Acta Univ. Sapientiae, Alimentaria, **17** (2024) 61–82

DOI: 10.47745/ausal-2024-0004

Technological aspects of lactic acid bacteria originated from artisanal cheeses

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Abstract. Well-characterized, genetically stable starter cultures are used to produce safe fermented dairy products of consistent quality. Lactic acid bacteria play several roles in cheese production. The lactic acid produced influences the firmness of the curd, the yield, and the rheological properties of the cheese. Starter cultures contribute to the formation of flavour and aroma compounds in the product.

The aim of the research is to select and determine the technological aspects of lactic acid bacteria isolated from fresh traditional cheese varieties, which could potentially be used as cheese starter. The 13 strains of the more than 50 lactic acid bacteria studied showed different proteolytic activities and moderate acidifier capacity, contributed to the suppression of pathogenic or spoilage bacteria, and, with cell autolysis, accelerated cheese ripening through the release of enzyme. There are species that convert non-carbohydrate compounds into aroma compounds such as diacetyl. The identified bacteria are *Lactiplantibacillus pentosus, Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei*, and *Lactiplantibacillus argentoratensis*.

Based on the results, we can confirm that some of the lactic acid bacteria isolated from fresh cow and goat milk cheese can be potentially applied as starter cultures in cheese production such as *Lacticaseibacillus paracasei* L13C, *Lactiplantibacillus pentosus* L10G, *Lactiplantibacillus plantarum* L7C, and *Lactiplantibacillus argentoratensis* L2C.

Keywords and phrases: lactic acid bacteria, cheese, technological aspects, salt tolerance, antibacterial activity

1. Introduction

Artisanal cheeses are part of the culinary culture of regions in the Eastern Carpathians of Transylvania, obtained from locally produced milk, using small-scale equipment and traditional processing methods (*Demeter et al.*, 2011). This fermented food has been part of that community diet since the beginning of their civilization. Particular quality of artisanal cheese is linked to natural resources of the region and to complex microbiota as native lactic acid bacteria (LAB) of milk (*Caldeira et al.*, 2024; *Rangel-Ortega et al.,* 2023). The primary role of starter cultures is to control the fermentation process. As a result of the conversion of lactose to lactic acid, the raw material transforms into fermented product (*Altieri et al.*, 2017; *Blaya et al.*, 2018; *Binda and Ouwehand*, 2019; *Ağagündüz et al.*, 2022; *Sharma et al.*, 2023). The autochthonous LAB are responsible for the specific and unique characteristics of artisanal cheese, including the technological, sensory, and safety traits. Due to their characteristics, they are involved in the maturation process, flavour (sensorial quality) and texture development. LAB play an important role in the secondary proteolysis of cheese, liberating low molecular weight peptides and free amino acids. These compounds serve as precursors in the formation of flavouring compounds contributing to more intense sensory properties. Bioactive peptides contribute to the safety of the product (*Guo and Liu* 2023; *Vallejo-Cordoba et al.*, 2023; *Abarquero et al.*, 2024; *Valdiviezo-Marcelo et al.*, 2023, *Hosken et al.*, 2023).

LAB are a group of bacteria with common metabolic and physiological characteristics. In general, these bacteria are Gram-positive, non-spore-forming bacteria, catalase and oxidase negative rods or cocci (*Deák* 2006; *von Wright and Axelsson*, 2019). The non-starter LAB are mesophilic LAB, mostly belonging to the genus *Lactobacillus*, but other species of *Pediococcus* or *Micrococcus* genera also appear (*Meng et al.*, 2018; *Grujović et al.*, 2022). They are involved in maturation through metabolic activity (proteolysis, amino acid catabolism, and lipolysis) and with enzymes released as a result of cell autolysis (*Carafa et al.*, 2016; *Randazzo et al.*, 2021; *Albayrak and Duran*, 2021; *Blaya, et al.*, 2018; *Chourasia et al.*, 2022).

The technological properties of LAB are acidifying capacity, growth at different temperatures, metabolic activity, production of different aroma and flavour compounds, or antimicrobial activity. The free amino acids are involved in flavour development. There is a correlation between cell autolysis and flavour development (*Altieri et al.*, 2017; *Abarquero et al.*, 2023).

In the process of selecting useful LAB strains for product development, the knowledge of species-specific metabolic and technological properties is an essential aspect. The limited number of strains from traditional cheese with excellent technological properties and the constant threat of bacteriophages encourages researchers to isolate new starter cultures. In addition, the new strains meet the

increasing and emerging consumer expectations and contribute to developing different product varieties with sensorial and nutritional complexity. The steps of starter selection involve the determination of technological and probiotic properties and their application at the laboratory and industrial level.

