over a 7-day storage period. Our results show a significant drop in TPC measured at 4 °C on day 3 followed by an increase on day 5 irrespective of the coating. At day 7, only the HMC-AsA-GSEO increased progressively during storage at 4 °C (597 mg GAE/L), revealing a significant improvement over the control (Figure 4a). The significant main effect of coating Treatment (p < 0.0001*) and Treatment × Time (p = <0.0001*) is also displayed at 8 °C by the similar patterns in TPC over time, with HMC-AsA-GSEO registering higher levels than the control throughout storage (454.4–736.8 mg GAE/L).

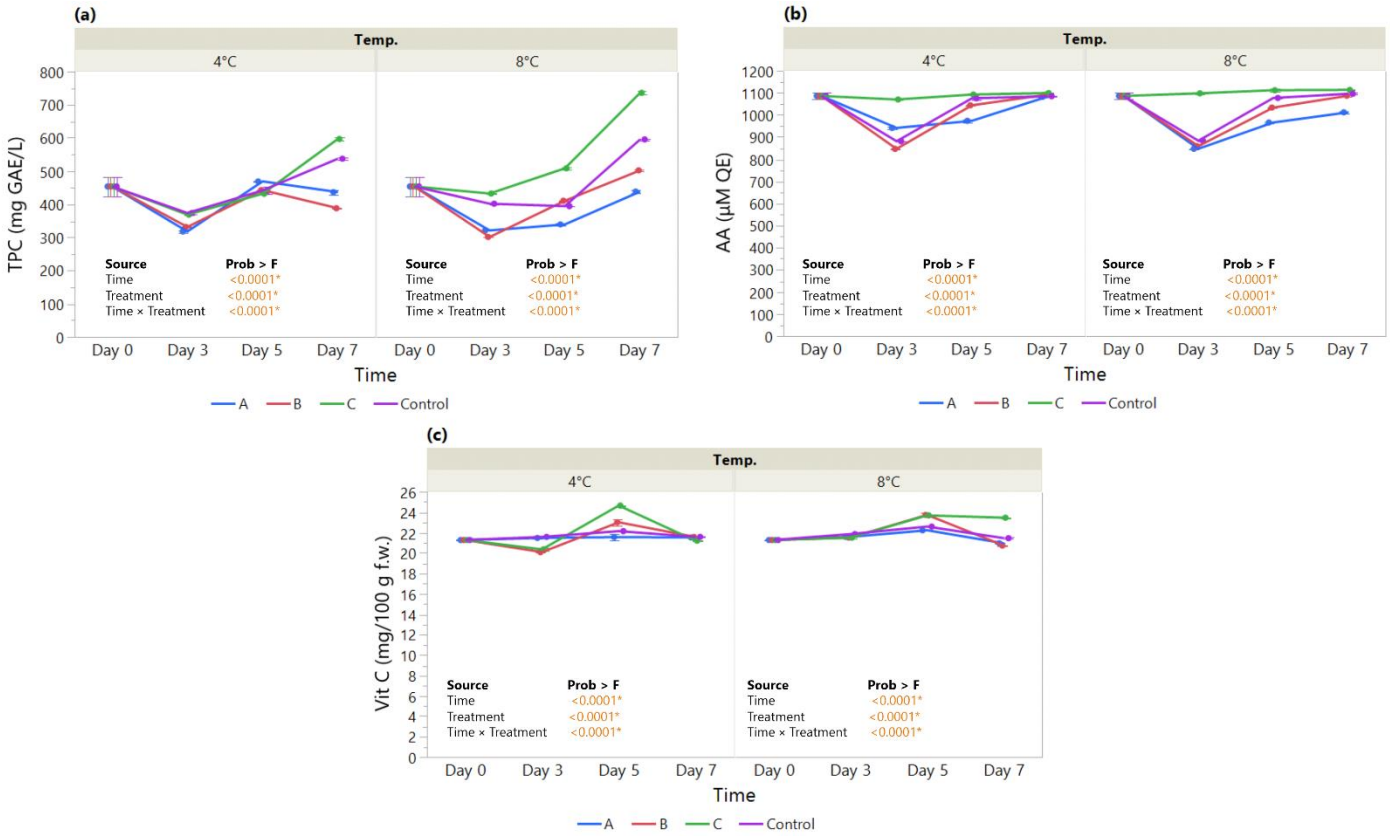


Figure 4. The effect of coating treatment on strawberry antioxidant status during a 7-day storage period at 4 °C and 8 °C. (**a**) Total Polyphenol Content (TPC—mg GAE/L); (**b**) Antioxidant Activity (AA—µM QE); and (**c**) Vitamin C content (Vit C—mg/100 g fresh weight). The p-values for the effects of Treatment (coating), Time and Interaction Treatment × Time are shown. Data are means ± SEM (n = 3). Asterisk (*) indicates significance (p < 0.05*).

Similarly, the antioxidant activity (AA) shows close patterns over time between the MMC coatings and the control measured both at 4 °C (Treatment × Time, p < 0.0001*) and 8 °C (Treatment × Time, p < 0.0001*) (Figure 4b); the control and the MMC coatings registered a drop at day 3 at both temperatures, while the HMC-AsA-GSEO displayed steady levels maintained until day 7. At the same time, the vitamin C content (Figure 4c) measured at 4 °C (20.11–24.67 mg/100 g f.w.) registered a drop on day 3 for the MMC-AcA-SBEO- and HMC-AsA-GSEO-coated fruit compared to the control, followed by an increase on day 5 and another drop on day 7 matching the control (Treatment × Time, p < 0.0001*). On the other hand, when measured at 8 °C (20.75–23.76 mg/100 g f.w.), the HMC-AsA-GSEO maintained higher levels than the control toward the end of the study period (Treatment × Time, p < 0.0001*).

Despite the overall decrease in DM% in coated strawberries, along with the maintenance of a_w levels below that of the control, we noted that only the HMC-AsA-GSEO treatment exhibited a significant improvement in TPC together with AA (Figure 4a,b). In addition to the concentration of the solids, this effect might be ascribed to the more compact coating in comparison with the MMC treatments, which provided an enhanced protective barrier against polyphenol oxidative reactions [76,77]. Similarly, Dashipour et al. (2015) observed higher efficiency for TPC and AA in *Zataria multiflora* EO coatings [78].

Moreover, the incorporation of EOs has been considered to interact with the fruit tissue, as well as with the internal atmosphere; hence, it provides a suitable environment toward the inhibition of biochemical reactions and scavenging of hydroxyl radicals [79,80].

In this context, the oxidation/degradation of ascorbic acid is also affected, pertaining to a decelerated ripening process through the coating [74]. As presented above, our findings indicate that the vitamin C levels in the strawberry population differ as a function of coating treatment depending on time; we noted a significant effect of both of our main effects, coating and time, as well as their interaction with HMC-AsA-GSEO as the most efficient. It stands to reason that, despite the presence of EOs in all coating treatments, the HM chitosan is responsible for the delayed respiration rate and metabolic activity [76,79,81]. Similar results were observed by de Oliveira Filho et al. (2022) who analyzed strawberry samples during a 12 days period. The coated samples presented improved shelf life, antioxidant activity, phenolic compounds, and ascorbic acid content compared with uncoated strawberries. The coatings that contained essential oils proved to have antimicrobial activity [82]. In addition, an edible coating developed from chia seed mucilage and bacterial cellulose nanofiber was analyzed in order to determine its effect on the bioactive compounds and antioxidant enzyme activity of the strawberry samples. The coated strawberry samples proved to preserve the ascorbic acid content, protein content, and antioxidant activity, as well as the phenolic and flavonoid compounds [83]. In line with these, Martínez et al. (2018) described the protective effect of the *Thymus capitatus* essential oil incorporated in a chitosan edible coating on strawberries, delaying the reduction in antioxidant properties and extending the shelf life of the coated fruit to 15 days [45].

3.3. Effect of Edible Coating on Fruit Quality Parameters in Apples

3.3.1. Dry Matter and Water Activity

Observations were recorded for apple slices on the coating treatment they were assigned and their response for dry matter (%) and water activity (a_w) for a period of 9 days (Figure 5).

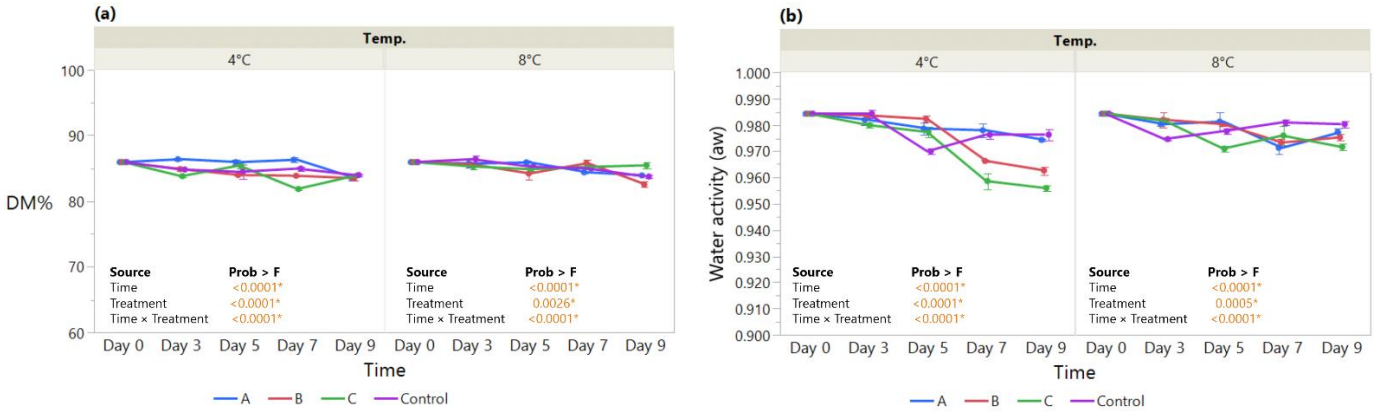


Figure 5. The effect of coating treatment on apple slices (**a**) Dry matter (DM%) and (**b**) Water activity (a_w) during a 9-day storage period at 4 °C and 8 °C. The *p*-values for the effects of Treatment (coating), Time and Interaction Treatment × Time are shown. Data are means ± SEM (n = 3). Asterisk (*) indicates significance ($p < 0.05$*).

The assessment of the effect of the different coatings on DM% levels measured at both 4 °C and 8 °C indicates that there is a different effect of treatment depending on time (Treatment × Time, $p < 0.0001$* at 4 °C and 8 °C, respectively) (Figure 5a). For a 9-day storage assessment, the effect appears identical for the tested coatings, showing steady overall patterns (81.8–86.9%); for HMC-AsA-GSEO; however, a drop in DM% is delineated at day 7 (4 °C).

For water activity, ranging between 0.96 and 0.98, the pattern of effect of time depending on the different coating treatments is more pronounced at 4 °C than at 8 °C (Figure 5b). The delayed decrease in a_w levels was noted only for MMC-AcA-SBEO and HMC-AsA-GSEO at 4 °C, whereas it manifested for all coating treatments at 8 °C when compared with the control.

Unlike strawberries, apples contain fewer amounts of water. However, when sliced, it is only natural that moisture loss occurs [84]. The apparent improvement depicted through the tendency for a delayed weight loss (Figure 5a) is further substantiated when HMC-AsA-GSEO and MMC-AcA-SBEO (treatments C and B, respectively) exert a positive decrease in aw values at 4 °C (Figure 5b). It might be attributed to differences in the hydrophobicity of the coating treatments due to the addition of EOs. To a certain degree, a cross-linking effect might be responsible for a reduced interaction between the coating surface and the water molecules [74,76,78]. Like the case of strawberries, our findings on cold-stored apples indicate that coating permeability might be responsible for the plain overall improvement, revealed also at 8 °C. As a consequence of coating treatment application, a higher relative humidity surrounding the apple slices mitigates water loss [84].

3.3.2. Total Polyphenol Content, Antioxidant Activity, and Vitamin C Content

The Least Squares Means Plot (Figure 6) shows the results of the test for the fixed factors, in this case, the two main effects for Treatment and Time. In addition, the interaction of Coating Treatment and Time is showing the differences by time broken apart by treatment. TPC levels measured at 4 °C (89–242 mgGAE/L) showed an increasing tendency for HMC-AsA-GSEO until day 7, after which it registered a significant drop below the control on day 9 (Figure 6a). By contrast, the two MMC coatings had contents lower than the control overall. As such, we can see a statistically significant effect for Treatment ($p < 0.0001$*) on TPC registered and for Time ($p < 0.0001$*) on TPC registered, as well as evidence that there is an interaction between Treatment and Time ($p < 0.0001$*). When evaluated at 8 °C (107–240 mg GAE/L), the evolution of TPC showed similar trends for the GSEO-treated fruit, with the HMC coating well above the MMC one, and higher than the control overall (Treatment × Time, $p < 0.0001$*). The MMC-AcA-SBEO did not manage to improve the TPC of apple slices over time, registering the lowest content at the end of the storage period.

Our results show the AA levels drop at 4 °C for all treatments on day 3, followed by a progressive increase over time for the coatings containing GSEO (Treatment × Time, $p < 0.0001$*) (Figure 6b). The MMC-AcA-GSEO-coated fruit revealed a notable evolution in TPC, standing out at the end of cold storage, followed closely by HMC-AsA-GSEO (Treatment, $p < 0.0001$*). By increasing the storage temperature (8 °C), we observed different trends in the overall coating effect, as evidenced by the Treatment × Time interaction ($p < 0.0001$*). Nonetheless, by the end of the study period, a similar disposition in TPC was achieved, highlighted by the elevated levels of GSEO coatings compared with the control.

Similarly, for the vitamin C content (Figure 6c), we evaluated whether there is anything about the effect of time that changes the effect of coating treatment. In this case, the effect of time is to have the vitamin C be constant, with a notable increase from day 3 observed at both 4 °C and 8 °C. Furthermore, the control fruit stored at 4 °C showed a slight increase; however, it was lower than the coated fruit. Nonetheless, when comparing the differences at each time point measured at 8 °C, the effect of treatment for the uncoated apple slices is the same at all time periods.

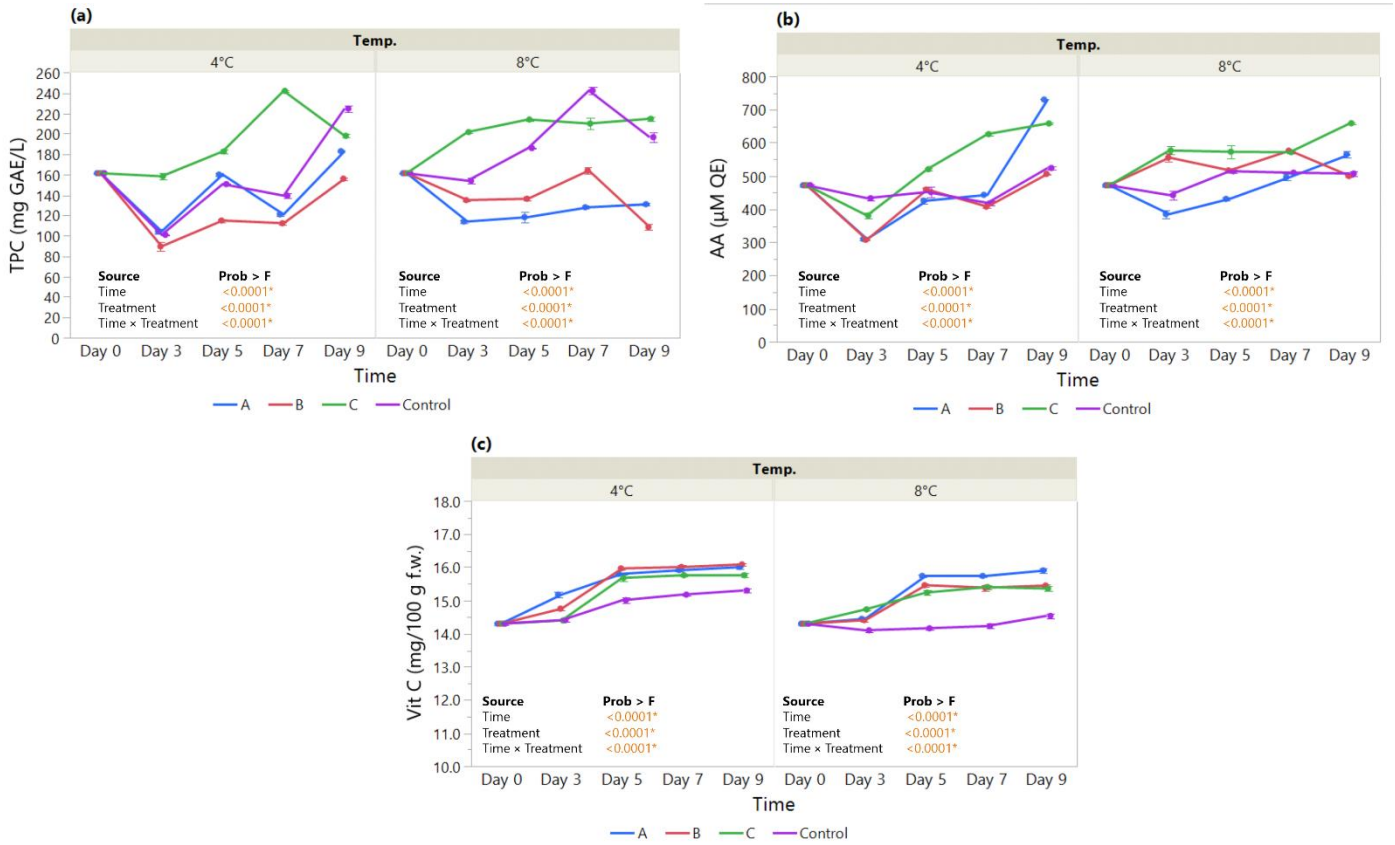


Figure 6. The effect of coating treatment on apple slices antioxidant status during a 9-day storage period at 4 °C and 8 °C. (**a**) Total Polyphenol Content (TPC—mg GAE/L); (**b**) Antioxidant Activity (AA—µM QE); and (**c**) Vitamin C content (Vit C—mg/100 g fresh weight). The *p*-values for the effects of Treatment (coating), Time and Interaction Treatment × Time are shown. Data are means ± SEM (n = 3). Asterisk (*) indicates significance ($p < 0.05$*).

Present in variable concentrations in apples, phenolic compounds together with ascorbic acid, account to a great extent for the antioxidant activity of food and promote human health [85,86]. Minimally processed apples are accompanied by an enzymatic synthesis of polyphenols through the activity of phenylalanine ammonia lyase (PAL); at the same time, polyphenol oxidase (PPO) causes the degradation of phenolic compounds [85,87]. In addition, the content of acidic compounds during ripening has a decreasing tendency over time, which might be attributed to the oxidation of organic acids along with the rise in pH values [84,88]. The tandem use of edible coatings and antioxidants (essential oils) is able to inhibit these degradation processes [87,89]. In this context, our findings suggest that the incorporation of GSEO into the HMC coating enabled the stabilization of pH and decreased the activity of PPO, which in turn contributed to the higher TPC and AA monitored at both 4 °C and 8 °C. Given that gas permeability reduction is promoted by using edible coatings, fruit are accompanied by a decrease in respiration rate during cold storage; accordingly, ascorbic acid degradation is also diminished [86]. Accordingly, the overlapping patterns for vitamin C (Figure 6c) provide further insight on the considerable improvement pertaining to the antioxidant activity levels. It can be inferred that the assessed coatings are not interacting with the vitamin C content, irrespective of the treatment, and are able to maintain this parameter above the physiological postharvest levels. Similar results were obtained when rose apples (*Cv. Tabtimchan*) were coated with a sodium alginate-based edible coating in order to maintain the postharvest quality when stored at low temperatures. The coated samples had a significantly reduced respiration rate and weight loss throughout the 10 days of storage. The total phenolic content and antioxidant activity presented higher

values compared to the control on day 10 [90]. In addition, Kumar et al. (2018) reported the improvement of shelf life of fresh-cut Royal Delicious apple wedges when applied an edible coating containing antibrowning agents. Their application indicated a significant effect on the apples' weight loss and enzymatic browning and retarded microorganism growth during a 7-day storage period at 5 ± 2 °C [91]. In this context, similar findings were indicated by Farina et al. (2020) when lemon essential oil was incorporated in aloe vera gel-based edible coatings. The postharvest quality of fresh-cut Fuji apples was improved in terms of soluble solids, titratable acidity, and pH and managed to maintain the overall nutrients and vitamin content throughout storage [92].

3.4. Microbiological Analysis

Strawberries are fruits that have a short shelf life because of their high water content and high physiological activities, thus being susceptible to several types of microorganisms, the main one being *Botrytis cinerea* [56]. The results of the microbiological analysis of yeasts and molds are quite straightforward; the microbial load was significantly decreased throughout the storage period at both temperatures, 4 °C and 8 °C, as shown in Figure 7. All types of coatings, compared to the control samples that were uncoated, had significantly lower levels of microbial load. However, strawberries coated with chitosan, acetic acid solutions, and sea buckthorn oil provided the lowest count of molds and yeasts until day 7 of the analysis. The results are in accordance with the lower water activity presented in this study by the coated samples, thus providing an unsuitable environment for microbial growth. These results are in agreement with those obtained by Xin et al. (2022) [93], Lukša et al. (2018) [94], and de Mata et al. (2022) [95] who studied the antifungal effect of sea buckthorn and grape seed EOs, and their findings confirmed their antifungal activity over several fungi strains. Moreover, Velickova et al. (2013) [96] show that chitosan-based edible films present antimicrobial growth and extend the shelf life of strawberries.

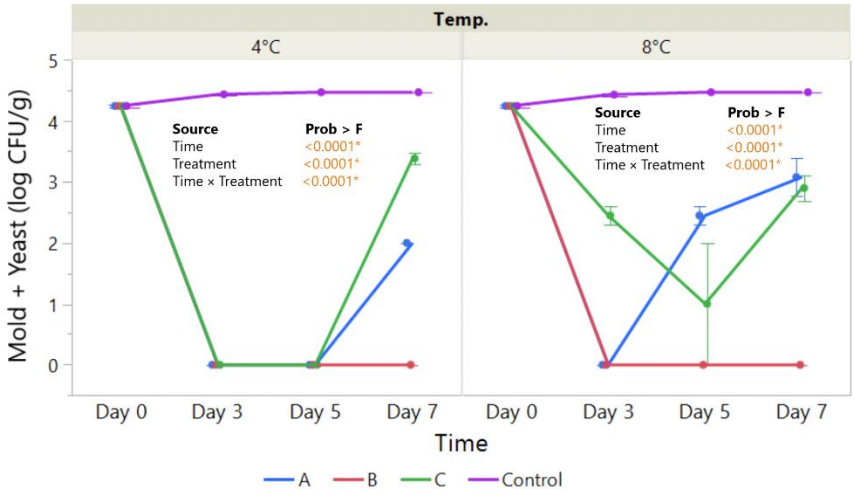


Figure 7. The effect of coating treatment on total number of strawberry mold and yeast colonies (log CFU/g) for a 10^{-2} dilution during a 7-day storage period at 4 °C and 8 °C. The *p*-values for the effects of Treatment (coating), Time and Interaction Treatment × Time are shown. Data are means ± SEM (n = 2). Asterisk (*) indicates significance ($p < 0.05$*).

In the case of the microbiological assay of the apple samples, it is noted that no microbial load was observed at the tested dilutions.

3.5. Study Limitations

The present study was intended to stand as a potential tool in support to maintaining the postharvest quality of fruit. Yet, given the nature of the employed methods, some drawbacks of our study include (a) missing sensorial evaluation and (b), arguably, a lack

of measurements of the tested parameters at more realistic temperatures (e.g., 23 °C). Nonetheless, taking into account the adjunction of functional ingredients pertaining to added-value features (e.g., antimicrobial activity), edible coatings represent a suitable choice towards maintaining the characteristics of fresh-cut fruit, a well-established challenge for food processors.

In order to attain adequacy from the economical perspective and to provide pertinent insight toward real-life applications, consistent research and development on edible coating methods is needed [7]. Moreover, consumers are progressively demanding healthy foods and become more educated; consequently, from a practical perspective, one of the approaches employed to extend the storability of perishable produce is the application of edible coatings, followed by cold storage, deemed more realistic than at room temperature [30–32].

With regard to the taste of the coated fruit, we did not evaluate the sensorial attributed by employing a trained panel. The addition of ascorbic or acetic acid along with the sea buckthorn or grape seed essential oils may indeed have the potential of altering the taste of the resulting coated fruit. However, given that postharvest losses of fruits are due to fungal infection, physiological disorders, and physical injuries, edible coatings can be used as a protective barrier to reduce respiration rates, retard microbial growth, and improve texture quality [30–32]. As such, if the coating film applied herein were to be unable to maintain the firmness and microbial load of the samples after refrigerated storage, off-odor/off-flavor may have indeed occurred. In addition, the use of essential oils as antimicrobial agents prolonged the desirable appearance of the tested fruit and retarded the development of microorganisms; these adjuvants have been previously deemed acceptable for consumers [3,7]. In a similar manner, Pavinatto et al. (2020) observed that a chitosan/glycerol 30% coating had an insignificant effect on the flavor, appearance, aroma, and texture of coated strawberries [55].

A fair assumption would be that the measurements could seem short and some results scattered and perceived as systematic errors, which may not be representative enough. Nonetheless, having achieved mechanical/biological protection/extension afforded herein by the coating in conjunction with the inclusion of essential oils, with no disproportionate response of the measurements corroborated with other scientific works, the benefits of the proposed approach would be desirable. Moreover, having assumed a proper tandem assessment of the physicochemical parameters at two different temperatures of cold storage (4 °C and 8 °C), our observations could provide supplementary information as to what extent the functional coatings can explain the GSEO and SBEO potential and render the measurements of the study relevant pertaining to the retention of postharvest quality. Taking into account both the drawbacks and the strengths of the study, these observations warrant their practical relevance for the purpose at hand and merit further exploration with regard to their potential application.

4. Conclusions

The developed chitosan-based and EO edible coatings managed to improve the postharvest quality and shelf life of the strawberries and apple slices during the storage period, minimizing physicochemical changes and maintaining a significantly lower microbial growth compared to the uncoated control samples. Coating strawberries and apples slices with the three chitosan-based edible coatings presented a beneficial impact in preserving the ascorbic acid, total phenolic content, and antioxidant activity in fruits during the cold storage period. Strawberries and apples coated with HMC-AsA-GSEO presented the best results regarding the ascorbic acid content and the antioxidant activity during the shelf-life period. In the case of strawberry samples, after 7 days of cold storage, the chitosan-based edible coatings functionalized with essential oils were able to reduce the microbial growth significantly, thus maintaining their postharvest and shelf life. It can be concluded that the developed chitosan-based coatings represent a promising technique to attain mechanical protection and preserve the postharvest quality parameters and shelf life of strawberries and apple slices during cold storage.

Author Contributions: Conceptualization, P.-A.P. and L.M.P.; methodology, P.-A.P. and L.M.P.; validation, P.-A.P., L.M.P., E.E.P., M.E.P., A.C.M., and F.M.; formal analysis, L.M.P.; investigation, P.-A.P. and I.-C.N.; resources, P.-A.P. and M.E.P.; data curation, P.-A.P. and L.M.P.; writing—original draft preparation, P.-A.P., L.M.P., and E.E.P.; writing—review and editing, A.C.M., E.E.P., F.M., and M.E.P.; visualization, P.-A.P. and L.M.P.; supervision, M.E.P., A.C.M., and F.M.; project administration, M.C.D.; funding acquisition, P.-A.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by a grant of the University of Agronomic Sciences and Veterinary Medicine of Bucharest, contract number 1281/30.07.2021, acronym EDIFOODMAT, within Joint Junior Competition 2021 and project FDI-2021-0430.

Data Availability Statement: The authors confirm that the data supporting the findings of this study are available within the article.

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

References

1. Yousuf, B.; Qadri, O.S. 11—Preservation of fresh-cut fruits and vegetables by edible coatings. In *Fresh-Cut Fruits and Vegetables Technologies and Mechanisms for Safety Control*; Siddiqui, M.W., Ed.; Academic Press: Cambridge, MA, USA, 2020; pp. 225–242. ISBN 978-0-12-816184-5.
2. TENEA, G.N. Postbiotics: A Solution to Protect Tropical Fruits towards Postharvest Adulteration. *AgroLife Sci. J.* **2021**, *10*, 189–196.
3. Yousuf, B.; Qadri, O.S.; Srivastava, A.K. Recent developments in shelf-life extension of fresh-cut fruits and vegetables by application of different edible coatings: A review. *LWT* **2018**, *89*, 198–209. [CrossRef]
4. Hassan, B.; Chatha, S.A.S.; Hussain, A.I.; Zia, K.M.; Akhtar, N. Recent advances on polysaccharides, lipids and protein based edible films and coatings: A review. *Int. J. Biol. Macromol.* **2018**, *109*, 1095–1107. [CrossRef] [PubMed]
5. Oberti, I.; Paciello, A. Bioplastic as a Substitute for Plastic in Construction Industry. *Encyclopedia* **2022**, *2*, 1408–1420. [CrossRef]
6. Yun, X.; Wang, Y.; Li, M.; Jin, Y.; Han, Y.; Dong, T. Application of permselective poly (ε-caprolactone) film for equilibrium-modified atmosphere packaging of strawberry in cold storage. *J. Food Process. Preserv.* **2017**, *41*, e13247. [CrossRef]
7. Maringgal, B.; Hashim, N.; Tawakkal, I.S.M.A.; Mohamed, M.T.M. Recent advance in edible coating and its effect on fresh/fresh-cut fruits quality. *Trends Food Sci. Technol.* **2020**, *96*, 253–267. [CrossRef]
8. Mir, N.; Beaudry, R.M. Modified Atmosphere Packaging. In *The Commercial Storage of Fruits, Vegetables, and Florist and Nursery Stocks*; Gross, K., Wang, C.Y., Saltveit, M., Eds.; Agricultural Research Service, United States Department of Agriculture: Washington, DC, USA, 2016; pp. 42–53. ISBN 3015046128.
9. Opara, U.L.; Caleb, O.J.; Belay, Z.A. 7—Modified atmosphere packaging for food preservation. In *Food Quality and Shelf Life*; Galanakis, C.M., Ed.; Academic Press: Cambridge, MA, USA, 2019; pp. 235–259. ISBN 978-0-12-817190-5.
10. Markevičiūtė, Z.; Varžinskas, V. Smart Material Choice: The Importance of Circular Design Strategy Applications for Bio-Based Food Packaging Preproduction and End-of-Life Life Cycle Stages. *Sustainability* **2022**, *14*, 6366. [CrossRef]
11. de Oliveira, W.Q.; de Azeredo, H.M.C.; Neri-Numa, I.A.; Pastore, G.M. Food packaging wastes amid the COVID-19 pandemic: Trends and challenges. *Trends Food Sci. Technol.* **2021**, *116*, 1195–1199. [CrossRef]
12. Singh, G.P.; Bangar, S.P.; Yang, T.; Trif, M.; Kumar, V.; Kumar, D. Effect on the Properties of Edible Starch-Based Films by the Incorporation of Additives: A Review. *Polymers* **2022**, *14*, 1987. [CrossRef]
13. European Commission. Regulation (EC) No 1935/2004 of the European Parliament and of the Council of 27 October 2004 on materials and articles intended to come into contact with food and repealing Directives 80/590/EEC and 89/109/EEC. *Off. J. Eur. Union* **2004**, *338*, 4–17.
14. European Commission. Commission Regulation (EU) No 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food Text with EEA relevance. *Off. J. Eur. Union* **2011**, *45*, 42–130.
15. Aiduang, W.; Chanthaluck, A.; Kumla, J.; Jatuwong, K.; Srinuanpan, S.; Waroonkun, T.; Oranratmanee, R.; Lumyong, S.; Suwannarach, N. Amazing Fungi for Eco-Friendly Composite Materials : A Comprehensive Review. *J. Fungi* **2022**, *8*, 842. [CrossRef]
16. Salazar, R.; Salas-Gomez, V.; Alvarado, A.A.; Baykara, H. Preparation, Characterization and Evaluation of Antibacterial Properties of Polylactide-Polyethylene Glycol-Chitosan Active Composite Films. *Polymers* **2022**, *14*, 2266. [CrossRef] [PubMed]
17. Davis, G.; Song, J.H. Biodegradable packaging based on raw materials from crops and their impact on waste management. *Ind. Crop. Prod.* **2006**, *23*, 147–161. [CrossRef]
18. Salehi, F. Edible Coating of Fruits and Vegetables Using Natural Gums: A Review. *Int. J. Fruit Sci.* **2020**, *20*, S570–S589. [CrossRef]
19. Valdés, A.; Ramos, M.; Beltrán, A.; Jiménez, A.; Garrigós, M.C. State of the art of antimicrobial edible coatings for food packaging applications. *Coatings* **2017**, *7*, 56. [CrossRef]
20. Paidari, S.; Zamindar, N.; Tahergorabi, R.; Kargar, M.; Ezzati, S.; Shirani, N.; Musavi, S.H. Edible coating and films as promising packaging: A mini review. *J. Food Meas. Charact.* **2021**, *15*, 4205–4214. [CrossRef]

