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# Probiotic Properties, Safety Assessment, and Aroma-Generating Attributes of Some Lactic Acid Bacteria Isolated from Iranian Traditional Cheese

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**Abstract:** Artisanal cheeses are known as the source of beneficial lactic acid bacteria (LAB). Therefore, this study aimed to isolate and characterize LAB with different proteolytic activities from Iranian artisanal white cheeses. The isolates were classified into low, medium, and high proteolytic activity clusters via K-means clustering and identified as *Lactiplantibacillus* (*Lpb.*) *pentosus* L11, *Lpb. plantarum* L33, and *Enterococcus faecium* L13, respectively. Some safety tests (such as resistance to antibiotics, hemolytic activity, and biogenic amine production), probiotic properties (including cell surface hydrophobicity, auto/co-aggregation, and antibacterial activity), and production of volatile compounds were evaluated. These were non-hemolytic and non-biogenic amine producers, and showed no irregular antibiotic resistance. *Lpb. plantarum* L33 had the highest hydrophobicity (30.55%) and auto-aggregation (49.56%), and the highest co-aggregation was observed for *Lpb. pentosus* L11 with *Staphylococcus aureus* (61.51%). The isolates also showed a remarkable antibacterial effect against pathogenic bacteria. Moreover, *Lpb. pentosus* L11 and *Lpb. plantarum* L33 with low and medium proteolytic activity produced a wider range of volatile compounds in milk compared to the strain with a high proteolytic effect. The results showed that a probiotic strain with low or medium proteolytic activity could improve the flavor characteristics of fermented milk.

**Keywords:** traditional cheese; probiotic; safety; proteolysis; cheese aroma



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## 1. Introduction

Lactic acid bacteria (LAB) have the ability to produce a number of desirable substances that can improve the flavor, texture, nutritional value, and shelf life of foods [1]. Antimicrobial and antioxidant properties and some health benefits of LAB including effects on lowering blood pressure, reducing serum cholesterol levels, and stimulating the immune system have been well-reported [2]. Apart from the production of vitamins and short-chain fatty acids, most recently, LAB is gaining more attention because of their organic acid production, which also has different therapeutic applications such as being anti-obesity and anti-diabetic [3]. The majority of LAB have well-established proteolytic systems [4]. The proteolytic system of LAB influences their growth and affects flavor compounds of fermented products [5]. Moreover, the proteolytic system of LAB participates in a reduction in protein complexity for the protein metabolism in LAB, but in the process, it generates simple peptides with a large variety of bioactivities such as ACE-inhibitory, antioxidant, and antimicrobial activities [6–8].

Artisanal cheeses can be good sources of probiotic LAB with suitable biofunctional properties such as multifunctional cultures, and this subject has been studied by many

scientists from different perspectives [9]. For example, Domingos-Lopes et al. [10] reported that some proteolytic LAB strains isolated from cheese during ripening had a great potential ability to produce diacetyl. Albayrak and Duran [9] and Mohammed and Çon [11] isolated aroma-producer, proteolytic, and probiotic LAB from artisanal cheese and white cheese, respectively. Margalho et al. [12] isolated 220 LAB strains from 10 types of Brazilian artisanal cheeses and evaluated their safety, EPS production from different sugar sources, diacetyl formation as a precursor of aromatic compounds, and bacteriocinogenic activity. Additionally, different LAB strains were isolated from traditional Iranian cheeses and their probiotic and functional properties were evaluated [13–15]. However, to the best of our knowledge, the proteolytic activity of LAB isolated from white Iranian cheese and their ability to produce aroma have rarely been investigated. This study was therefore aimed to isolate potential LAB strains from white Iranian cheese, classify them into three levels of low, medium, and high proteolytic activity, and evaluate the safety, probiotic, and aroma-producing properties of the selected isolates.

## 2. Materials and Methods

### *2.1. Chemicals and Bacterial Strains*

Microbial culture media and other chemicals used in this study were purchased from Merck Co. (Darmstadt, Hesse, Germany). All bacterial strains had been previously isolated from white Iranian cheese, evaluated morphologically and biochemically, and kept in the microbial collection (Department of Food Science and Technology, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran) as potential LAB.

### *2.2. LAB Screening Based on Proteolytic Activity*

A well-diffusion method was used to assess the proteolytic activity of 40 isolates, according to a method described by Karimian et al. [16]. To achieve this, 70 µL of overnight culture was added to the well on skim milk agar and the plates were incubated for 24 h at 37 °C. The diameters of clear zones around the wells were regarded as the criteria for proteolytic activity. The strains were then classified into three groups with high, medium, and low proteolytic activity.

### *2.3. Molecular Identification of Selected Isolates*

Three isolates (L11, L13, and L33) were identified by using the polymerase chain reaction (PCR). According to the manufacturer's instructions, genomic DNA was extracted using a DNA extraction kit. Amplification of 16S rDNA was performed using universal primers 27F (50-AGAGTTTGA TCCTGGCTCAG-30) and 1492R (50-TACGGCTACCTTGTTACGACTT-30). The following program was used to amplify the DNA in a PCR thermocycler: 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 40 s, and extension at 72 °C for 30 s. Finally, a 10-min extension cycle was performed at 72 °C. For the confirmation of the amplification performance and fragment size, agarose gel electrophoresis (1%) was used. Sequencing was performed on the purified PCR products. Finally, "https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 21 October 2022)" and "ezbiocloud.net/identify (accessed on 21 October 2022)" were used to compare nucleotide sequences in the Gen Bank database for similarity [17].