The indigenous microbiota of spontaneously fermented dairy products improves food safety by enhancing the technological and organoleptic functions of the product. These bacteria are competitive in the fermentation of traditional foods, preserving the sensory aspects of the product (*Domingos-Lopes et al.* 2017; *Wang et al.*, 2022; *Abarquero et al.*, 2022; *Abarquero et al.*, 2024).

The isolation and characterization of autochthonous bacteria from traditional products made by spontaneous fermentation contribute to the maintenance of microbiological diversity and to the production of new, higher-quality products relevant to a specific region (*Araújo-Rodrigue et al.*, 2021; *Ruvalcaba-Gómez et al.*, 2022; *Caldeira et al.*, 2024).

There is a lack of scientific information on the complex and diverse microbiota of artisanal cheeses. The selection of autochthonous starter cultures with desirable characteristics is the first tool to obtain a regionally specific cheese of lasting quality from milk from animals with a specific grassland habitat. The main objective of the present study was the selection and characterization of LAB isolated from locally obtained fresh cow and goat milk cheeses produced without commercially available starter cultures and traditional methods. During this study, the technological properties of the LAB, their resistance to antibiotics, and the detection of their antimicrobial effects were evaluated.

2. Materials and methods

2.1. Lactic acid bacteria isolation and identification

In the course of our work, more than 50 lactic acid bacteria isolates were isolated on de Man, Rogosa, and Sharpe (MRS Agar Fluka Analytical) agar from eight different artisanal fresh rennet-coagulated soft cheeses made from cow and goat milk without commercially available starter cultures. The cheese originates from the villages of the Csík Basin. This is one of the great tectonic basins of the Eastern Carpathians. From the samples, 10 g were smashed in a sterile mortar and homogenized with 90 ml physiological solution. From these samples, serial dilutions were made, and 0.1 ml from each mixture was spread on the prepared MRS agar medium surface. The inoculated media was incubated at 37 °C for 24 hrs in aerobic conditions. Fifty bacterial colonies with high numbers and characteristic colony morphology were isolated. Pure cultures were prepared.

Thirteen isolates were selected based on biochemical and phenotypic characteristics (salt tolerance, Gram staining, catalase test, acid and gas production) and were analysed for the most representative technological characteristics of LAB.

Total genomic DNA was extracted and purified according to the manufacturer's protocol (Bioneer AccuPrep® Genomic DNA Extraction Kit). Polymerase chain reaction for 16S rDNA amplification was performed as described by *György and Laslo* (2021).

2.2. Proteolytic activity

The proteolytic activity of LAB isolates was determined with two methods. The identified bacterial strains were inoculated on the surface of MRS supplemented with peptone 10 g/L and gelatine 30 g/L and incubated at 37 °C for 16–18 hrs followed by incubation at 4 °C for 5 hrs. The proteolytic activity of LAB was indicated by the turbidity that appeared around the colonies (*Landeta et al.*, 2013). The well diffusion method was used on modified skimmed milk agar medium (5 g/L casein, 2.5 g/L yeast extract, 1 g/L dextrose, 28 g/L skim milk powder, and 15 g/L agar). 50 µl of supernatant of bacterial isolates were inoculated in the hole in the modified skimmed milk containing agar medium. The incubation was performed at 37 °C for 48 hrs. The proteolytic activity was determined in accordance with the appeared clear zone surrounding each culture.

2.3. Acidifying activity

Acidifying activity was determined by monitoring pH change in 10 ml UHT milk inoculated with the tested LAB (1% v/v) and incubated at 37 °C. Over 48 hrs, the change in pH was measured (*Ribeiro et al.*, 2014).

2.4. Autolysis of LAB

The autolytic phenotype of identified LAB strains was evaluated in 50 mM/L pH 6.5 phosphate buffer. The buffer solution was inoculated with 2 g/100 ml LAB cultures. The optical density of the samples was measured at 600 nm for 72 hrs. The autolysis of lactic acid bacteria cells was expressed as percentage (%) of the initial change in optical density OD (OD 0/OD t*100, where OD 0 is the OD measured at the initial time point, and OD t is the OD measured at the time point under study) (*Mora et al.*, *2003*).

2.5. Diacetyl production

Diacetyl formation by the tested LAB was determined according to *Ribeiro et al.* (2014). An aliquot of 10 ml UHT milk was inoculated with the tested LAB (1%

v/v). The samples were incubated at 30 °C for 24 hrs. Subsequently, an amount of 0.5 ml of α-naphthol (1% w/v) and KOH solution (16% w/v) were added to one ml of each sample and incubated at 30 °C for 10 min. Diacetyl production was recorded by the forming of a red ring at the top of the tubes. The lighter red indicated medium capacity.