21. Shahidi, F.; Hossain, A. Preservation of aquatic food using edible films and coatings containing essential oils: A review. *Crit. Rev. Food Sci. Nutr.* **2022**, *62*, 66–105. [CrossRef]
22. Mistriotis, A.; Briassoulis, D.; Giannoulis, A.; D'Aquino, S. Design of biodegradable bio-based equilibrium modified atmosphere packaging (EMAP) for fresh fruits and vegetables by using micro-perforated poly-lactic acid (PLA) films. *Postharvest Biol. Technol.* **2016**, *111*, 380–389. [CrossRef]
23. Jalali, A.; Linke, M.; Geyer, M.; Mahajan, P.V. Shelf life prediction model for strawberry based on respiration and transpiration processes. *Food Packag. Shelf Life* **2020**, *25*, 100525. [CrossRef]
24. Pop, O.L.; Pop, C.R.; Dufrechou, M.; Vodnar, D.C.; Socaci, S.A.; Dulf, F.V.; Minervini, F.; Suharoschi, R. Edible films and coatings functionalization by probiotic incorporation: A review. *Polymers* **2020**, *12*, 12. [CrossRef] [PubMed]
25. Lin, D.; Zhao, Y. Innovations in the Development and Application of Edible Coatings for Fresh and Minimally Processed Fruits and Vegetables. *Compr. Rev. Food Sci. Food Saf.* **2007**, *6*, 60–75. [CrossRef]
26. Singh, P.; Magalhães, S.; Alves, L.; Antunes, F.; Miguel, M.; Lindman, B.; Medronho, B. Cellulose-based edible films for probiotic entrapment. *Food Hydrocoll.* **2019**, *88*, 68–74. [CrossRef]
27. Tavassoli-Kafrani, E.; Shekarchizadeh, H.; Masoudpour-Behabadi, M. Development of edible films and coatings from alginates and carrageenans. *Carbohydr. Polym.* **2016**, *137*, 360–374. [CrossRef]
28. Duran, A.; Kahve, H.I. The effect of chitosan coating and vacuum packaging on the microbiological and chemical properties of beef. *Meat Sci.* **2020**, *162*, 107961. [CrossRef]
29. Guerreiro, A.C.; Gago, C.M.L.; Faleiro, M.L.; Miguel, M.G.C.; Antunes, M.D.C. The effect of alginate-based edible coatings enriched with essential oils constituents on *Arbutus unedo* L. fresh fruit storage. *Postharvest Biol. Technol.* **2015**, *100*, 226–233. [CrossRef]
30. No, H.K.; Meyers, S.P.; Prinyawiwatkul, W.; Xu, Z. Applications of Chitosan for Improvement of Quality and Shelf Life of Foods: A Review. *J. Food Sci.* **2007**, *72*, R87–R100. [CrossRef]
31. Etxabide, A.; Uranga, J.; Guerrero, P.; de la Caba, K. Development of active gelatin films by means of valorisation of food processing waste: A review. *Food Hydrocoll.* **2017**, *68*, 192–198. [CrossRef]
32. Díaz-Montes, E.; Castro-Muñoz, R. Edible films and coatings as food-quality preservers: An overview. *Foods* **2021**, *10*, 249. [CrossRef] [PubMed]
33. Galus, S.; Kibar, E.A.A.; Gniewosz, M.; Kraśniewska, K. Novel materials in the preparation of edible films and coatings—A review. *Coatings* **2020**, *10*, 674. [CrossRef]
34. Bi, J.; Tian, C.; Zhang, G.-L.; Hao, H.; Hou, H.-M. Novel procyanidins-loaded chitosan-graft-polyvinyl alcohol film with sustained antibacterial activity for food packaging. *Food Chem.* **2021**, *365*, 130534. [CrossRef] [PubMed]
35. Song, X.; Wang, L.; Liu, L.; Li, J.; Wu, X. Impact of tea tree essential oil and citric acid/choline chloride on physical, structural and antibacterial properties of chitosan-based films. *Food Control* **2022**, *141*, 109186. [CrossRef]
36. Sen, S.K.; Tripathi, R.; Mandal, P.; Choudhuri, C. Assessment of antifungal activity of nano-chitosan against mung bean seed borne pathogen Aspergillus flavus through solid matrix priming. *S. Afr. J. Bot.* **2022**, *150*, 372–386. [CrossRef]
37. Garcia, L.G.S.; de Melo Guedes, G.M.; Fonseca, X.M.Q.C.; Pereira-Neto, W.A.; Castelo-Branco, D.S.C.M.; Sidrim, J.J.C.; de Aguiar Cordeiro, R.; Rocha, M.F.G.; Vieira, R.S.; Brilhante, R.S.N. Antifungal activity of different molecular weight chitosans against planktonic cells and biofilm of *Sporothrix brasiliensis*. *Int. J. Biol. Macromol.* **2020**, *143*, 341–348. [CrossRef]
38. Hasheminejad, N.; Khodaiyan, F.; Safari, M. Improving the antifungal activity of clove essential oil encapsulated by chitosan nanoparticles. *Food Chem.* **2019**, *275*, 113–122. [CrossRef] [PubMed]
39. Bonilla, J.; Fortunati, E.; Atarés, L.; Chiralt, A.; Kenny, J.M. Physical, structural and antimicrobial properties of poly vinyl alcohol—Chitosan biodegradable films. *Food Hydrocoll.* **2014**, *35*, 463–470. [CrossRef]
40. Oberlintner, A.; Bajić, M.; Kalčíková, G.; Likozar, B.; Novak, U. Biodegradability study of active chitosan biopolymer films enriched with *Quercus* polyphenol extract in different soil types. *Environ. Technol. Innov.* **2021**, *21*, 101318. [CrossRef]
41. Priyadarshi, R.; Rhim, J.-W. Chitosan-based biodegradable functional films for food packaging applications. *Innov. Food Sci. Emerg. Technol.* **2020**, *62*, 102346. [CrossRef]
42. Jafarzadeh, S.; Nafchi, A.M.; Salehabadi, A.; Oladzad-abbasabadi, N.; Jafari, S.M. Application of bio-nanocomposite films and edible coatings for extending the shelf life of fresh fruits and vegetables. *Adv. Colloid Interface Sci.* **2021**, *291*, 102405. [CrossRef] [PubMed]
43. Bahmani, R.; Razavi, F.; Mortazavi, S.N.; Gohari, G.; Juárez-Maldonado, A. Evaluation of Proline-Coated Chitosan Nanoparticles on Decay Control and Quality Preservation of Strawberry Fruit (cv. Camarosa) during Cold Storage. *Horticulturae* **2022**, *8*, 648. [CrossRef]
44. Salha, B.A.; Gedanken, A. Extending the Shelf Life of Strawberries by the Sonochemical Coating of their Surface with Nanoparticles of an Edible Anti-Bacterial Compound. *Appl. Nano* **2021**, *2*, 14–24. [CrossRef]
45. Martínez, K.; Ortiz, M.; Albis, A.; Castañeda, C.G.G.; Valencia, M.E.; Tovar, C.D.G. The effect of edible chitosan coatings incorporated with *Thymus capitatus* essential oil on the shelf-life of strawberry (*Fragaria x ananassa*) during cold storage. *Biomolecules* **2018**, *8*, 155. [CrossRef] [PubMed]
46. Lee, J.Y.; Park, H.J.; Lee, C.Y.; Choi, W.Y. Extending shelf-life of minimally processed apples with edible coatings and antibrowning agents. *LWT Food Sci. Technol.* **2003**, *36*, 323–329. [CrossRef]

47. Salvia-Trujillo, L.; Rojas-Graü, M.A.; Soliva-Fortuny, R.; Martín-Belloso, O. Use of antimicrobial nanoemulsions as edible coatings: Impact on safety and quality attributes of fresh-cut Fuji apples. *Postharvest Biol. Technol.* **2015**, *105*, 8–16. [CrossRef]
48. Álvarez, A.; Manjarres, J.J.; Ramírez, C.; Bolívar, G. Use of an exopolysaccharide-based edible coating and lactic acid bacteria with antifungal activity to preserve the postharvest quality of cherry tomato. *LWT* **2021**, *151*, 112225. [CrossRef]
49. Yaman, Ö.; Bayoındırlı, L. Effects of an Edible Coating and Cold Storage on Shelf-life and Quality of Cherries. *LWT Food Sci. Technol.* **2002**, *35*, 146–150. [CrossRef]
50. Manzoor, S.; Gull, A.; Wani, S.M.; Ganaie, T.A.; Masoodi, F.A.; Bashir, K.; Malik, A.R.; Dar, B.N. Improving the shelf life of fresh cut kiwi using nanoemulsion coatings with antioxidant and antimicrobial agents. *Food Biosci.* **2021**, *41*, 101015. [CrossRef]
51. Ortiz-Duarte, G.; Pérez-Cabrera, L.E.; Artés-Hernández, F.; Martínez-Hernández, G.B. Ag-chitosan nanocomposites in edible coatings affect the quality of fresh-cut melon. *Postharvest Biol. Technol.* **2019**, *147*, 174–184. [CrossRef]
52. Hira, N.; Mitalo, O.W.; Okada, R.; Sangawa, M.; Masuda, K.; Fujita, N.; Ushijima, K.; Akagi, T.; Kubo, Y. The effect of layer-by-layer edible coating on the shelf life and transcriptome of 'Kosui' Japanese pear fruit. *Postharvest Biol. Technol.* **2022**, *185*, 111787. [CrossRef]
53. Amal, S.H.; El-Mogy, M.M.; Aboul-Anean, H.E.; Alsanius, B.W. Improving Strawberry Fruit Storability by Edible Coating as a Carrier of Thymol or Calcium Chloride. *J. Hortic. Sci. Ornam. Plant.* **2010**, *2*, 88–97.
54. Hernández-Muñoz, P.; Almenar, E.; Ocio, M.J.; Gavara, R. Effect of calcium dips and chitosan coatings on postharvest life of strawberries (*Fragaria* x *ananassa*). *Postharvest Biol. Technol.* **2006**, *39*, 247–253. [CrossRef]
55. Pavinatto, A.; de Almeida Mattos, A.V.; Malpass, A.C.G.; Okura, M.H.; Balogh, D.T.; Sanfelice, R.C. Coating with chitosan-based edible films for mechanical/biological protection of strawberries. *Int. J. Biol. Macromol.* **2020**, *151*, 1004–1011. [CrossRef] [PubMed]
56. Khodaei, D.; Hamidi-Esfahani, Z.; Rahmati, E. Effect of edible coatings on the shelf-life of fresh strawberries: A comparative study using TOPSIS-Shannon entropy method. *NFS J.* **2021**, *23*, 17–23. [CrossRef]
57. Jin, T.Z.; Chen, W.; Gurtler, J.B.; Fan, X. Effectiveness of edible coatings to inhibit browning and inactivate foodborne pathogens on fresh-cut apples. *J. Food Saf.* **2020**, *40*, e12802. [CrossRef]
58. Kalinowska, M.; Bielawska, A.; Lewandowska-Siwkiewicz, H.; Priebe, W.; Lewandowski, W. Apples: Content of phenolic compounds vs. variety, part of apple and cultivation model, extraction of phenolic compounds, biological properties. *Plant Physiol. Biochem.* **2014**, *84*, 169–188. [CrossRef] [PubMed]
59. Karagöz, Ş.; Demirdöven, A. Effect of chitosan coatings with and without Stevia rebaudiana and modified atmosphere packaging on quality of cold stored fresh-cut apples. *LWT* **2019**, *108*, 332–337. [CrossRef]
60. Zhang, W.; Shu, C.; Chen, Q.; Cao, J.; Jiang, W. The multi-layer film system improved the release and retention properties of cinnamon essential oil and its application as coating in inhibition to penicillium expansion of apple fruit. *Food Chem.* **2019**, *299*, 125109. [CrossRef] [PubMed]
61. Pilon, L.; Spricigo, P.C.; Miranda, M.; de Moura, M.R.; Assis, O.B.G.; Mattoso, L.H.C.; Ferreira, M.D. Chitosan nanoparticle coatings reduce microbial growth on fresh-cut apples while not affecting quality attributes. *Int. J. Food Sci. Technol.* **2015**, *50*, 440–448. [CrossRef]
62. Khalifa, I.; Barakat, H.; El-Mansy, H.A.; Soliman, S.A. Preserving apple (*Malus domestica* var. Anna) fruit bioactive substances using olive wastes extract-chitosan film coating. *Inf. Process. Agric.* **2017**, *4*, 90–99. [CrossRef]
63. Popescu, P.-A.; Popa, V.I.; Frîncu, M.; Miteluț, A.C.; Popa, E.E.; Popa, M.E. In Vitro Research Study on the Antimicrobial Activity of Sea Buckthorn, Black Cumin and Grape Seed Essential Oils Against Selected Food Spoilage Fungi. *Sci. Pap. Ser. B Hortic.* **2022**, *66*, 857–862.
64. Singleton, V.L.; Rossi, J.A. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158.
65. Arnous, A.; Makris, D.P.; Kefalas, P. Effect of Principal Polyphenolic Components in Relation to Antioxidant Characteristics of Aged Red Wines. *J. Agric. Food Chem.* **2001**, *49*, 5736–5742. [CrossRef]
66. Villaño, D.; Fernández-Pachón, M.S.; Moyá, M.L.; Troncoso, A.M.; García-Parrilla, M.C. Radical scavenging ability of polyphenolic compounds towards DPPH free radical. *Talanta* **2007**, *71*, 230–235. [CrossRef] [PubMed]
67. Robinson, W.B.; Stotz, E. The Indophenol-Xylene Extraction Method for Ascorbic Acid and Modifications for Interfering Substances. *J. Biol. Chem.* **1945**, *160*, 217–225. [CrossRef]
68. Jafari, S.; Jouki, M.; Soltani, M. Modification of physicochemical, structural, rheological, and organoleptic properties of sweetened condensed milk by maltodextrin, fructose, and lactose. *J. Food Meas. Charact.* **2021**, *15*, 3800–3810. [CrossRef]
69. Eshghi, S.; Karimi, R.; Shiri, A.; Karami, M.; Moradi, M. Effects of polysaccharide-based coatings on postharvest storage life of grape: Measuring the changes in nutritional, antioxidant and phenolic compounds. *J. Food Meas. Charact.* **2022**, *16*, 1159–1170. [CrossRef]
70. Biquet, B.; Labuza, T.P. Evaluation of the Moisture Permeability Characteristics of Chocolate Films as an Edible Moisture Barrier. *J. Food Sci.* **1988**, *53*, 989–998. [CrossRef]
71. Alzamora, S.M.; Tapia, M.S.; López-Malo, A.; Welti-Chanes, J. Chapter 8—The control of water activity. In *Food Preservation Techniques*; Zeuthen, P., Bøgh-Sørensen, L., Eds.; Woodhead Publishing: Sawston, UK, 2003; pp. 126–153. ISBN 978-1-85573-530-9.
72. Parreidt, T.S.; Lindner, M.; Rothkopf, I.; Schmid, M.; Müller, K. The development of a uniform alginate-based coating for cantaloupe and strawberries and the characterization of water barrier properties. *Foods* **2019**, *8*, 21. [CrossRef]

73. Mosquera, L.H.; Moraga, G.; Martínez-Navarrete, N. Critical water activity and critical water content of freeze-dried strawberry powder as affected by maltodextrin and arabic gum. *Food Res. Int.* **2012**, *47*, 201–206. [CrossRef]
74. Riaz, A.; Aadil, R.M.; Amoussa, A.M.O.; Bashari, M.; Abid, M.; Hashim, M.M. Application of chitosan-based apple peel polyphenols edible coating on the preservation of strawberry (*Fragaria ananassa* cv Hongyan) fruit. *J. Food Process. Preserv.* **2021**, *45*, e15018. [CrossRef]
75. Choi, W.S.; Singh, S.; Lee, Y.S. Characterization of edible film containing essential oils in hydroxypropyl methylcellulose and its effect on quality attributes of 'Formosa' plum (*Prunus salicina* L.). *LWT* **2016**, *70*, 213–222. [CrossRef]
76. Dong, F.; Wang, X. Effects of carboxymethyl cellulose incorporated with garlic essential oil composite coatings for improving quality of strawberries. *Int. J. Biol. Macromol.* **2017**, *104*, 821–826. [CrossRef] [PubMed]
77. Aminifard, M.H.; Mohammadi, S. Essential oils to control Botrytis cinerea in vitro and in vivo on plum fruits. *J. Sci. Food Agric.* **2013**, *93*, 348–353. [CrossRef] [PubMed]
78. Dashipour, A.; Razavilar, V.; Hosseini, H.; Shojaee-Aliabadi, S.; German, J.B.; Ghanati, K.; Khakpour, M.; Khaksar, R. Antioxidant and antimicrobial carboxymethyl cellulose films containing Zataria multiflora essential oil. *Int. J. Biol. Macromol.* **2015**, *72*, 606–613. [CrossRef] [PubMed]
79. Perdones, A.; Sánchez-González, L.; Chiralt, A.; Vargas, M. Effect of chitosan—Lemon essential oil coatings on storage-keeping quality of strawberry. *Postharvest Biol. Technol.* **2012**, *70*, 32–41. [CrossRef]
80. Teixeira, B.; Marques, A.; Pires, C.; Ramos, C.; Batista, I.; Saraiva, J.A.; Nunes, M.L. Characterization of fish protein films incorporated with essential oils of clove, garlic and origanum: Physical, antioxidant and antibacterial properties. *LWT Food Sci. Technol.* **2014**, *59*, 533–539. [CrossRef]
81. Gol, N.B.; Patel, P.R.; Rao, T.V.R. Improvement of quality and shelf-life of strawberries with edible coatings enriched with chitosan. *Postharvest Biol. Technol.* **2013**, *85*, 185–195. [CrossRef]
82. de Oliveira Filho, J.G.; Albiero, B.R.; Calisto, Í.H.; Bertolo, M.R.V.; Oldoni, F.C.A.; Egea, M.B.; Bogusz Junior, S.; de Azeredo, H.M.C.; Ferreira, M.D. Bio-nanocomposite edible coatings based on arrowroot starch/cellulose nanocrystals/carnauba wax nanoemulsion containing essential oils to preserve quality and improve shelf life of strawberry. *Int. J. Biol. Macromol.* **2022**, *219*, 812–823. [CrossRef] [PubMed]
83. Mousavi, S.R.; Rahmati-Joneidabad, M.; Noshad, M. Effect of chia seed mucilage/bacterial cellulose edible coating on bioactive compounds and antioxidant activity of strawberries during cold storage. *Int. J. Biol. Macromol.* **2021**, *190*, 618–623. [CrossRef] [PubMed]
84. Cofelice, M.; Lopez, F.; Cuomo, F. Quality control of fresh-cut apples after coating application. *Foods* **2019**, *8*, 189. [CrossRef]
85. Solís-Contreras, G.A.; Rodríguez-Guillermo, M.C.; de la Luz Reyes-Vega, M.; Aguilar, C.N.; Rebolloso-Padilla, O.N.; Corona-Flores, J.; de Abril Alexandra Soriano-Melgar, L.; Ruelas-Chacon, X. Extending shelf-life and quality of minimally processed golden delicious apples with three bioactive coatings combined with cinnamon essential oil. *Foods* **2021**, *10*, 597. [CrossRef] [PubMed]
86. Thomas, A.B.; Nassur, R.D.C.M.R.; Boas, A.C.V.; Lima, L.C.D.O. Cassava starch edible coating incorporated with propolis on bioactive compounds in strawberries. *Ciência E Agrotecnologia* **2016**, *40*, 87–96. [CrossRef]
87. Guerreiro, A.C.; Gago, C.M.L.; Faleiro, M.L.; Miguel, M.G.C.; Antunes, M.D.C. The effect of edible coatings on the nutritional quality of 'Bravo de Esmolfe' fresh-cut apple through shelf-life. *LWT* **2017**, *75*, 210–219. [CrossRef]
88. Dávila-Aviña, J.E.; Villa-Rodríguez, J.A.; Villegas-Ochoa, M.A.; Tortoledo-Ortiz, O.; Olivas, G.I.; Ayala-Zavala, J.F.; González-Aguilar, G.A. Effect of edible coatings on bioactive compounds and antioxidant capacity of tomatoes at different maturity stages. *J. Food Sci. Technol.* **2014**, *51*, 2706–2712. [CrossRef] [PubMed]
89. Rocha, A.M.C.; Morais, A.M.M. Shelf life of minimally processed apple (cv. Jonagored) determined by colour changes. *Food Control* **2003**, *14*, 13–20. [CrossRef]
90. Duong, N.T.C.; Uthairatanakij, A.; Laohakunjit, N.; Jitareerat, P.; Kaisangsri, N. An innovative single step of cross-linked alginate-based edible coating for maintaining postharvest quality and reducing chilling injury in rose apple cv. "Tabtimchan" (*Syzygium samarangenese*). *Sci. Hortic.* **2022**, *292*, 110648. [CrossRef]
91. Kumar, P.; Sethi, S.; Sharma, R.R.; Singh, S.; Varghese, E. Improving the shelf life of fresh-cut 'Royal Delicious' apple with edible coatings and anti-browning agents. *J. Food Sci. Technol.* **2018**, *55*, 3767–3778. [CrossRef] [PubMed]
92. Farina, V.; Passafiume, R.; Tinebra, I.; Palazzolo, E.; Sortino, G. Use of Aloe vera gel-based edible coating with natural anti-browning and anti-oxidant additives to improve post-harvest quality of fresh-cut "Fuji" apple. *Agronomy* **2020**, *10*, 515. [CrossRef]
93. Xin, Y.; Yang, J.; Zhao, S.; Chen, K.; Zhao, Y.; Zhang, T.; Zhang, J. Sea buckthorn (*Hippophae rhamnoides* L.) oil exhibits antifungal activity against *Aspergillus flavus* via disrupting mitochondrial function. *Czech J. Food Sci.* **2022**, *40*, 179–186. [CrossRef]
94. Lukša, J.; Vepštaitė-Monstavičė, I.; Yurchenko, V.; Serva, S.; Servienė, E. High content analysis of sea buckthorn, black chokeberry, red and white currants microbiota—A pilot study. *Food Res. Int.* **2018**, *111*, 597–606. [CrossRef] [PubMed]
95. da Mata, I.R.; Bosco, S.M.D.; Garavaglia, J. Chapter 17—Different biological activities (antimicrobial, antitumoral, and antioxidant activities) of grape seed oil. In *Multiple Biological Activities of Unconventional Seed Oils*; Mariod, A., Ed.; Academic Press: Cambridge, MA, USA, 2022; pp. 215–227. ISBN 978-0-12-824135-6.
96. Velickova, E.; Winkelhausen, E.; Kuzmanova, S.; Alves, V.D.; Moldão-Martins, M. Impact of chitosan-beeswax edible coatings on the quality of fresh strawberries (*Fragaria ananassa* cv Camarosa) under commercial storage conditions. *LWT Food Sci. Technol.* **2013**, *52*, 80–92. [CrossRef]

foods

MDPI

Article

The Biotechnological Potential of *Pediococcus* spp. Isolated from Kombucha Microbial Consortium

Camelia Filofteia Diguță, George Daniel Nițoi, Florentina Matei *, Gabriela Luță and Călina Petruța Cornea

Faculty of Biotechnologies, University of Agronomic Sciences and Veterinary Medicine Bucharest, 59 Mărăști Blvd., 011464 Bucharest, Romania; camifilo@yahoo.com (C.F.D.); daniel.nitoi0@gmail.com (G.D.N.); glutza@yahoo.com (G.L.); pccornea@yahoo.com (C.P.C.)

* Correspondence: florentina.matei@biotehnologii.usamv.ro

Received: 29 October 2020; Accepted: 28 November 2020; Published: 1 December 2020

Abstract: In the past decade, the probiotic market has grown rapidly, both for foods and supplements intended to enhance wellness in healthy individuals. Different lactic acid bacteria (LAB), especially *Lactobacillus* spp., of different origins have already been used to develop commercial probiotic products. Nowadays, LAB new alternative sources, such as non-dairy fermented food products, are being exploited. One such source is Kombucha, a fermented low-alcohol beverage made of tea leaves. In this regard, we tested seven *Pediococcus* spp. strains isolated from a local industrial Kombucha for their biotechnological potential. Two, out of the seven isolates, identified as *Pediococcus pentosaceus* (L3) and *Pediococcus acidiliactici* (L5), were selected as successful candidates for the food industry, due to their probiotic and technological properties. In regard to their resistance in the gastro-intestinal tract, both selected strains were tolerant to a pH of 3.5, presence of 0.3% pepsin, and 0.5% bile salt concentration. On the antagonistic side, the fresh suspension of selected isolates had high inhibitory activity against pathogenic bacteria, such as *Salmonella enterica* Typhimurium, *Listeria monocytogenes, Listeria ivanovii, Bacillus cereus, Proteus hauseri,* and methicillin resistant *Staphylococcus aureus*. In addition, moderate to high inhibitory activity was noticed against foodborne molds (e.g., *Penicillium expansum* and *Penicillium digitatum*). These safety issues were supported by their negative hemolytic activity and good antioxidant potential (56–58%). Selected isolates were sensitive to ampicillin, penicillin, erythromycin, and lincomycin, while a broad range of other antibiotics were not effective inhibitors. On the technological side, both strains tolerated 5% NaCl and, during the freeze-drying process, had a good survival rate (86–92%). The selected *Pediococcus* strains have proven properties to be used for further development of functional products.

Keywords: lactic acid bacteria (LAB); *Pediococcus* spp.; Kombucha; probiotic; antimicrobial; lyophilization

1. Introduction

In 2002, the United Nations' Food and Agriculture Organization and the World Health Organization [1] defined probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host". From that moment until the present, important efforts have been made to develop probiotic commercial products, as well as products with prebiotics and synbiotics. It is generally accepted that these are likely to provide general health benefits for humans and animals, such as restoring the disturbed gut microbiota, regulating intestinal transit, competitively excluding pathogens from adhesion sites, and producing short chain fatty acids [2]. Consequently, the probiotic market has grown rapidly both for foods and supplements intended to enhance wellness in healthy individuals [3]. Different microorganisms of different origins have already been used to develop commercial probiotic products. The most common commercially available strains belong

to the *Lactobacillus* species (*casei, acidophilus, fermentum, gasseri, johnsonii, reuteri, plantarum, paracasei, rhamnosus* or *salivarius*) and *Bifidobacterium* species (*bifidum, breve, adolescentis, animalis*, or *longum*) [4]. The probiotic market trend indicates that there is still room for new products made of alternative probiotic sources that bode extended shelf lives, chemical stability, and are reasonably priced [5], all while reducing the risk of cholesterol problems in lactose intolerant people [6]. Different alternative sources have been exploited, i.e., mainly non-dairy fermented food products, such as traditional fermented foods, traditional fermented drinks, vegetables, and fruit juice. It has been demonstrated that the differences in raw materials and ingredients used to prepare such products are the main factors that lead to the different available species or strains of probiotics in food sources [7].

The main lactic acid bacteria (LAB) genera isolated from fermented plants and fermented meats is *Lactobacillus*. The most common LAB genera isolated from fermented seafoods is *Enterococcus* [7]. For example, the main LAB isolated from black olives were *L. pentosus* and *Leuconostoc mesenteroides* and from green olives were *L. pentosus*, *L. plantarum*, and *L. paracasei* [8,9]. *Pediococcus pentosaceous* strains were isolated from some traditional Thai fermented foods containing fish and pork [10] or from traditional Ethiopian fermented beef sausage [11].

Non-dairy fermented beverages, such as *Pozol*, *Bushera*, *Boza*, *Mahewu*, and *Togwa,* made of cereals, millets, legumes, fruits, and vegetables, are frequently reported as good sources of probiotics [12]. In the past decade, special attention has been paid to Kombucha, a fermented low-alcohol beverage made of tea leaves. Kombucha is reported to have several health benefits, such as antioxidant potential, antibacterial activity, and antiproliferative activity against cancer cell lines [13]. Different sources of Kombucha were characterized for their biochemical composition and complex microbial biodiversity (characterized by the presence of acetic acid bacteria, yeasts, and lactic acid bacteria). Generally, the consortium is dominated by acetic bacteria such as *Komagataeibacter* sp. and *Gluconobacter* sp., as well as yeast such as *Brettanomyces, Hanseniaspora*, *Saccharomyces, Torulaspora,* or *Schizosaccharomyces* [14,15]. Few studies reported on the interactions inside such complex microbial biodiversity and the focus was mainly on the acetic bacteria-yeast interaction [16]. The LAB are often, but not always, reported in Kombucha, and the main identified genera are *Lactobacillus* spp., *Lactococcus* spp., or *Leuconostoc* spp. [17,18]. Occasionally, *Pediococcus* spp. has been reported to belong to such consortium in industrial Kombucha sources [19]. It has been demonstrated that the viability of probiotic microorganisms is more difficult to maintain in non-dairy matrices than in dairy matrices and the physicochemical parameters must be carefully controlled to guarantee the probiotic viability [20]. Different strains of *Pediococcus* spp. isolated from other traditional fermented sources were reported as potential probiotics: *P. pentosaceus* from *Idly* batter, a traditional fermented food in South India [21]; *P. pentosaceus* and *P. acidiliactici* from *Omegisool*, a traditionally fermented millet alcoholic beverage in Korea [22]; *P. pentosaceous* strains isolated from *Wakalim*, the traditional Ethiopian fermented beef sausage [11]; or from *Kunu-zaki*, a Nigerian traditional fermented drink [23].

In the developmental process of new probiotic food products, the selection of probiotic microorganisms is the main challenge for food industries. The selection of probiotics from different sources requests screening for non-pathogenic microorganisms, which are further evaluated for some basic properties, such as the tolerance to gastro-intestinal environments, the ability to inhibit pathogens in the gastro-intestinal tract, resistance to antibiotics, adhesion potential, etc. Another important feature for industrial use is the viability during processing treatments and storage [24]. Our demarche targeted the screening of *Pediococcus* strains, formerly isolated from industrial Kombucha, for their probiotic potential and technological aspects. On the probiotic side, the isolates were tested for their resistance to the gastro-intestinal environment (i.e., low pH, pepsin, and bile salt presence), antagonism against different groups of pathogens, safety aspects (e.g., hemolytic activity, antioxidant activity), and antibiotic resistance. The technological aspects covered tolerance to NaCl presence, as a food additive, and the influence of the freeze-drying procedure on the cell viability, as a conditioning method for probiotics' industrial use.

2. Materials and Methods

2.1. Microorganisms

The tested LAB were formerly isolated in our laboratory from an industrial Kombucha source, made of green tea leaves, and provided by Medica FarmImpex SRL, Otopeni, Romania. All isolates (S1, S2, S3, L3, and F1) have been previously identified by sequencing and reported as belonging to the species *P. pentosaceus* [19], except two isolates (L5 and F2), which reclassified after further investigations as *P. acidilactici* (for F1 and F2 isolates the sequences analysis is provided in the Supplementary Information, Table S1).

In the case of the antagonistic interactions' study, different groups of pathogens were taken into account, from human pathogenic bacteria to foodborne molds, as detailed in Table 1.

Table 1. Pathogenic microorganisms used in the antagonistic tests.

Strain	Origin	Special Characteristics
Bacteria		
Escherichia coli ATCC 8739	ATCC®	-
Salmonella enterica Typhimurium ATCC 14028	ATCC®	-
Staphylococcus epidermidis ATCC 12228	ATCC®	vancomycin sensitive
Staphylococcus epidermidis ATCC 51625	ATCC®	Methicillin resistant
Staphylococcus aureus ATCC 25923	ATCC®	methicillin sensitive
Staphylococcus aureus ATCC 6538	ATCC®	methicillin sensitive
Staphylococcus aureus ATCC 43300	ATCC®	methicillin and oxacilin -resistant
Staphylococcus aureus ATCC 33592	ATCC®	methicillin and gentamicin -resistant
Listeria ivanovii ATCC 19119	ATCC®	resistant in acid medium
Listeria monocytogenes ATCC 7644	ATCC®	serogroup 1/2c
Proteus hauseri (vulgaris) ATCC 13315	ATCC®	-
Streptococcus pyogenes ATCC 19615	ATCC®	β-hemolytic
Bacillus cereus CP1	UASVM Bucharest	-
Yeast		
Candida albicans ATCC 10231	ATCC®	serotype A
Candida parapsilosis ATCC 20019	ATCC®	-
Candida guilermondii MI 40	UASVM Bucharest	-
Candida krusei MI 41	UASVM Bucharest	-
Molds		
Aspergillus niger M4	UASVM Bucharest	
Aspergillus carbonarius MI 15	UASVM Bucharest	
Aspergillus flavus MI 24	UASVM Bucharest	
Penicillium digitatum MI 22	UASVM Bucharest	
Penicillium expansum MI BB Huși	UASVM Bucharest	

2.2. Testing LAB Tolerance to Pepsin in Acidic pH

The tolerance to pepsin's presence and acidic pH was tested with an adapted method described by Chen et al. 2018 [25]. Fresh culture of LAB cultivated in a Man–Rogosa–Sharpe (MRS) broth medium (VWR, UK) for 18 h at 37 °C was centrifuged at 2000 g/10 min and the cell pellets was re-suspended and washed twice, under aseptic conditions, with sterile physiological saline solution (NaCl 0.9%). The LAB biomass was re-suspended in PBS (phosphate-buffered saline) supplemented with 0.3% pepsin (Merck); the pH was adjusted to 2.5 with 1 N HCl. The suspension was incubated at 37 °C and cell viability was calculated according to the formula:

$$\%\ viability = \frac{logCFU\ Nt}{logCFU\ Ni} \times 100$$

where *Ni* is the initial number of viable cells in the suspension and *Nt* the total viable cells after a specific incubation time. Colonies of the surviving LAB were counted after on-plate cultivation at

37 °C during 24 h, on MRS medium (VWR, UK). Samples were counted after 90 and 180 minutes' exposure to low pH and pepsin.

2.3. Testing LAB Tolerance to Bile Salts Presence

Bile salts influence on LAB was tested according to the method described by Adetoye et al. (2018) [26] with some modifications. The Kombucha LAB isolates were cultivated in MRS broth (VWR, UK) at 37 °C/4 h supplemented with different bile salt (Oxoid, UK) concentrations (0.5 and 1%). The sampling was done after 2 and 4 h of cultivation; dilutions were performed in sterile peptone (Oxoid, UK) water and spread on MRS-agar plate. Cell levels dynamic was measured by calculating CFU/mL After counting the colonies by the aid of a Colony Counter (Boeco CC-1, UK). All samples were performed in triplicate.

2.4. Testing the Inhibitory Activity of the LAB on Pathogenic Microorganisms by Disk-Diffusion Assay

The antagonistic activity of the Kombucha LAB on the pathogenic microorganisms has been tested by an adapted method of the disk-diffusion assay (Kirby–Bauer test). A double layer media was used; the bottom layer was MRS with 2% agar, while the upper layer contained 1% agar. For the pathogenic bacteria the employed medium was nutrient-agar, while for the yeast and molds PDA (potato dextrose agar) (VWR, UK). Fresh suspension (10 µL) of LAB cultivated on MRS broth for 18 h at 37 °C was inoculated in small wholes made in the first layer medium. After 48 h of LAB incubation at 35 °C, the pathogenic microorganisms were incorporated in the second layer of the media, containing 1% agar. After other 48 h of incubation at 35 °C, the inhibition zones were measured and all plates were performed in triplicate.

2.5. Testing the LAB Hemolytic Activity

The hemolytic activity of the LAB isolates was determined using the procedure described by Yadav et al. (2016) [27]. All the isolates tested were streaked onto blood agar plates (Oxoid, UK) containing 5% (*w*/*v*) sheep blood and incubated at 37 °C for 48 h. After incubation, the plates were examined for the zone of hemolysis around bacterial growth. If the zone around bacterial growth was clear, the bacteria were susceptible of β-hemolysis; when the zone become greenish, the tested bacteria produced α-hemolysis; if the microorganism did not produce hemolysins and did not break down the blood cells, no clearing occurred.

2.6. Antioxidant Assay

The free radical scavenging activities of the LAB suspension (10^9 CFU/mL) was determined using the stable free radical diphenyl picryl hydrazyl (DPPH) method according to the procedure adapted by Brand-Williams et al. (1995) [28] for complexes matrices. Briefly, 2 mL of 100 µM methanolic solution of DPPH was mixed with 1 mL of different LAB suspensions. The mixture was shaken vigorously and incubated for 30 min at room temperature, in a dark place. The change in color from deep violet to light yellow was then measured at 515 nm (A_{sample}). The percentage of the radical scavenging activity (RSA) was calculated by using the following equation: % RSA = $(1- [A_{sample}/A_{control\ t=0}])/100$. DPPH 100 µM solution in 80% methanol was used as a negative control.

2.7. Antibiotic Susceptibility Test

From the seven Kombucha LAB isolates, due to their biotechnological potential, two of them (L3 and L5) were used further to be tested for their antibiotic susceptibility different antibiotics (BioAnalyse, Turkey). The susceptibility of the LAB isolates was assessed on MRS agar plates using the antibiotic disc diffusion method. The overnight LAB cultures (100 µL) were spread on MRS agar plates and allowed to dry. The antibiotic discs were placed on the inoculated plates and incubated at 37 °C for 48 h. In the test we used ampicillin (AM-10), penicillin (P-2U), cephalexin (CL-30), cefaloridine (CPH-30), cefuroxime (sodium) (CXM-30), ceftriaxone (CRO-30), norfloxacin

(NOR-30), nalidixic acid (NA-30), amikacin (AK-10), erythromycin (E-10), gentamicin (CN-30), lincomicina (L-10), streptomycin (S-10), chloramphenicol (C-30), trimethoprim/sulphamethoxazole (SXT-1,25/23,75, SXT-25), nitrofurantoin (F-300), colistin (CT-10), vancomycin (VA-10), bacitracin (B-10U), amoxicillin/clavulanic acid, (AMC-20/10, AMC-30), tetracycline (TE-30), kanamycin (K-30), oxitetracycline (T-30), and fluconazole (FLU-10). The diameter of the zone of inhibition was measured using the antibiotic zone scale (CLSI scale). The results obtained are presented in terms of susceptibility, moderate susceptibility, or resistance. These results were compared with the interpretative zone diameters as described in the Performance Standards for Antimicrobial Disc Susceptibility Tests [29].