### *2.4. Safety Evaluation*

#### 2.4.1. Resistance to Antibiotics

The agar overlay diffusion method was used to assess the antibiotic resistance of selected LAB isolates against eight common antibiotics including ampicillin (10 µg), clindamycin (2 mg), erythromycin (30 µg), penicillin (10 µg), tetracycline (30 µg), gentamycin (10 µg), vancomycin (30 mg), and chloramphenicol (30 mg). The bacterial suspension (106–107 cells/mL, 200 µL) was added to 4 mL of soft agar (0.8 % *w*/*v*) and overlaid on a 15 mL MRS plate. Then, the antibiotic discs were placed on the culture and plates incubated for 24 h at 37 °C. Finally, the diameter of the inhibition zone around the discs was

measured and classified as resistant (<10.5 mm), intermediate (10.5–20.5 mm), and sensitive (>20.5 mm) [18].

#### 2.4.2. Hemolytic Activity

The hemolytic activity was assessed by streaking overnight cell cultures on blood agar plates containing 5% sheep blood. The hemolytic activity was optically evaluated after 48 h at 37 °C and distinguished as β-hemolysis, α-hemolysis, or γ-hemolysis based on the presence of clear zones, green haloes, or no color around the colonies, respectively. *Staphylococcus aureus* and *Listeria monocytogenes* were used as positive controls [19].

#### 2.4.3. Biogenic Amine Production

The production of tyramine, putrescine, and histamine was estimated by adding 0.5% (*w*/*v*) of the amino acid precursors including L-tyrosine, L-ornithine, and L-histidine to MRS agar plates containing 0.06% bromocresol purple. The appearance of the purple color indicated the production of biogenic amines [14].

### *2.5. Probiotic Properties*

#### 2.5.1. Cell Surface Hydrophobicity

After growing LAB in MRS at 37 °C for 16–18 h, the cells were washed with phosphate buffer (pH 7.1) and resuspended in the same buffer. At 600 nm, the absorbance of cell suspension was adjusted to 0.8–0.9 ($A_{Initial}$). Then, 3 mL of the LAB isolate suspension was added to 1 mL of hexane and mixed. Afterward, the mixture was incubated at 37 °C without agitation for 3 h. Finally, the absorbance ($A_{Final}$) of the aqueous phase (1 mL) was measured at 600 nm, and the cell surface hydrophobicity (%) was determined according to the formula below [20]:

$$\text{Hydrophobicity (\%)} = [1 - (A_{Final}/A_{Initial}) \times 100] \quad (1)$$

#### 2.5.2. Auto-Aggregation

The method of Reuben et al. [21] was applied to evaluate the auto-aggregation property of the bacterial strains. A total of 5 mL of the bacterial suspension ($10^8$ CFU ml/L) was vortexed for 10 s, and the absorbance was measured at 600 nm ($A_i$). After 2 h of incubation at 37 °C, the supernatant absorbance was determined at the same wavelength ($A_{2h}$). The auto-aggregation coefficient was calculated using the following formula:

$$\text{Auto-aggregation coefficient (\%)} = [1 - (A_{2h}/A_i)] \times 100 \quad (2)$$

#### 2.5.3. Co-Aggregation

The co-aggregation of LAB and pathogenic strains (*Salmonella typhimurium*, *Staphylococcus aureus*, and *Listeria monocytogenes*) was evaluated according to the method described by Motey et al. [22] with some modification. In this way, the absorbance of LAB strains ($A_x$), pathogenic strains (Ay), and the mixture of LAB and pathogenic strains ($A_{x+y}$) was determined at 600 nm after incubation at 0 h and 5 h. Co-aggregation was calculated as:

$$\text{Co-aggregation \%} = [(A_x + A_y)/2 - A_{x+y}]/[(A_x + A_y)/2] \times 100 \quad (3)$$

#### 2.5.4. Antibacterial Activity

The modified agar overlay was used to determine the antimicrobial activity of LAB. The MRS agar plate was inoculated with loops of LAB grown in MRS broth, and incubated under microaerophilic conditions at 37 °C for 24 h. Following incubation, a broth culture of the pathogens inoculated in 10 mL of nutrient soft agar (0.8% agar) was overlaid onto the MRS agar plates. Under aerobic conditions, the overlay was incubated at 37 °C for 24 h and the zones of inhibition were measured [23].

### *2.6. Analysis of Volatile Compounds*

The ultra-high temperature processed milk (25 mL) was poured into a sterilized flat bottom flask, inoculated with selected LAB strains (1% $v/v$; $1 \times 10^7$ CFU/mL), and incubated for 24 h at 37 °C. The fermented milk was then analyzed for the aroma profile [24].