2.6. Antibacterial activity

The antibacterial activity of LAB cell-free supernatant was determined with the agar diffusion method on different pathogenic and spoilage indicator strains (from the microbiological laboratory of the University) as *Escherichia coli*, *Bacillus cereus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Staphylococcus aureus*. The bacterial cultures were harvested for 24 hrs at 28 °C and 37 °C on nutrient agar. An amount of 0.1 ml bacterial suspension (with OD at 600 nm = 1) was spread on nutrient agar medium surface. 50 µl of the cell-free supernatant of LAB (14,000 rpm, 5 min) was dropped in the hole (d = 8 mm) cut with a sterile test-tube. The incubation was performed for 24 hrs at 28 °C / 37 °C. The antimicrobial effect of the LAB isolates was expressed in accordance with the diameter of the inhibition zone.

2.7. Antibiotic susceptibility

The determination of antimicrobial susceptibilities of LAB was performed according to the guideline reported in *EFSA* (2012). For the assessment of the susceptibility to nine antibiotics: penicillin, ampicillin, gentamicin, streptomycin, erythromycin, chloramphenicol, kanamycin, tetracycline, and clindamycin, two-fold serial dilutions were realized ranging from 0 up to 128 µg/ml in MRS broth *(Laslo et al.*, 2015).

2.8. Stability of lactic acid bacteria to freeze-drying

The stability of lactic acid bacteria to freeze-drying process was assessed using a modified method previously reported by *Carafa et al.* (2016). LAB from liquid culture was harvested by centrifugation (4,000 rpm for 20 min, 4 °C). The pellets were suspended in sterile freeze-drying medium and finally dried in a Cryodos 45 freeze dryer. For determination of the viability of freeze-dried LAB, an aliquot of 0.1 ml was suspended in a volume of 1 ml physiological solution. From the dilution series, 0.1 ml was spread on the surface of MRS agar medium. Incubation was performed at 37 °C for 24 hrs.

2.9. Statistical analysis

Analyses were performed in triplicate. The results of each determination were expressed as the mean ± standard deviation. Some results were analysed using principal component analysis, with the PAST software package (*https://past.en.lo4d.com/windows*), and some of the results were analysed with Tukey's test using IBM SPSS Statistics v22; $p < 0.05$ was considered statistically significant.

3. Results and discussions

Based on 16S rDNA, the selected 13 lactic acid bacterial strains from fresh cheese belong to the *Lactiplantibacillus* genus reclassified (*Zheng et al.*, 2020*)* (*Table 1*). The sequencing results showed that 38.46% of the total isolated bacteria were identified as *Lactiplantibacillus argentoratensis* (46.16%), as *Lactiplantibacillus pentosus* (7.69%), as *Lactiplantibacillus plantarum*, and as *Lacticaseibacillus paracasei* (7.69%).

Table 1. Molecular identification of the lactic acid bacteria isolates based on partial 16S rDNA analysis

Size in points	Isolates code	Bacterial source type of cheese	Most closely related organism	% Gene identity
1	L1C	Cow's milk cheese	*Lactiplantibacillus argentoratensis* ON527796.1	100%
2	L2C	Cow's milk cheese	*Lactiplantibacillus argentoratensis* ON527796.1	100%
3	L3C	Cow's milk cheese	*Lactiplantibacillus argentoratensis* ON527796.1	99.89%
4	L4C	Cow's milk cheese	*Lactiplantibacillus argentoratensis* ON527796.1	100%
5	L5C	Cow's milk cheese	*Lactiplantibacillus pentosus* ON387456.1	99.88%
6	L6C	Cow's milk cheese	*Lactiplantibacillus pentosus* MT229656.1	99.88%
7	L7C	Cow's milk cheese	*Lactiplantibacillus plantarum* CP052869.1	100%

Size in points	Isolates code	Bacterial source type of cheese	Most closely related organism	% Gene identity
8	L8G	Goat milk cheese	*Lactiplantibacillus argentoratensis* ON387453.1	99.89%
9	L9G	Goat milk cheese	*Lactiplantibacillus pentosus* ON495423.1	100%
10	L10G	Goat milk cheese	*Lactiplantibacillus pentosus* ON495423.1	99.89%
11	L11G	Goat milk cheese	*Lactiplantibacillus pentosus* ON495423.1	100%
12	L12C	Cows' milk cheese	*Lactiplantibacillus pentosus* ON495423.1	99.88%
13	L13C	Cows' milk cheese	*Lacticaseibacillus paracasei* ON387664.1	100%