2.8. Testing the Kombucha Selected LAB for NaCl and pH Tolerance

The selected LAB (L3 and L5) were tested for their tolerance to different NaCl concentration and different pH, according to the method described by Prabhurajeshwar and Chandrakanth, (2019) [30] with some modifications. All tests were conducted in MRS broth. The NaCl (VWR, UK) concentrations was adjusted to 2.5%, 5%, 7.5%, 9.5%, and 11.5%; the pH tested values were 3.5, 5.5, 7.5, and 9.5. After the inoculation with 10^6 CFU/mL, the cultures were incubated at 37 °C for 72 h. The bacterial population evolution was performed by measuring the optical density at 600 nm and by counting the CFU/mL on plates with MRS agar. All experiments were performed in triplicates and ±SD was taken into account.

2.9. Testing the Behavior of Kombucha Selected LAB to the Lyophilization Procedure

Both selected isolates, L3 and L5, were subject to the lyophilization procedure. Two different cryoprotectors were used: sterile solution of 5% glucose and 5% sucrose (Oxoid, UK). As a control, sterile distilled water was employed. The lyophilization procedure included the following steps: bacterial biomass obtained after cultivation in MRS broth (37 °C/48 h) was recovered and washed twice with sterile physiological saline solution (NaCl 0.9%); after centrifugation at 5000 rpm/5 min/4 °C, the sediment was transferred in the cryoprotector solution and freezed overnight at −24 °C. As equipment was used a 6 L benchtop freeze-dryer from Labconco, USA. The lyophilization parameters were 0.04 mbar pressure and −55 °C temperature for 6 h. The cell viability was tested before and after the lyophilization procedure. Supplementary, the lyophilized biomass was tested for its antagonistic activity against several pathogenic microorganisms, as described above (Section 2.4).

2.10. Statistical Analysis

All of the parameters investigated were evaluated in a minimum of three independent determinations, and the results were expressed as the mean ± standard deviation (SD). The differences were analyzed by one-way analysis of variance (ANOVA). The significance level for the calculations was set as follows: significant, $p \leq 0.05$; very significant, $p \leq 0.01$; and highly significant, $p \leq 0.001$.

3. Results

3.1. LAB Tolerance to Pepsin Presence in Acid pH

In the intestinal tract, the potential probiotic microorganisms, should tolerate the environmental conditions found in the superior part of the tract, where the pH is very acidic and the pepsin is present. In our study, conducted on a pH of 2.5 and in the presence of 0.3% pepsin, it was noticed that, after 90 min exposure, the viable cell levels substantially decreased from 10^8–10^9 CFU/mL to 10^4 CFU/mL. After a longer exposure, of 90 cultivation minutes (a total of 3 h), the population level decreased with other one logarithmic unit, to 10^3 CFU/mL (Figure 1).) Isolates like L3, L5, and F2 have better adapted to a very acidic medium than the other isolates.

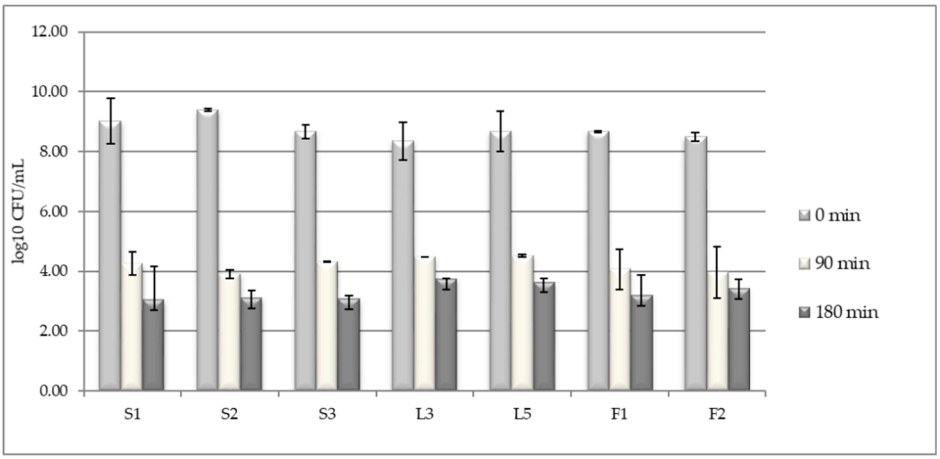
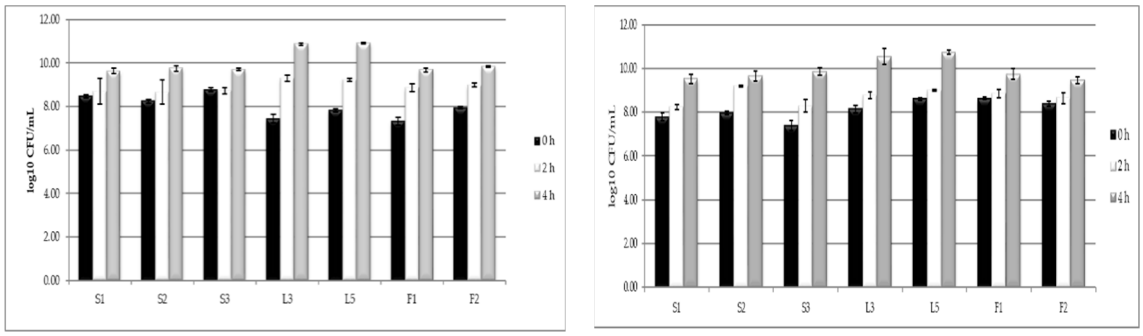


Figure 1. Tolerance of Kombucha lactic acid bacteria (LAB) isolates when cultivating at pH 2.5 and 0.3% pepsin. Data shown are mean ± standard deviation (SD) of triplicate values of independent experiments.

3.2. Testing LAB Tolerance to Bile Salts Presence

When taking into account the study of microorganisms, LAB has probiotic potential and thus different characteristics should be taken in account, as described above. The bile salt tolerance was tested at concentrations of 0.5% and 1%, while bile salts vary between 0.3% and 0.5% in the human digestive tract. The initial cell levels were 10^7–10^8 CFU/mL in all samples; in the first two hours of incubation, the cell levels increased significantly ($p \leq 0.05$) with one logarithmic unit (10^8–10^9 CFU/mL) when cultivating under 0.5% bile salts. At 1% salt concentration, no significant increases were measured (Figure 2). Under both concentrations, the isolates L3 and L5 proved to be tolerant to the bile salts presence, with a population level increased ($p \leq 0.01$) to 10^{10} CFU/mL after 4 cultivation hours.



(**a**) (**b**)

Figure 2. Tolerance of Kombucha LAB isolates to different bile salts concentration: (**a**) −0.5%; (**b**) −1%.

3.3. Inhibitory Activity of Kombucha LAB on Pathogenic Microorganisms

All lab isolates were tested for their inhibitory activity against microorganisms with pathogenic potentials. It was noticed that all isolates had high inhibitory activity against the major foodborne pathogens (Table 2) such as *Salmonella enterica* Typhimurium, *Listeria monocytogenes*, *Listeria ivanovii*, *Bacillus cereus*, *Proteus hauseri*, and other human pathogens responsible for major illnesses, such as methicillin resistant or methicillin sensible *Staphylococcus aureus*. The lower inhibitory activity was registered in the case of *E. coli*. Low to moderate inhibitory activity was noticed against the main potential pathogenic *Candida* spp., including a serotype A *Candida albicans*; the isolates S2, L3, and L5 are potential candidates for *C. albicans* moderate inhibition (Figure S1). No inhibitory activity was noticed against *Candida krusei*.

Table 2. Inhibitory activity of Kombucha LAB isolates on pathogenic microorganisms were tested using an adapted disk diffusion method.

Pathogenic Microorganisms	S1	S2	S3	L3	L5	F1	F2
Bacteria							
Escherichia coli ATCC 8739	+	+	+	+	+	+	+
Salmonella enterica Typhimurium ATCC 14028	+ + +	+ ++	+ + +	+ + +	+ +	+ + +	+ + +
Staphylococcus epidermidis ATCC 12228	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Staphylococcus epidermidis ATCC 51625	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Staphylococcus aureus ATCC 25923	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Staphylococcus aureus ATCC 6538	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ +
Staphylococcus aureus ATCC 43300	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Staphylococcus aureus ATCC 33592	+ +	+ +	+ +	+ + +	+ + +	+ +	+ + +
Listeria ivanovii ATCC 19119	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Listeria monocytogenes ATCC 7644	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Proteus hauseri (vulgaris) ATCC 13315	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Streptococcus pyogenes ATCC 19615	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Bacillus cereus CP1	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Yeast							
Candida albicans ATCC 10231	+	++	+	++	++	+	+
Candida parapsilosis ATCC 20019	+	+	+	++	++	+	+
Candida guilermondii MI 40	+	+	+	++	++	+	+
Candida krusei MI 41	-	-	-	-	-	-	-
Molds							
Aspergillus niger M4	+	+	-	+	+	-	+
Aspergillus carbonarius MI 15	+	+	+	++	++	++	+
Aspergillus flavus MI 24	+	+	-	+	+	-	-
Penicillium digitatum MI 22	+++	++	++	+++	+++	+++	+++
Penicillium expansum MI BB Huși	+++	+++	++	++	+++	+++	+++

Legend: (-) = no halo formation; (+) inhibition halo of 1–5 mm diameter; (++) halo of 6–17 mm diameter; (+++) halo of 18–29 mm diameter.

In relation to the foodborne molds, low to medium inhibition was noticed in the case of Aspergilli group. The highest inhibitory activity was exhibited by L3, L5, and F1 isolates on *Aspergillus carbonarius* (Figure S2). On both *Penicillium expansum* and *Penicillium digitatum,* the mycelial growth was inhibited moderately to high by the LAB isolates, except for the S3 isolate.

3.4. Hemolytic and Antioxidant Activity of Kombucha LAB Strains

The safety evaluation of the isolates was primarily determined by detecting their hemolytic activity, which proved the nonpathogenic status of the probiotic isolates. The results revealed no hemolytic activity (Figure S3), which was confirmed by the "no zone" in the test plates inoculated with all the isolates studied.

Regarding antioxidant activity, the control (MRS media without any inoculum) reached a 39.2% radical scavenging activity. The Kombucha LAB isolates radical scavenging activity varied between 48.8% and 58.0%. Significant differences ($p \leq 0.05$) were noticed between Control and all LAB tested isolates. No significant differences were observed between the isolates L5 (58%), F2 (57.6%), and L3 (56.6%), which showed the highest antioxidant activity (Figure 3).

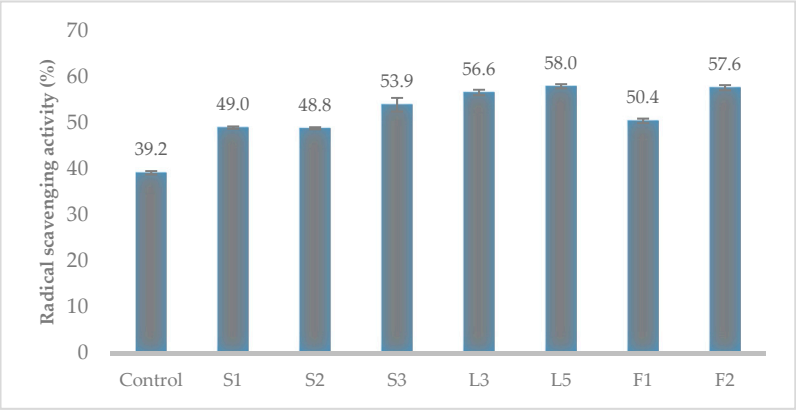


Figure 3. Radical scavenging activity of LAB strains isolated from industrial Kombucha. Data shown are mean ± SD of triplicate values of independent experiments.

3.5. Antibiotic Susceptibility of Selected LAB Isolates

The antibiotic susceptibility of the selected LAB isolates L3 and L5 was assessed on MRS agar plates using the antibiotic disc diffusion method using abroad range of antibiotics' classes. The variability of the antibiotic susceptibility can be observed in Table 3. The isolates have relatively similar patterns in their antibiotic susceptibility, with few exceptions, in which L3 isolate showed higher resistance. For both isolates, an important range from the tested antibiotics were not effective inhibitors, including fluoroquinolones, amoxicillin/clavulanic acid, cephalexin, cefuroxime, amikacin, streptomycin, kanamycin, sulphamethoxazole, vancomycin, tetracycline, colistin, and fluconazole. Kombucha's selected LAB were sensitive to ampicillin, penicillin, erythromycin, and lincomycin.

Table 3. Antibiotic susceptibility of LAB strains of *Pediococcus* spp. isolated from industrial Kombucha.

Antibiotic Classes/Antibiotic	LAB Isolates	
	L3 *P. pentosaceus*	**L5** *P.acidilactici*
Penicillins		
Ampicillin 10 µg/disc	MS	S
Penicillin 2 µg/disc	R	S
Amoxicillin/Clavulanic acid 20/10 µg/disc	R	R
Cephalosporins		
Cephalexin 30 µg/disc	R	R
Cefuroxime 30 µg/disc	R	R
Ceftriaxone 30 µg/disc	MS	MS
Fluoroquinolones		
Ciprofloxacin 1 µg/disc	R	R
Norfloxacin 30 µg/disc	R	R
Nalidixic acid 30 µg/disc	R	R
Aminoglycosides		
Amikacin 10 µg/disc	R	R
Gentamicin 10 µg/disc	MS	MS
Streptomycin 10 µg/disc	R	R
Kanamycin 30 µg/disc	R	R
Macrolides		
Erythromycin 10 µg/disc	S	S

Table 3. *Cont.*

Antibiotic Classes/Antibiotic	LAB Isolates	
	L3 *P. pentosaceus*	**L5** *P.acidilactici*
Lincosamide		
Lincomycin 10 µg/disc	S	S
Sulfonamides		
Sulphamethoxazole 25 µg/disc	R	R
Glycopeptides		
Vancomycin 10 µg/disc	R	R
Tetracyclines		
Tetracycline 30 µg/disc	R	MS
Oxytetracycline 30 µg/disc	MS	MS
Other		
Chloramphenicol 30 µg/disc	R	MS
Colistin 10 µg/disc	R	R
Bacitracin 10 U	R	MS
Fluconazole 10 µg/disc	R	R
Nitrofurantoin 300 µg/disc	R	MS

Legend: R, resistant; S, sensitive; MS, moderately sensitive.

3.6. Tolerance of the Selected LAB to NaCl and pH

Both L3 and L5 were tested for their behavior under different NaCl concentrations. The bacterial population dynamic of L3 under such environmental condition is represented in Figure 4. It was noticed that both L3 and L5 tolerate concentrations of 2.5% and 5% NaCl during 72 h of cultivation ($p \leq 0.05$); at the 7.5% NaCl concentration, a slight increase of the population in the case of L3 can be observed, while the increase for L5 was consistent (double that of L3). The level of the viable cells after 72 cultivation hours at 37 °C reached a significant level ($p \leq 0.05$) of 10^{12} CFU/mL in the control and at 2.5% NaCl, at 5% the level was 10^{1o} CFU/mL, for both L3 and L5. At 7.5% NaCl, the maximum population level was 10^{7} CFU/mL, while for L5 it was 10^{9} CFU/mL (data not shown). Regarding the pH tolerance, at pH 3.5 the isolate L5 behaved better (higher population level) than L3, while at higher pH (5, 7.5 and 9.5) both isolates exhibited similar behaviors. The significantly ($p \leq 0.05$) higher population levels (10^{12} CFU/mL) were counted for both isolates in the case of the pH of 7.5.

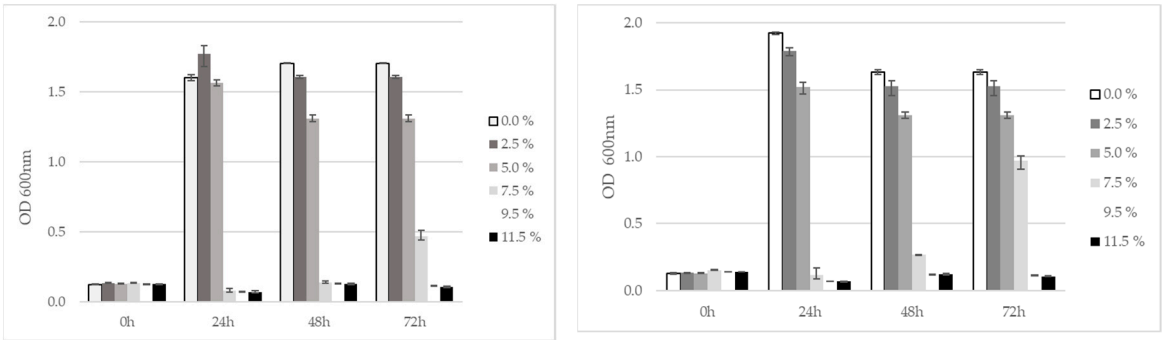


Figure 4. Tolerance to different NaCl concentrations of Kombucha L3 (**left**) and L5 (**right**) LAB isolates. Data shown are mean ± SD of triplicate values of independent experiments.

3.7. The LAB Behavior under Lyophilization Procedure

The isolates L3 and L5 were lyophilized in the presence of two different cryoprotectors (glucose and sucrose). The initial viable biomass for both isolates was adjusted to an initial content of 10^{10} CFU/mL. After the lyophilization, in the control (sterile distilled water) the viable cells levels was 10^5 CFU/mL (a 50% survival), whereas in both sugar solutions, L3 survival was 85–86% and L5 was slightly higher (91–92%). The lyophilized biomass was rehydrated and tested for the inhibitory activity against several pathogens. The severe environmental conditions applied during lyophilization did not affect the inhibitory activity of the rehydrated bacterial biomass on the tested pathogenic microorganisms, as provided in Table 4 (*Listeria* ssp., *Salmonella enterica Thyphimurium, Stahylococcus aureus* methicillin sensible and methicillin-resistant, *Bacillus cereus*, and *Candida albicans).*

Table 4. Inhibitory activity of lyophilized Kombucha LAB isolates L3 and L5 on pathogenic microorganisms tested by adapted disk diffusion method.

Pathogenic Microorganisms	L3	L5
Listeria ivanovii ATCC 19119	+ + +	+ + +
Listeria monocytogenes ATCC 7644	+ + +	+ + +
Salmonella enetrica Typhimurium ATCC 14028	+ + +	+ +
Staphylococcus aureus ATCC 25923	+ + +	+ + +
Staphylococcus aureus ATCC 6538	+ + +	+ + +
Staphylococcus aureus ATCC 43300	+ + +	+ + +
Staphylococcus aureus ATCC 33592	+ + +	+ + +
Bacillus cereus CP1	+ + +	+ + +
Candida albicans ATCC 10231	++	++

Legend: (-) = no halo formation; (+) inhibition halo of 1–5 mm diameter; (++) halo of 6–17 mm diameter; (+++) halo of 18–29 mm diameter.

4. Discussion

Nowadays, alternative isolation sources of probiotics, such as non-dairy fermented food products, are increasingly exploited. Kombucha, a fermented beverage made of tea leaves (*Camellia sinensis*) is one such source because of its complex SCOBY (symbiotic consortia of bacteria and yeast). The main reported LAB in this SCOBY are *Lactobacillus* spp., *Lactococcus* spp., or *Lecunosctoc* spp., while *Pediococcus* spp. was only occasionally isolated [17–19]. Our goal was to test the biotechnological potential of seven *Pediococcus* spp. strains isolated from a local industrial Kombucha, and identified formerly as *Pediococcus pentosaceus* [19] and *Pediococcus acidiliactici*. In an initial stage, all isolates were screened for a series of probiotics properties, such as their resistance in the gastro-intestinal tract, antagonistic activity against human/animal pathogens, foodborne molds, antioxidant potential, and hemolytic activity. Two isolates, L3 (*Pediococcus pentosaceus*) and L5 (*Pediococcus acidilactici*), were selected as potential probiotic candidates and further investigated for their antibiotic resistance and for their behavior against some technological parameters. In terms of technological aspects, the strains were tested for their tolerance to a food preservative (sodium chloride) and for their viability after applying extreme freeze-dried conditions, which is a frequent method used industrially to process probiotics. Former data [31] showed that L5 isolate have a good capacity to adhere in vitro to the surface of the Caco-2 cellular monolayer; it was proven that after four incubation hours, the bacterial cells start to form aggregates, suggesting a diffuse-aggregative adherence pattern.

The resistance in the gastro-intestinal tract in the presence of bile salts, pepsin, and acidic pH is one of the most important characteristics of the probiotic microorganisms. *P. pentosaceus* strains isolated from *Wakalim*, a traditional Ethiopian fermented beef sausage, are reported as tolerant to a pH of 3 and a 0.3% bile salt concentration [9], while strains isolated from *Kunu-zaki*, a Nigerian traditional fermented drink made from non-germinated sorghum and millet cereal grains proved to resist at the same pH but to a higher bile salt concentration of 3% [19]. In our case, the selected strains (L3 and L5) were tolerant to a pH of 2.5, 0.3% pepsin and 0.5% bile salt concentrations.

Numerous strains isolated from fermented beverages were reported as having antagonistic effect on pathogenic microorganism, mainly due to the production of organic acids and hydrogen peroxide. LAB strains of *Lactobacillus plantarum* isolated from a Turkish traditional fermented drink (*Boza*) showed antagonistic activity against pathogenic bacteria such as *Listeria monocytogenes, Bacillus subtilis, Bacillus cereus, Yersinia enterocolitica, Pseudomonas aeruginosa, E. coli, Salmonella enterica* Typhimurium, and *Klebsiella pneumonia*, even after the neutralization of the cell free supernatant. The authors imply the presence of bacteriocin's production [32]. *Kunu-zaki*, the Nigerian traditional fermented beverage, had isolated probiotic strains of *Lactobacillus, Pediococcus,* and *Lactococcus,* all of which were reported with inhibitory activity on *Pseudomonas aeruginosa, Staphylococcus aureus*, *E. coli*, and *Enterococcus faecalis* when used for active cell suspension [23]. The authors suggested that the results were caused by bacteriocins and organic acids production. *P. pentosaceus* isolated from other natural fermented sources, such as Kimchi (traditional Korean fermented vegetable), has been reported to inhibit *Listeria monocytogenes* due to the production of class II bacteriocins [33]. The species of *Pediococcus* genera are recognized for their ability to produce bacteriocin. Oher strains of *P. pentosaceus* isolated from paocai (a Chinese fermented vegetable) have been proven to have inhibitory activity against *E. coli* and *Salmonella enterica* Typhimurium [34]. It has also proven that *P. pentosaceus* strains isolated from traditional Thai meat fermented foods inhibited the growth of some pathogenic bacteria, such as *Salmonella enterica* Typhimurium, *Pseudomonas aeruginosa*, *Bacillus cereus, E. coli*, *Staphylococcus epidermidis,* or *Vibrio cholera* [10]. Our team has formerly reported bacteriocin production in the case of L5 Kombucha isolate [19], tested against *Streptococcus thermophilus.* Such strains and their bacteriocin can be used in food and feed industries as natural biopreservatives and for probiotic application to humans or livestock, including functional foods. All Kombucha isolates proved to have high inhibitory activity against the major foodborne pathogens, like *Salmonella enterica* Typhimurium, *Listeria monocytogenes, Listeria ivanovii, Bacillus cereus, Proteus hauseri,* as well as on resistant or methicillin sensible *Staphylococcus aureus*. As a novelty, we have proven the inhibitory activity of the *P. pentosaceus* isolated from Kombucha on the human emerging pathogen, *Listeria ivanovii,* known as ruminants' pathogen, as it can cause epidemic abortion, stillbirths, and encephalitis [35].

The LAB inhibitory activity on foodborne molds has often been reported on *Lactobacillus* spp. [36] and only occasionally in the case of *P. pentosacesus* or *P. acidilactici* isolated from natural sources. For instance, bacteriocin produced by *P. acidilactici* isolated from vacuum packed fermented meat products inhibited *A. fumigatus, A. parasiticus, F. oxyporum,* and *Penicillium* spp. [37], while *P. pentosaceus* isolated from malted cereals inhibited *Pencillium expansum* [38]. Strains from stored wheat samples showed antagonistic activity against different species such as *Alternaria alternata, Penicillium chrysogenum,* and *Aspergillus carbonarius* [39]. Although the same authors did not report any inhibition of *P. pentosacesus* on *A. niger,* our experiments found that the Kombucha LAB isolates exhibited low inhibitory activity, probably due to the low pH level of the suspension (pH of 4–4.5). There might be a correlation between our results and data reported on how Kombucha itself, made of green tea, and with a final pH of 3.5, have moderate inhibitory potential on molds like *Botrytis cinerea, Aspergillus carbonarius*, or *Penicillium expansum,* which proved on-plate and by in vivo artificial infections on grapes [40]. Both selected *Pediococcus* isolates (L3 and L5) have moderate to high inhibitory activity against *Penicillium expansum* and *Penicillium digitatum*, which suggest that our strains can be used in agricultural practices to control post-harvest mold development

The radical scavenging activity of LAB isolates is due to the colonization of viable cells and their propagation in the gut. Our results are comparable with studies reported before and Kombucha *Pediococcus* strains, such as L5, F2, and L3 LAB, which have an antioxidant activity of 56–58%. LAB isolated from *Neera* (fermented coconut palm nectar) can reach hydroxyl-scavenging activity of 32–77% [41], while for *Pediococcus* strains from *Omegisool* the DPPH radical-scavenging activity ranged between 30% and 39% [22].

Due to safety considerations, the obtained isolates were also tested for antibiotic resistance. The transmission of antibiotic resistance genes to potentially pathogenic bacteria in the gut is

a major health concern related to the probiotic application [42]. The European Food Safety Authority (EFSA) recommends that bacterial strains harboring transferable antibiotic resistance genes should not be used in animal feeds or fermented and probiotic foods for human use. For an appropriate selection of functional strains, two main antibiotics' groups are recommended in EFSA guidelines to be tested, such as inhibitors of protein synthesis (chloramphenicol, gentamycin, clindamycin, erythromycin, streptomycin, kanamycin, and tetracycline) and inhibitors of cell wall synthesis (ampicillin and vancomycin). In the case of LAB isolated from different natural sources, such as fermented coconut palm nectar, and belonging to other species (*Lactobacillus brevis, Enterococcus durans, Leuconostoc lactis, Enterococcus lactis, and Enterococcus faecium*), chloramphenicol, vancomycin, and streptomycin were effective inhibitors [41]. *Pediococcus* isolated from *Omegisool* are shown to be resistant to chloramphenicol [22]. Both Kombucha *Pediococcus* isolates (L3 and L5) are sensitive to ampicillin, penicillin, erythromycin, and lincomycin, while a broad range of other antibiotics are not effective inhibitors (fluoroquinolones, amoxicillin/clavulanic acid, cephalexin, cefuroxime, amikacin, streptomycin, kanamycin, sulphamethoxazole, vancomycin, and tetracycline). In this regard, before using these isolates in food or feed formulations per EFSA guidelines, virulence and antimicrobial resistance genes should be verified to prevent the horizontal gene transfer for antibiotic resistance.

The incorporation of probiotic bacteria to food products represents a major technological challenge because of the known sensitivity of these microorganisms to salt, spices, and other substances used in its formulation. In relation to the NaCl tolerance, the most halophilic LAB (*Enterococcus* ssp., *Lactobacillus* ssp.) were isolated from seafoods and fermented meats, being able to grew under a NaCl concentration of more than 22% [7]. Meanwhile *L. fermentum* and *L. plantarum* isolated from fermented plants grew under NaCl concentrations of less than 6% [43]. Strains of *P. pentosaceus* isolated from various traditional Thai fermented foods containing fish and pork were reported as tolerant up to 14% NaCl in an acid medium (pH 2) with 0.3–0.5% bile salt [10]. Our Kombucha isolates (L3 and L5) tolerated 5% NaCl. Furthermore, L5, after a period of adaptation, is tolerant to 7.5% NaCl. Thus, we concluded that L3 and L5 were resistant to salt concentrations used in industrial levels and maintained a concentration suitable for carrying probiotic effect.

According to Champagne et al. (2011) [44], a product containing probiotic organisms is efficient if it contains a number of viable cells higher than 10^6–10^8 CFU/g. However, viability and optimum concentration of probiotic microorganism is still under debate, but the trend is to have a minimum of one billion viable cells per 100 g of product to declare it as a probiotic functional product [45]. Lyophilization is one of the procedures used to deliver probiotics for commercial products. Regarding the lyophilization procedure, the survival rate may be improved by the use of other cryoprotectants, such as poly-glutamic acid, which was successfully used for the protection of probiotic *Lactobacillii* [45]. In the case of our *Pediococcus* isolates, by the use of glucose or sucrose as cryopretectant, we obtained a good viability rate of 86–92%. In addition, lyophilization did not affect the inhibitory activity on the tested pathogenic microorganisms. The initial biomass of 10^{10} CFU/mL conditioned by freeze-drying procedure, recovered levels of 10^9 CFU/g after rehydration. Rehydration is a normal industrial step when using such dried strains, and has a major influence on the CFU readings obtained. We concluded that our strains were suitable to be conditioned by lyophilization and employed industrially after rehydration.

From a larger biotechnological point of view, probiotic bacteria can also be used as ingredients in cosmetic products. *P. acidilactici* strain isolated from Korean Perilla Leaf Kimchi was proven to a have direct melanin-degrading and tyrosinase-inhibiting effects, given that it has high value as a raw material for melanin degradation drugs and cosmetics [46]. However, there are still technical barriers to incorporate live probiotics into conventional skincare products with a reasonable shelf life. Some results are reported when using *Lactobacilli*, and the solution was to add probiotic ingredients which were not alive or viable to form colonies to the formulation at the end of the manufacturing process. Another strain of *P. pentosaceus* isolated from kaki fruit increased the antioxidative and aging activities of the *Lavandula angustifolia* extract through fermentation, so it was proposed to be used as an

anti-aging agent [47]. Our *Pediococcus* isolates are expected to be further tested for such properties for further industrial application.

5. Conclusions

In our effort to find new natural resources and to develop new biotechnological solutions for food and pharmaceutical industries, we investigated several *Pediococcus* strains isolated from a local Kombucha source. Two of the strains, L3 (*P. penteosaceus*) and L5 (*P. acidilactici*), proved to have properties related to their potential use in the development of functional products, due to their tolerance to acid pH, the presence of pepsin and bile salts, resistance to a large range of different antibiotic classes, high antioxidant potential, and inhibitory activity among a large range of foodborne bacteria and fungi. Both strains were resistant to high NaCl concentrations and to the invasive lyophilization procedure, being good candidates for the industrial use.

Supplementary Materials: The following are available online at http://www.mdpi.com/2304-8158/9/12/1780/s1, Figure S1: On-plate aspects of the Kombucha LAB inhibitory activity on *Candida albicans* ATCC 10231 (Ca), *Staphylococcus aureus* ATCC 33592 methicilin resistant (Sa 33592), *Staphylococcus aureus* ATCC 25923 methicilin sensitive (Sa 25923), *Salmonella typhimurium* ATCC 14028 (St) tested by adapted disk diffusion assay, Figure S2: Aspects of the Kombucha LAB inhibitory activity on *Aspergillus carbonarius* MI 15 (left) and *Penicillium digitatum* MI 22 (right) tested by adapted disk diffusion assay, Figure S3: On-plate test of haemolytic activity of Kombucha LAB isolates on blood agar mediu, Table S1: Results of the molecular identification of Kombucha LAB F1 and F2 by 16S rDNA sequencing with Lac primers.

Author Contributions: Conceptualization, F.M and C.P.C.; methodology, G.D.N., C.F.D., and G.L; validation, F.M. and C.P.C.; formal analysis, G.D.N. and C.F.D.; investigation, G.D.N., C.F.D., and G.L.; resources, F.M. and C.F.D.; data curation, F.M and C.F.D.; writing—original draft preparation, G.D.N.; writing—review and editing, F.M and C.F.D; visualization, F.M.; supervision, C.P.C.; project administration, F.M.; funding acquisition, C.P.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partially funded by the Romanian national funding program PN-III-CERC-CO-BG-2016 through the project 6BG/2016 (3-BIOTIC).

Acknowledgments: Special thanks go to Ionut Moraru from Medica Farmipex SRL Otopeni, Romania who kindly provided the Kombucha SCOBY consortium.