Solid-phase microextraction (SPME) fibers with GC-MS were used to analyze the volatile compounds in the fermented milk samples (Agilent 6890 gas chromatograph equipped with a 5973 network mass selective detector) according to the method of Bulat and Topcu [25] with some modification. In each vial, 80 µL of 2-heptanone (20 µL/L in water) was added as an internal standard (IS). The bi-metal magnetic crimp-cap seal with PTFE/silicone septa was equilibrated at 45 °C for 30 min with pulsed agitation at 250 rpm for 4 s. The SPME fiber ((1 cm, 75 µm), StableFlex carboxen/polydimethylsiloxane, Supelco, Bellefonte, PA, USA) was exposed to the headspace of the vial for 30 min at 50 °C. A manual sampler was used for equilibration, extraction, and injection. In splitless mode, the volatile compounds were desorbed from the fiber at 260 °C for 3 min. Separation was achieved by CP-Sil8 CB-MS (50 m × 0.25 mm I.D. × 0.25 µm film thickness, Agilent Technologies, Santa Clara, CA, USA). A constant flow of 1.0 mL/min of helium was used as the carrier gas. The initial temperature was 40 °C for 10 min, followed by 5 °C/min increases to 250 °C for 10 min, and finally 280 °C for 5 min in the GC oven. The GC-MS transfer line and ion source temperatures were 260 °C. Scan mode mass spectral data were collected within a mass range of 33–400 amu at 4 scans/s. The peaks were identified using mass spectral libraries from WILEY and NIST as well as external standards. Retention indices were confirmed using the n-alkane (C8–C20) standard. Quantities for each compound were calculated using the ratio of the compound peak area/IS peak area.

### *2.7. Statistical Analysis*

Data were analyzed using SPSS software (version 26) by one-way analysis of variance. The Duncan multiple range test was used to compare the mean values ($p \leq 0.05$). K-means clustering was performed by SPSS software. ClustVis online software ("https://biit.cs.ut.ee/clustvis/ (accessed on 17 November 2022)") was used to obtain principal component analysis (PCA) and cluster analysis (heatmaps), according to a method reported by Metsalu and Vilo [26].

## 3. Results and Discussion

### *3.1. Proteolytic Activity*

Proteolytic activity is necessary for LAB growth in fermented foods and improving the organoleptic properties of these products [27]. According to Vuillemard et al. [28], strains with a diameter zone between 15 and 21 mm on skim milk agar can be considered as proteolytic isolates. In this study, the proteolytic activity of 40 isolates was measured, and eight isolates did not show any proteolytic activity; they were therefore excluded from statistical analysis. The proteolytic activity results of 32 isolates are indicated in Figure 1. As can be seen, the proteolytic activity varied among different isolates, as L11 had the lowest proteolytic activity, and L13 and S3 showed the highest activity. Three isolates including L33, MDM21, and L1 had medium activity.

It is known that LAB strains have the ability to hydrolyze proteins into amino acids and peptides, and subsequent amino acid catabolism results in the formation of aromatic compounds such as α-keto acids, hydroxy acids, aldehydes, ketones, alcohols, carboxylic acids, and esters [29]. Therefore, the isolates were classified into three groups with low, medium, and high proteolytic activities via the K-means clustering approach. In the first (low proteolytic activity) and second (high proteolytic activity) clusters, the highest distance to the cluster mean was considered, whereas the intermediate distance was considered as the third cluster (medium proteolytic activity) [30]. The following strains were selected for their probiotic and aroma-producing properties:

Low proteolytic activity: L11;
Medium proteolytic activity: L33;

High proteolytic activity: L13.

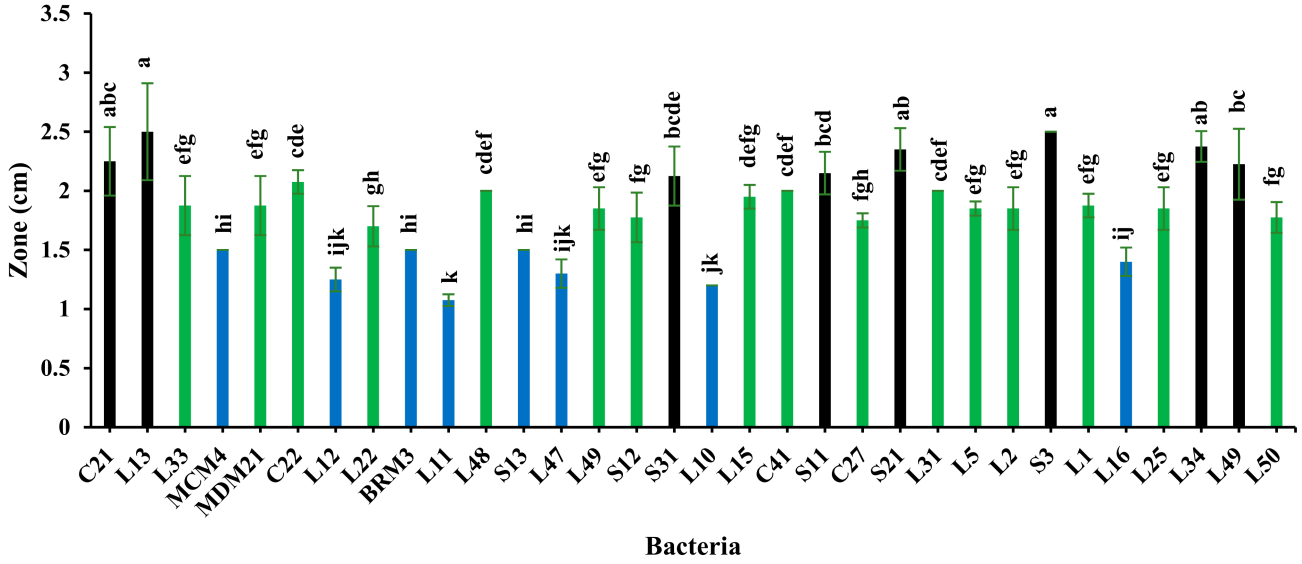


**Figure 1.** Proteolytic activity of the lactic acid bacteria (LAB) strains isolated from Iranian traditional cheese. Black-colored columns: high proteolytic activity; green-colored columns: medium proteolytic activity; blue-colored columns: low proteolytic activity. Different letters indicate significant differences between samples at $p \leq 0.05$.