Traditional foods obtained by spontaneous fermentation are a promising source of new, competitive starter cultures. The quality, flavour of artisanal cheese highly correlates with bacterial diversity (*Zhang et al.*, 2021; *Grujović et al.*, 2022; *Guo and Liu* 2023). In the different types of cheese, *Lactiplantibacillus plantarum* was the most frequently occurring non-starter LAB*. L. plantarum subsp. plantarum* and *L. plantarum subsp. argentoratensis* are known for their health benefits as probiotics, as they are safe and thus urged to be used in dietary supplements or dairy products (*Choi et al.*, 2021; *Yilmaz et al.*, 2022; *Guo and Liu* 2023).

The studied strains cultured in media with protein supplementation showed different proteolytic activities. These strains have different proteolytic capacities depending on the used proteins. 38% of the studied bacterial strains showed the most favourable result. *Lactiplantibacillus argentoratensis* L2C*, Lactiplantibacillus pentosus* L6C*, Lactiplantibacillus pentosus* L9G, *Lactiplantibacillus pentosus* L10G, and *Lactiplantibacillus pentosus* L11G could hydrolase the most of the proteins, and the halozone was the most interpretable in these cases.

During ripening, proteolysis is a complex biochemical process that determines the texture and softness of cheese (*Ribeiro et al.*, 2014; *Pagthinathan and Nafees*, 2015). LAB proteinases and peptidases produce peptides and amino acids during ripening (*Ardö et al.*, 2017). These compounds serve as precursors for flavour development. Autochthonous LAB has been shown to contribute to higher proteolysis in traditional cheese products (*de Aguiar e Câmara et al.*,

2022). *Lb. plantarum* strains in yogurt showed the highest proteolytic activity and contributed to developing texture and volatile flavour (*Yilmaz et al.*, 2022).

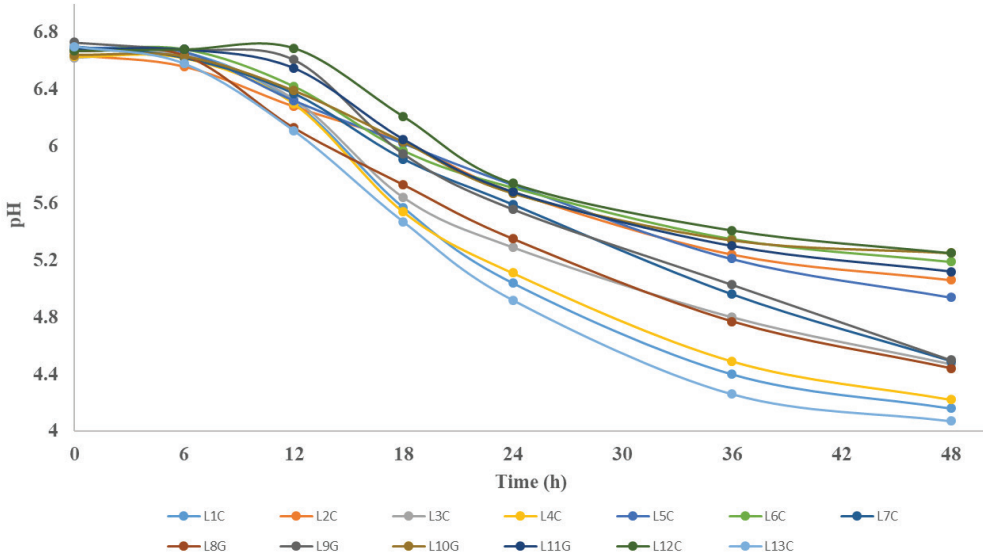

Figure 1. Acidifying capacity of the studied bacterial strains

The pH values of the inoculated UHT milk are shown in *Figure 1*. The majority of the bacterial strains were the slowest acid producers. In this case, it took 36 or 48 hours for the pH to decrease below 5. *Lacticaseibacillus paracasei* L13C reduced the pH of the UHT milk after 24 hrs. The pH values after 18 hrs were 5.57 in the case of *Lactiplantibacillus argentoratensis* L1C, 5.64 in the case of *Lactiplantibacillus argentoratensis* L3C, 5.54 in the case of *Lactiplantibacillus argentoratensis* L4C, and the *Lactiplantibacillus argentoratensis* L8G reduced the pH to 5.73. After 48 hours, the pH values were approximately 4 for three strains: 4 in the case of *Lacticaseibacillus paracasei* L13C, 4.16 in the case of *Lactiplantibacillus argentoratensis* L1C, and for *Lactiplantibacillus argentoratensis* L4C it was 4.22. The mentioned bacterial strains have an excellent acidifying capacity as starter cultures for cheese. This is due to their ability to reduce the pH by 0.05–0.2 within the first 120 minutes, which is favourable for different types of cheese.