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

References

1. FAO/WHO. *FAO/WHO Joint Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food (30 April 2002 and 1 May 2002)*; Scientific Research Publishing: London, ON, Canada, 2002.
2. Hill, C.; Guarner, F.; Reid, G.; Gibson, G.R.; Merenstein, D.J.; Pot, B.; Morelli, L.; Canani, R.B.; Flint, H.J.; Salminen, S.; et al. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* **2014**, *11*, 506–514. [CrossRef] [PubMed]
3. De Simone, C. The Unregulated Probiotic Market. *Clin. Gastroenterol. Hepatol.* **2019**, *17*, 809–817. [CrossRef] [PubMed]
4. Panghal, A.; Janghu, S.; Virkar, K.; Gat, Y.; Kumar, V.; Chhikara, N. Potential non-dairy probiotic products—A healthy approach. *Food Biosci.* **2018**, *21*, 80–89. [CrossRef]
5. Dey, G. Non-dairy Probiotic Foods: Innovations and Market Trends. In *Innovations in Technologies for Fermented Food and Beverage Industries. Food Microbiology and Food Safety*; Panda, S., Shetty, P., Eds.; Springer: Cham, Switzerland, 2018. [CrossRef]
6. Oak, S.J.; Jha, R. The effects of probiotics in lactose intolerance: A systematic review. *Crit. Rev. Food Sci. Nutr.* **2019**, *59*, 1675–1683. [CrossRef]
7. Sornplang, P.; Piyadeatsoontorn, S. Probiotic isolates from unconventional sources: A review. *J. Anim. Sci. Technol.* **2016**, *58*, 1–11. [CrossRef]
8. Argyri, A.A.; Zoumpopoulou, G.; Karatzas, K.-A.G.; Tsakalidou, E.; Nychas, G.-J.E.; Panagou, E.Z.; Tassou, C.C. Selection of potential probiotic lactic acid bacteria from fermented olives by in vitro tests. *Food Microbiol.* **2013**, *33*, 282–291. [CrossRef] [PubMed]

9. Doulgeraki, A.I.; Pramateftaki, P.; Argyri, A.A.; Nychas, G.-J.E.; Tassou, C.C.; Panagou, E.Z. Molecular characterization of lactic acid bacteria isolated from industrially fermented Greek table olives. *LWT* **2013**, *50*, 353–356. [CrossRef]
10. Siripornadulsil, W.; Tasaku, S.; Buahorm, J.; Siripornadulsil, S. Probiotic properties of lactic acid bacteria isolated from fermented food. *Intl. J. Biol. Food Vet Agric. Eng.* **2014**, *8*, 364–366.
11. Bacha, K.; Mehari, T.; Ashenafi, M. In-vitro probiotic potential of lactic acid bacteria isolated from 'Wakalim', a traditional Ethiopian fermented beef sausage. *Ethiop. J. Health Sci.* **2009**, *19*, 21–29.
12. Krishnamoorthy, M.; Arjun, P. Probiotic and antimicrobial activity of bacteria from fermented toddy of *Cocus nucifera*. *J. Acad. Indus. Res.* **2012**, *1*, 127–131.
13. Cardoso, R.R.; Neto, R.O.; D'Almeida, C.T.D.S.; Nascimento, T.P.D.; Pressete, C.G.; Azevedo, L.; Martino, H.S.D.; Cameron, L.C.; Ferreira, M.S.L.; Barros, F. Kombuchas from green and black teas have different phenolic profile, which impacts their antioxidant capacities, antibacterial and antiproliferative activities. *Food Res. Int.* **2020**, *128*, 108782. [CrossRef] [PubMed]
14. Jayabalan, R.; Malbaša, R.V.; Lončar, E.S.; Vitas, J.S.; Sathishkumar, M. A Review on Kombucha Tea-Microbiology, Composition, Fermentation, Beneficial Effects, Toxicity, and Tea Fungus. *Compr. Rev. Food Sci. Food Saf.* **2014**, *13*, 538–550. [CrossRef]
15. Villarreal-Soto, S.A.; Beaufort, S.; Bouajila, J.; Souchard, J.-P.; Taillandier, P. Understanding Kombucha Tea. Fermentation: A Review. *J. Food Sci.* **2018**, *83*, 580–588. [CrossRef] [PubMed]
16. Tran, T.; Grandvalet, C.; Verdier, F.; Martin, A.; Alexandre, H.; Tourdot-Maréchal, R. Microbial Dynamics between Yeasts and Acetic Acid Bacteria in Kombucha: Impacts on the Chemical Composition of the Beverage. *Foods* **2020**, *9*, 963. [CrossRef]
17. Marsh, A.J.; O'Sullivan, O.; Hill, C.; Ross, R.P.; Cotter, P.D. Sequence-based analysis of the bacterial and fungal compositions of multiple kombucha (tea fungus) samples. *Food Microbiol.* **2014**, *38*, 171–178. [CrossRef] [PubMed]
18. Chakravorty, S.; Bhattacharya, S.; Chatzinotas, A.; Chakraborty, W.; Bhattacharya, D.; Gachhui, R. Kombucha tea fermentation: Microbial and biochemical dynamics. *Int. J. Food Microbiol.* **2016**, *220*, 63–72. [CrossRef]
19. Matei, B.; Salzat, J.; Diguta, C.F.; Cornea, C.P.; Luta, G.; Utoiu, E.R.; Matei, F. Lactic acid bacteria isolated from Kombucha as potential probiotics. Rom. Biotech. *Letters* **2018**, *23*, 13592–13598. [CrossRef]
20. Marsh, A.J.; Hill, C.; Ross, R.P.; Cotter, P.D. Fermented beverages with health-promoting potential: Past and future perspectives. *Trends Food Sci. Technol.* **2014**, *38*, 113–124. [CrossRef]
21. Vidhyasagar, V.; Jeevaratnam, K. Evaluation of Pediococcus pentosaceus strains isolated from Idly batter for probiotic properties in vitro. *J. Funct. Foods* **2013**, *5*, 235–243. [CrossRef]
22. Oh, Y.J.; Jung, D.S. Evaluation of probiotic properties of Lactobacillus and Pediococcus strains isolated from Omegisool, a traditionally fermented millet alcoholic beverage in Korea. *LWT* **2015**, *63*, 437–444. [CrossRef]
23. Oluwajoba, S.O.; Akinyosoye, F.A.; Oyetayo, V.O. In Vitro Screening and Selection of Probiotic Lactic Acid Bacteria Isolated from Spontaneously Fermenting Kunu-Zaki. *Adv. Microbiol.* **2013**, *3*, 309–316. [CrossRef]
24. Ventura, M.; Perozzi, G. Introduction to the special issue "Probiotic bacteria and human gut microbiota & rdquo. *Genes Nutr.* **2011**, *6*, 203–204. [CrossRef] [PubMed]
25. Chen, S.; Chen, L.; Chen, L.; Ren, X.; Ge, H.; Li, B.; Ma, G.; Ke, X.; Zhu, J.; Li, L.; et al. Potential probiotic characterization of Lactobacillus reuteri from traditional Chinese highland barley wine and application for room-temperature-storage drinkable yogurt. *J. Dairy Sci.* **2018**, *101*, 5780–5788. [CrossRef] [PubMed]
26. Adetoye, A.; Pinloche, E.; Adeniyi, B.A.; Ayeni, F.A. Characterization and anti-salmonella activities of lactic acid bacteria isolated from cattle faeces. *BMC Microbiol.* **2018**, *18*, 96. [CrossRef] [PubMed]
27. Yadav, R.; Puniya, A.K.; Shukla, P. Probiotic Properties of Lactobacillus plantarum RYPR1 from an Indigenous Fermented Beverage Raabadi. *Front. Microbiol.* **2016**, *7*, 1683. [CrossRef]
28. Brand-Williams, W.; Cuvelier, M.; Berset, C. Use of a free radical method to evaluate antioxidant activity. *LWT* **1995**, *28*, 25–30. [CrossRef]
29. CLSI. *CLSI Supplement M100S. Performance Standards for Antimicrobial Susceptibility Testing*, 26th ed.; Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2016.
30. Prabhurajeshwar, C.; Chandrakanth, K. Evaluation of antimicrobial properties and their substances against pathogenic bacteria in-vitro by probiotic Lactobacilli strains isolated from commercial yoghurt. *Clin. Nutr. Exp.* **2019**, *23*, 97–115. [CrossRef]

31. Utoiu, E.; Oancea, A.; Stanciuc, A.M.; Stefan, M.L.; Toma, A.; Moraru, A.; Diguta, C.F.; Matei, F.; Cornea, C.P.; Oancea, F. Prebiotic content and probiotic effect of kombucha fermented pollen. *AgroLife Sci. J.* **2018**, *7*, 149–156.
32. Kivanc, M.; Yilmaz, M.; Cakir, E. Isolation and identification of lactic acid bacteria from Boza, and their microbial activity against several reporter strains. *Turk. J. Biol.* **2011**, *35*, 313–324. [CrossRef]
33. Shin, M.; Han, S.; Ryu, J.; Kim, K.; Wan-Kyu, L. Isolation and partial characterization of a bacteriocin produced by Pediococcus pentosaceusK23-2 isolated from Kimchi. *J. Appl. Microbiol.* **2008**, *105*, 331–339. [CrossRef]
34. Cao, Z.; Pan, H.; Tong, H.; Gu, D.; Li, S.; Xu, Y.; Ge, C.; Lin, Q. In vitro evaluation of probiotic potential of Pediococcus pentosaceus L1 isolated from paocai—a Chinese fermented vegetable. *Ann. Microbiol.* **2015**, *66*, 963–971. [CrossRef]
35. Chen, J.-Q.; Regan, P.; Laksanalamai, P.; Healey, S.; Hu, Z. Prevalence and methodologies for detection, characterization and subtyping of Listeria monocytogenes and L. ivanovii in foods and environmental sources. *Food Sci. Hum. Wellness* **2017**, *6*, 97–120. [CrossRef]
36. Dalié, D.; Deschamps, A.; Richard-Forget, F. Lactic acid bacteria—Potential for control of mould growth and mycotoxins: A review. *Food Control.* **2010**, *21*, 370–380. [CrossRef]
37. Mandal, V.; Sen, S.K.; Mandal, N.C. Detection, Isolation and Partial Characterization of Antifungal Compound(s) Produced by Pediococcus acidilactici LAB 5. *Nat. Prod. Commun.* **2007**, *2*, 671–674. [CrossRef]
38. Rouse, S.; Harnett, D.; Vaughan, A.; Van Sinderen, D. Lactic acid bacteria with potential to eliminate fungal spoilage in foods. *J. Appl. Microbiol.* **2008**, *104*, 915–923. [CrossRef]
39. Belkacem-Hanfi, N.; Fhoula, I.; Semmar, N.; Guesmi, A.; Perraud-Gaime, I.; Ouzari, H.-I.; Boudabous, A.; Roussos, S. Lactic acid bacteria against post-harvest moulds and ochratoxin A isolated from stored wheat. *Biol. Control.* **2014**, *76*, 52–59. [CrossRef]
40. Matei, B.; Matei, F.; Diguta, C.; Popa, O. Potential use of Kombucha crude extract in postharvest grape moulds control. *Sci. Bull. Ser. F Biotechnol.* **2017**, *XXI*, 77–80.
41. Somashekaraiah, R.; Shruthi, B.; Deepthi, B.V.; Sreenivasa, M.Y. Probiotic Properties of Lactic Acid Bacteria Isolated from Neera: A Naturally Fermenting Coconut Palm Nectar. *Front. Microbiol.* **2019**, *10*, 1382. [CrossRef]
42. Sharma, P.; Tomar, S.K.; Goswami, P.; Sangwan, V.; Singh, R. Antibiotic resistance among commercially available probiotics. *Food Res. Int.* **2014**, *57*, 176–195. [CrossRef]
43. Settanni, L.; Moschetti, G. Non-starter lactic acid bacteria used to improve cheese quality and provide health benefits. *Food Microbiol.* **2010**, *27*, 691–697. [CrossRef]
44. Champagne, C.P.; Ross, R.P.; Saarela, M.; Hansen, K.F.; Charalampopoulos, D. Recommendations for the viability assessment of probiotics as concentrated cultures and in food matrices. *Int. J. Food Microbiol.* **2011**, *149*, 185–193. [CrossRef] [PubMed]
45. Gomaa, E.Z. Cryoprotection of probiotic bacteria with poly-γ-glutamic acid produced by Bacillus subtilis and Bacillus licheniformis. *J. Genet. Eng. Biotechnol.* **2016**, *14*, 269–279. [CrossRef] [PubMed]
46. Kim, S.; Seo, H.; Al Mahmud, H.; Islam, I.; Sultana, O.F.; Lee, Y.; Kim, M.; Song, M.K.A.H.-Y. Melanin Bleaching and Melanogenesis Inhibition Effects of Pediococcus acidilactici PMC48 Isolated from Korean Perilla Leaf Kimchi. *J. Microbiol. Biotechnol.* **2020**, *30*, 1051–1059. [CrossRef] [PubMed]
47. Ha, J.H.; Kim, A.R.; Lee, K.-S.; Xuan, S.H.; Kang, H.C.; Lee, D.H.; Cha, M.Y.; Kim, H.J.; An, M.; Park, S.N. Anti-Aging Activity of Lavandula angustifolia Extract Fermented with Pediococcus pentosaceus DK1 Isolated from Diospyros kaki Fruit in UVB-Irradiated Human Skin Fibroblasts and Analysis of Principal Components. *J. Microbiol. Biotechnol.* **2019**, *29*, 21–29. [CrossRef] [PubMed]

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

nutrients

MDPI

Article

Bee Collected Pollen with Enhanced Health Benefits, Produced by Fermentation with a Kombucha Consortium

Elena Uțoiu [1,2,†], Florentina Matei [1,†], Agnes Toma [2], Camelia Filofteia Diguță [1], Laura Mihaela Ștefan [2], Sorin Mănoiu [2], Virgil Valeriu Vrăjmașu [1], Ionuț Moraru [3], Anca Oancea [2], Florentina Israel-Roming [1], Călina Petruța Cornea [1], Diana Constantinescu-Aruxandei [4], Angela Moraru [1,3] and Florin Oancea [1,4,*]

1 Faculty of Biotechnologies, University of Agronomic Sciences and Veterinary Medicine of Bucharest, 011464 Elena, Romania; Elena.Utoiu@gmail.com (E.U.); Florentina.Matei@biotehnologii.usamv.ro (F.M.); camifilo@yahoo.com (C.F.D.); VirgilVrajamasu@yahoo.com (V.V.V.); florentinarom@yahoo.com (F.I.-R.); pccornea@yahoo.com (C.P.C.); Angela.Moraru@medicagroup.ro (A.M.)

2 Department of Cellular and Molecular Biology, National Institute of Research and Development for Biological Sciences, 060031 Elena, Romania; agnes12ro@yahoo.com.au (A.T.); LauraMihaelaStefan@yahoo.com (L.M.S.); spagiricus@yahoo.com (S.M.); Oancea.Anca@gmail.com (A.O.)

3 Medica Laboratories Srl, 075100 Elena, Romania; IonutMoraru@pro-natura.ro

4 Departments of Biotechnology and Bioresources, National Institute for Research & Development in Chemistry and Petrochemistry-ICECHIM, 060021 Elena, Romania; doro31@gmail.com

* Correspondence: Florin.Oancea@icechim.ro; Tel.: +40-21-316-3071

† These authors contributed equally.

Received: 21 August 2018; Accepted: 16 September 2018; Published: 23 September 2018

Abstract: The bioavailability of pollen bioactive compounds for humans is limited. In this study, our aim was to enhance the health-related benefits of pollen by fermentation with a Kombucha/SCOBY (symbiotic culture of bacteria and yeasts) consortium. We performed the fermentation of pollen suspended from the beginning with SCOBY on sweetened green tea or on Kombucha vinegar, by adding pollen after 20 days of Kombucha fermentation. We analyzed: formation of bioactive compounds (anti-oxidant polyphenols, soluble silicon, hydroxy-acids, short chain fatty acids—SCFA); parameters related to Kombucha fermentation (dynamics of lactic acid bacteria—LAB, formation of organic acids, soluble sugar evolution on Kombucha vinegar); the influence of Kombucha fermentation on pollen morphology and ultrastructure; in vitro cytotoxic and antitumoral effects of the Kombucha fermented pollen. The pollen addition increases LAB proportion in the total number of SCOBY microbial strains. SEM images highlight the adhesion of the SCOBY bacteria to pollen. Ultrastructural analysis reveals the release of the pollen content. The content of bioactive compounds (polyphenols, soluble silicon species and SCFA) is higher in the fermented pollen and the product shows a moderate antitumoral effect on Caco-2 cells. The health benefits of pollen are enhanced by fermentation with a Kombucha consortium.

Keywords: pollen; multi-floral; bee collected; fermentation; symbiotic culture of bacteria and yeasts—SCOBY; lactic acid bacteria—LAB; anti-oxidant polyphenols; soluble silicon; short-chain fatty acids—SCFA

1. Introduction

Bee collected pollen is a "superfood", a nutraceutical with high biological value ingredients, e.g., essential amino acids, (pro)vitamins, essential fatty acids, minerals and anti-oxidant polyphenols [1].

Due to such phytonutrients with high biological value, commercial mixtures of multi-floral, bee collected pollen, have been proven to have anti-microbial, antimutagenic, antioxidant and anti-inflammatory effects [2]. Antitumoral, immuno-modulatory and anti-metabolic syndrome (cardio-protective, anti-hypertensive, anti-atherosclerotic and anti-diabetic) activities were also reported for bee (collected) pollen [3]. Recently, a modulation effect exerted by carbohydrates from bee pollen on gut microbiota was suggested [4]. However, the bioavailability of phytonutrients from bee pollen is significantly limited by the complex structure of the pollen grain wall and the recalcitrance to biodegradation of the rigid pollen outer wall, exine [5]. The exine resistance results from its major component, sporopollenin, a biopolymer with extremely high stability and very high resistance to (bio)degradation, including to the action of digestive enzymes [6]. Only the animals which developed mechanisms for phytonutrients extraction from pollen grains (e.g., mechanical crack-up, pseudogermination/fake germination, osmotic shock, outer wall piercing sharp mouth parts) are able to use more than 50% of the pollen grain content [7]. Humans have not developed such mechanisms.

Various mechanical, chemical and biotechnological processes have been proposed to enhance the bioavailability of phytonutrients from bee collected pollen. A non-exhaustive list includes: high-shearing [8], thermal shocks, alone or combined with enzymatic treatment [5], wet milling [9], ultrasonication [10] combined with high-shearing [11] or enzymatic treatment [12], chemical extraction of polyphenols with supercritical fluid [13], fermentation [14–18].

Pollen fermentation is similar to the naturally evolved processes, targeted on enhancement of pollen nutritional value. In the bee hive, pollen is fermented and stored with honey, into wax sealed honey combs. The resulting products, the bee bread, combine the pollen phytonutrients (with increased bioavailability) and bioactive compounds formed during pollen fermentation [19]. As a result of such a combination, bee bread has a higher content in essential amino acids and vitamins (e.g., from B and K groups) and more bioactive polyphenols [20]. Lactic acid bacteria [21], yeasts [22] and endospore forming gram negative bacteria [23] are involved into pollen fermentation and bee brood formation.

Biotechnological alternatives to bee bread have been proposed. The bee bread is not produced in large quantities and it is difficult to harvest from the honey combs. Several processes of (semi)solid pollen fermentation were developed in an attempt to produce products similar to bee bread [20]. Enhancement of antioxidant polyphenols in fermented pollen was previously reported [5,14]. The addition of pollen to products resultant from lactic acid bacteria and/or yeasts fermentation has been also tested. One of the main results of such biotechnological processes was a better quality of the fermented products. Better white wines were produced after inclusion of pollen into white grape must [24]. Pollen addition also increases the quality of the yogurt [25] and of the other milk fermented beverages [25].

Our aim was to obtain a complex product, which includes various health-related compounds, by fermentation of the bee collected pollen with a Kombucha consortium, a symbiotic colony of bacteria and yeast (SCOBY). Kombucha is an oriental traditional beverage, produced from sweetened green or black tea by fermentation with a symbiotic consortium of bacteria and yeast (SCOBY). The consortium, including anaerobic and aerobic microbial strains, is embedded within a cellulose membrane, which floats on the fermented tea beverage (the soup) and which is a good source of bacterial nanocellulose [26]. The SCOBY fermentation process is static and the usual fermentation time is from 7 up to 30 days at room temperature [27]. Consumption of Kombucha is associated with various health benefits: protection against various pathologies induced by the radical oxygen species (ROS); detoxifying activities, mainly because of the accumulation of organic acids (acetic, gluconic. glucuronic, lactic); antimicrobial effects, resultant from polyphenols and antibiotics/bacteriocins; increased immunity, because of both antioxidant activity and bioactive ingredients, such as B and C vitamins [28–30]. Generally, the SCOBY consortium is dominated by acetic bacteria like *Gluconoacetobacter* sp. and yeast like *Zygosaccharomyces* or *Dekkera* [31–33]. Lactic acid bacteria (LAB) have been reported in both Kombucha layers, soup and pellicle, counting up to 30% of the SCOBY microbial cells [32]. Different LAB species have been identified in SCOBY, such as *Lactobacillus* sp.,

Lactococcus sp., *Leuconostoc* sp. or *Pediococcus pentosaceus* [32–34]. However, very few reports refer to LAB from SCOBY as potential probiotics [33,35]. To the best of our knowledge, there are no studies related to postbiotic compounds produced by LAB fermentation from SCOBY. Postbiotics are soluble compounds from probiotics, i.e., microbial metabolites, including short chain fatty acids such as acetic, propionic and butyric acids, and microbial components, such as (lipo)teichoic acids, peptidoglycans/mural peptides, cell surface proteins, with potential significant health benefits [36,37].

To demonstrate the enhancement of health benefits on pollen fermented by the Kombucha consortium, we analyzed the following: the formation of bioactive compounds (anti-oxidant polyphenols, soluble silicon, postbiotic short-chain fatty acids); parameters related to SCOBY/Kombucha fermentation (dynamics of LAB in SCOBY, formation of specific organic acids, evolution of soluble sugars in Kombucha vinegar); the influence of Kombucha fermentation on pollen morphology and ultrastructure; in vitro cytotoxic and antitumoral effects of the resultant Kombucha fermented pollen. Our focus was not only on anti-oxidant polyphenols, as such compounds have already been demonstrated to be enhanced during pollen fermentation [5,14,19]. We were also interested in biosilica solubilization and post-biotic short chain fatty acids (SCFA). Silicon is one of the main components of the pollen cell wall [38]. In the plant cell wall, silicon is present as biosilica, $SiO_2 \cdot nH_2O$, which is formed after orthosilicic acid polycondensation and precipitation [39]. Biosilica increases plant cell wall resistance to (bio)degradation [40]. Its solubilization weakens the pollen structure and provides an additional health-related benefit, due to the formation of soluble silicon species. These soluble silicon species, especially orthosilicic acid, H_4SiO_4, have been proven to exert several beneficial effects on humans, such as maintenance of bone health [41], including osteoporosis prevention [42,43], or optimal connective tissue function, stimulation of the immune system and Alzheimer's disease prevention [44,45]. Post-biotic short chains fatty acids (e.g., acetic, propionic, butyric acids, produced by the prebiotic strains) are important for proper colonic function, protection against colorectal cancer and modulation of intestinal immune, inflammatory and metabolic functions [37].

2. Materials and Methods

2.1. Biological Material

Samples of bee pollen were harvested from a pollen trap mounted in the beehives from the Bucharest-Ilfov area, in late spring to early summer of 2018. The samples were removed weekly and stored in a freezer (−18 °C). The composition of the multiflora bee collected pollen was microscopically evaluated, by using the unacetolysed method [46], with a total magnification (*x*40). For ultrastructural evaluation on SEM mono-floral pollen (apple pollen), more uniform in shape was used. The identifications were done by comparison to references from European pollen atlases [47,48]. The SCOBY/Kombucha consortium contained: lactic acid bacteria, acetobacteria, from two main genera, *Gluconoacetobacter* and *Komagataeibacter*, and yeasts from several genera, including *Brettanomyces/Dekkera*, *Zygosaccharomyces* and *Pichia* [26]. For the in vitro evaluation of the Kombucha fermented pollen, normal mouse fibroblast cell line, NCTC clone L929 (for biocompatibility), and two transformed human cell lines, laryngeal epidermoid carcinoma cells (Hep-2) and human colon adenocarcinoma cells (Caco-2) (for antitumor activity determination) were used. All cell lines were purchased from the European Collection of Cell Culture (ECACC).

2.2. Pollen Fermentation with the Kombucha Consortium

For Kombucha/SCOBY fermentation the tea infusion was produced from 5 g of green tea (Basilur green tea, Ceylon) infused for 15 min into 1 L of boiling sterile water. The non-extracted material was removed by filtration on Whatman no. 5 filter paper. The resultant tea infusion was sweetened with 70 g/L of sucrose (commercial crystalline sugar). For laboratory level investigations, 2 L of sweetened green tea infusion were aseptically distributed into a sterile brown glass bottle of 3 L. For the process scale-up larger Simax® glass vessels (Kavalier, Prague, Czech Republic), of 150 L total capacity, with

100 L working volume each, were used. For the experiments related to pollen biosilica solubilization, a 2.2 L plastic bottle made from fluoropolymers, (Nalgene™ Wide-Mouth EP Tox/TCLP Teflon™ FEP Bottle, with PFA-lined Closure, Nalge Nunc—Thermo Fisher, Rochester, NY, USA) was filled with 1.5 L of sweetened green tea infusion. For the inoculation, 10% of a previously fermented Kombucha tea (without pollen) was used. For each liter of SCOBY inoculated infusion, 50 g of multi-floral, bee collected pollen was added. Pollen was added immediately after Kombucha inoculation or after 20 days of fermentation at 28 °C (pollen addition to Kombucha vinegar). The pollen fermentation process was conducted at 28 °C, in static, microaerophilic conditions, without agitation, for a period of 30 days (for the pollen added immediately after Kombucha inoculation) and for additional 10 days, for the pollen added into Kombucha vinegar. Samples were taken under axenic conditions from the Kombucha liquid phase. The concentration of reducing sugars from the fermentation of pollen with Kombucha vinegar was determined with dinitrosalicylic acid (98%, Sigma-Aldrich, Merck Group, Darmastad, Germany) [49]. On pilot scale, the floating cellulose pellicle was collected separately at the end of the fermentation period and the remaining Kombucha liquid phase with pollen was subjected to pressure homogenization on a piston homogenizer, at 1000 bars (Pony 1200, GEA Niro Soavi, Parma, Italy). The resultant suspension was spray dried (FSD Production Minor™ Spray Dryer, GEA Niro Process Engineering, Soeborg, Denmark), under mild conditions, 135–140 °C entrance temperature, 70–75 °C exit temperature, with a flow rate of 25 L of water evaporated per hour.

2.3. Pollen Biosilica Solubilization

The total silicon of the initial pollen, after non-oxidative alkaline sample digestion [50], was analyzed by inductively coupled plasma optical emission spectroscopy (PerkinElmer® Optima™ 7000 DV, Perkin Elmer, Waltham, MA, USA). The soluble silicon (monomers and dimers of silicic acid) was determined for samples taken every two days from the Kombucha liquid phase, using the blue silicomolybdic spectrophotometric method [51], measuring the optical density at λ = 810 nm, with a standard curve of silicic acid (pro analysis—Spectroquant®, EMD Millipore, Merck Group), from 5×10^{-4} to 5×10^{-5} mol/L.

2.4. Determination of the Total Phenolic and Flavonoid Compounds

The total polyphenols were determined using the Folin–Ciocâlteu method [52], with some modifications [53]: 750 µL of Folin–Ciocâlteu reagent, 4 mL of 15% Na_2CO_3 and distilled water were added to 150 µL of sample, to a final volume of 15 mL; after 2 h incubation at room temperature, the absorbance at λ = 756 nm was measured. The total phenolic compounds were expressed as caffeic acid equivalents based on a calibration curve made with known concentrations of caffeic acid. The aluminum chloride colorimetric method was used for flavonoids assay [54]: 0.5 mL of sample was mixed with 1.5 mL ethanol, 0.1 mL of 1 M potassium acetate, 0.1 mL of 10% aluminum chloride, and 2.8 mL of distilled water; after 30 min incubation at room temperature the absorbance at λ = 415 nm was measured. The flavonoids content was expressed as quercetin equivalents using a calibration curve made with known concentrations of quercetin. All reagents used were analytical grade reagents, purchased from Sigma-Algrich, Merck Group.

2.5. Antioxidant Activity

The antioxidant activity was determined by two complementary assays: DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) and TEAC (Trolox equivalent antioxidant capacity). The capacity to inhibit the DPPH radical is based on the fact that the purple color of DPPH changes to yellow when the full amount of free radicals is blocked by the antioxidants The DPPH scavenging activity was measured by using the method described by Huang et al. [52]: 150 µL DPPH solution (0.25 mM) in methanol was vigorously mixed with 15 µL of sample and 90 µL of 0.1 M Tris-HCl and the resultant mixture was incubated at 37 °C for 30 min in the dark. As positive control butylated hydroxytoluene (BHT) was used. The sample absorbance (Asample) was read at λ = 520 nm against methanol blank (Ablank),

using a microplate reader (Sunrise, Tecan, Männedorf, Switzerland). The DPPH Inhibition (%) was calculated using the following formula:

$$\% \text{ Inhibition} = (1 - \text{Asample}/\text{Ablank}) \times 100 \quad (1)$$

IC50 value, i.e., the concentration necessary to inhibit 50% of the DPPH radical was calculated using linear regression, obtained by plotting concentration vs. inhibition.

The TEAC assay is based on sample ability to inhibit the ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) radical compared to a standard antioxidant, Trolox, used as reference. The antioxidant capacity (TEAC) was measured based on the method of Re et al. [55], with some modifications. The ABTS radical cation was generated by reacting a 7 mM 2,2′-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS) solution with 2.45 mM potassium persulfate solution (1:1, v/v). The mixture was incubated in the dark at room temperature for 16 h. The initial absorbance of ABTS radical solution was equilibrated to a value of 0.7 ± 0.02 at $\lambda = 734$ nm. Next, 1 mL of ABTS radical solution was mixed with 0.1 mL test sample and after incubation at room temperature for 6 min, the absorbance was measured at $\lambda = 734$ nm. A calibration curve of Trolox (0–250 μM) was used to convert the absorbance into the equivalent activity of Trolox per mL sample (μg Trolox/mL).

2.6. Dynamics of Lactic Acid Bacteria Population During Product Fermentation

The dynamics of lactic acid bacteria population was measured using two methods: targeted Q-PCR total LAB level and direct on-plate counting of viable cells.

2.6.1. Real Time PCR (qPCR) Technique

Lactobacillus acidophilus ATCC 4356 was used as the reference strain. For DNA extractions, *L. acidophilus* ATCC 4356 was grown in 10 mL MRS (De Man, Rogosa, Sharpe) Broth (Oxoid, Thermo Scientific, Hampshire, UK), at 37 °C, for 24 h. The bacterial cells were collected by centrifugation at 4000 rpm for 10 min and a working cell suspension of 10^7 cells/mL was used to generate the standard curve. The DNA from *Lactobacillus acidophilus cells* and Kombucha samples was extracted by using the QIAamp®cador® Pathogen Mini Kit (Qiagen, Hilden, Germany) and the presence of DNA was verified by electrophoresis with ethidium bromide. The DNA quality was checked using a spectrophotometer (Biophotometer plus, Eppendorf, Hamburg, Germany). The DNA samples were treated with Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Hampton, NH, USA), in the presence of LAB specific primers LacF (5-AGCAGTAGGGAATCTTCCA-3) and LacR (5-ATTYCACCGCTACACATG-3) recommended by Ritchie et al., 2010 [55]. The reaction was performed in a Real-time PCR System (7900 Fast Real-time PCR System, Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA) following the program: UNG (Uracil-N-Glycozylase) treatment 2 min at 50 °C; initial denaturation 10 min at 95 °C; 40 cycles of denaturation 15 s at 95 °C followed by an extension of 60 s at 60 °C. Each sample was amplified in duplicate in every experiment. To generate the standard curve, a 10-fold dilution series of DNA from *Lactobacillus acidophilus* ATCC 4356 was subjected to qPCR under the same conditions as described above. A threshold cycle Ct, for the beginning of exponential phase/detection limit was established at 30. All reagents used were molecular biology grade reagents.

2.6.2. On-plate LAB Counting

The LAB suspensions were counted in Petri dishes with 1% agar MRS (Man, Rogosa, Sharpe, Oxoid), after decimal dilutions, by the incorporation techniques which assure the microaerophilic conditions. The development of the fungi in the medium was inhibited by the addition of cycloheximide (0.5 mg/mL in 96% ethanol). The bacteria were cultivated at 37 °C for 2–3 days, until the colonies could be counted. Reagent suitable for bacteria cultivation was used.

2.7. Analysis of Organic Acids

The Kombucha liquid phase was centrifuged at 5000 rpm, for 10 min. and the resultant supernatant was stored at −18 °C. Prior to analysis, the samples were diluted (1:10) with ultrapure water and the diluted samples were passed through 0.2 μm hydrophilic PTFE Millex syringe filter (Merck Millipore, Darmstad, Germany). Two different types of organic acids were analyzed by HPLC: the (poly)hydroxy-acids (lactic, gluconic, citric) and the short chains fatty acids (acetic, propionic and butyric).

2.7.1. Analysis of Hydroxy-Acids

A volume of 10 μL of the filtrate was injected into the HPLC system and the chromatographic analysis was carried out using a Waters Alliance system (Waters Corporation, Milford MA, USA), with a 2695 separation module and a 2487 UV detector (Waters, Milford). The system included a Supelcogel H column (250 mm × 4.6 mm) fitted with Supelcogel H Guard Column (50 mm × 4.6 mm) for the analytic separation, using 0.1% H_3PO_4 as mobile phase and0.17 mL/min flow rate. The UV detection was performed at 210 nm. Data were collected and analyzed with the Empower 2.3 system (Waters, Milford). The organic hydroxy-acids were identified according to their retention times: 10.7 min for citric acid, 11.2 min for gluconic acid, 15.1 min for lactic acid. The compounds peak area was used for quantification, based on a calibration curve obtained by injecting different volumes of a standard solution containing 30 ng/μL lactic acid, 25 ng/μL citric acid and 77 ng/μL gluconic acid. LOD/LOQ were 1.2/3.6 ng/μL for lactic acid, 1.1/2.2 ng/μL for citric acid and 1.9/5.7 ng/μL for gluconic acid. All used reagents were HPLC grade reagents, purchased from EMD Millipore, Merck Group.

2.7.2. Analysis of Short Chains Fatty Acids

For this determination an Agilent HPLC 1200 system (Agilent, Palo Alto, CA, USA), comprising a DAD detector, quaternary pump, and a thermostatic auto sampler, was used. A Luna 2 Phenomenex (size: Φ 4.6 × 150 mm) column served as a stationary phase at 25 °C. The mobile phase consisted of: (A) 10mM $NaH_2PO_4 \cdot H_2O$ (pH 2.50 ± 0.02) and (B) acetonitrile. A volume of 10 μL was injected into the system and the elution was performed using an isocratic method with 90% (A) and a variable flow: 0.6 mL/min until 1.5 min, 0.4 mL/min at 2 min, 0.25 mL/min from 3 to 15 min, 0.3 mL/min at 16 min, 0.6 mL/min at 17 min, 1 mL/min from 18 to 23 min, 0.6 mL/min until 25 min, for a better separation. The absorbance was monitored at 220 nm. The separation of organic acids was achieved in 25 min and the identification was obtained by comparing their retention times with those of standards using a Chemstation (Agilent, Palo Alto, CA, USA) software. All used reagents were HPLC grade reagents, purchased from EMD Millipore, Merck Group.

2.8. Ultrastructural and Morphological Analysis

For ultrastructural analysis, the fermented pollen samples were investigated by transmission electron microscopy (TEM), using a Philips EM 208S electron microscope equipped with Veleta video camera and imaging software iTEM Olympus Soft Imaging System. The morphological analysis of the samples was performed by scanning electron microscopy (SEM), using a SEM-HITACHI SU-1510 microscope. The samples were centrifuged at 10,000 rpm and the resultant sediments were subjected to a plant-specific fixation process with 3% glutaraldehyde, 1.5% paraformaldehyde, 1 M Na_3PO_4, for 2 h, to create cross-links between the constituent molecules of the processed material and to avoid the destruction of the ultrastructural architecture. The fixation step was followed by rinsing with 0,5 M Na_3PO_4, 3 times at 4 °C. For the post-fixation step the sample was transferred in a solution of 1% OsO_4 and 0.5 M Na_3PO_4, for 1 h in the dark. After the post-fixation step, the samples were washed in water at 4 °C (3 times for 10 min) and dehydrated by successive washings with ethanol, 10 min each: 12.5%, 25%, 35%, 50%, 70%, 80%, 90%, 95% and 100%. To replace the old solution and ethanol completely with an acetone-resin mixture, the dehydrated samples were incubated successively with acetone-resin

(1:1) for 1 h, acetone-resin (1:2) for $\frac{1}{2}$ h, resin 100% for 1 h, and again fresh resin 100% over night. Next, the sample was placed in fresh resin (100%), for 60 h at 50 °C, to perform the polymerization step. The samples were cut with an ultramicrotome Leica UC6 (Leica Biosystem, Wetzlar, Germany), with a diamond knife and the resultant sections were placed on copper grids of 200 mesh, covered with a formvar pellicle. The samples were stained for 7 min with 5% uranyl acetate in absolute methanol, to increase the contrast. Then the samples were washed in distilled water and re-stained for another 7 min, with a solution of lead nitrate 4.4%, 0.2 M trisodium citrate monohydrate in distilled water (with 1% NaOH to clarify the solution). All reagents were of electron microscopy grade, supplied by Sigma-Aldrich (Sigma, Merck Millipore, Darmstadt, Germany).

2.9. Biocompatibility and Antitumoral Activity

The spray dried product resultant from the process scale-up in 100 L fermentation vessels was evaluated in vitro, for biocompatibility, on normal mouse fibroblast cell line, NCTC clone L929, and for antitumoral activity, on two transformed human cell lines, laryngeal epidermoid carcinoma cells (Hep-2) and human colon adenocarcinoma cells (Caco-2). The cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin, streptomycin and neomycin) at 37 °C, in a humidified atmosphere with 5% CO_2. The spray dried Kombucha fermented pollen was solubilized in MEM at a concentration of 50 mg/mL and this stock suspension was sterilized by passage through a 0.2 µm hydrophilic PTFE Millex syringe filter (Merck Millipore). The cells were treated with different concentrations of the suspension (5, 6, 8, 10, 15, 20, 25 and 30 mg/mL) and the treated cells were incubated for 24 and 72 h at 37 °C, in a humidified atmosphere with 5% CO_2. All reagents used were suitable for cell culture and were purchased from Sigma–Merck Group.