In this selective mode, strains with the greatest proteolytic difference were selected so that the proteolytic effect of each strain on the aroma profiles of the fermented milk could be clearly determined.

### *3.2. Molecular Identification*

Based on the analysis of sequences, the isolates were identified as *Lactiplantibacillus* (*Lpb.*) *pentosus* (L11), *Lpb. plantarum* (L33), and *Enterococcus faecium* (L13). Different strains from *Enterococcus* and *Lactobacillus* genera have been identified from traditional cheeses in other studies [31,32]. For example, *E. faecium*, *Lpb. plantarum*, and *L. paraplantarum* have been identified from artisanal cheeses [33,34].

### *3.3. Safety Evaluation*

#### 3.3.1. Resistance to Antibiotics

The antibiotic resistance of LAB isolates is shown in Table 1. It should be noted that isolates with ≤15 mm, 15–21 mm, and ≥21 mm diameter zones can be considered as resistant, intermediate (semi-sensitive), and sensitive isolates, respectively [35,36]. All the isolates were resistant to vancomycin, sensitive to chloramphenicol, erythromycin, penicillin, and ampicillin, and semi-sensitive to tetracycline. Additionally, *Lpb. pentosus* L11 showed intermediate sensitivity to gentamycin and clindamycin, *Lpb. plantarum* L33 was resistant to gentamycin and sensitive to clindamycin, and *E. faecium* L13 was resistant to gentamycin and clindamycin. Similar results have been reported in the literature [37,38]. Antibiotic resistance of LAB strains varies with species and strains [39]. Furthermore, the condition of culture including the inoculum level and culture media, the position of resistance gene, which can be in the plasmid or chromosome, and the participation of other mechanisms affect the different results [40]. The use of LAB for fermented food and beverages has a long and reliable history, but recent studies have suggested that some of these bacteria may carry antibiotic-resistance genes when used in starter cultures or co-cultures. It is important to note that in numerous species of LAB, resistance genes are located on the chromosome, which are intrinsic and cannot be transferred [18].

**Table 1.** Antibiotic resistance of selected LAB isolates against various antibiotics.

| LAB Isolates | Antibiotic | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Vancomycin | Chloramphenicol | Erythromycin | Penicillin | Ampicillin | Gentamycin | Tetracycline | Clindamycin |
| *Lpb. pentosus* | R * | S | S | S | S | I | I | I |
| *Lpb. plantarum* | R | S | S | S | S | R | I | S |
| *E. faecium* | R | S | S | S | S | R | I | R |

* R: resistant, I: intermediate, and S: sensitive.

#### 3.3.2. Hemolytic Activity

Hemolytic activity is necessary to evaluate the safety of probiotic isolates [41]. In this study, no clear lysis zone appeared on the blood agar plates for *Lpb. pentosus* L11, *Lpb. plantarum* L33, and *E. faecium* L13. In line with our results, the non-hemolytic activity of *Lpb. plantarum*, *E. faecium*, and *L. paraplantarum* isolated from traditional cheeses has been reported in the literature [42–44].

#### 3.3.3. Biogenic Amine Production

In addition to its potential health concerns, the biogenic amine content in fermented foods is also of great economic importance. Fermented foods, however, contain small concentrations of biogenic amines [45]. Therefore, the ability of LAB isolates to produce the biogenic amine was assayed. In this study, none of the isolates showed biogenic amine formation capabilities. Similarly, Colombo et al. [46] reported that 15 LAB strains isolated from dairies showed no biogenic amine production. Additionally, it has been reported that some of the LAB species such as *Enterococcus lactis* and *Lactobacillus plantarum* isolated from camel milk did not produce biogenic amines and can be used as safe starters [47].

### *3.4. Probiotic Properties*

#### 3.4.1. Cell Surface Hydrophobicity

The highest hydrophobicity was observed in *Lpb. plantarum* L33, followed by *Lpb. pentosus* L11 and *E. faecium* L13 ($p > 0.05$) (Figure 2). Similarly, LAB strains isolated from milk and cheese showed 48–83% surface hydrophobicity [48,49]. It is possible for microorganisms to bind covalently to the epithelial surfaces of the intestinal tract due to the hydrophobic compounds on their surface such as polysaccharides, proteins, fatty acids, and teichoic acids. In fact, probiotic strains and intestinal cells can adhere to each other because of the hydrophobic residues that recognized carbohydrate residues such as fructose, glycoproteins, mannose of glycolipids, and galactose on the surface of the cells [50]. Due to the bacteria's net negative surface charge, they behave as hydrophobic particles, and this hydrophobicity is typically associated with bacterial adhesiveness, which varies from strain to strain and is affected by the growth medium, bacterial age, and bacterial surface structure [51].