2.9.1. Cell Viability

The cell viability was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. This assay, based on the reduction of a yellow tetrazolium salt into purple formazan dye by active mitochondria [56], was adapted to our laboratory conditions [57]. Briefly, the cells were seeded in 96-well culture plates, at a density of 5×10^4 cells/mL (for L929 and Hep-2) and 1×10^5 cells/mL (for Caco-2) and incubated at 37 °C, to allow cell adhesion. After 24 h, the culture medium was discarded and replaced with fresh medium, containing different concentrations of spray-dried Kombucha fermented pollen. After 24 and 72 h of incubation, the cells were rinsed with phosphate buffered saline solution (PBS), pH 7.4, and incubated with 100 µL of MTT working solution (0.25 mg/mL) for 3 h at 37 °C. At the end of incubation period the medium was removed, and the formazan crystals were dissolved in 100 µL isopropanol. The optical density of the solution was measured after 15 min of incubation at room temperature, with gently stirring, using a microplate reader (Sunrise, Tecan, Männedorf). The concentration of converted MTT dye directly correlates with the number of metabolically active cells. The cell viability was expressed as a percentage of the control cells (untreated cells) considered 100% viable. All samples were tested in triplicate. All reagents used were suitable for cell culture and were purchased from Sigma–Merck Group.

2.9.2. Cell Morphology

The culture plates of normal L929 cells and of the transformed Hep-2 and Caco-2 cells were examined after 72 h of incubation in the presence of the pollen suspension. Images of the cells were acquired using a Zeiss Axio Observer microscope, with Axio Vision 4.6 software (Carl Zeiss, Oberkochen, Germany).

2.10. Statistical Analysis

Each assay was performed in triplicate. The Student t-test from the SPPS 21 Software package (IBM, Armonk, NY, USA) was used to analyze the relevance of the quantitative data, with the significance level set at $p < 0.05$.

3. Results

The major components of the multi-floral bee collected pollen were from the families: Brassicaceae (*Brassica* sp.) Asteraceae (*Carduus* sp., *Helianthus annus*, *Taraxacum officinale*), Rosaceae (*Prunus* sp., *Crataegus* sp.), Malvaceae (*Tilia* sp.) and Poaceae (*Zea mays*). Such composition is similar to that reported for other batches of multi-floral, bee collected pollen, from the same region of Romania [58].

3.1. Release of Pollen Biosilica, Polyphenols and Flavonoids During Fermentation with Kombucha

The total polyphenol content of the bee collected pollen batches used for our experiments was 12.73 ± 3.38 mg/g dry weight (d.w.). Such content is in line with those reported already for Romanian pollen [59]. We determined a total silicon content of 4.47 ± 0.78 mg/g d.w., on the same level as the reported values for pollen from the same geographic region—Eastern Europe/Asia Minor [60,61]. The biosilica from pollen wall was solubilized during Kombucha fermentation. The solubilization was more significant after 18 days of fermentation (Figure 1) and it is probably a result of the accumulation of organic acids [62].

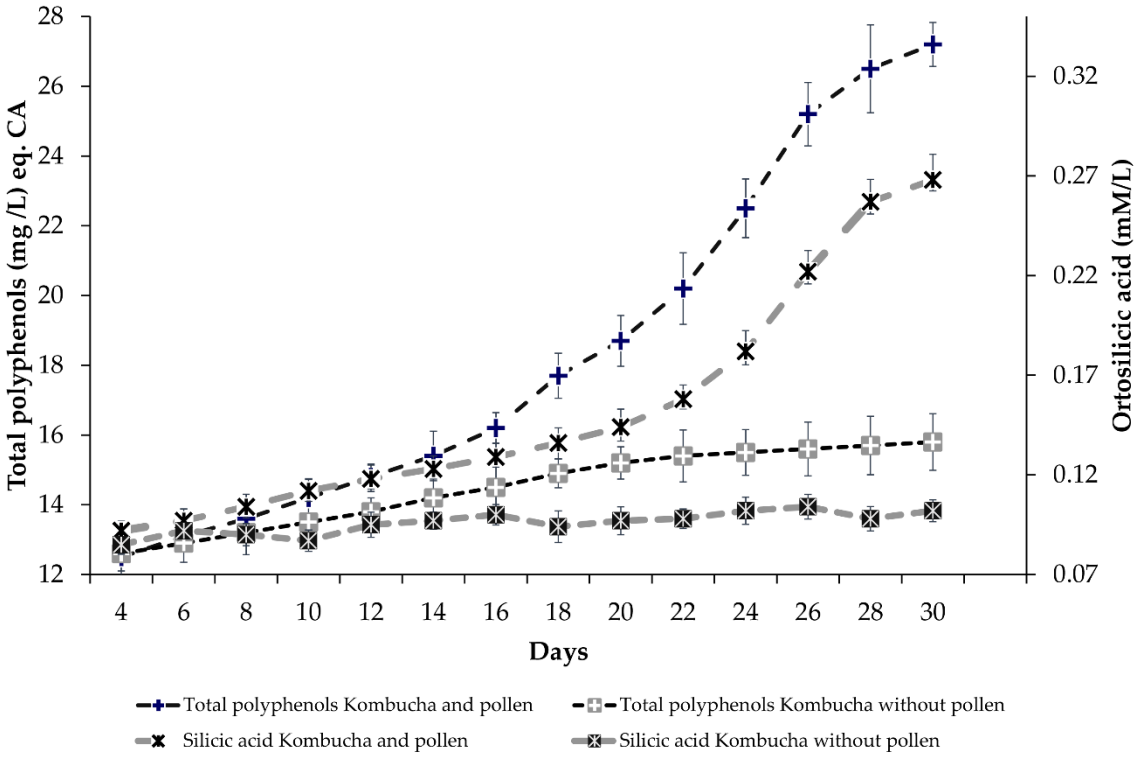


Figure 1. Evolution of soluble silicon and total polyphenols in Kombucha liquid phase, with and without addition of bee collected pollen.

The release of polyphenols into Kombucha liquid phase during the fermentation of pollen followed the same pattern as the solubilization of biosilica, with a more significant and accelerated release after 15–18 days of fermentation (Figure 1). It was already reported that the content of polyphenols is increased during Kombucha fermentation [63]. The addition of another vegetal source of polyphenols, i.e., wheatgrass juice, significantly increased the polyphenol content of the Kombucha [64].

The phytonutrients from pollen are released mainly after 18–20 days of fermentation in the Kombucha liquid phase. The addition of pollen into Kombucha vinegar after 20 days of fermentation determined a significant increase in the content of total polyphenols and flavonoids in the liquid phase (Figure 2). At the same time, the content of reducing sugars decreased, after a small increase during the first days (Figure 2), showing that the microorganisms from the Kombucha consortium are still active during this late fermentation stage. The small increase in sugar reducing content during the first days after pollen addition suggests a release of such compounds from pollen.

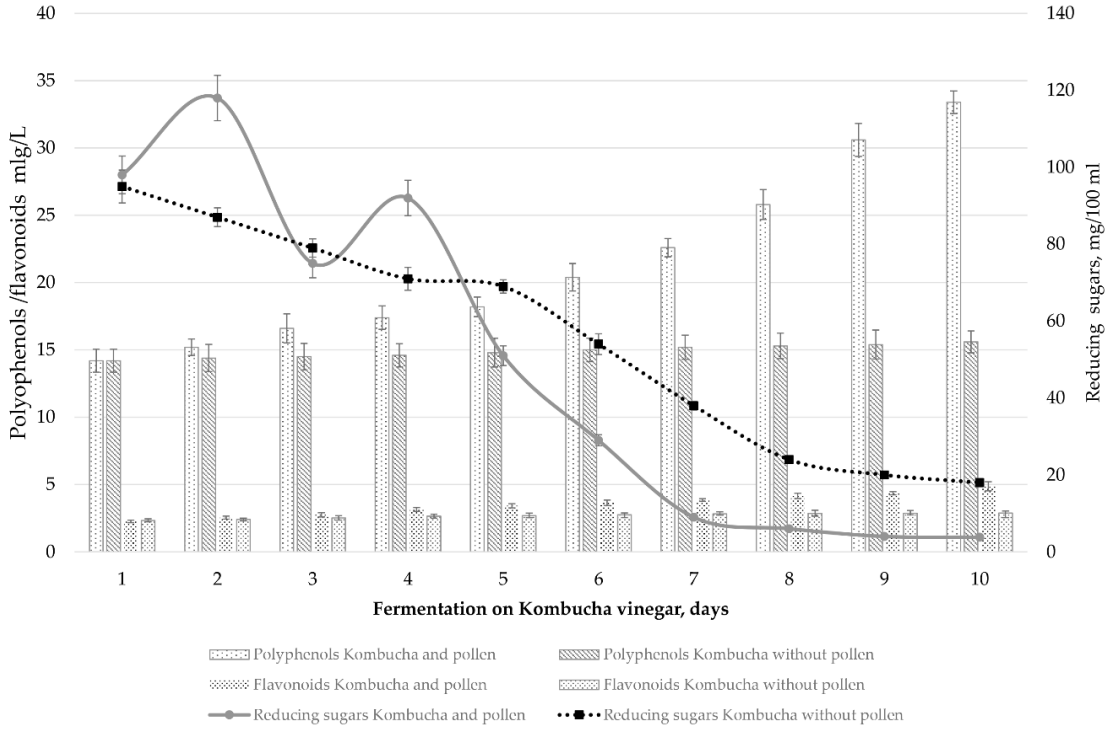


Figure 2. Evolution of polyphenols, flavonoids and reducing sugars of Kombucha vinegar wherein bee collected pollen was introduced.

The values of the DPPH and TEAC assays for each day of pollen fermentation in Kombucha vinegar are presented in Table 1, and they show an increase in the recorded values, and therefore of the antioxidant activity as the fermentation process unfolds. The antioxidant activity profile had a similar pattern of evolution to that of the total content of polyphenols and flavonoids. The IC50 value, calculated for the DPPH free radical scavenging activity, decreased continuously from the first fermentation day to the 10th day.

Table 1. Time-dependent antioxidant capacity of Kombucha vinegar with added pollen.

Fermentation Time (days)	TEAC (µg Trolox/mL)	DPPH (Inhibition Grade%/mL)	IC_{50} (mg/mL)
1	8.83 ± 0.17	1.35 ± 0.1	15.16
2	3.54 ± 0.63	1.32 ± 0.13	13.76
3	3.9 ± 0.18	1.62 ± 0.04	14
4	4.21 ± 0.21	1.64 ± 0.03	12.6
5	5.61 ± 0.22	2.16 ± 0.06	14.96
6	7.8 ± 0.26	2.85 ± 0.07	13.76
7	7.23 ± 0.31	3.79 ± 0.07	11.97
8	16.24 ± 0.34	4.87 ± 0.04	11.95
9	20.94 ± 0.67	4.86 ± 0.11	11.68
10	22.95 ± 0.77	4.91 ± 0.11	10.56

3.2. Dynamic of Lactic Acid Bacteria Population During Product Fermentation

LAB were previously reported to comprise up to 30% of the bacterial population of Kombucha cultures/SCOBY consortium [32]. In our study we used qPCR to quantify the LAB in different Kombucha samples with or without added pollen. The molecular data were compared with results obtained by on-plate cultivation of LAB in specific media, respectively MRS.

The first applications of qPCR in microbial ecology were reported in 2000, and it is still considered a fast and effective method enabling the quantification of transcript numbers within environmental samples, providing high specificity and sensitivity to targeted sequences present in mixed community background using specific primers [65]. This method has been previously used for detection of lactic acid bacteria dynamics during fermentation, such as *Streptococcus thermophilus* and *Lactococcus lactis* in dairy [66], *L. plantarum* and *L. fermentum* in cocoa bean fermentation [67], *L. curvatus*, *L. brevis*, *L. pontis*, *Pediococcus pentosaceus* in sourdough [68,69]. Under our experimental conditions, the correlation coefficient was R^2 = 0.9948 for the standard curve. The detection limit showed a maximum Ct (threshold cycle, related to the number of amplification cycles needed for the exponential amplification phase to begin) of 30, which was needed for a positive reaction with SYBR-Green I. The amount of LAB specific DNA present in an unknown sample was obtained by interpolating its Ct value against the standard curve, and it was expressed in log10 CFU/mL.

As seen in Figure 3, immediately after inoculation with SCOBY during Kombucha fermentation, the level of LAB measured by both methods is close to 10^5 CFU/mL, level which remains constant during the first five fermentation days and goes down to 10^3–10^4 CFU/mL by the end of the fermentative process. When adding pollen in the beginning of the fermentation, the initial LAB level raised almost two logarithmic units, up to 10^7 CFU/mL.

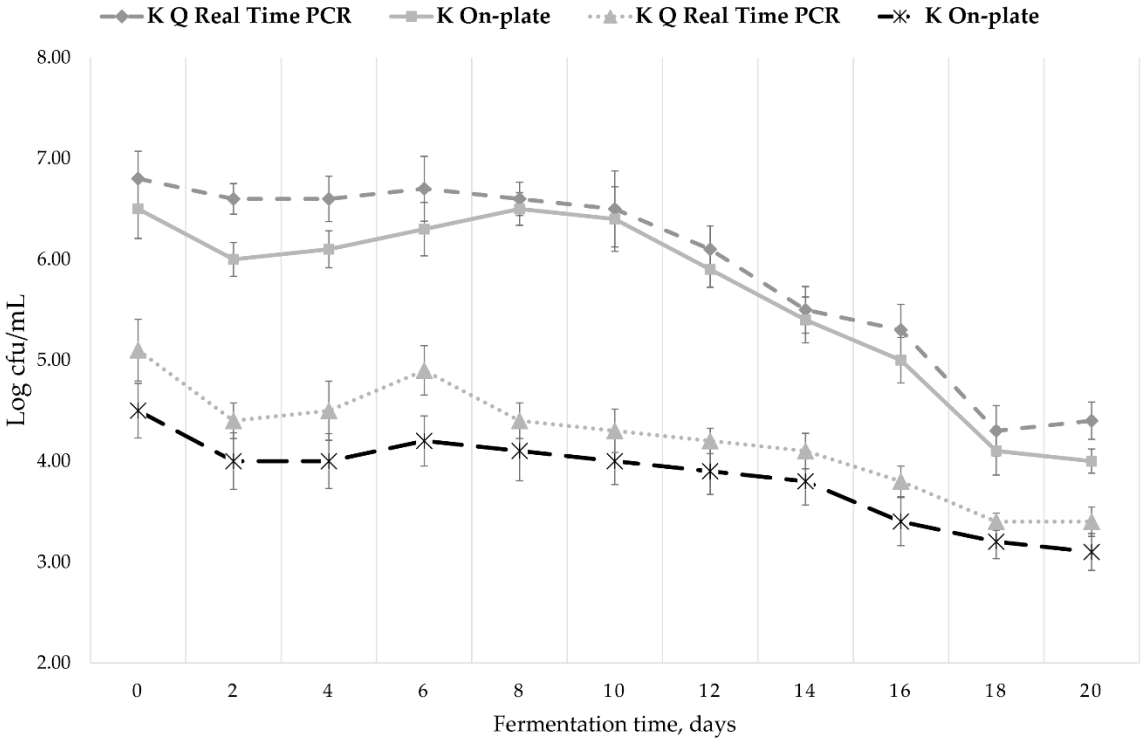


Figure 3. Lactic acid bacteria population during Kombucha without (K) or with pollen (KP) fermentation. Data obtained by q-Real Time PCR versus on-plate quantification.

For the initial level of LAB in the pollen, three different samples of multi-flower pollen used on an industrial level were investigated by q-PCR technique. The LAB content varied between 10^5–10^6 CFU/mL total cells. Until now, few studies have focused on pollen LAB payload. Our result is in

line with the only data already reported, respectively a level of viable cells (on-plate counting) from 10^4–10^6 CFU/mL [65]. The most common specie identified by molecular tools in the pollen was reported previously to be *Lactobacillus* species and in particular *Lactobacillus kunkeei* [66]. Therefore, the important bounce of the lactic acid bacteria population upon pollen addition could be caused by the pollen contribution, considering the reports which emphasize such transfer of lactic bacteria of genus *Lactobacillus* sp, *Bifidobacterium* sp. from the bees' digestive system into the bee pollen, being involved in bee bread fermentation [21,67].

Since we did not register significant differences between the level of LAB measured by qPCR and on-plate technique (colony forming units), respectively, between the total LAB cells and the viable LAB cells, we will only use qPCR in further studies, as it is faster and more reliable.

When adding pollen in Kombucha vinegar (Kombucha previously fermented for 20 days) we did not notice significant differences in LAB population level between the first and the last maceration day. The experiment on the laboratory scale in 2 L working volume induced a lower level (10^7 CFU/mL) than that resulted from pilot trials of 100 L (10^9 CFU/mL) (Figure 4). This may be linked to the specific ratio area/volume and interfacial specific area on large scale level, which assure better conditions for LAB development. Up-scale enhances the pollen effect of boosting LAB microbial population. Further investigations should be performed in this respect.

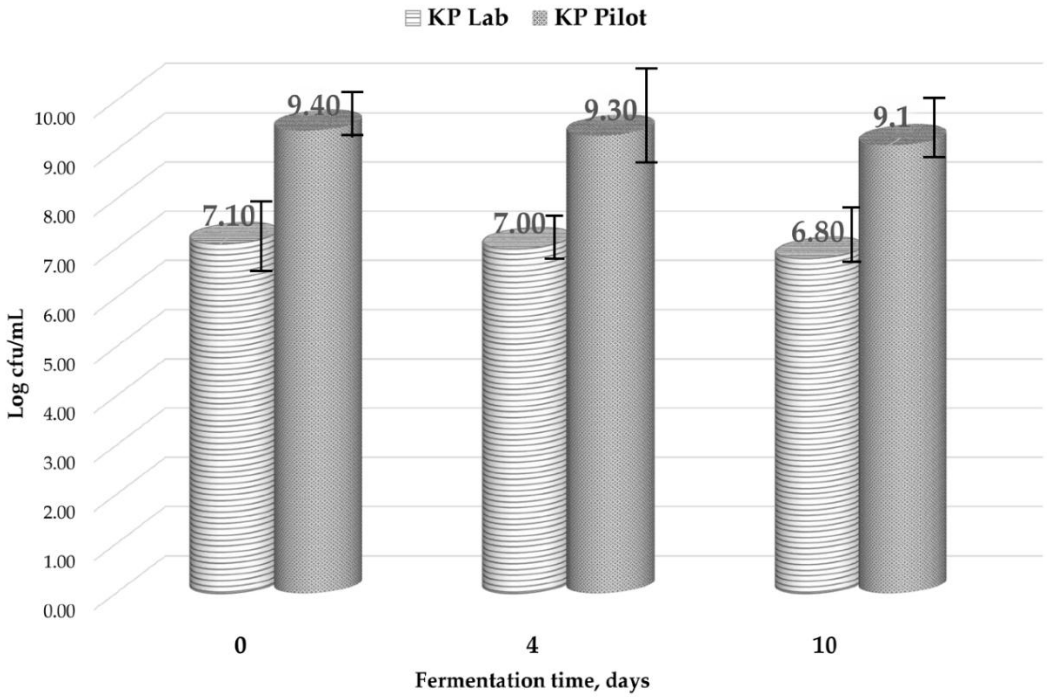


Figure 4. Lactic acid bacteria level during the fermentation process of Kombucha with pollen. Laboratory (Lab) versus Pilot Plant (Pilot) scale.

3.3. Dynamics of Organic Acids During Product Fermentation

We determined the dynamics of several organic acids, hydroxy acids (citric, gluconic and lactic) and short-chain fatty acids (acetic, propionic and butyric). Pollen addition determined an enhancement in the production of the organic acids (Table 2).

Table 2. Organic acids content during Kombucha fermentation with or w/o pollen addition.

Sample	Day	Hydroxy-Acids			Short-Chain Fatty Acids		
		Citric Acid, g/L	Gluconic Acid, g/L	Lactic Acid, g/L	Acetic Acid, g/L	Propionic Acid, g/L	butyric Acid, g/L
K Lab	0	ND	0.545 ± 0.006	0.38 ± 0.01	0.375 ± 0.005	ND	ND
	5	ND	0.56 ± 0.006	0.36 ± 0.006	0.605 ± 0.004	ND	0.14 ± 0.011
	9	ND	0.555 ± 0.014	0.36 ± 0.006	1.32 ± 0.03	0.12 ± 0.033	0.18 ± 0.014
	13	ND	0.94 ± 0.03	0.37 ± 0.006	2.32 ± 0.015	0.16 ± 0.024	0.28 ± 0.017
	17	ND	1.59 ± 0.043	0.375 ± 0.003	4.66 ± 0.025	0.24 ± 0.016	0.30 ± 0.021
KP Lab	0	0.02 ± 0.005	2.795 ± 0.015	0.46 ± 0.015	0.415 ± 0.005	0.095 ± 0.012	0.12 ± 0.038
	5	0.02 ± 0.005	3.155 ± 0.016	0.44 ± 0.01	0.515 ± 0.007	0.205 ± 0.034	0.44 ± 0.032
	9	0.045 ± 0.005	3.59 ± 0.090	0.57 ± 0.02	2.275 ± 0.013	0.31 ± 0.023	0.74 ± 0.028
	13	0.04 ± 0.003	2.545 ± 0.005	0.72 ± 0.01	2.25 ± 0.04	0.42 ± 0.014	1.06 ± 0.063
	17	0.055 ± 0.005	2.26 ± 0.02	0.77 ± 0.01	3.51 ± 0.11	0.56 ± 0.041	1.78 ± 0.054
K Pilot	18	ND	2.645 ± 0.14	1.75 ± 0.15	17.64 ± 0.21	0.27 ± 0.032	0.84 ± 0.037
KP Pilot	18	0.09 ± 0.001	3.975 ± 0.32	10.265 ± 0.42	19.56 ± 0.18	0.66 ± 0.037	1.92 ± 0.033

Legend: K—fermented Kombucha; KP—Kombucha fermented with pollen; Pilot—large-scale.

Such enhancement of organic acid production is most probably related to the significant increase of SCOBY biodiversity, with a higher weight/proportion of LAB. Different types of fermentation occur at the same time in Kombucha with pollen. As seen in Table 1, citric acid was not detectable (ND) in any of the samples without pollen and only in the case of Kombucha fermented with pollen (KP) some small values were detected, increasing from 0.02 g/L in the beginning of the fermentation to 0.055 g/L by the end of the fermentation. The presence of citric acid in Kombucha was previously reported by Jayabalan et al. [31], who found a content of 0.03 g/L in Kombucha prepared with green tea, on the 3rd day of fermentation and 0.11 g/L in the product obtained from black tea. Regarding lactic acid, higher levels were obtained in the samples fermented with pollen, almost double in the final product (0.37 g/L without pollen and 0.7 g/L with pollen), which could be correlated with the higher levels [68] of lactic acid bacteria population brought by the pollen addition. Malbasa et al. (2008) [31] reported a maximum of 0.54 g/L *L*-lactic acid in Kombucha made of black tea, which is in the same range with our results.

In the product fermented without pollen, the gluconic acid content increased by the end of the processes, reaching 1.5 g/L, while when adding pollen, a small increase in the first 9 days was noticed, followed by its decrease. Gluconic acid is produced mainly by acetic bacteria and the high level of lactic acid bacteria may inhibit the growth of acetic bacteria. Chen and Liu (2000) [69] reported a higher content in Kombucha made with black tea, 39 g/L, but after a much longer fermentation period.

Acetic acid is the main organic acid in all samples and increases during the fermentation, due to the activity of acetic bacteria. In the laboratory samples, fermented with and without pollen, the final content of acetic acid was 3.51 and 4.61 g/L, respectively, which is in the range with the data already reported [63], respectively 4.69 g/L after 18 days of fermentation. However, the samples obtained under pilot plant conditions contained a substantial higher level of acetic acid than laboratory samples, reaching levels of 17–19 g/L acetic acid, most probably due to the same surface-volume difference between laboratory and larger scale level.

To the best of our knowledge, the presence of propionic and butyric acids has not been reported yet in Kombucha beverages. However, the presence of these SCFA is plausible and it was probably not reported because it was not extensively studied. Such SCFA are usually produced by the probiotic microorganisms, including lactic acid bacteria, and are an important part of postbiotics [36,37]. The higher content of LAB within SCOBY developed by tea with pollen could explain the higher production of such SCFA in Kombucha with pollen.

3.4. Morphological and Structural Analysis of Fermented Pollen

The ultrastructure analysis with transmission electron microscopy (TEM) highlighted differences between the control (dry) pollen (Figure 5) and pollen undergoing a fermentation process in Kombucha

vinegar, for samples at 3rd, 5th, 7th and 9th fermentation day (Figure 6). A very important feature of the pollen grain is the resistant outer coat named exine, which has a role in protecting the reproductive cells and is important for the attachment to insect pollinators and adhesion to the stigmatic surfaces. The exine is composed of sexine—the ornamental external part, and the nexine, the basal layer. Beneath the exine, a second major wall layer, the intine, surrounds the pollen grain protoplasm. The exine is composed of sporopollenin, a highly resistant biopolymer containing fatty acids, phenylpropanoids, phenolics and carotenoids, and the intine is largely composed of pectin and cellulose.

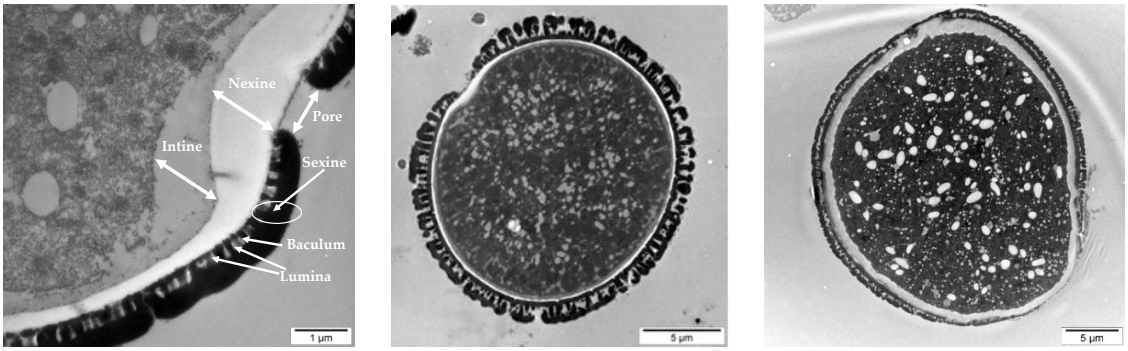
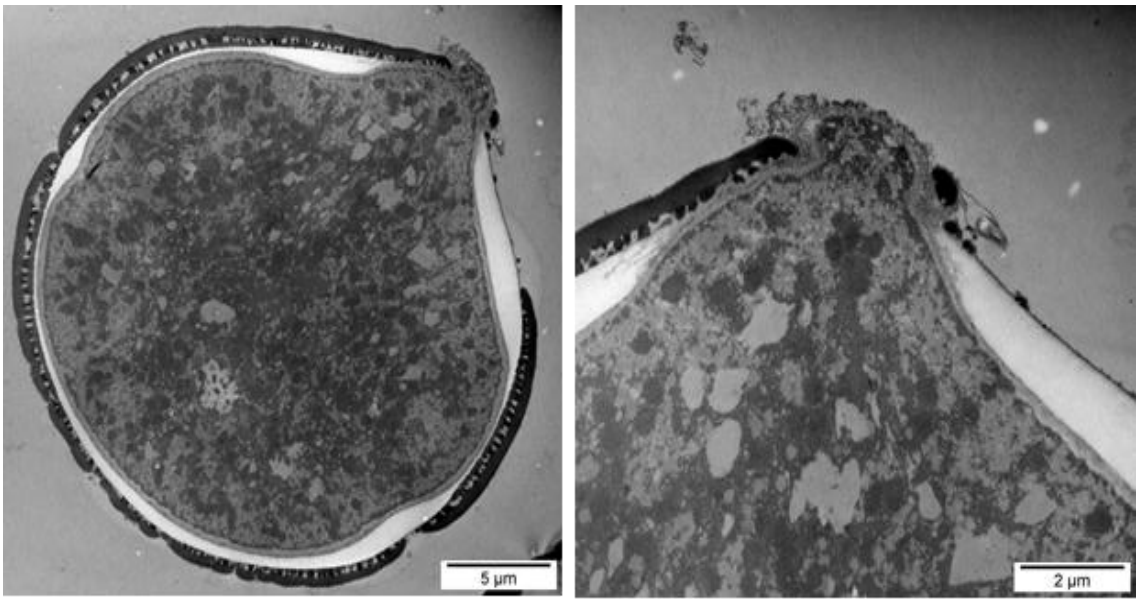


Figure 5. Control sample of unfermented pollen. The arrows show the external wall exine, consisting of sexine and nexine, and the inner wall intine. Additional ultrastructural characteristics of pollen, lumina and baculum are spotlighted. Middle and right pictures illustrate representative aspects of the analyzed pollen grains.



(**a**)

Figure 6. *Cont.*

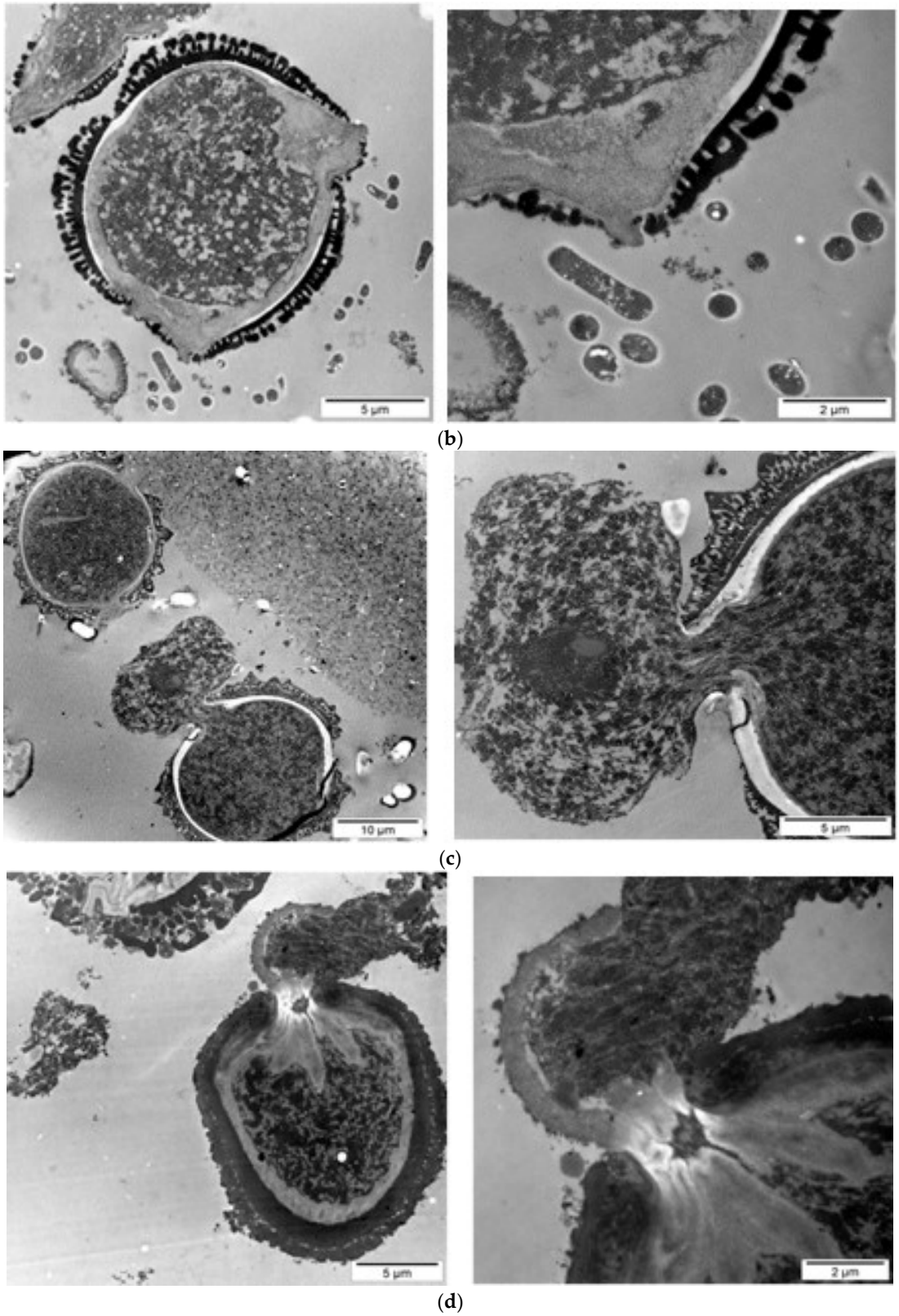


Figure 6. Fermented pollen in kombucha vinegar: (**a**) 3 days, (**b**) 5 days, (**c**) 7 days, (**d**) 9 days.

There is a progressive dynamic of destruction of the pollen complex wall structure, starting both from exterior and interior of the pollen grain. TEM images show that there are visible modifications

of the structural units of the pollen shells (external and internal pollen coating—exine and intine). The pollen grains undergo wall destruction and release of the cellular content in the Kombucha vinegar.

During the first days of fermentation the swelling of the pollen granule, the breaking of the exine and the opening of the pores were observed (Figure 6a,b). Planktonic bacteria are present near the pollen grain (Figure 6b). Most probably, the observed bacilli and cocci are lactic acid bacteria, e.g., *Lactobacillus* sp. or *Pediococcus* sp.—our group recently reported the presence of *Pediococcus pentosaceus* in our local SCOBY/Kombucha consortium. Our hypothesis is that these planktonic bacteria are involved in the breakage of the pollen complex wall structure.

The exine starts to erode and to break, further exposing the pores and the intine. The swelling, created also due to the difference in osmotic pressure, generates an internal pressure, which is pushing on pores. As we approach the 10th day of fermentation, we notice the extensive breakage of the intine. The intine destruction results in significant release of the internal content (Figure 6c,d). Our observations on ultrastructural changes support the biochemical results which show an increase in the content of bioactive compounds (flavonoids, polyphenols, antioxidant activity) in Kombucha liquid phase wherein pollen is included, as compared with the Kombucha without pollen. Most probably, the additional bioactive ingredients from Kombucha fermented with pollen are released from the pollen grains that undergo destruction of the complex wall structure.

SEM images highlight the adhesion of the bacteria from SCOBY consortium microorganisms to the surface of the pollen granules (Figure 7b, arrows). Such a finding supports our hypothesis regarding the involvement of the planktonic microorganisms from Kombucha/SCOBY consortium (the microorganisms which are not included into the cellulose biofilm) in the pollen degradation. Most likely, different microorganisms from Kombucha/SCOBY consortium develop on the surface of pollen grains and promote the formation of small pores into exine, through local oxidative degradation. The SEM analysis also illustrates the morphological changes of pollen granules after Kombucha fermentation, such as swelling and tearing.

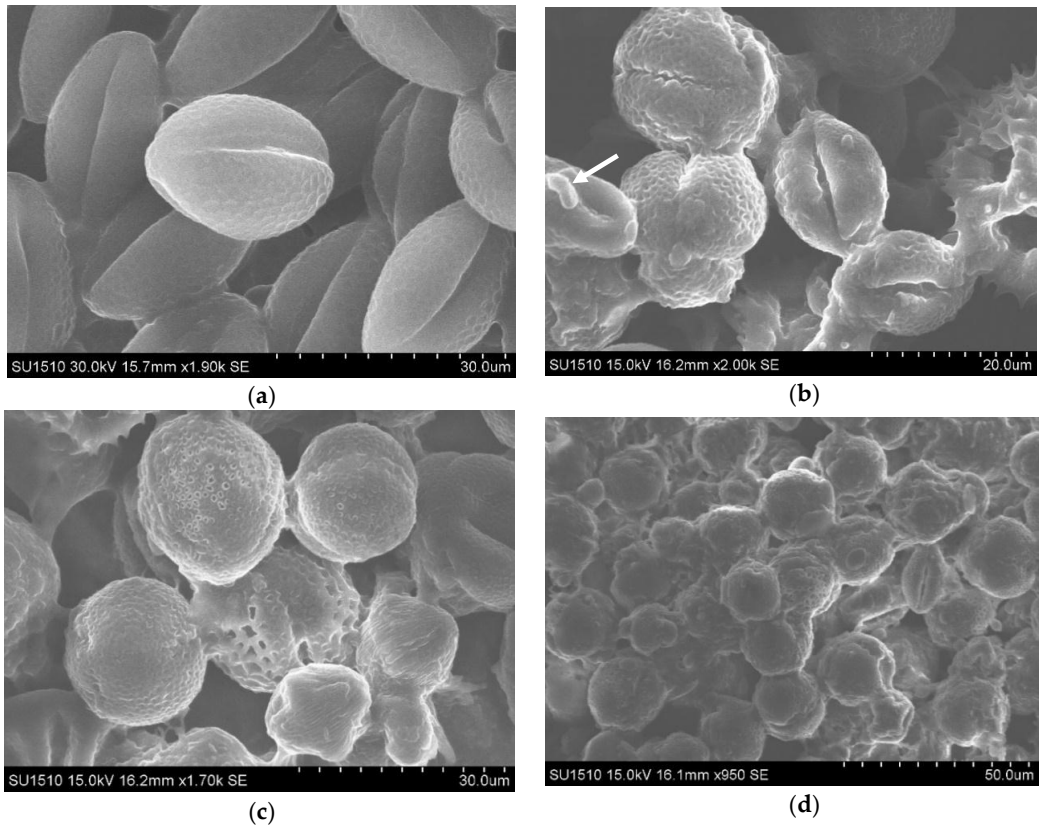
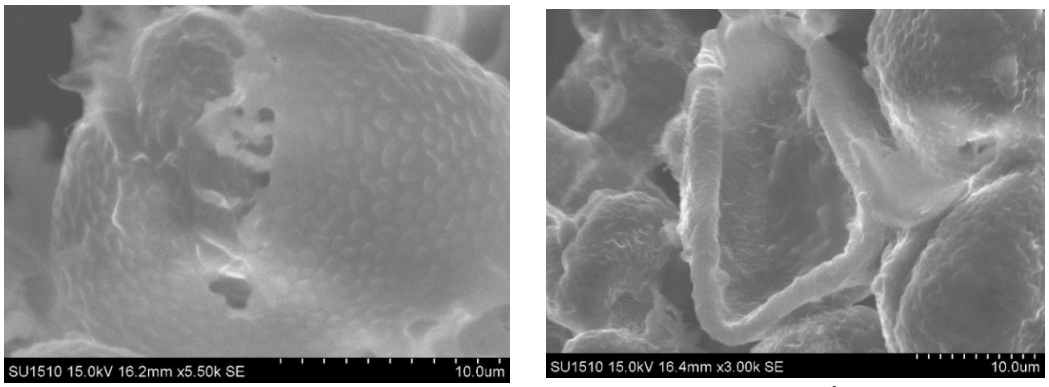


Figure 7. *Cont.*



(**e**) (**f**)

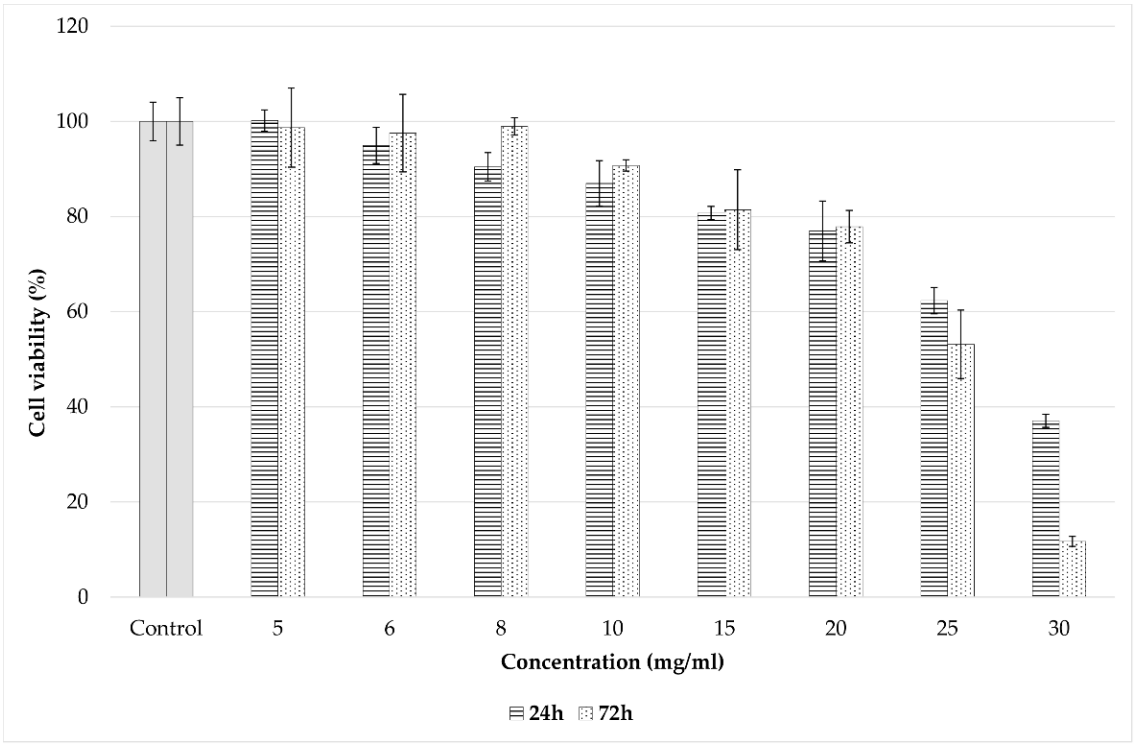
Figure 7. SEM evaluation of the ultrastructure of mono-floral, apple pollen, which has a more uniform shape. (**a**) unfermented pollen; (**b**) 1-day fermented pollen, with attached bacteria (arrow); (**c**,**d**) 7 days fermented pollen; (**e**) detail: pollen granule cracked after 10 days of the fermentation process; (**f**) detail: pollen granule drained because of the10 day of fermentation process.

Both the morphological and ultrastructural analyzes present evidences for an increased bioavailability of the pollen grain content after fermentation with SCOBY/Kombucha consortia. Such a fermentation process determines the morphological and ultrastructural alterations of the pollen outer and inner membrane and leads to the release of the pollen granule content.

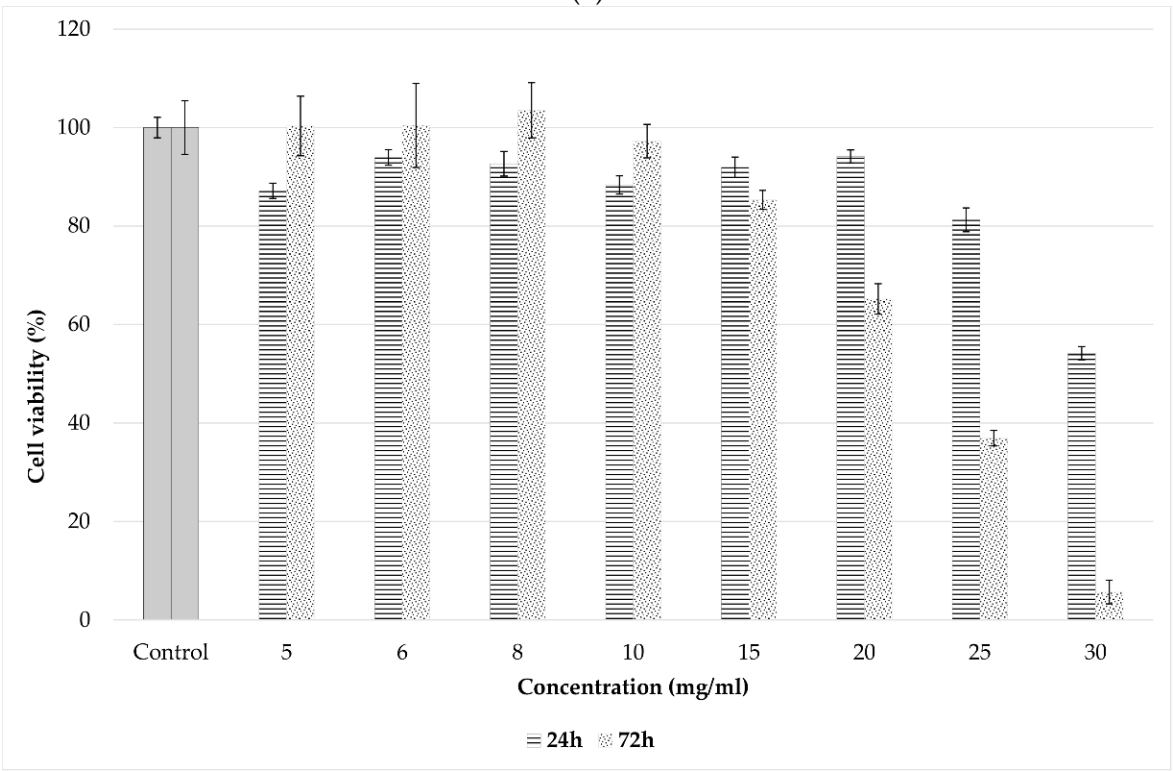
3.5. Cytotoxic and Antitumoral Effects of Spray-Dried Product

We used spray-dried pollen processed on pilot plant level, 100 L working volume, to determine the cytotoxic and antitumoral effects. The in vitro biocompatibility of the spray-dried sample was assessed using the MTT assay, which evaluates the activity of mitochondrial dehydrogenases. The results obtained after 24 h and 72 h of cell incubation in the presence of different concentrations of the sample indicated that the spray-dried product (fermented pollen with Kombucha consortium soluble products, metabolites and cell components) presented a good biocompatibility within the concentration range of 5–20 mg/mL. At these concentrations, the cell viability was above 80%, which is considered non-cytotoxic (Figure 8a). At concentration of 30 mg/mL, the cell viability decreased to 37% and 11.71% after 24 h and 72 h of treatment, respectively. The extracts/products added into the cell culture media at concentrations higher than 2%, respectively 20 mg/mL, modify the characteristics of the cells.

Regarding the antitumoral activity, after 24 h of treatment, the viability of Hep-2 cells was maintained over 80% at concentrations ranging between 5–25 mg/mL but dropped significantly below 65% after 72 h starting with the concentration of 20 mg/mL (Figure 8b). Better results were obtained on Caco-2 intestinal tumor cells. Thus, the values of cell viability slightly decreased below 80% (75.56%) after 24 h of treatment at the concentration of 15 mg/mL, whereas at higher concentrations, significantly lower values were obtained (below 20%) (Figure 8c). Similar results were found after 72 h, although the values of the cell viability were even lower at all tested concentrations compared to those obtained after 24 h. Thus, the cell viability dropped to 52.65% at the concentration of 15 mg/mL and below 10% at higher concentrations (20–30 mg/mL).

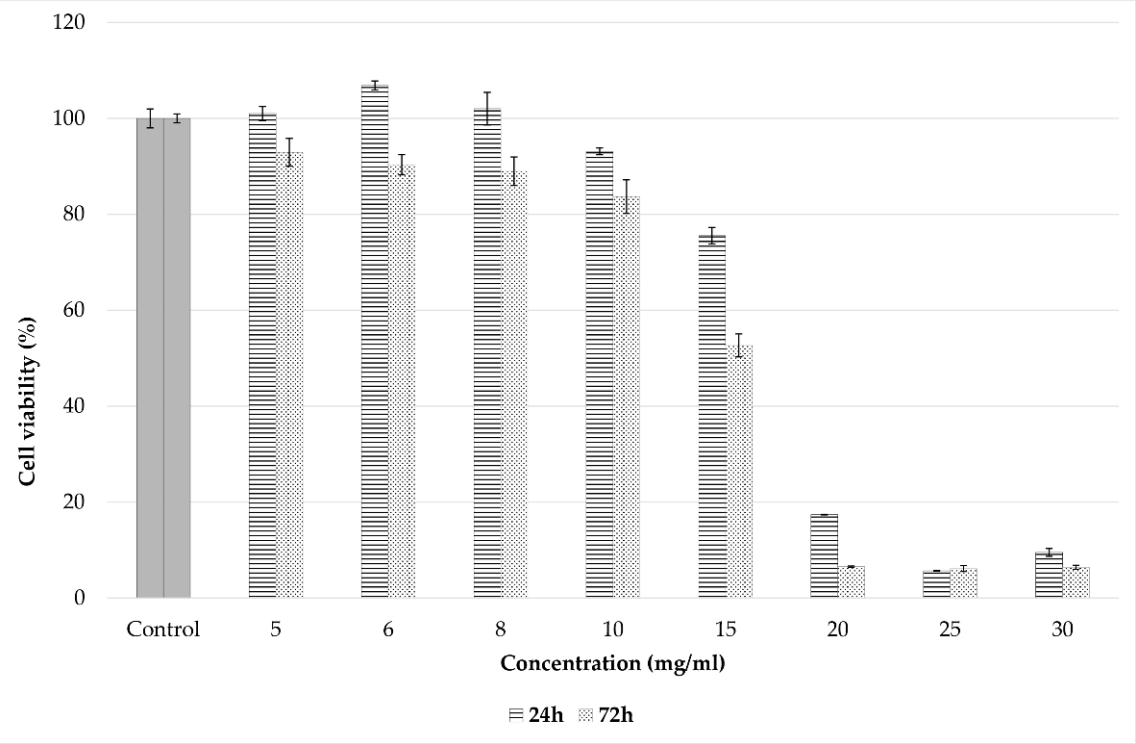


(**a**)



(**b**)

Figure 8. *Cont.*



(**c**)

Figure 8. Effect of treatment with the spray-dried, large-scale obtained product on cell viability of (**a**) NCTC clone L929, (**b**) Hep-2 cells and (**c**) Caco-2 cells, after 24 and 72 h, evaluated by the MTT assay. The experiment was carried out in triplicate. Data are presented as mean ± SD.

In conclusion, the tested sample showed no cytotoxic effect within the concentration range of 5–20 mg/mL and exhibited a moderate antitumoral activity starting with the concentration of 20 mg/mL for Hep-2 cells and 15 mg/mL for Caco-2 intestinal tumor cells.

Cell Morphology

Optic microscopy allowed for observations of cell morphology changes induced in the cell membrane, cytoplasm and nuclei after treatment with different concentrations of atomized sample. Light microscopy images were taken for all three types of cell lines (NCTC clone L929, Hep-2 and Caco-2) incubated in the presence of the tested sample for 72 h (Figures S1–S3). The micrographs of the NCTC clone L929 control culture showed that the cells maintained their normal fibroblastic phenotype with euchromatic nuclei and clear cytoplasm (Figure S1a). The morphology of L929 cells treated with the spray-dried product was similar to that of the untreated control up to the concentration of 20 mg/mL and the cell density reached a complete monolayer similar to the control cells (Figure S1b). Evident morphological changes in the cell morphology, such as round cells and giant cells with multiple nuclei and granular cytoplasm, were observed at higher concentrations (25–30 mg/mL), when the cell viability dropped below 55% (Figure S1c,d).

The light microscope images of Hep-2 cells cultivated in the presence of the spray-dried product revealed a normal morphology (epithelial-like cells) like that of the untreated cells up to the concentration of 15 mg/mL (Figure S2a,b). Moreover, the density of treated cells was also like that of the control cells, reaching an almost complete monolayer (90–95% surface covered by cells). Morphological changes in the cell membrane, cytoplasm and nuclei were observed starting with the concentration of 20 mg/mL, when cell viability decreased significantly, down to 5.66% at the concentration of 30 mg/mL (Figure S2c,d).

Finally, Caco-2 cells treated with different concentrations of the product showed a normal epithelial-like phenotype (cuboidal shape, similar to intestinal enterocytes) just as the untreated cells, within the concentration range of 5–10 mg/mL, when the cell density reached an almost complete monolayer (85–90% surface covered by cells) (Figure S3a,b). Significant morphological modifications were observed at higher concentrations, with an increased percentage of rounded cells and low cell density (Figure S3c,d).

In conclusion, the quantitative results obtained by the MTT assay correlated well with cell morphology observations based on optical microscopy and both highlighted the antitumoral effect of the tested sample.

4. Discussion

The results demonstrate that the process which we proposed, fermentation of bee collected pollen with Kombucha, increases the bioavailability of bioactive compounds from pollen. The fermentation of pollen with Kombucha releases important amounts of soluble silicon from biosilica embedded into the wall. It is known that plant biosilica presents two pools, a concentrated one, in the form of SiO_2xnH_2O aggregates and a dispersed one, poly-condensed silica complexed within the cell wall [70]. Concentrated forms of biosilica aggregates have not been described in pollen. Thus, most probably, the soluble silicon species which are released from pollen into Kombucha liquid phase result from the dissolution of pollen wall matrix in the liquid phase. Weakening of the pollen wall due to silicon solubilization increased the amount of released nutrients into Kombucha liquid phase. We found an increase in the polyphenols content during the pollen fermentation with Kombucha. Pollen proteins/amino acids are also most probably released after pollen grain breakage. Both polyphenols and amino acids were reported to solubilize biosilica through a surface complexation reaction [71]. This suggests an interconnected, positive feed-back process, by which compounds released from pollen could accelerate further the dissolution of wall matrix. Such processes could explain the exponential phase release of pollen bioactive compounds, which we found both for pollen fermented in Kombucha from the very beginning and for pollen fermented/macerated in Kombucha vinegar.

Pollen addition to Kombucha determined a better development of LAB. In Kombucha fermented on industrial scale, the metabarcoding technique from a previous study revealed values from 6 to 8 units log CFU/mL, in the case of fermentation of green tea, from the 1st to the 8th day of fermentation [72]. In our experimental large-scale fermentation of Kombucha with pollen, for 100 L working volume, we determined more than 9 units log CFU/mL, even on the 10th day of fermentation. The larger weight of the LAN population into SCOBY consortium from Kombucha with added pollen is correlated with higher formation of lactic acid.

Large-scale fermentation of pollen with Kombucha proved to be more effective than laboratory, small scale batches. The main reason is probably related to the interfacial transfer of oxygen. During Kombucha fermentation the SCOBY consortium accomplishes different types of fermentation in the same time, an aerobic one (acetic acid fermentation, oxygen being needed to transform ethanol to acetic acid) and anaerobic fermentations—alcoholic and lactic [73]. Oxygen transfer should be enough for acetic acid fermentation, but not in excess to inhibit alcoholic and/or lactic acid fermentation. The oxygen transfer rate in the static culture of Kombucha is determined by the interfacial specific area [74], i.e., the ratio between the surface exposed to air/oxygen and the total working volume. The higher this ratio, the faster is the rate of oxygen transfer and the production of acetic acids. However, on a too large area, the oxygen level in fermentation broths/liquid phase could limit significantly the anaerobic fermentation. Thus, there are some limits for interfacial specific area, between 0.0232 and 0.0681 cm^{-1}, for a proper Kombucha fermentation process [75]. The vessel which we used for large-scale pollen fermentation with Kombucha has a specific interfacial ratio of 0.0573 cm^{-1}, which assures better oxygen transfer than the cylindrical bottles used under laboratory conditions, with a specific interfacial ratio of 0.0308 cm^{-1}.

The addition of pollen seems to increase the proportion of lactic acid producers. The better development of LAB on large scale fermentation with Kombucha could be explained by the larger microbiota diversity associated with the diverse bee collected batches used on large scale. It has been already demonstrated that introduced LAB are well tolerated by SCOBY consortium; For the hybrid Kombucha culture, sweetened tea—sweetened cabbage brine, the microbial consortium maintained the large amount of LAB introduced by the cabbage brine [76]. Most probably, bee collected pollen brings to the SCOBY community fructophilic lactic acid bacteria, specific to honey bee and honey bee products [77]. Fructophilic lactic acid bacteria are fitted to establish and develop into SCOBY consortium/Kombucha habitat: are tolerant to oxygen and high level of sugars [75], metabolize fructose [77] and use polyphenols as electron acceptors [78]. All these features support them to proliferate into sweetened tea, with high content of soluble sugars (including fructose, produced by yeast invertase from sucrose) and polyphenols. Additional polyphenols released from pollen should further support fructophilic LAB. Further studies will help to elucidate these observations.

Larger microbial diversity of Kombucha with added pollen supports also the formation of short-chain fatty acids—SCFA. SCFA are representative for postbiotics, bioactive metabolites produced by probiotics [36]. Postbiotics are among the main active ingredients of paraprobiotics, products which contain inactivated probiotics/beneficial microbial strains. Typical examples of paraprobiotics are the milk-based product fermented with *Bacteroides xylanisolvens* DSM23964. Such products result from cultivation of *B. xylanisolvens* on (skimmed) milk, followed by a thermal inactivation (including by spray-drying) of the resultant fermentation product. Spray-drying in mild conditions does not affect the bioactive compounds produced by *B. xylanisolvens* and reduces the concerns regarding potential hazards of active growing microbial strains for consumers with (auto)immune disorders and inflammatory conditions. We used the same approach to limit the concerns regarding the safety of our pollen fermented with Kombucha. The cell culture tests proved a good biocompatibility. Even after inclusion of 20 mg/mL in the culture media (i.e., 2%) the spray-dried pollen fermented with the Kombucha soup is still biocompatible. Usually, the biocompatibility of herbal extracts on cell culture of NCTC clone L929 is investigated at concentrations between 0,1 and 0,5%, respectively from 1 to 5 mg/mL, and the products with limited influence at 0.5% concentration on growing medium are considered safe [79]. In the case of transformed cells of intestinal origins, we noted an inhibitory effect starting from 10–15 mg/mL. This antitumoral slight effect might be related to the presence of postbiotics into the tested products, as such compounds were demonstrated to have antitumoral effect against intestinal transformed cells [36].

5. Conclusions

The fermentation with a Kombucha consortium enhances pollen phytonutrients bioavailability. However, the pollen fermentation with Kombucha leads not only to enhanced bioavailability of pollen phytonutrients. Pollen has already been demonstrated to be a good fermentation activator for mead and white wine. Our data presented here show that pollen is also a good activator of Kombucha/SCOBY fermentation. The addition of the bee collected pollen also improves Kombucha fermentation and the formation of Kombucha health-related compounds. At the end of the pollen fermentation with Kombucha consortium, a product with enhanced health benefits is formed, with complementary bioactive ingredients.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/10/10/1365/s1, Figure S1: Light micrographs of NCTC clone L929 cells untreated (a) and treated with different concentrations of atomized product: 20 mg/mL (b), 25 mg/mL (c) and 30 mg/mL (d); Figure S2: Light micrographs of Hep-2 cells untreated (a) and treated with different concentrations of atomized product: 15 mg/mL (b), 20 mg/mL (c) and 30 mg/mL (d); Figure S3: Light micrographs of Caco-2 cells untreated (a) and treated with different concentrations of atomized product: 10 mg/mL (b), 15 mg/mL (c) and 20 mg/mL (d).

Author Contributions: Conceptualization, F.O. and I.M.; I.M. had the initial idea to ferment pollen with Kombucha; Methodology and Investigation, E.U. organized pollen fermentation on Kombucha vinegar and determined SCFA, F.M. completed the experiments related to q-Real Time PCR, A.T. completed the analysis of

polyphenols, flavonoids and antioxidants, C.F.D. determined the LAB by plate-method, V.V.V. extracted DNA and participated in q-Real Time PCR, F.I.-R. determined hydroxy-acids, L.M.S. and A.O. performed the cell culture experiments, SM did ultrastructural and morphological evaluation, D.C.-A. did experiments related to biosilica dissolutionn, A.M. performed the large-scale, pilot experiments, C.P.C. organized the pollen fermentation on lab scale; Supervision and/or Data curation, F.O. the overall experiments, C.P.C. microbiological analysis, including q-Real Time PCR, F.I.-R. the HPLC determinations, A.O. the cell culture tests, I.M. process scale-up; Writing and Draft Preparation, E.U. and F.M. wrote the draft reports; Writing, Review and Editing, D.C.-A. and F.O. wrote and review the final paper, Submission, F.O.

Funding: This research was funded by grants of the Romanian Ministry of Research and Innovation, CCCDI–UEFISCDI, project ERA.NET ERA-IB-15-129 Convert-Si, contract No. 62/2016, and PN-III-P2-2.1-BG-2016-0051 contract 6BG/2016, within PNCDI IIII. The APC was funded from contract 62/2016 Convert-Si.

Acknowledgments: Access to research infrastructure developed at the National Institute for Research & Development in Chemistry and Petrochemistry-ICECHIM through the European Social Fund for Regional Development, Programme POS.CCE, Project Agri-flux (ID 1938, Contract 645/18.03.2014) is gratefully acknowledged.

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

References

1. Denisow, B.; Denisow-Pietrzyk, M. Biological and therapeutic properties of bee pollen: A review. *J. Sci. Food Agric.* **2016**, *96*, 4303–4309. [CrossRef] [PubMed]
2. Pascoal, A.; Rodrigues, S.; Teixeira, A.; Feás, X.; Estevinho, L.M. Biological activities of commercial bee pollens: Antimicrobial, antimutagenic, antioxidant and anti-inflammatory. *Food Chem. Toxicol.* **2014**, *63*, 233–239. [CrossRef] [PubMed]
3. Cornara, L.; Biagi, M.; Xiao, J.; Burlando, B. Therapeutic properties of bioactive compounds from different honeybee products. *Front. Pharmacol.* **2017**, *8*. [CrossRef] [PubMed]
4. Zhou, W.; Yan, Y.; Mi, J.; Zhang, H.; Lu, L.; Luo, Q.; Li, X.; Zeng, X.; Cao, Y. Simulated digestion and fermentation in vitro by human gut microbiota of polysaccharides from bee collected pollen of chinese wolfberry. *J. Agric. Food Chem.* **2018**, *66*, 898–907. [CrossRef] [PubMed]
5. Zuluaga, C.M.; Serrato, J.C.; Quicazan, M.C. Bee-pollen structure modification by physical and biotechnological processing: Influence on the availability of nutrients and bioactive compounds. In Proceedings of the 12th International Conference on Chemical & Process Engineering (ICHEAP12), Milano, Italy, 19–22 May 2015; Aidic Servizi Srl: Milano, Italy, 2015; Volume 43, pp. 79–84.
6. Mackenzie, G.; Boa, A.N.; Diego-Taboada, A.; Atkin, S.L.; Sathyapalan, T. Sporopollenin, the least known yet toughest natural biopolymer. *Front. Mater.* **2015**, *2*. [CrossRef]
7. Roulston, T.A.H.; Cane, J.H. Pollen nutritional content and digestibility for animals. *Plant Syst. Evol.* **2000**, *222*, 187–209. [CrossRef]
8. Feng, Z.-F.; Chen, X.-F.; Di, D.-L. Online extraction and isolation of highly polar chemical constituents from brassica napus l. Pollen by high shear technique coupled with high-performance counter-current chromatography. *J. Sep. Sci.* **2012**, *35*, 625–632. [CrossRef] [PubMed]
9. Choi, Y.-S.; Suh, H.-J.; Chung, I.K. Enhanced extraction of bioactive compounds from bee pollen by wet-grinding technology. *J. Life Sci.* **2016**, *26*, 651–656. [CrossRef]
10. Liu, X.-D.; Zhang, F.-B.; Zhou, B.; Shan, H.; Chen, P.-Y. Effect of sonication on different quality parameters of pinus massoniana pollen. *Ultrason. Sonochem.* **2015**, *22*, 174–181. [CrossRef] [PubMed]
11. Wu, W.; Wang, K.; Qiao, J.; Dong, J.; Li, Z.; Zhang, H. Improving nutrient release of wall-disrupted bee pollen with a combination of ultrasonication and high shear technique. *J. Sci. Food Agric.* **2018**. [CrossRef] [PubMed]
12. Dong, J.; Gao, K.; Wang, K.; Xu, X.; Zhang, H. Cell wall disruption of rape bee pollen treated with combination of protamex hydrolysis and ultrasonication. *Food Res. Int.* **2015**, *75*, 123–130. [CrossRef] [PubMed]
13. Xu, X.; Sun, L.; Dong, J.; Zhang, H. Breaking the cells of rape bee pollen and consecutive extraction of functional oil with supercritical carbon dioxide. *Innov. Food Sci. Emerg. Technol.* **2009**, *10*, 42–46. [CrossRef]
14. Kaskoniene, V.; Katileviciute, A.; Kaskonas, P.; Maruska, A. The impact of solid-state fermentation on bee pollen phenolic compounds and radical scavenging capacity. *Chem. Pap.* **2018**, *72*, 2115–2120. [CrossRef]
15. Yang, S.Y.; Wu, Y.Y.; Luo, C.B.; Di, Z.H.; Wu, Y.P.; Zhang, J.; Xu, Y.M. A *Bacillus* coagulans and its application in pine pollen fermentation. *Food Sci. Biotechnol.* **2015**, *24*, 2129–2135. [CrossRef]

16. Yang, S.Y.; Li, H.L. Optimization of pine pollen fermentation conditions using lactobacillus paracasei. *Food Sci. Biotechnol.* **2015**, *24*, 155–160. [CrossRef]
17. Zhang, Z.; Cao, H.G.; Chen, C.; Chen, X.; Wei, Q.; Zhao, F.Y. Effects of fermentation by ganoderma lucidum and saccharomyces cerevisiae on rape pollen morphology and its wall. *J. Food Sci. Technol.* **2017**, *54*, 4026–4034. [CrossRef] [PubMed]
18. Salazar-Gonzalez, C.; Diaz-Moreno, C. The nutritional and bioactive aptitude of bee pollen for a solid-state fermentation process. *J. Apic. Res.* **2016**, *55*, 161–175. [CrossRef]
19. Tomás, A.; Falcão, S.I.; Russo-Almeida, P.; Vilas-Boas, M. Potentialities of beebread as a food supplement and source of nutraceuticals: Botanical origin, nutritional composition and antioxidant activity. *J. Apic. Res.* **2017**, *56*, 219–230. [CrossRef]
20. Kieliszek, M.; Piwowarek, K.; Kot, A.M.; Błażejak, S.; Chlebowska-Śmigiel, A.; Wolska, I. Pollen and bee bread as new health-oriented products: A review. *Trends Food Sci. Technol.* **2018**, *71*, 170–180. [CrossRef]
21. Vasquez, A.; Olofsson, T.C. The lactic acid bacteria involved in the production of bee pollen and bee bread. *J. Apic. Res.* **2009**, *48*, 189–195. [CrossRef]
22. Gilliam, M. Microbiology of pollen and bee bread: The yeasts. *Apidologie* **1979**, *10*, 43–53. [CrossRef]
23. Gilliam, M. Microbiology of pollen and bee bread: The genus bacillus. *Apidologie* **1979**, *10*, 269–274. [CrossRef]
24. Amores-Arrocha, A.; Roldan, A.; Jimenez-Cantizano, A.; Caro, I.; Palacios, V. Effect on white grape must of multiflora bee pollen addition during the alcoholic fermentation process. *Molecules* **2018**, *23*, 1321. [CrossRef] [PubMed]
25. Zlatev, Z.; Taneva, I.; Baycheva, S.; Petev, M. A comparative analysis of physico-chemical indicators and sensory characteristics of yogurt with added honey and bee pollen. *Bulg. J. Agric. Sci.* **2018**, *24*, 132–144.
26. Dima, S.-O.; Panaitescu, D.-M.; Orban, C.; Ghiurea, M.; Doncea, S.-M.; Fierascu, R.C.; Nistor, C.L.; Alexandrescu, E.; Nicolae, C.-A.; Trică, B.; et al. Bacterial nanocellulose from side-streams of kombucha beverages production: Preparation and physical-chemical properties. *Polymers* **2017**, *9*, 374. [CrossRef]
27. Lončar, E.; Djurić, M.; Malbaša, R.; Kolarov, L.J.; Klašnja, M. Influence of working conditions upon kombucha conducted fermentation of black tea. *Food Bioprod. Process.* **2006**, *84*, 186–192. [CrossRef]
28. Jayabalan, R.; Malbaša, R.V.; Lončar, E.S.; Vitas, J.S.; Sathishkumar, M. A review on kombucha tea-microbiology, composition, fermentation, beneficial effects, toxicity, and tea fungus. *Compr. Rev. Food Sci. Food Saf.* **2014**, *13*, 538–550. [CrossRef]
29. Vīna, I.; Semjonovs, P.; Linde, R.; Deniņa, I. Current evidence on physiological activity and expected health effects of kombucha fermented beverage. *J. Med. Food* **2014**, *17*, 179–188. [CrossRef] [PubMed]
30. Martínez Leal, J.; Valenzuela Suárez, L.; Jayabalan, R.; Huerta Oros, J.; Escalante-Aburto, A. A review on health benefits of kombucha nutritional compounds and metabolites. *CyTA J. Food* **2018**, *16*, 390–399. [CrossRef]
31. Marsh, A.J.; O'Sullivan, O.; Hill, C.; Ross, R.P.; Cotter, P.D. Sequence-based analysis of the bacterial and fungal compositions of multiple kombucha (tea fungus) samples. *Food Microbiol.* **2014**, *38*, 171–178. [CrossRef] [PubMed]
32. Matei, B.; Salzat, J.; Diguta, F.C.; Cornea, P.C.; Luta, G.; Utoiu, R.E.; Matei, F. Lactic acid bacteria strains isolated from kombucha with potential probiotic effect. *Romanian Biotechnol. Lett.* **2018**, *23*, 13592–13598.
33. Chakravorty, S.; Bhattacharya, S.; Chatzinotas, A.; Chakraborty, W.; Bhattacharya, D.; Gachhui, R. Kombucha tea fermentation: Microbial and biochemical dynamics. *Int. J. Food Microbiol.* **2016**, *220*, 63–72. [CrossRef] [PubMed]
34. Kozyrovska, N.O.; Reva, O.M.; Goginyan, V.B.; Vera, J.-P.D. Kombucha microbiome as a probiotic: A view from the perspective of post-genomics and synthetic ecology. *Biopolym. Cell* **2012**, *28*, 103–113. [CrossRef]
35. Aguilar-Toalá, J.E.; Garcia-Varela, R.; Garcia, H.S.; Mata-Haro, V.; González-Córdova, A.F.; Vallejo-Cordoba, B.; Hernández-Mendoza, A. Postbiotics: An evolving term within the functional foods field. *Trends Food Sci. Technol.* **2018**, *75*, 105–114. [CrossRef]
36. Gill, P.A.; van Zelm, M.C.; Muir, J.G.; Gibson, P.R. Short chain fatty acids as potential therapeutic agents in human gastrointestinal and inflammatory disorders. *Aliment. Pharmacol. Ther.* **2018**, *48*, 15–34. [CrossRef] [PubMed]
37. Crang, R.E.; May, G. Evidence for silicon as a prevalent elemental component in pollen wall structure. *Can. J. Bot.* **1974**, *52*, 2171–2174. [CrossRef]