#### 3.4.2. Auto-Aggregation

The aggregation is strain-specific and can even be different in the same classification. The ability to attach to the intestinal mucous surface is an important factor to select a bacteria as a probiotic strain. The intestine colonization of probiotic strains in the intestine shows an important role in biological reactions because it affects the immune system, increasing the competition with pathogens and protecting them from GIT peristalsis [52]. In this study, the LAB isolates showed a remarkable auto-aggregation property ($p \leq 0.05$) (Figure 3). Among the strains, *Lpb. plantarum* L33 showed the highest (49.56%) and *E. faecium* L13 showed the lowest auto-aggregation (6%). In the examination of the cell surface properties of LAB isolated from traditional fermented food, it was reported that 18 LAB strains showed auto-aggregation activity from 5.92% to 23.32% [53]. Hashemi et al. [54] also reported that all *L. paraplantarum* strains showed a high auto-aggregation from 77.3 to 80.2% after 4 h incubation. Moreover, the auto-aggregation of *Lpb. plantarum* has been reported to be from 20% to 99% [55]. It has also been demonstrated that auto-aggregation depends on

bacterial strains and can be different within taxonomic groups, and this property ranges from 50 to 96% for *E. faecium* strains [56].

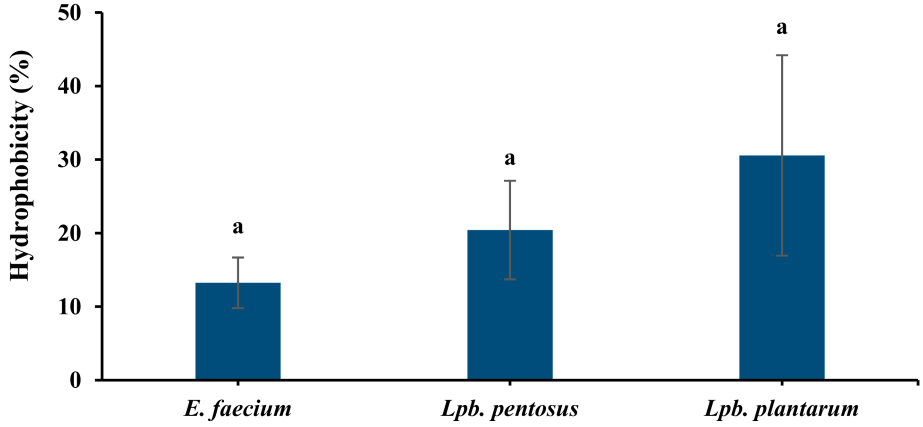


**Figure 2.** Cell surface hydrophobicity value of *E. faecium*, *Lpb. plantarum*, and *Lpb. pentosus*. There were no significant differences between the samples at $p \leq 0.05$.

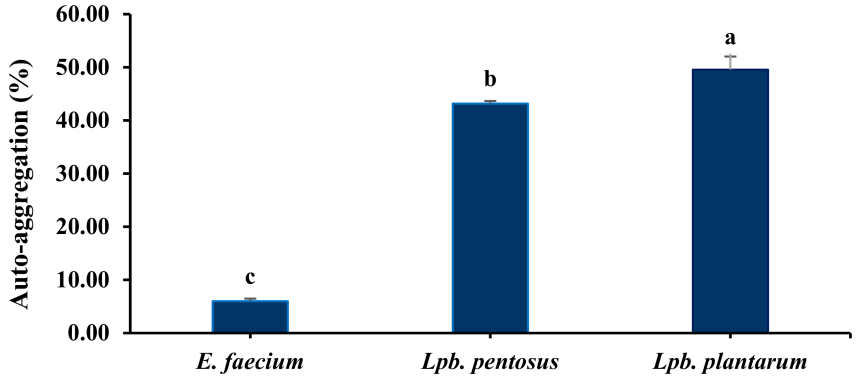


**Figure 3.** Auto-aggregation of *E. faecium*, *Lpb. plantarum*, and *Lpb. pentosus*. Different letters indicate significant differences between samples at $p \leq 0.05$.

A relationship between the attachment capacity and hydrophobicity of LAB has been reported. Collado et al. [57] demonstrated that *L. plantarum* IS-10506 and *L. plantarum* IS-20506 with the highest and the lowest percentages of adhesion to hydrocarbons showed the highest and the lowest auto-aggregation, respectively. The auto-aggregation mechanisms of LAB remain unclear. According to most studies, the auto-aggregation ability is species- and environment-specific. This ability is the result of complex physical and chemical interactions. Cells that are larger and heavier will precipitate more quickly. It is likely, however, that the components of the cell surface and the cell surface charge play a major role in the ability to auto-aggregate. Furthermore, several studies have described the genes that encode aggregation-promoting factors in Lactobacillus species [58]. Generally, bacteria with auto-aggregative potential can inhibit pathogenic bacteria from colonizing intestinal mucosa by forming a barrier through auto-aggregation [56].

### 3.4.3. Co-Aggregation

The co-aggregation capacity of LAB isolates with *S. typhimurium*, *L. monocytogenes*, and *S. aureus* is illustrated in Figure 4. *E. faecium* L13, *Lpb. pentosus* L11, and *Lpb. plantarum* L33 showed significant co-aggregation with *S. typhimurium* and *L. monocytogenes*. Additionally, there was no significant difference between the co-aggregation of *E. faecium* L13 and *Lpb. plantarum* L33 with *S. aureus*, but the co-aggregation of these isolates was significantly different to *Lpb. pentosus* L11 and *S. aureus*. The highest co-aggregation was observed for

*Lpb. pentosus* L11 with *S. aureus* (61.51%), and *E. faecium* L13 with *L. monocytogenes* presented the lowest co-aggregation (50.36%). Ou et al. [59] reported that *Lpb. plantarum* showed a 50% co-aggregation activity after 3 h. The value of the co-aggregation of *E. faecium* AQ71 with *L. monocytogenes* strains has been reported between 30% and about 53% [60]. *L. paraplantarum* KM0 isolated from milk presented 20% co-aggregation with *L. monocytogenes* [61]. Co-aggregation of LAB isolates with pathogens may be due to cell surface components, but further study is needed to understand how this occurs. This may be caused by interactions of carbohydrate-lectin and proteinaceous components on the cell surface [62]. As LAB and Gram-positive pathogenic bacteria have similar cell wall morphologies, they have a thick layer of peptidoglycan, and their hydrophobic nature makes it easier for them to bond together, which could explain their efficient co-aggregation ability against Gram-positive bacteria [61]. However, the incubation time and strain (probiotic and pathogen) affect the co-aggregation percentages [62].