38. Exley, C. A possible mechanism of biological silicification in plants. *Front. Plant Sci.* **2015**, *6*. [CrossRef] [PubMed]
39. Guerriero, G.; Hausman, J.-F.; Legay, S. Silicon and the plant extracellular matrix. *Front. Plant Sci.* **2016**, *7*. [CrossRef] [PubMed]
40. Jugdaohsingh, R. Silicon and bone health. *J. Nutr. Health Aging* **2007**, *11*, 99–110. [PubMed]
41. Price, C.T.; Koval, K.J.; Langford, J.R. Silicon: A review of its potential role in the prevention and treatment of postmenopausal osteoporosis. *Int. J. Endocrinol.* **2013**. [CrossRef] [PubMed]
42. Rodella, L.F.; Bonazza, V.; Labanca, M.; Lonati, C.; Rezzani, R. A review of the effects of dietary silicon intake on bone homeostasis and regeneration. *J. Nutr. Health Aging* **2014**, *18*, 820–826. [CrossRef] [PubMed]
43. Jurkic, L.M.; Cepanec, I.; Pavelic, S.K.; Pavelic, K. Biological and therapeutic effects of ortho-silicic acid and some ortho-silicic acid-releasing compounds: New perspectives for therapy. *Nutr. Metab.* **2013**, *10*. [CrossRef] [PubMed]
44. Nielsen, F.H. Update on the possible nutritional importance of silicon. *J. Trace Elements Med. Biol.* **2014**, *28*, 379–382. [CrossRef] [PubMed]
45. Barth, O.M.; Freitas, A.S.; Oliveira, E.S.; Silva, R.A.; Maester, F.M.; Andrella, R.R.S.; Cardozo, G. Evaluation of the botanical origin of commercial dry bee pollen load batches using pollen analysis: A proposal for technical standardization. *An. Acad. Bras. Cienc.* **2010**, *82*, 893–902. [CrossRef] [PubMed]
46. Maurizio, A.; Louveaux, J. *Pollens de Plantes Mellifères D'Europe*; Union des Groupements Apicoles Français: Paris, France, 1965.
47. Ricciardelli, D.A.G. *Textbook of Melissopalynology*; Apimondia Publishing House: Bucharest, Romania, 1997.
48. Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426–428. [CrossRef]
49. Hauptkorn, S.; Pavel, J.; Seltner, H. Determination of silicon in biological samples by icp-oes after non-oxidative decomposition under alkaline conditions. *Fresenius J. Anal. Chem.* **2001**, *370*, 246–250. [CrossRef] [PubMed]
50. Coradin, T.; Eglin, D.; Livage, J. The silicomolybdic acid spectrophotometric method and its application to silicate/biopolymer interaction studies. *J. Spectrosc.* **2004**, *18*, 567–576. [CrossRef]
51. Huang, D.; Ou, B.; Prior, R.L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53*, 1841–1856. [CrossRef] [PubMed]
52. Craciunescu, O.; Constantin, D.; Gaspar, A.; Toma, L.; Utoiu, E.; Moldovan, L. Evaluation of antioxidant and cytoprotective activities of *Arnica montana* L. and *Artemisia absinthium* L. ethanolic extracts. *Chem. Cent. J.* **2012**, *6*. [CrossRef] [PubMed]
53. Chang, C.-C.; Yang, M.-H.; Wen, H.-M.; Chern, J.-C. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J. Food Drug Anal.* **2002**, *10*, 178–182.
54. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved abts radical cation decolorization assay. *Free Radic. Biol. Med.* **1999**, *26*, 1231–1237. [CrossRef]
55. Ritchie, L.E.; Burke, K.F.; Garcia-Mazcorro, J.F.; Steiner, J.M.; Suchodolski, J.S. Characterization of fecal microbiota in cats using universal 16s rRNA gene and group-specific primers for *Lactobacillus* and *Bifidobacterium* spp. *Vet. Microbiol.* **2010**, *144*, 140–146. [CrossRef] [PubMed]
56. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55–63. [CrossRef]
57. Craciunescu, O.; Gaspar, A.; Trif, M.; Moisei, M.; Oancea, A.; Moldovan, L.; Zarnescu, O. Preparation and characterization of a collagen-liposome-chondroitin sulfate matrix with potential application for inflammatory disorders treatment. *J. Nanomater.* **2014**, *2014*, 1–9. [CrossRef]
58. Spulber, R.; Dogaroglu, M.; Babeanu, N.; Popa, O. Physicochemical characteristics of fresh bee pollen from different botanical origins. *Romanian Biotechnol. Lett.* **2018**, *23*, 13357–13365.
59. Mărghitaş, L.A.; Stanciu, O.G.; Dezmirean, D.S.; Bobiş, O.; Popescu, O.; Bogdanov, S.; Campos, M.G. In vitro antioxidant capacity of honeybee-collected pollen of selected floral origin harvested from Romania. *Food Chem.* **2009**, *115*, 878–883. [CrossRef]
60. Altunatmaz, S.S.; Tarhan, D.; Aksu, F.; Barutçu, U.B.; Or, M.E. Mineral element and heavy metal (cadmium, lead and arsenic) levels of bee pollen in turkey. *Food Sci. Technol.* **2017**, *37*, 136–141. [CrossRef]
61. Kolesnikov, M.; Gins, V. Flavonoids and silicon in certain plant pollen. *Chem. Nat. Compd.* **1999**, *35*, 520–523. [CrossRef]

62. Faizul, C.P.; Abdullah, C.; Fazlul, B. Review of extraction of silica from agricultural wastes using acid leaching treatment. In *Advanced Materials Engineering and Technology*; Abdullah, M.M.A., Jamaludin, L., Razak, R.A., Yahya, Z., Hussin, K., Eds.; Trans Tech Publications Ltd.: Stafa-Zurich, Switzerland, 2012; Volume 626, pp. 997–1000.
63. Jayabalan, R.; Marimuthu, S.; Swaminathan, K. Changes in content of organic acids and tea polyphenols during kombucha tea fermentation. *Food Chem.* **2007**, *102*, 392–398. [CrossRef]
64. Sun, T.-Y.; Li, J.-S.; Chen, C. Effects of blending wheatgrass juice on enhancing phenolic compounds and antioxidant activities of traditional kombucha beverage. *J. Food Drug Anal.* **2015**, *23*, 709–718. [CrossRef] [PubMed]
65. Mauriello, G.; Prisco, A.D.; Di Prisco, G.; La Storia, A.; Caprio, E.; Galdiero, M. Microbial characterization of bee pollen from the Vesuvius area collected by using three different traps. *PLoS ONE* **2017**, *12*, e0183208. [CrossRef] [PubMed]
66. Corby-Harris, V.; Maes, P.; Anderson, K.E.; Gerardo, N.M. The bacterial communities associated with honey bee (*Apis mellifera*) foragers. *PLoS ONE* **2014**, *9*, e95056. [CrossRef] [PubMed]
67. Vásquez, A.; Forsgren, E.; Fries, I.; Paxton, R.J.; Flaberg, E.; Szekely, L.; Olofsson, T.C.; Ahmed, N. Symbionts as major modulators of insect health: Lactic acid bacteria and honeybees. *PLoS ONE* **2012**, *7*, e33188. [CrossRef]
68. Malbaša, R.; Lončar, E.; Djurić, M. Comparison of the products of kombucha fermentation on sucrose and molasses. *Food Chem.* **2008**, *106*, 1039–1045. [CrossRef]
69. Chen, C.; Liu, B.Y. Changes in major components of tea fungus metabolites during prolonged fermentation. *J. Appl. Microbiol.* **2000**, *89*, 834–839. [CrossRef] [PubMed]
70. Currie, H.A.; Perry, C.C. Silica in plants: Biological, biochemical and chemical studies. *Ann. Bot.* **2007**, *100*, 1383–1389. [CrossRef] [PubMed]
71. Ehrlich, H.; Demadis, K.D.; Pokrovsky, O.S.; Koutsoukos, P.G. Modern views on desilicification: Biosilica and abiotic silica dissolution in natural and artificial environments. *Chem. Rev.* **2010**, *110*, 4656–4689. [CrossRef] [PubMed]
72. Coton, M.; Pawtowski, A.; Taminiau, B.; Burgaud, G.; Deniel, F.; Coulloumme-Labarthe, L.; Fall, A.; Daube, G.; Coton, E. Unraveling microbial ecology of industrial-scale kombucha fermentations by metabarcoding and culture-based methods. *FEMS Microbiol. Ecol.* **2017**, *93*. [CrossRef] [PubMed]
73. Villarreal-Soto, S.A.; Beaufort, S.; Bouajila, J.; Souchard, J.P.; Taillandier, P. Understanding kombucha tea fermentation: A review. *J. Food Sci.* **2018**, *83*, 580–588. [CrossRef] [PubMed]
74. Cvetković, D.; Markov, S.; Djurić, M.; Savić, D.; Velićanski, A. Specific interfacial area as a key variable in scaling-up kombucha fermentation. *J. Food Eng.* **2008**, *85*, 387–392. [CrossRef]
75. Endo, A.; Futagawa-Endo, Y.; Dicks, L.M. Isolation and characterization of fructophilic lactic acid bacteria from fructose-rich niches. *Syst. Appl. Microbiol.* **2009**, *32*, 593–600. [CrossRef] [PubMed]
76. Reva, O.N.; Zaets, I.E.; Ovcharenko, L.P.; Kukharenko, O.E.; Shpylova, S.P.; Podolich, O.V.; de Vera, J.-P.; Kozyrovska, N.O. Metabarcoding of the kombucha microbial community grown in different microenvironments. *AMB Express* **2015**, *5*. [CrossRef] [PubMed]
77. Endo, A.; Maeno, S.; Tanizawa, Y.; Kneifel, W.; Arita, M.; Dicks, L.; Salminen, S. Fructophilic lactic acid bacteria, a unique group of fructose-fermenting microbes. *Appl. Environ. Microbiol.* **2018**. [CrossRef] [PubMed]
78. Filannino, P.; Di Cagno, R.; Addante, R.; Pontonio, E.; Gobbetti, M. Metabolism of fructophilic lactic acid bacteria isolated from *Apis mellifera* L. Bee-gut: A focus on the phenolic acids as external electron acceptors. *Appl. Environ. Microbiol.* **2016**. [CrossRef] [PubMed]
79. Vajrabhaya, L.-O.; Korsuwannawong, S. Cytotoxicity evaluation of a thai herb using tetrazolium (MTT) and sulforhodamine b (SRB) assays. *J. Anal. Sci. Technol.* **2018**, *9*, 15. [CrossRef]

© 2018 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

Food Biotechnology

ISSN: 0890-5436 (Print) 1532-4249 (Online) Journal homepage: http://www.tandfonline.com/loi/lfbt20

Antifungal Activity of Lactic Acid Bacteria Isolated from Peanuts, Gari, and Orange Fruit Juice against Food Aflatoxigenic Molds

Kaktcham Pierre Marie, Zambou Ngoufack François, Foko Kouam Edith Marius, Oana Ciobotaru, Florentina Matei, Calina Petruta Cornea & Florentina Israel-Roming

To cite this article: Kaktcham Pierre Marie, Zambou Ngoufack François, Foko Kouam Edith Marius, Oana Ciobotaru, Florentina Matei, Calina Petruta Cornea & Florentina Israel-Roming (2018) Antifungal Activity of Lactic Acid Bacteria Isolated from Peanuts, Gari, and Orange Fruit Juice against Food Aflatoxigenic Molds, Food Biotechnology, 32:4, 237-256, DOI: 10.1080/08905436.2018.1519443

To link to this article: https://doi.org/10.1080/08905436.2018.1519443

Published online: 27 Nov 2018.

Submit your article to this journal

View Crossmark data

Full Terms & Conditions of access and use can be found at
http://www.tandfonline.com/action/journalInformation?journalCode=lfbt20

FOOD BIOTECHNOLOGY
2018, VOL. 32, NO. 4, 237–256
https://doi.org/10.1080/08905436.2018.1519443

Taylor & Francis
Taylor & Francis Group

Check for updates

Antifungal Activity of Lactic Acid Bacteria Isolated from Peanuts, Gari, and Orange Fruit Juice against Food Aflatoxigenic Molds

Kaktcham Pierre Marie [a,b], Zambou Ngoufack François[a], Foko Kouam Edith Marius[a], Oana Ciobotaru[b], Florentina Matei[b], Calina Petruta Cornea[b], and Florentina Israel-Roming[b]

[a]Laboratory of Biochemistry, Food Science and Nutrition (LABPMAN), Department of Biochemistry, Faculty of Science, University of Dschang, Dschang, Cameroon; [b]Faculty of Biotechnology, University of Agronomic Sciences and Veterinary Medicine (U.S.A.M.V of Bucharest. 59), Bucharest, Romania

ABSTRACT

This study aims to evaluate the antifungal activity of lactic acid bacteria (LAB) from some Cameroonian food commodities against mycotoxigenic and spoilage molds. Following LAB isolation, the antifungal activity of the isolates was assessed. The organic acids were quantified using high-performance liquid chromatography and the ability of the LAB to reduce mold biomass and aflatoxin production was evaluated. The LAB were identified and the biopreservative potential of strain LO3 was evaluated on tomato paste. Nine percent of the strains isolated showed broad antifungal activity. The activity was due to the effect of organic acids comprising lactic, acetic, 4-hydroxy-3-phenyllactic and 3-phenyllactic acids. Lactobacillus plantarum LO3 exhibiting the highest and broadest antifungal activity was selected and showed the capacity to inhibit fungal growth and aflatoxin production in vitro. Moreover, this strain and its cell-free supernatant showed the ability to prevent aflatoxigenic mold growth in tomato paste without altering its physico-chemical and organoleptic properties.

KEYWORDS

Aflatoxigenic and spoilage molds; *L. plantarum*; antifungal activity; organic acids; molecular identification

Introduction

Molds are the main spoilage organisms of various food products, causing substantial economic losses (Pitt and Hocking 2009). Moreover, they are one of the major worldwide causes of mutagenic, carcinogenic, and teratogenic diseases, due to their capacity to produce mycotoxins. They thus constitute a serious health risk for humans and animals. The problem of food and feed contamination with spoilage and mycotoxigenic molds still prevails worldwide. As a remedy, the biological control

CONTACT Kaktcham Pierre Marie pierre.kaktcham@univ-dschang.org; pierre.kaktcham@univ-dschang.org; pierre.kaktcham@univ-dschang.org Department of Biochemistry, University of Dschang, P.O Box 67 Dschang, Cameroon

Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/lfbt.

© 2018 Taylor & Francis Group, LLC

of food mycotoxigenic and spoilage molds has usually been considered as one of the best approaches to impede these harmful effects (Dalié, Deschamps and Richard-Forget 2010). In this respect, strategies involving chemical preservatives added directly to food have been used in the farming and food processing industries. However, the development of chemical-resistant molds is becoming problematic (Viljoen 2001). In addition, the increasing consumers' trend toward high-quality and chemical preservative-free foods, coupled with the ban of many chemical-based preservatives by the European legislation (Directive (CE) No 1107/2009 of the European Union), have prompted the quest for more natural preservation approaches (Official Journal of the European Union 2009). In response, biopreservation has been of growing interest during the last decade. Lactic acid bacteria (LAB) are of particular interest as biopreservation agents, as many of them have been granted the generally recognized as safe (Von Wright 2005) status and qualified presumption of safety status (EFSA, 2013). They possess antifungal properties, as reported by numerous existing studies (Gerez et al. 2013; Lavermicocca et al. 2000; Schnürer and Magnusson 2005; Ström et al. 2002; Wang et al. 2012; Yang and Chang 2010; Yang, Kim and Chang 2011). In fact, LAB can produce different kinds of antifungal bioactive molecules, such as hydroxyfatty acids, phenyllactic acids (P.L.A), cyclic dipeptides, 3,6-bis (2-methylpropyl)-2,5-piperazinedion and δ-dodecalacetone proteinaceous compounds, and bacteriocin-like substances. The application of antifungal metabolite-producing LAB in food preservation has also been well documented to date, as evidenced by an increasing number of studies (Coda et al. 2011; Crowley, Mahony and van Sinderen 2012; Gerez et al. 2009; Rouse et al. 2008; Yang and Chang 2010). However, in some developing countries where regulations are nonexistent or not enforced, the majority of inhabitants are exposed to the consumption of mycotoxin-contaminated products, either directly or at various points of the food chain. In Cameroon for instance, the isolation and characterization of mycotoxin-producing mold strains from certain commonly consumed food commodities have been previously reported (Abia et al. 2013; Leong et al. 2012; Ngoko et al. 2008; Njobeh et al. 2009). Therefore, the search for means of their biological control ought to be urgently addressed.

The purpose of the present study was to isolate LAB from Cameroonian food commodities and evaluate their antifungal activity against mycotoxigenic and spoilage molds. The aflatoxin (AF) production potential of molds strains, the *in vitro* antifungal activity and ability to reduce AF production, the identification of antifungal metabolites, and the biopreservation potential of promising LAB strains were investigated.

Materials and methods

Mold strains, culture media, and culture conditions

The mold strains used in this study are listed in Table 1. They were formerly isolated from frequently consumed food commodities in Cameroon, and from cereals in Romania. They were found to produce mycotoxins on coconut agar medium, and their identification was earlier achieved based on cultural and microscopic characteristics, following the methods described by Pitt and Hocking (2009). Before use, they were grown on potato dextrose agar (PDA, Carl Roth, GmbH+ Co, Karlsruhe, Germany) plates at 29°C until sporulation (5–7 d). Mold spores suspensions were prepared by scraping spores from the surface of PDA culture medium and resuspending in PBS solution (pH 7.0). These suspensions were filtered twice using sterile cheesecloth and adjusted to the final concentration of 10^5–10^6 spores/ml using a Neubauer hemocytometer.

Determination of the mold strains' mycotoxigenic potential: Detection and quantification of total AFs and ochratoxin A

The ability of *Aspergillus flavus* strains to produce AFs *in vitro* was tested using the methodology described by Reddy et al. (2009) with slight modifications. Briefly, the mold strains were individually cultured on yeast extract sucrose agar for 7 d at 25°C in the dark. Then, plugs of medium (5 g) were cut from each Petri dish and ground for 1 h in 3 ml methanol/H_2O (80:20 v/v) solution to extract total AFs. The resultant mixtures were subsequently filtered using Whatman paper No 4, and the filtrates were analyzed for the presence of total AFs by ELISA using the RIDASCREEN® FAST Aflatoxin kit (R-Biopharm, Darmstadt, Germany), according to the manufacturer's standard protocol.

In the second set of the experiment, *Penicillium citrinum* AR2 and *Aspergillus niger* AR4 were assayed for OTA production according to the slightly modified method of Koteswara Rao et al. (2013). The molds were

Table 1. Mold strains used in this study.

Fungi	Source (Origin)
Aspergillus flavus AR1	Peanuts (Cameroon)
A. flavus OG2	Orange (Cameroon)
A. flavus MB5	Dried fish (Cameroon)
A. flavus PN4	Spices (Cameroon)
A. flavus MUCL 18903	Culture collection[a] (Romania)
A. flavus GE2	Wheat (Romania)
A. flavus OR 31	Barley (Romania)
A. flavus T11	Triticale (Romania)
Aspergillus niger AR4	Peanuts (Cameroon)
Penicillium citrinum AR2	Peanuts (Cameroon)
Penicillium verrucosum AR5	Peanuts (Cameroon)

[a]Laboratory of Microbiology of the University of Agronomic Sciences and Veterinary Medicine of Bucharest.

inoculated on Czapek yeast extract agar (Fluka Chemie AG, Buchs, Switzerland) plates and subsequently incubated at 25°C for 10 d in the dark. Five grams of agar medium together with mycelium were collected from the culture plates and ground in 20 ml of acetonitrile/H_2O (60:40 v/v) using a blender. The preparations were filtered as previously detailed, and the filtrates were subsequently analyzed for the presence of OTA using HPLC as described by Rotaru et al. (2011).

Isolation of LAB

Three frequently consumed food commodities (peanuts, gari, i.e., starch-depleted cassava granular flour, and oranges) were collected from the local markets in the western highland regions of Cameroon. Orange fruits were cleaned using 70% ethanol and rinsed with sterile distilled water. They were then aseptically cut and squeezed to obtain the juice. Meanwhile, peanuts and gari samples were ground in a sterile blender. LAB were isolated after plating tenfold serial dilution of each sample onto MRS agar (Liofilchem, Srl, Roseto degli Abruzzi, Italy). Preliminary identification of LAB isolates were done by catalase test, oxidase test, Gram staining, and microscopy and then maintained either on MRS agar slants at 4°C with a monthly based sub-cultivation until assayed, or at −20°C for long-term conservation in MRS broth containing 25% (v/v) glycerol as cryoprotectant.

The strain *Lactobacillus plantarum* IC12353 (from the culture collection of the Laboratory of Microbiology of the University of Agronomic Sciences and Veterinary Medicine of Bucharest) was used as the reference strain for the molecular identification of LAB isolates.

Antifungal overlay assay

The ability of the LAB isolates to inhibit mold growth was evaluated using the overlay method on solid media as described by Magnusson et al. (2003). Briefly, LAB were inoculated in two 2-cm lines on MRS agar plates and allowed to grow at 30°C for 48 h in candle jars. The plates were subsequently overlaid with 10 ml of potato dextrose soft agar (0.08% agar) containing approximately 10^4 indicator mold spores (conidia) per ml. After aerobic incubation at 30°C for 48 h, the zone of inhibition was measured. The indicator molds are listed in Table 1. The degree of inhibition was calculated as a ratio or proportion (%) of the surface area of the zone of growth inhibition relative to the total surface area of the Petri dish, and the scale was the following: (-) = no visible inhibition, (+) = no fungal growth on 0.1–3% of plate area/bacterial streak, (++) = no fungal growth on 3–8% of plate area/bacterial streak, (+++) = no fungal growth on >8% of plate area/bacterial streak.

Identification and quantification of the antifungal compounds

Here, the identification, as well as quantitative analyses of the antifungal compounds produced, was assessed. Briefly, each of the nine LAB isolates that showed a strong inhibition of mold growth in the agar overlay assay was cultivated in MRS broth for 24 h at 37°C, and then centrifuged at 3,000 ×*g* for 10 min, 4°C. The cell-free supernatants (CFS) were collected, filtered (0.20-µm Millipore Nylon syringe filters) and subsequently subjected to HPLC analysis for organic acid detection and quantification. Thus, lactic, acetic, propionic, 4-hydroxy-3-phenyllactic [2-hydroxy-3-(4-hydroxyphenyl) propanoic acid, (OH) $C_6H_5CH_2CH(OH)COOH$] and 3-phenyllactic [2-hydroxy-3-phenylpropionic acid, $C_6H_5CH_2CH(OH)COOH$] acids were quantified by a Waters Alliance HPLC system using a reverse phase column (SUPELCOGEL column, 25-cm length × 4.6-mm inner diameter, SUPELCO Analytical, Bellefonte, Pennsylvania 16823, USA). The conditions were as follows: injection volume 10 µl; mobile phase 0.1% phosphoric acid in ultrapure water; flow rate 0.17 ml/min; isocratic elution; temperature of column 40°C; a UV (210 nm) detector (Waters 2487 model) connected to the hardware system. A standard curve was plotted, and the data acquisition and management were completed using the Empower 2 software. After a first screening, five LAB isolates were selected following their ability to produce various organic acids and were subjected to further analyses. All the standards and reagents originated from Sigma (Sigma-Aldrich, Saint Louis, Missouri, USA).

Effect of LAB on A. flavus AR1 mycelium growth and AF production

The effects of isolates LO3 and LA5 on *A. flavus* AR1 mycelium growth and AF production were assessed by coculture experiment as described by Coman et al. (2014) with slight modifications. The experiments were performed in a modified MRS medium named mMRS (MRS broth/potato dextrose broth 1:1 v/v), capable of sustaining the growth of both types of microorganisms. In the first experiment, three 100-ml Erlenmeyer flask containing each 25 ml aliquot of mMRS were inoculated separately so as to obtain: 10^5 spores/ml of *A. flavus* AR1 strain (Control 1); 10^8 CFU/ml of each LAB strains (control 2); 10^8 CFU/ml of each LAB strains and 10^5 spores/ml of *A. flavus* AR1 strain (test). Incubations were carried out aerobically without shaking at 25°C for up to 15 d. The LAB cell suspensions were prepared in PBS following centrifugation (3,000 ×*g* for 10 min, 4°C), two washes in PBS, and resuspension in the same buffer solution. Their cell concentrations were determined by plate count onto MRS agar. In the second experiment, each LAB isolate was firstly cultured in MRS broth at 30°C for 24 h. Then, *A. flavus* AR1 (chosen based on its high AF production ability) strain was inoculated in each of the 24-h-old culture

and incubated at 25°C for 15 d. At the end of the incubation periods, the mold growth in mMRS medium and the paired cultures with LAB (first and second experiments) was expressed as the percentage of the difference in mycelia growth (expressed in mm as measured on the wall of the tube using a Vernier caliper) compared to the positive mold control. Thereafter, the mycelia were collected and weighed before and after drying (at 105°C until constant weight in an oven). In addition, quantitative analysis of total AFs in mMRS media from the first and second experiments was carried out as described in the second subheading of the "Materials and methods" section. Prior to this analysis, total AFs were extracted from broth medium using methanol/H_2O (80:20 v/v) solution. The mold biomass reduction was expressed as the percentage of the mycelia dry weight (g) compared to the positive mold control. The total AF reduction was expressed as the percentage of AF production compared to the control.

Phenotypic and genotypic identification of some LAB isolates

Phenotypic identification

Physiological characteristics such as the production of gas from glucose and the ability to grow in MRS broth at 10°C for 10 d, 45°C and pH 9.6 for 48 h, as well as in MRS broth + 4% and 6.5% NaCl were evaluated. Moreover, isolates were biochemically identified using the API 50CHL kit (BioMérieux, Marcy-l'Etoile, France). The API (Analytical Profile Index) 50 CHL kit test results were analyzed using the APIWEB® software.

DNA extraction

Genomic DNA was extracted from LAB cells according to the protocol of Gevers, Huys, and Swings (2001).

BOX-PCR and ERIC-PCR genotyping

For the BOX-PCR assay, the primer used was BOXA1R (5′-CTACGGCAAGGCGACGCTGACG-3′), whereas the primers pair ERIC-I (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC-II (5′-AAGTAAGTGA CTGGGGTGAGCG-3′) was used for the ERIC-PCR assay. Amplifications were performed as described by Mohammed et al. (2009) and PCR products were visualized after electrophoresis on 2% agarose gel (run at 90 V for 90 min) and staining with ethidium bromide using Gel Doc XR + Imaging System (BioRad, Madrid, Spain).

Species-specific PCR assay

In this experiment, the rec-A gene-based primer, planF (5′-CGCTTTATGCGGQQCQCCTQ-3′), specific for *L. plantarum*, was used

and the amplification was carried out as described by Torriani, Felis, and Dellaglio (2001). The PCR products were examined as described above. *L. plantarum* IC12353 was the reference strain in all the PCR assays.

16S rRNA gene sequencing

The LAB strain LO3 was further identified by 16S rRNA gene sequencing. Briefly, genomic DNA was extracted from pure cultures by the standard phenol-chloroform-isoamyl alcohol method described by Ampe et al. (1999). The 16S rRNA gene PCR amplification was performed using the following forward and reverse primers respectively: **pA**: 5′-AGAGTTTGATCCTGGCTCAG-3′ and **3**: 5′-GTTGCGCTCGTTGCGGGACT-3′. The DNA sample was subjected to an initial denaturation cycle (3 min at 94°C), followed by 34 cycles of denaturation (1 min at 94°C), annealing (1.5 min at 65°C), extension (2 min at 72°C), ending with 2 final extension cycles (15 min at 72°C each). The PCR product was analyzed as described previously and subsequently purified using Montage^{TM} GENOMICS Kit (Millipore Corporation, Bedford, USA). The sequencing of the purified PCR products, targeting the V1-V2 hypervariable regions of the 16S rDNA, was performed by the commercial services of Macrogen Inc. (Seoul, Korea). Chimeras were trimmed from the sequences by using the bioinformatics software ChromasPro 1.7.7 and the assignment of the isolate to the closest related taxon was achieved using BLAST analysis tool in the NCBI GenBank database (www.ncbi.nih.gov). The 16S rDNA sequence of strain LO3 was deposited into the NCBI GenBank database to obtain an accession number.

Biocontrol activity of L. plantarum *LO3 and its CFS on aflatoxigenic* A. flavus *AR1*

The biopreservation potential of *L. plantarum* LO3 and its CFS was assessed on tomato paste. Fresh tomato fruits (*Solanum lycopersicum* L.) were purchased from the markets in the western highland regions of Cameroon. They were carefully hand-selected, surface-sterilized by dipping for 5 min in 2% of sodium hypochlorite, rinsed with sterile distilled water, and air-dried under the hood. Afterwards, the fruits were dipped in boiling water for 3 min, subsequently immersed in cold water, ground in a sterilized grinder to obtain the paste and finally pasteurized (heating in boiling water at 71°C for 5 min followed by immediate cooling at 4°C). The pasteurized paste was divided into four batches each constituting of 24 screw-capped test tubes containing 20 g of paste per tube. The control batches were inoculated with 10^5 spores/ml of *A. flavus* AR1 strain (positive control) or not (negative control). One of the test batches was inoculated with 10^5 spores/ml of *A. flavus* AR1 and 10^8 CFU/ml of *L. plantarum* LO3 (T1), while the other was inoculated with 10^5 spores/ml of *A. flavus* AR1 and then received 1 ml of CFS (T2). All samples were incubated at 30°C

for 9 d, observed for spore germination (visual growth) and also analyzed for pH as well as microbiological and sensory properties at d 0, 3, 6, and 9. For the microbiological analysis, the presence of molds and LAB in the samples was evaluated by plating the samples onto PDA and MRS agar, respectively. For the sensory analyses, a descriptive quantitative test was carried out using a panel of 15 trained persons. They were recruited based on criteria such as availability, interest and motivation, intellectual ability, the state of physiological condition, absence of anomalies or alterations affecting the senses. They were trained to assess the intensity of each of the descriptors (Color, texture, and odor), with the objective of being able to determine small differences in intensity and to give identical answers for the same product (repeatability). Using a graphic scale of 1–5, the panel evaluated the color (1 = scarlet red; 2 = red; 3 = cardinal red, 4 = red carmine; 5 = red burgundy), texture (1 = pasty; 2 = very heavy; 3 = heavy, 4 = slightly heavy; 5 = liquid), and odor (1 = excellent; 2 = very good; 3 = good, 4 = bad; 5 = very bad).

Statistical analysis

The data of total AF concentrations, organic acid concentrations, pH as well as sensory parameters were presented as the mean ± standard deviation and analyzed by one-way ANOVA. When differences were significant between means, the latter were separated using Student's *t*-test. The analyses were performed using GraphPad InStat software, and the differences were considered significant for p-values <0.05.

Results

Mycotoxins production by the mold strains

The measured total AF (AFB_1, AFB_2, AFG_1, and AFG_2) levels produced by the tested *A. flavus* strains are shown in Fig. 1. All the strains tested showed the ability to produce significant concentrations (ppb) of AFs in the agar medium. Mold strains AR1 and MUCL 18903 showed levels that are above the maximum permissible level set by the European Union for their residues in food and feed (15 µg/kg). Ochratoxin A (OTA) was not found to be produced by the mold strains tested.

Isolation of LAB and screening for antifungal activity

Of the 100 LAB isolated, 9 were able to show high and broad antifungal activity against the tested mold strains (Table 2). It is noteworthy that only isolates LO3 and LA5 were able to inhibit all the tested mold strains, showing high (+++) to moderate (++) antifungal activities. *P. citrinum* AR2, *P. verrucosum* AR5, and *A.*

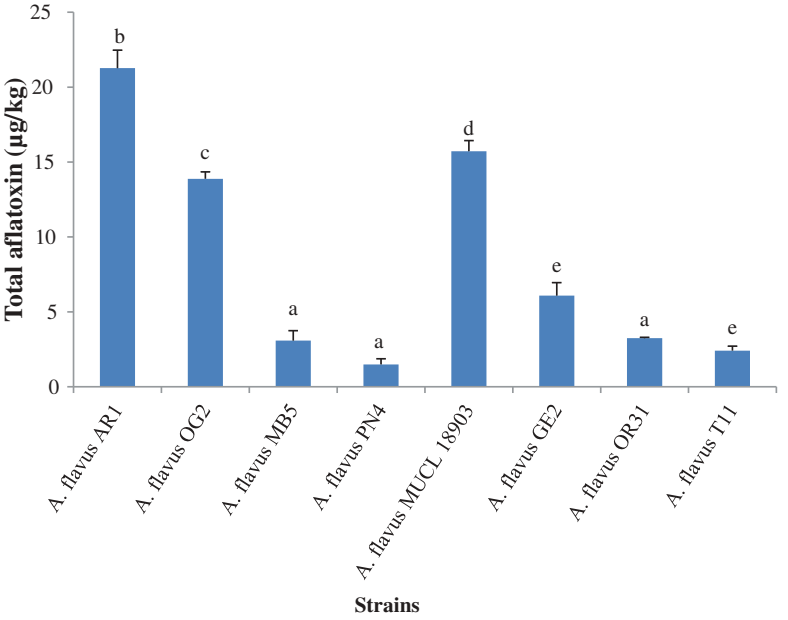


Figure 1. Total aflatoxin production by *Aspergillus flavus* strains grown on yeast extract sucrose agar for 7 d at 25°C.

[a]: The values with this symbol do not differ from each other ($p > 0.05$), but differ significantly from the others ($p < 0.01$).
[b, c, d, e]: The values carrying these symbols differ significantly ($p < 0.05$) from each other and from the others.
Aflatoxins were extracted from agar medium plugs with methanol/water (80:20), filtered, and quantified using RIDASCREEN® FAST Aflatoxin kit. The test was performed in duplicate.

Table 2. Inhibitory activity of nine preselected LAB isolates against the aflatoxigenic and non-aflatoxigenic fungi on MRS agar overlaid with soft PDA containing the indicator molds spores.

	Fungal indicator strains										
	A. flavus								*A. niger*	*P. citrinum*	*P. verrucosum*
LAB	AR1	OG2	MB5	PN4	GE2	OR31	T11	MUCL 18903	AR4	AR2	AR5
LA1	-	++	+++	++	-	+	-	+	-	++	++
LA2	-	+++	+++	++	-	-	-	+	-	++	++
LA3	+	-	-	+	-	-	+	-	+	+++	+
LA4	+	-	-	+	-	-	+	-	++	+++	++
LA5	++	++	++	++	+	+	+	++	+	+++	+++
LA6	++	-	-	+	-	+	+	-	+	++	++
LO1	-	+	+	+	-	-	-	-	+	+	++
LO2	+	++	++	+	+	-	-	+	+	+++	+++
LO3	+++	+++	+++	+++	++	+	++	++	+++	+++	+++

*bacterial culture overlays: (-) = no visible inhibition, (+) = no fungal growth on 0.1–3% of plate area/bacterial streak, (++) = no fungal growth on 3–8% of plate area/bacterial streak, (+++) = no fungal growth on >8% of plate area/bacterial streak. The test was performed in duplicate. Strains with code LA originate from peanut and those with code LO from orange fruit juice.

flavus PN4 were the most sensitive mold strains, while *A. flavus* AR1, *A. flavus* OG2, *A. flavus* MB5, *A. flavus* T11, and *A. flavus* MUCL 18903 were inhibited by at least 50% of the LAB isolates. *A. flavus* GE2 and *A. flavus* OR31 were found to be resistant to inhibition by the LAB isolates.

Five LAB isolates (LA1, LA2, LA5, LO2, LO3) that showed moderate to high antifungal activities were thereafter selected for evaluation of organic acids production and their quantification.

Identification and quantification of the antifungal compounds

The HPLC analysis revealed that lactic acid (L.A), acetic acid (A.A), 4-hydroxy-3-phenyllactic acid (HO-P.L.A), and P.L.A were produced by the LAB isolates assayed. As shown in Fig. 2, the significantly lowest ($p < 0.05$) concentration of L.A (82.51 ± 3.09 mmol/L) was found in the CFS of isolate LA2, whereas the concentrations in the CFS of isolates LA1, LA5, LO2, and LO3 were not significantly different ($p > 0.05$), with the highest being 122.32 ± 1.39 mmol/L. MRS contains 111.1 mmol/L of glucose and any L. A concentration above 111.1 mmol/L represents a >50% bioconversion of substrate.

Only one isolate (LO3) was able to produce A.A (9.55 ± 0.75 mmol/l), while none of them produced propionic acid (a known mold inhibitor). Isolates LA5 and LO3 were the best producers of HO-P.L.A (0.71 ± 0.04 mmol/L and 0.35 ± 0.01 mmol/L, respectively) and P.L.A (1.11 ± 0.10 mmol/L and 0.54 ± 0.02 mmol/L, respectively). It is important to mention that for each isolate, the organic acid concentrations in the CFS from 24-, 48-, and 72-h cultures were also determined, but were not significantly different ($p > 0.05$). Based on their high antifungal activity, coupled with their high production of organic acids, the isolates LO3 and LA5 were selected for the next experiments.

Effect of LAB on A. flavus AR1 mycelium growth and AF production

The effects of the two LAB isolates on the reduction of *A. flavus* AR1 mycelium growth and AF production were studied in two growth phases, and the results are presented in Table 3.

In cocultures (simultaneous mold-LAB inoculation and mold strain inoculation into a 24-h-old LAB culture), the LAB isolates LO3 and LA5 completely inhibited the mycelium growth of *A. flavus* AR1 after 7 d of aerobic incubation. During this same period, AF production was reduced at a 100% level by each of the LAB isolates. After 15 d of incubation, isolate LO3 was able to completely reduce (100%) mold biomass and AF production following *A. flavus* AR1 inoculation into 24-h-old LO3 culture. While, during simultaneous mold and strain LO3 inoculation, respectively, 85% and 96%

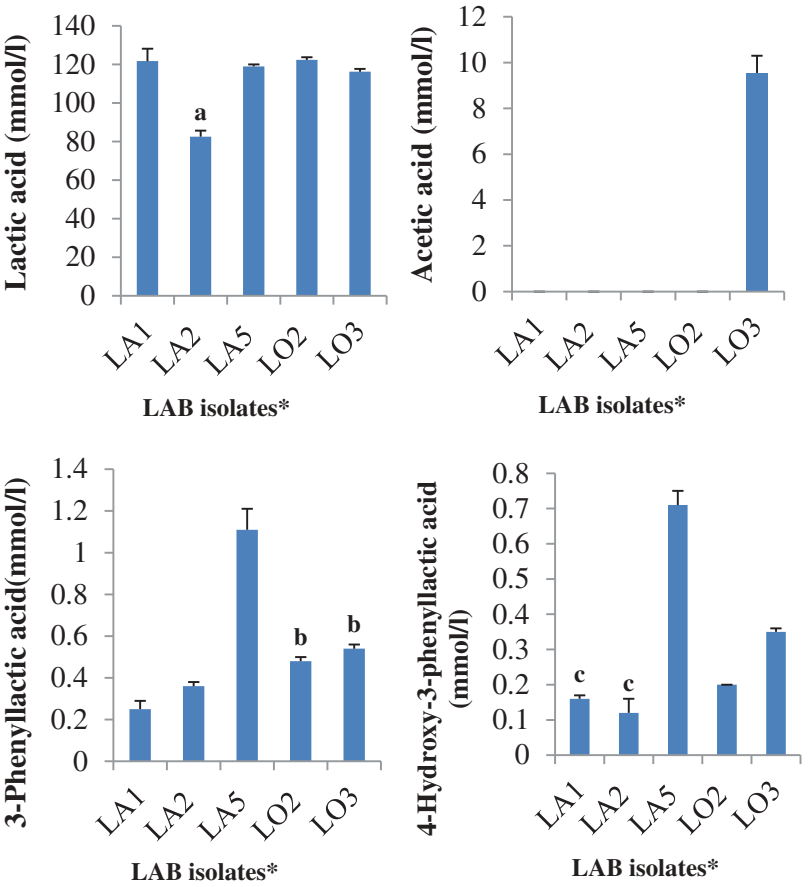


Figure 2. Concentrations of the organic acids produced by the selected antifungal-LAB isolates as determined by HPLC.

*Organic acids were quantified in the crude cell-free supernatant (CFS) from a 24-h culture of each isolate. The Mean pH of each CFS was LA1 (pH 4.34); LA2 (pH 4.49); LA5 (pH 4.35); LO2 (pH 4.33); LO3 (pH 4.29).
[a]Only the value carrying this symbol differs significantly ($p < 0.001$) from the others.
[b]At the exception of the values carrying this symbol, all the other values in this column are significantly different ($p < 0.01$) from each other.
[c]The values with this symbol are significantly different ($p < 0.05$) from the others.
The test was performed in duplicate.

mold biomass and AF production reduction levels were recorded. For isolate LA5, mold biomass and AF production reduction levels of 75% and 86%, respectively, were recorded following simultaneous inoculation with *A. flavus* AR1, against 80% and 92%, respectively, after inoculation of *A. flavus* AR1 into 24-h-old LA5 culture. The reduction of AF production thus followed the same pace as the reduction of mold biomass. The A.A produced by isolate LO3 hence contributed substantially to its antifungal activity. This isolate was finally selected as the best.

Table 3. *A. flavus* AR1 biomass and aflatoxin production reductions in mixed cultures with LAB after 7 and 15 d incubation at 30°C.

	AR1(control 1)		LO3 + AR1		LA5 + AR1	
	0 h	24 h	0 h	24 h	0 h	24 h
7 d						
MDW (g)	77.63	79.47	0	0	0	0
AFs (μg kg^{-1})	12.16	14.07	0	0	0	0
FB red (%)	0%	0%	100%	100%	100%	100%
AFs red (%)	0%	0%	100%	100%	100%	100%
15 d						
MDW (g)	77.81	79.78	11.43	0	19.45	15.96
AFs (μg kg^{-1})	13.87	13.29	0.55	0	1.86	1.06
FB red (%)	0%	0%	85%	100%	75%	80%
AFs red (%)	0%	0%	96%	100%	86%	92%

0 h: simultaneous inoculation of LAB + Fungal strain. 24 h: inoculation of LAB 24 h prior to fungal inoculation.

AR1: *A. flavus* AR1; LO3 + AR1: *A. flavus* AR1 in mixed culture with LAB strain LO3; LA5 + AR1: *A. flavus* AR1 in mixed culture with LAB strain LA5; MDW: mold dry weight; FB red: fungal biomass reduction; AFs: total aflatoxin production; AFs red: total aflatoxin reduction. The test was performed in duplicate.

Phenotypic and genotypic identification of some LAB isolates

The physiological tests showed that among the five selected isolates with high to moderate antifungal activities, four of them (LA1, LA2, LO2, and LO3) corresponded well to *Lactobacillus* Group II (facultative heterofermentatives), whereas isolate LA5 was assigned to *Lactococcus* sp. Based on biochemical characterization using API 50 CHL kit, isolates LA1, LO2, and LO3 were classified to the species *L. plantarum* (99.90% similarity). While, isolate LA5 was classified to the subspecies *Lactococcus lactis* subsp. *lactis* (99.80% similarity). The results of the BOX-PCR assay of the nine isolates tested in the overlay assay (Fig. 3a) demonstrated that the isolates LA1, LO2, and LO3 looked alike and were also similar to the reference strain *L. plantarum* IC12353, thus indicating that they effectively belong to *L. plantarum* species. Moreover, the differences in the BOX-PCR profiles of isolates LA2 and LA5 showed that they are different from each other.

The species-specific PCR analysis of isolate LO3 showed amplified products with a size of about 322 bp which is identical to the amplified product of the reference strain *L. plantarum* IC12353 (Fig. 3b). The phenotypic similarity and the outcome of this analysis allow us to confirm the initial affiliation of the isolate LO3 to the species *L. plantarum*. Also, the comparative analysis of the 16S rRNA gene sequence revealed that isolate LO3 (327 bp) had 99% nucleotide base homology with *L. plantarum*, with a 100% query coverage. The NCBI GenBank accession number of the sequence is KU921701.

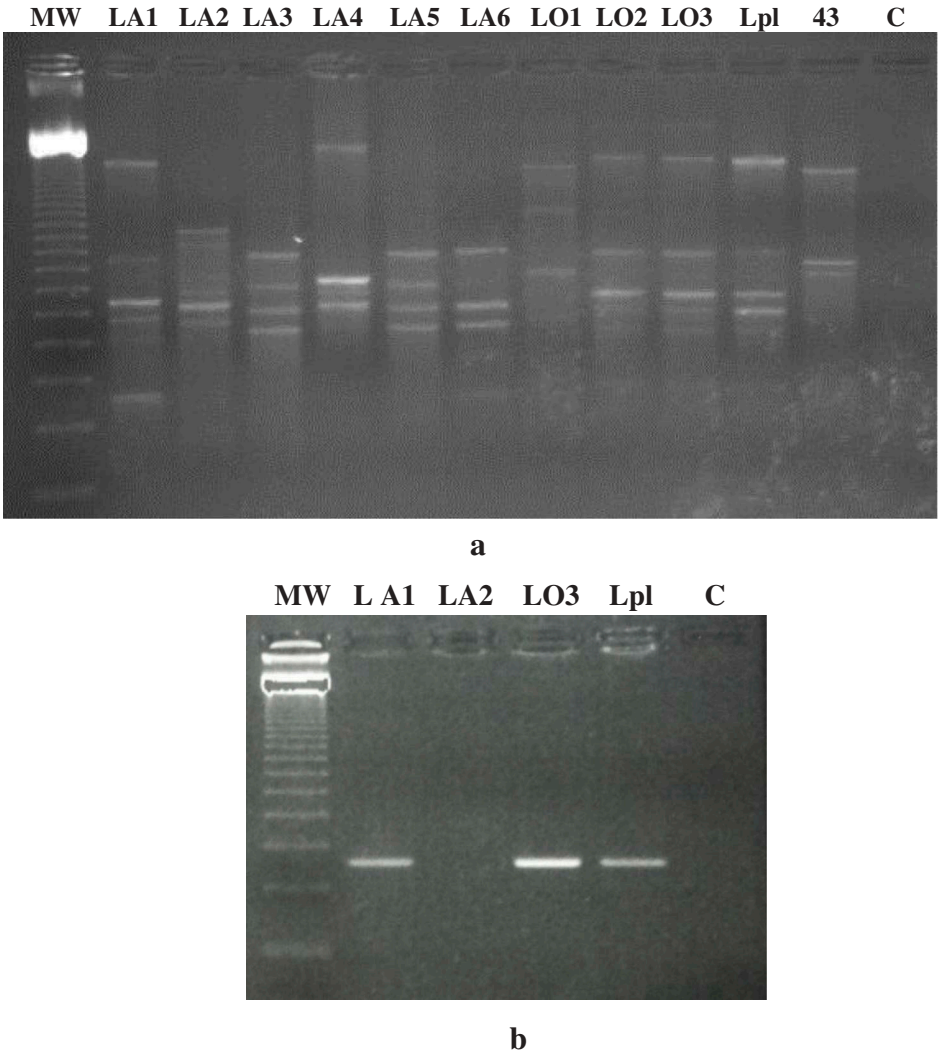


Figure 3. (a) BOXA1R-PCR profiles of LAB isolates LA1, LA2, LA3, LA4, LA5, LO1, LO2, and LO3 obtained after electrophoretic migration of their BOX-PCR products. (b) Amplicons obtained from species-specific PCR of isolates LA1, LA2, and LO3 using the primer planF derived from recA gene of *Lactobacillus plantarum* strains. Lane MW corresponds to a 123 bp DNA ladder. Lane Lpl: *Lactobacillus plantarum* IC12353 reference strain; Lane C: MilliQ water used as negative control.

Biocontrol activity of L. plantarum *LO3 and its CFS on aflatoxigenic* A. flavus *AR1 using tomato paste as a food model*

The biopreservation potential of *L. plantarum* LO3 and its CFS was assessed on tomato paste infected with spores of the aflatoxigenic strain *A. flavus* AR1. The pH of the paste as well as the *A. flavus* AR1 spore germination in the paste were monitored during storage, and the results are presented in Table 4. At d 0, only the pH of the sample T_2 (pasteurized tomato paste + *A. flavus* AR1 spores + CFS) was significantly different from the others, evidently due to the influence of the

Table 4. Effects of *L. plantarum* LO3 and its cell-free supernatant on spore germination of the aflatoxigenic *A. flavus* AR1 strain used to inoculate tomato paste.

		C-	C+	T_1	T_2
D 0	*pH*	4.41 ± 0.03^{Ab}	4.40 ± 0.23^{Bb}	4.41 ± 0.04^{Ab}	3.87 ± 0.27^{Aa}
	Spore germination	-	-	-	-
D 3	*pH*	4.42 ± 0.01^{Ab}	4.10 ± 0.01^{Bb}	3.80 ± 0.03^{Aa}	3.82 ± 0.03^{Aa}
	Spore germination	-	+	-	-
D 6	*pH*	4.40 ± 0.08^{Ac}	3.82 ± 0.05^{Abc}	3.57 ± 0.03^{Aa}	3.78 ± 0.03^{Ab}
	Spore germination	-	+	-	-
D 9	*pH*	4.41 ± 0.07^{Aa}	3.78 ± 0.30^{Aa}	3.40 ± 0.83^{Aa}	3.77 ± 0.03^{Aa}
	Spore germination	-	+	-	+

-: No visual mold growth. +: Visual mold growth.

$^{a-c}$The values carrying different letters on the same line differ significantly ($p < 0.05$).

^{A,B}The values carrying different capital letters on the same column for one parameter differ significantly ($p < 0.05$).

C-: Pasteurized tomato paste (negative control); C+: pasteurized tomato paste + *A. flavus* AR1 spores (positive control); T_1: pasteurized tomato paste + *A. flavus* AR1 spores + *L. plantarum* LO3; T_2: pasteurized tomato paste + *A. flavus* AR1 spores + cell-free supernatant. The tests were performed in duplicate. *A. flavus* AR1(from spore suspension in PBS) and *L. plantarum* LO3 (cells suspension in PBS) were added at final concentration of 10^5 spores/ml and 10^8 CFU/ml, respectively, and the incubation was held at 30°C.

initial pH of the supernatant added. From d 6 to 9, only the pH of sample C_+ (positive control: pasteurized tomato paste + *A. flavus* AR1 spores) was significantly different from the others. Spore germination, recorded as visual growth, was observed in sample C_+ from d 3 to 9, while it was observed in sample T_2 from d 6 to 9. Thus, *L. plantarum* LO3 inhibited *A. flavus* AR1 growth for 9 d, while its supernatant could do so for 6 d.

Also, the sensory properties of tomato paste samples were monitored during the storage period, and the results are shown in Table 5. It reveals that, compared to the other samples, only the color of sample C_+ (positive control: pasteurized tomato paste + *A. flavus* AR1 spores) was significantly affected during the storage (from d 6 to 9). The growth of *A. flavus* AR1 in the tomato paste (as testified by the result of spore germination) hence affected its color.

Discussion

In the present study, all the *A. flavus* strains tested showed the ability to produce AFs. The results are consistent with some previous studies that reported *A. flavus* strains as the high AF producers in peanuts and many other food commodities in Cameroon (Kana et al. 2013; Leong et al. 2012; Ngoko et al. 2008; Njobeh et al. 2009), and in the rest of the world as well (Cornea et al. 2013; Liu et al. 2013; Milhome et al. 2014; Muñoz et al. 2010; Yousef and Naresh 2010).

The LAB isolates from Cameroonian food commodities demonstrated high and broad antifungal activity against aflatoxigenic *A. flavus* strains as

Table 5. Effects of *L. plantarum* LO3 and its cell-free supernatant on the sensory properties of tomato paste infected with the aflatoxigenic *A. flavus* AR1 strain.

Parameters	*Storage duration*	C-	C+	T_1	T_2
Color	D 0	4.50 ± 0.53^{aA}	4.50 ± 0.53^{aA}	4.40 ± 0.52^{aA}	4.60 ± 0.56^{aA}
	D 3	4.40 ± 0.69^{aA}	4.50 ± 0.53^{aA}	4.40 ± 0.84^{aA}	4.30 ± 0.82^{aA}
	D 6	4.50 ± 0.53^{aA}	2.60 ± 0.52^{bB}	4.30 ± 1.06^{aA}	4.90 ± 0.32^{aA}
	D 9	4.50 ± 0.53^{aA}	2.40 ± 0.52^{bB}	4.40 ± 0.52^{aA}	4.90 ± 0.32^{aA}
Texture	D 0	3.10 ± 0.74^{aA}	2.90 ± 0.87^{aA}	3.10 ± 0.87^{aA}	3.10 ± 0.87^{aA}
	D 3	2.90 ± 0.74^{aA}	2.90 ± 0.57^{aA}	3.00 ± 0.47^{aA}	3.20 ± 0.42^{aA}
	D 6	3.20 ± 0.74^{aA}	3.10 ± 0.57^{aA}	2.90 ± 0.47^{aA}	3.10 ± 0.87^{aA}
	D 9	2.90 ± 0.87^{aA}	3.10 ± 0.74^{aA}	3.10 ± 0.87^{aA}	3.0 ± 0.00^{aA}
Odor	D 0	3.10 ± 0.87^{aA}	3.00 ± 0.82^{aA}	2.90 ± 0.87^{aA}	3.30 ± 0.82^{aA}
	D 3	3.10 ± 0.87^{aA}	3.00 ± 0.82^{aA}	2.90 ± 0.87^{aA}	3.30 ± 0.82^{aA}
	D 6	3.00 ± 0.82^{aA}	3.10 ± 0.87^{aA}	3.30 ± 0.82^{aA}	2.90 ± 0.87^{aA}
	D 9	3.00 ± 0.87^{aA}	3.00 ± 0.82^{aA}	3.10 ± 0.87^{aA}	3.20 ± 0.42^{aA}

[a,b]The values carrying different letters on the same line differ significantly ($p < 0.05$).

[A,B]The values carrying different capital letters on the same column for one parameter differ significantly ($p < 0.05$).

C-: Pasteurized tomato paste (negative control); C+: pasteurized tomato paste + *A. flavus* AR1 spores (positive control); T_1: pasteurized tomato paste + *A. flavus* AR1 spores + *L. plantarum* LO3; T_2: pasteurized tomato paste + *A. flavus* AR1 spores + cell-free supernatant. The tests were performed in duplicate. *A. flavus* AR1 (from spore suspension in PBS) and *L. plantarum* LO3 (cells suspension in PBS) were added at final concentration of 10^5 spores/ml and 10^8 CFU/ml, respectively, and the incubation was held at 30°C.

well as food spoilage *Penicillium* sp. The isolate LO3 was characterized by its highest antifungal activity as evidenced by the high concentrations of the various organic acids produced. Paradoxically, its L.A production was similar to those of isolates LA1, LA2, LA5, and LO2 which showed moderate antifungal activity. From this observation, we hypothesized that L.A to a lesser extent contributed to the antifungal activity, and also that A.A, OH-P. L.A, and P.L.A were mostly responsible for the varied levels of activity in the overlay assay. These results are in conformity with reports of several authors who previously assessed the *in vitro* antifungal effects of LAB on aflatoxigenic *Aspergillus* sp. and other mold strains (Gerbaldo et al. 2012; Kam, Bianchini and Bullerman 2007; Muñoz et al. 2010; Onilude et al. 2005; Prema et al. 2010; Sicuia et al. 2014; Tropcheva et al. 2014; Voulgari et al. 2010; Zara et al. 2003). While P.L.A is regarded as the most active compound against several fungal species, OH-P.L.A has also been known to be a potent antifungal compound produced by LAB (Coloretti et al. 2007; Lavermicocca et al. 2000; Mu et al. 2010; Valerio et al. 2004). This study also found an OH-PLA production by isolate LA5 that was greater than the highest concentration (0.41 mmol/L or 75 µg/ml) reported so far in the literature for *Lactobacillus* sp. (Mu et al. 2010). The simultaneous production of L.A, AA, P.L.A, and OH-P.L.A by isolate LO3 is, to the best of our knowledge, a rare observation.

Our results also pointed out the efficacy of two LAB strains (*L. plantarum* LO3 and *Lactococcus lactis* subsp. *lactis* LA5) to suppress mold growth as well as reduce AFs production over 7 d, following simultaneous

or late mold inoculation. However, *L. plantarum* LO3 strain showed a relatively higher reduction in mold biomass (100%) and AF production (100%) than *Lactococcus lactis* subsp. *lactis* LA5 (80% and 92% respectively) over 15 d, under simultaneous or late inoculation. Reduction in mold biomass was correlated to the reduction in AF production. This decrease in AF production could be explained by a direct inhibition of the mold by the various organic acids produced. These findings corroborate those of Belkacem-Hanfi et al. (2014) who reported high ability to reduce *Aspergillus carbonarius* ANC89 biomass and its OTA production in liquid medium at 28°C and 37°C by *L. plantarum* and *Lactobacillus graminis* (>97%) followed by *Pediococcus pentosaceus* (>81.5%) strains. *Lactobacillus brevis* and *L. plantarum* strains isolated from plant material in Romania were also found to inhibit *Aspergillus* and *Penicillium* mycotoxin-producing strains up to 2 weeks (Cornea et al. 2013). Our results are also consistent with those of Gerbaldo et al. (2012) who reported not only the complete inhibition of aflatoxigenic *A. flavus* strains by *Lactobacillus rhamnosus* L60 and *Lactobacillus fermentum* L23, but also the reduction of AF B1 production (95.7–99.8% with L60 and 27.5–100% with L23) during coculture in liquid medium. The difference in the efficacy of the two strains in reducing mold growth as well as mycotoxin production could be explained either by the production of AA by strain LO3 alone, or a synergistic action of A.A, OH-P.L.A, and P.L.A produced by this same strain. The reduction of *A. flavus* AR1 growth, as well as its AF production in the liquid medium, is important result, as AFs are the most important carcinogenic mycotoxins.

Using tomato paste as food model in our investigation, the aflatoxigenic strain *A. flavus* AR1 grew on it and caused some physicochemical (pH) and sensory changes (color). Our results also showed that the addition of *L. plantarum* LO3 or its CFS to tomato paste sample inoculated with the spores of *A. flavus* AR1 not only prevented the physicochemical and sensory changes but also inhibited spore germination. *L. plantarum* LO3 inhibited the spore germination for the entire duration of the experiment (9 d) while its CFS did as such for 6 d. These results corroborate the findings of Fatima, Mebrouk, and Miloud (2015) who reported the biopreservation potential of a *Leuconostoc* sp. and its metabolic extracts on tomato paste and sauce infected with a non-aflatoxigenic *A. flavus, E. coli*, and *Staphylococcus aureus*. They are also similar to several previous studies reporting the protective potential of an antifungal *L. plantarum* in yogurt and orange juice (Crowley, Mahony and van Sinderen 2012), the ability of *P. pentosaceus* to prevent growth of *Penicillium expansum* on apples (Rouse et al. 2008), and the capacity of a *L. plantarum* strain and its CFS to delay the spoilage of fresh vegetables infected with *A. flavus,*

Fusarium graminearum, Rhizopus stolonifer, and *Botrytis cinerea* (Sathe et al. 2007).

Conclusion

The current study shows that LAB isolated from peanuts, gari, and orange fruit juice exhibited antifungal activity against the aflatoxigenic molds evaluated. The inhibitory activity is caused by the various organic acids produced or their possible synergistic action. The strain *L. plantarum* LO3 showed the best antifungal activity as well as the ability to completely reduce the tested mold biomass and AFs production *in vitro*. Moreover, this strain and its CFS showed the ability to prevent aflatoxigenic mold growth as well as some physicochemical and sensory changes in tomato paste used as food model. The findings suggest that *L. plantarum* LO3 has potential applications in the biological control of some food and agricultural products against mycotoxigenic and spoilage molds.

Funding

The authors are grateful to the AUF-BECO (Agence Universitaire de la Francophonie, Bureau Europe Centrale et Orientale) and the Ministry of External Affairs of Romania, for their support through "Eugen Ionescu" postdoctoral fellowship [grant number CE/GR/662/2013] awarded to K.P.M.

ORCID

Kaktcham Pierre Marie http://orcid.org/0000-0003-0213-8858

References

Abia, A., B. Warth, M. Sulyok, R. Krska, N. Tchana, B. Njobeh, F. Dutton, and F. Moundipa. 2013. Determination of multi-mycotoxin occurrence in cereals, nuts and their products in Cameroon by liquid chromatography tandem mass spectrometry (LC-MS/MS). *Food Control* 31:438–453.

Ampe, F., N. Ben Omar, N. Moizan, C. Wacher, and J.-P. Guyot. 1999. Polyphasic study of the spatial distribution of microorganisms in mexican pozol, a fermented maize dough, demonstrates the need for cultivation-independent methods to investigate traditional fermentations. *Applied and Environmental Microbiology* 65 (12):5464–5473.

Belkacem-Hanfi, N., I. Fhoula, N. Semmar, A. Guesmi, I. Perraud-Gaime, H.-I. Ouzari, A. Boudabous, and S. Sevastianos Roussos. 2014. Lactic acid bacteria against post-harvest moulds and ochratoxin A isolated from stored wheat. *Biological Control* 76:52–59.

Coda, R., A. Cassone, C. G. Rizzello, L. Nionelli, G. Cardinali, and M. Gobbetti. 2011. Antifungal activity of Wickerhamomyces anomalus and Lactobacillus plantarum during sourdough fermentation: Identification of novel compounds and long-term effect during storage of wheat bread. *Applied and Environmental Microbiology* 77:3484–3492.

Coloretti, F., S. Carri, E. Armaforte, C. Chiavari, L. Grazia, and C. Zambonelli. 2007. Antifungal activity of lactobacilli isolated from salami. *FEMS Microbiologic Letters* 271 (2):245–250.

Coman, M. M., M. C. Verdenelli, C. Cecchini, S. Silvi, C. Orpianesi, N. Boyko, and A. Cresci. 2014. *In vitro* evaluation of antimicrobial activity of *Lactobacillus rhamnosus* IMC 501, *Lactobacillus paracasei* IMC 502 and SYNBIO against pathogens. *Journal of Applied Microbiology* 117 (2):518–527.

Cornea, C. P., O. A. Sicuia, G. Popa, F. Israel, and M. Zamfir. 2013. Screening of antifungal lactic acid bacteria isolated from plant materials. *Current Opinion in Biotechnology* 24S: S48–S143.

Crowley, S., J. Mahony, and D. van Sinderen. 2012. Comparative analysis of two antifungal Lactobacillus plantarum isolates and their application as bioprotectants in refrigerated foods. *Journal of Applied Microbiology* 113:1417–1427.

Dalié, D. K. D., A. M. F. Deschamps, and F. Richard-Forget. 2010. Lactic acid bacteria – Potential for control of mould growth and mycotoxins: A review. *Food Control* 21:370–380.

EFSA Panel on Biological Hazards. 2013. Scientific opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2013 update). *EFSA Journal* 11 (11):3449, 106.

Fatima, D., K. Mebrouk, and H. Miloud. 2015. Biopreservation of tomato paste and sauce with *Leuconostoc* spp. metabolites. *African Journal of Food Science* 9 (6):359–366.

Gerbaldo, G. A., C. Barberis, L. Pascual, A. Dalcero, and L. Barberis. 2012. Antifungal activity of two Lactobacillus strains with potential probiotic properties. *FEMS Microbiologic Letters* 332:27–33.

Gerez, C. L., M. J. Torres, G. Font de Valdez, and G. Rollán. 2013. Control of spoilage fungi by lactic acid bacteria. *Biological Control* 64:231–237.

Gerez, L. C., M. I. Torino, G. Rollan, and G. de Valdez. 2009. Prevention of bread mould spoilage using lactic acid bacteria with antifungal properties. *Food Control* 20:144–148.

Gevers, D., G. Huys, and J. Swings. 2001. Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. *FEMS Microbiologic Letters* 205:31–36.

Kam, P. V., A. Bianchini, and L. B. Bullerman. 2007. Inhibition of mould growth by sourdough bread cultures. *Rurals* 2:1–12.

Kana, J. R., B. G. J. Gnonlonfin, J. Jagger Harvey, J. Wainaina, I. Wanjuki, R. A. Skilton, and A. Teguia. 2013. Mycobiota and toxigenecity profile of *Aspergillus flavus* recovered from food and poultry feed mixtures in Cameroon. *Journal Animals Poultry Sciences* 2 (4):98–107.

Koteswara Rao, V., M. V. Ramana, S. Girisham, and S. M. Reddy. 2013. Culture media and factors influencing ochratoxin a production by two species of *Penicillium* isolated from poultry feeds. *National Academic Sciences Letters* 36 (1):101–110.

Lavermicocca, P., F. Valerio, A. Evidente, S. Lazzaroni, A. Corsetti, and M. Gobetti. 2000. Purification and characterisation of novel antifungal compounds from the sourdough *Lactobacillus plantarum* strain 21B. *Applied and Environmental Microbiology* 66:4084–4090.

Leong, S., A. T. Niba, S. Ny, and M. Olstorpe. 2012. Microbial populations during maize storage in Cameroon. *African Journal Biotechnology* 11 (35):8692–8697.

Liu, B., Y. Hsu, C. Lu, and F. Yu. 2013. Detecting aflatoxin B1 in foods and feeds by using sensitive rapid enzyme-linked immunosorbent assay and gold nanoparticle immunochromatographic strip. *Food Control* 30:184–189.

Magnusson, J., K. Ström, S. Ross, J. Sjögren, and J. Schnürer. 2003. Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. *FEMS Microbiologic Letters* 219:129–135.

Milhome, M. A. L., C. G. Lima, L. K. De Lima, F. A. F. Lima, D. O. B. Sousa, and R. F. Nascimento. 2014. Occurrence of aflatoxins in cashew nuts produced in northeastern Brazil. *Food Control* 42:34–37.

Mohammed, M., H. Abd El-Aziz, N. Omran, S. Anwar, S. Awad, and M. El-Soda. 2009. Rep-PCR characterisation and biochemical selection of lactic acid bacteria isolated from the Delta area of Egypt. *International Journal Food Microbiologic* 128:417–423.

Mu, W., Y. Yang, J. Jia, T. Zhang, and B. Jiang. 2010. Production of 4-hydroxyphenyllactic acid by *Lactobacillus* sp. SK007 fermentation. *Journal of Bioscience and Bioengineering* 109 (4):369–371.

Muñoz, R., M. E. Arena, J. Silva, and S. N. González. 2010. Inhibition of mycotoxin-producing *Aspergillus nomius* VSC 23 by lactic acid bacteria and *Saccharomyces cerevisiae*. *Brazilian Journal of Microbiology* 41:1019–1026.

Ngoko, Z., H. Daoudou, P. T. Imele, S. Kamga, M. Mendi, R. Mwangi, A. Bandyopadhyay, and W. F. O. Marasas. 2008. Fungi and mycotoxin associated with food commodities in Cameroon. *Journal Applications Biologic* 6:164–168.

Njobeh, P. B., M. F. Dutton, S. H. Koch, A. Chuturgoon, S. Stoev, and K. Seifert. 2009. Contamination with storage fungi of human food from Cameroon. *International Journal Food Microbiologic* 135:193–198.

Official Journal of the European Union (2009) Regulation (EC) No 1107/2009 of the European parliament and of the council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. OJ L 309, 24.11.2009, pp. 1–50.

Onilude, A. A., O. E. Fagade, M. M. Bello, and I. F. Fadahunsi. 2005. Inhibition of aflatoxin-producing aspergilli by lactic acid bacteria isolates from indigenously fermented cereal gruels. *African Journal Biotechnology* 4:1404–1408.

Pitt, J. I., and A. D. Hocking. 2009. *Fungi and food spoilage*, 519. 3rd ed. Spinger: (Springer Science + Business Media, LLC, 233 Spring Street, New York, NY 10013, USA).

Prema, P., D. Smila, A. Palavesam, and G. Immanuel. 2010. Production and characterisation of an antifungal compound (3-phenyllactic acid) produced by *Lactobacillus plantarum* strain. *Food Bioproc Technological* 3:379–386.

Reddy, K. R. N., P. Saritha, C. S. Reddy, and K. Muralidharan. 2009. Aflatoxin B1 producing potential of *Aspergillus flavus* strains isolated from stored rice grains. *African Journal of Biotechnology* 8 (14):3303–3308.

Rotaru, S., F. Israel-Roming, G. Campeanu, and G. Deciu. 2011. Correlation of ochratoxin A level in wine with vine environment. *Romanian Biotechnology Letters* 16 (6):126–130.

Rouse, S., D. Harnett, A. Vaughan, and D. van Sinderen. 2008. Lactic acid bacteria with potential to eliminate fungal spoilage in foods. *Journal of Applied Microbiology* 104:915–923.

Sathe, S. J., N. Nawani, N. P. K. Dhakephalkar, and B. P. Kapadnis. 2007. Antifungal lactic acid bacteria with potential to prolong shelf-life of fresh vegetables. *Journal of Applied Microbiology* 103:2622–2628.

Schnürer, J., and J. Magnusson. 2005. Antifungal lactic acid bacteria as biopreservatives. *Trends Food Sciences Technological* 16 (1):70–78.

Sicuia, O. A., F. Israel-Roming, O. Ciobotaru, A. Matei, M. Zamfir, M. Ciuca, and C. P. Cornea. 2014. Antifungal action of lactic acid bacteria isolated from plant materials against mycotoxigenic fungi. *Scientific Bulletin Series F Biotechnologies* 12:234–240.

Ström, K., J. Sjogren, A. Broberg, and J. Schnurer. 2002. *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Phe-trans-4-OH-L-Pro) and 3- phenyllactic acid. *Applied and Environmental Microbiology* 68:4322–4327.

Torriani, S., G. E. Felis, and F. Dellaglio. 2001. Differenciation of *Lactobacillus plantarum, L. pentosus*, and *L. paraplantarum* by *recA* gene sequence analysis and multiplex PCR assay with *recA* gene-derived primers. *Applied and Environmental Microbiology* 67 (8):3450–3454.

Tropcheva, R., D. Nikolova, Y. Evstatieva, and S. Danova. 2014. Antifungal activity and identification of lactobacilli, isolated from traditional dairy product "katak". *Anaerobe* 28:78–84.

Valerio, F., P. Lavermicocca, M. Pascale, and A. Visconti. 2004. Production of phenyllactic acid by lactic acid bacteria: An approach to the selection of strains contributing to food quality and preservation. *FEMS Microbiologic Letters* 233 (2):289–295.

Viljoen, C. B. 2001. The interaction between yeasts and bacteria in dairy environments. *International Journal of Food Microbiology* 69:37–44.

Von Wright, A. 2005. Regulating the safety of probiotics – The European approach. *Current Pharmaceutical Design* 11:17–23.

Voulgari, K., M. Hatzikamari, A. Delepoglou, P. Georgakopoulos, E. Litopoulou-Tzanetaki, and N. Tzanetakis. 2010. Antifungal activity of non-starter lactic acid bacteria isolates from dairy products. *Food Control* 21:136–142.

Wang, H., Y. Yan, J. Wang, H. Zhang, and W. Qi. 2012. Production and characterisation of antifungal compounds produced by *Lactobacillus plantarum* IMAU10014. *PLoS One* 7 (1): e29452.

Yang, E. J., and H. C. Chang. 2010. Purification of a new antifungal compound produced by *Lactobacillus plantarum* AF1 isolated from kimchi. *International Journal of Food Microbiology* 139:56–63.

Yang, E. J., Y. S. Kim, and H. C. Chang. 2011. Purification and Characterisation of Antifungal δ-Dodecalactone from *Lactobacillus plantarum* AF1 Isolated from Kimchi. *Journal of Food Protection* 74 (4):651–657.

Yousef, S., and M. Naresh. 2010. Mycotoxigenic fungi in peanuts from different geographic regions of Egypt. *Mycotoxin Research* 26:133–140.

Zara, M. L., N. Belc, G. Bahrim, and A. Vasile. 2003. The study of antifungal action expressed by some bacterial selected strains. *Food Technol* 6:80–84.