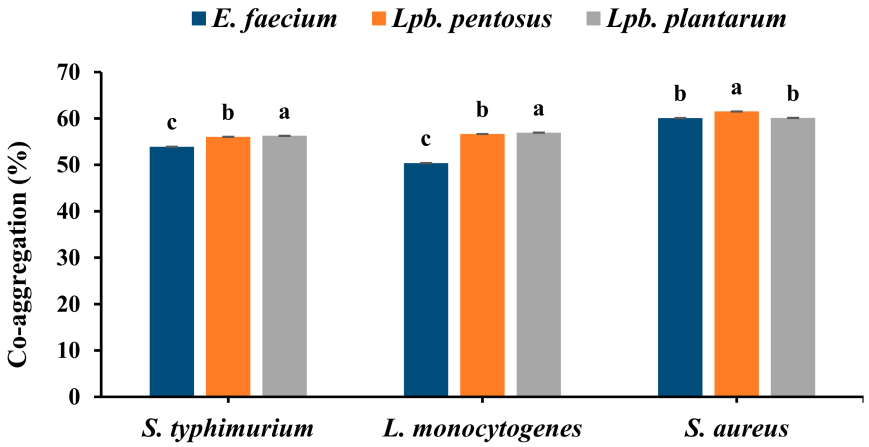


**Figure 4.** Co-aggregation of *E. faecium*, *Lpb. plantarum*, and *Lpb. pentosus*. Different letters indicate significant differences between samples at $p \leq 0.05$.

### 3.4.4. Antibacterial Activity

The results of the antimicrobial activity of LAB strains are shown in Table 2. As can be seen, *Lpb. plantarum* L33 and *E. faecium* L13 had antimicrobial effects on all pathogens, but *Lpb. pentosus* L11 only inhibited the growth of *S. typhimurium* and *L. monocytogenes*, and no zone was observed for S. aureus ($p > 0.05$). These findings are supported by other studies. Ahmadova et al. [60] demonstrated that *E. faecium* AQ71 had an appropriate antimicrobial activity against some pathogens such as *L. monocytogenes* and *Bacillus cereus*, but it could not inhibit the growth of *Escherichia coli* and *Salmonella*. Additionally, *Lpb. plantarum* isolated from kimchi showed high antimicrobial properties against six pathogens including *L. monocytogenes*, *Salmonella choleraesuis*, and *S. aureus* [63]. *L. paraplantarum* FT259, isolated from Brazilian semi-hard artisanal cheese, inhibited the growth of *Listeria innocua*, *L. monocytogenes*, and *Lactobacillus sakei* [64].

**Table 2.** Antimicrobial activity of LAB isolates.

| LAB Isolates | Inhibition Zone (mm) | | |
|---|---|---|---|
| | *S. typhimurium* | *S. aureus* | *L. monocytogenes* |
| *Lpb. pentosus* | 36.25 ± 0.18 [a] | - | 37.5 ± 0.36 [a] |
| *Lpb. plantarum* | 45 ± 0.71 | 37.5 ± 0.36 [a] | 41.25 ± 0.54 |
| *E. faecium* | 38.7 ± 0.54 | 42.5 ± 0.36 | 50 ± 0.71 |

[a]; There were non-significant difference among the values in each column ($p \leq 0.05$).

As a barrier against pathogens and food spoilage caused by bacteria, LAB-produced antimicrobial compounds are highly effective. Different factors such as organic acid pro-

duction (lactic acid and acetic acid) and nutritional competition, proteinaceous compounds, diacetyl, fatty acids, hydrogen peroxide, phenolic acids, bacteriocins, and bactericidal proteins contribute to LAB antimicrobial action. Aside from extending food shelf life and inhibiting pathogenic organism growth, these metabolic products have a positive impact on texture, smell, taste, and color [55,65,66]. The antimicrobial activity against Gram-positive pathogens is primarily due to the bactericidal effect of protease-sensitive bacteriocins, whereas the antagonistic effects against Gram-negative pathogens could be due to the production of hydrogen peroxide and organic acids [67]. Additionally, it is possible for LABs to produce extracellular substances that can act as inhibitors against competing microorganisms by secreting or tying them to the cell wall [55].

These isolates appear to meet the functional criteria necessary for being health-promoting probiotics. It might therefore be possible to use the isolates in various food products as novel probiotic candidates.

### *3.5. Aroma-Producing Ability*

The identification of volatile compounds can aid in understanding the flavor characteristics of milk fermented by LAB strains. Compounds were grouped by chemical families including aldehydes (pyruvaldehyde), ketones (acetone, butylideneacetone, acetoin, and diacetyl), alcohols (2-heptanol, cholest-5-en-3-ol, cholesta-4,6-dien-3-ol, and farnesol), acids (acetic acid, hexanoic acid, and nonahexacontanoic acid), esters (pentyl acetate), and lactones (isochiapin B) (Figure 5). It is known that LAB can contribute to flavor development by producing aromatic substances such as diacetyl (2,3-butanedione) and acetoin (3-hydroxy-2-butanone), two major flavor compounds in dairy products [65,68]. As found in the heatmap visualization (Figure 5a) and PCA plot (Figure 5b), the milk sample fermented by *Lpb. pentosus* L11 showed a different aroma profile compared with those fermented by *Lpb. plantarum* L33 and *E. faecium* L13. However, the PCA plot revealed that *Lpb. plantarum* L33 had a greater impact on aroma production in the milk samples than *E. faecium* L13.

It can be seen in the hierarchical clustering analysis (heatmap) results that *Lpb. plantarum* L33 had the highest contribution to pyruvaldehyde formation compared to other pairs. This dicarbonyl compound is produced by LAB and plays a role in the production of cheesy flavor [69]. The highest amount of acetone was produced by *Lpb. plantarum* L33. *Lpb. pentosus* L11 produced the highest level of butylideneacetone (3-Hepten-2-one) and acetoin.

Moreover, *Lpb. plantarum* L33 and *Lpb. pentosus* L11 were regarded as high- and low-level diacetyl producers, respectively. Ketones are most commonly known for their aroma-enhancing effect on dairy products because of their low perception threshold. Diacetyl is a sweet buttery and vanilla aromatic compound that is found in many fermented dairy products; it is considered the major aromatic compound in fresh cheese, and it has been identified as a key aroma component of Emmental, Cheddar, and Camembert cheeses [70]. The ability to produce diacetyl from milk was found to be strain dependent. In line with our results, it has been observed that diacetyl production varies from strain to strain, even within the same species [71]. For instance, the production of diacetyl has been observed in most *Lb. plantarum*, *Lb. paracasei* subsp. *paracasei*, and *E. faecalis* strains isolated from artisanal Pico cheese, but none of the *Lb. paraplantarum* strains showed diacetyl-producing activity [10]. Lactobacilli and enterococci play a significant role in developing the distinctive organoleptic properties of fermented dairy products because they produce the highest levels of diacetyl. It has been found that diacetyl-producing strains are effective starter cultures for fermented milk, increasing the buttery flavors of the final product [72]. In fermented milk, acetoin imparts a weak creamy flavor and assists in reducing the strong cream odor caused by diacetyl [73]. In general, diacetyl is found in a range of 0.02–16.7 ppm, whereas acetoin is about 10–50-fold higher. However, diacetyl has a 100-fold stronger buttery flavor than acetoin when it is present in concentrations of 1.5–5 ppm [74]. The chemical oxidation of diacetyl produces acetoin as a by-product of the metabolism of

LAB [2]. This could explain the reason why diacetyl was the least in the sample fermented by *Lpb. pentosus* L11, which had the highest level of acetoin among the samples.

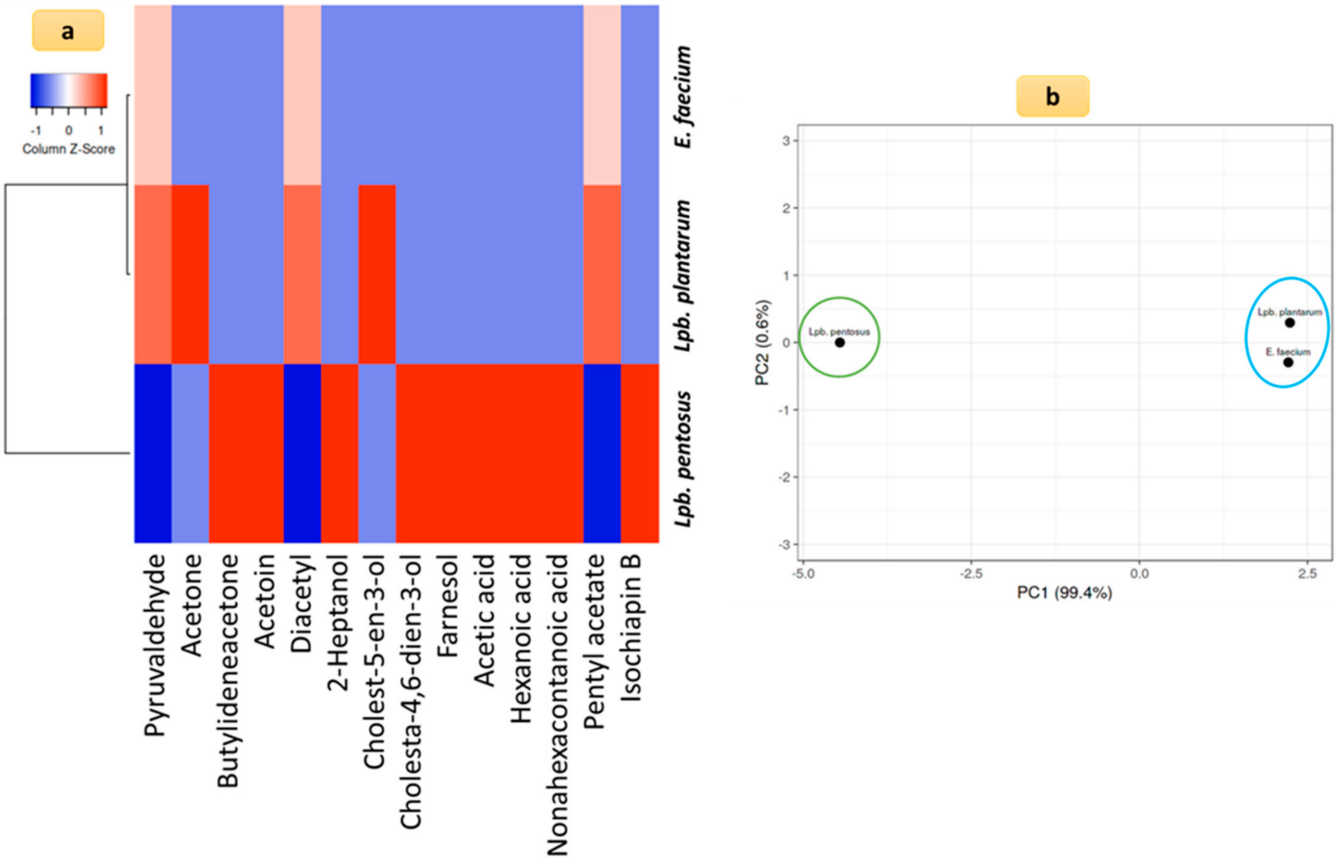


**Figure 5.** Heatmap visualization of the volatile compounds of fermented milk samples based on the hierarchical clustering analysis (**a**). Principal component analysis (PCA) of fermented milk based on their volatile compounds (**b**).

In fermented milk, alcohols may be associated with methyl ketone reduction, lactose metabolism, and amino acid metabolism [2]. The highest level of secondary alcohol 2-heptanol was observed in the *Lpb. pentosus* L11-fermented sample (Figure 5). As secondary alcohols, they can be formed by the enzymatic reduction of methyl ketones, which are themselves formed from β-ketoacids or from fatty acids by β-oxidation [70]. The highest cholest-5-en-3-ol was found in the sample fermented by *Lpb. plantarum* L33. Moreover, a relatively high concentration of cholesta-4,6-dien-3-ol and farnesol was detected in the *Lpb. pentosus* L11-fermented milk.

Fermented milk contains carboxylic acids that are usually produced by lipolysis, proteolysis, or lactose fermentation, contributing to its sourness [75]. The highest acetic acid, hexanoic acid, and nonahexacontanoic acid were detected in the sample fermented by *Lpb. pentosus* L11 (Figure 5). These results are in line with Chammas et al. [76], who detected acetic acid and hexanoic acid in milk fermented by LAB strains.

Because of lactose fermentation or amino acid catabolism, short- to medium-chain fatty acids undergo esterification reactions with primary and secondary alcohols to form esters [70]. The highest level of pentyl acetate, with a fruity note, was detected in milk samples fermented by *Lpb. plantarum* L33 (Figure 5). The majority of esters impart fruity or floral flavors to fermented milk as well as reducing the pungent and astringent aromas caused by fatty acids and amines [73]. Moreover, a remarkable level of lactone isochiapin B was found in the sample fermented by *Lpb. pentosus* L11 (Figure 5). Lactones in fermented milk products contribute to a buttery sensory perception and are thought to be

linked to lipid degradation as they are produced by the cyclization of γ- and δ-hydroxy acids [77]. In agreement with our results, the presence of aromatic alcohols, aldehydes, ketones, esters, and lactones has been reported in fermented milk products containing LAB strains [2,69,70,74].

It can be concluded that probiotic strains with low and medium proteolytic activity, particularly *Lpb. pentosus* L11, produce a wider range of volatile compounds in milk compared to the other strains with high proteolytic effects. This means that a high proteolytic activity does not necessarily lead to more aromatic compounds, and a probiotic strain with a low or medium proteolytic activity could be needed to improve the flavor characteristics of fermented milk. The ability of LAB strains in hydrolyzing proteins into peptides and amino acids and subsequent amino acid catabolism lead to the formation of various aromatic compounds such as α-keto acids, hydroxy acids, aldehydes, ketones, alcohols, carboxylic acids, and esters [29]. It is, however, necessary to point out that lipid degradation, rather than amino acid catabolism, could also be contributed to the aroma profile of fermented milk. Nonetheless, the hydrolysis of lipids is catalyzed by lipolytic esterases (also known as lipases), and with the exception of *L. fermentum*, which is known to possess a surface-associated esterase, most LAB only possess intracellular esterases. Therefore, most LAB esterases cannot hydrolyze food lipids until they are released from lysed cells. Additionally, LAB esterases have weak lipolytic activity [78].

## 4. Conclusions

This study confirmed that *Lpb. pentosus* L11, *Lpb. plantarum* L33, and *E. faecium* L13 (with low, medium, and high proteolytic activity, respectively) are promising probiotic strains. The isolates indicated remarkable hydrophobicity, auto-aggregation, co-aggregation, and antibacterial properties, and none of the isolates showed biogenic amine formation and hemolytic abilities. The potential probiotic strains contributed to flavor development by producing aromatic substances in milk such as diacetyl and acetoin as two major flavor compounds in dairy products. However, probiotic strains with low and medium proteolytic activities, particularly *Lpb. pentosus* L11, were able to produce a wider range of volatile compounds in milk in comparison to the strain with a high proteolytic effect. After further safety assessments are conducted, these multifunctional strains can be used in industrial cheese production and other dairy products. Moreover, new molecular tools for food safety and legislation could make these strains more applicable in the future.

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