Acid production is one of the fundamental properties of starter cultures. In some cheeses, it can contribute to sensory defects. For coagulation, denaturation, firmness of the curd/cheese, and control of undesirable microbes, it is essential to lower the pH as quickly as possible. Generally, *Lactiplantibacillus* species are moderate acidifiers, with some of them being slow (*Ribeiro et al.*, 2013; *Todorov et al.*, 2017; *Meng et al.*, 2018).

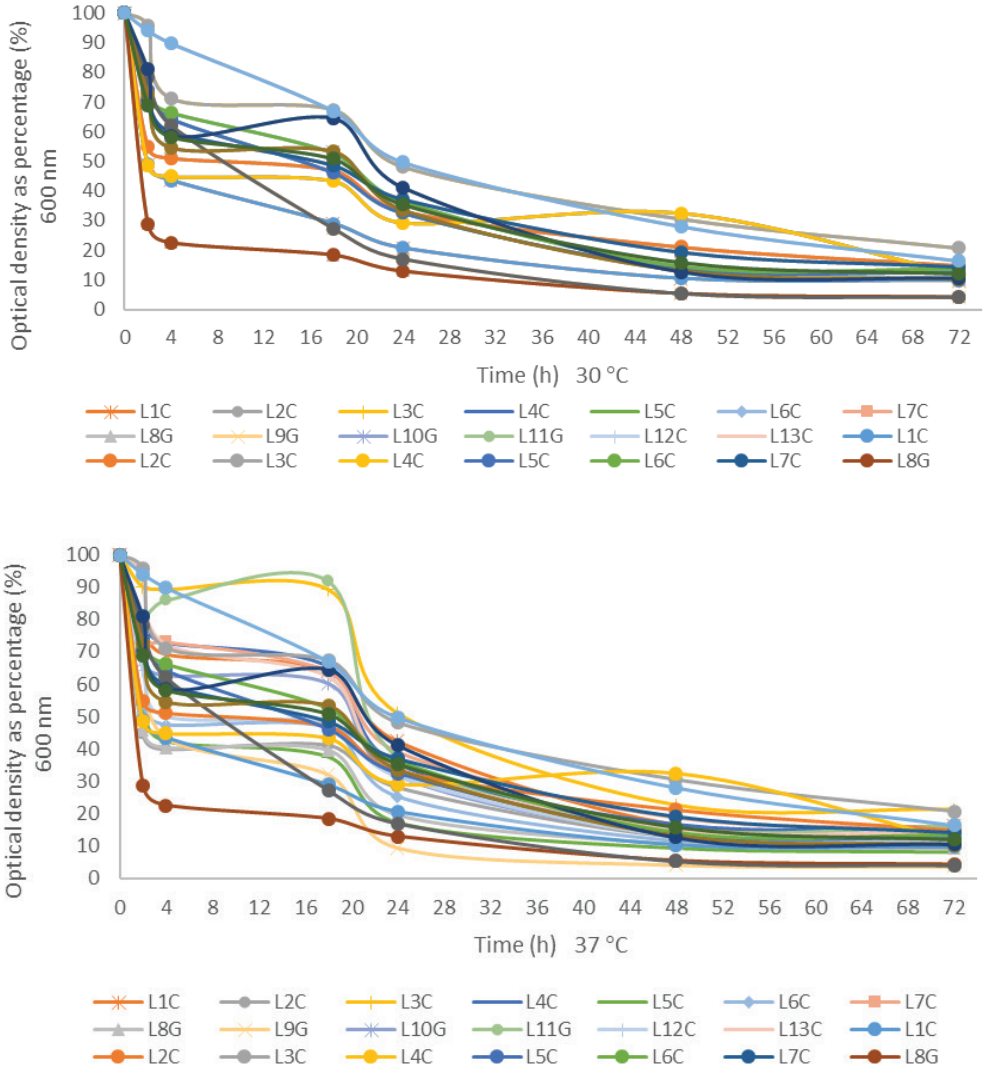

Figure 2. Autolysis of the LAB cells cultured at 30 °C (*A*) and at 37 °C (*B*)

Another technological aspect of LAB is cell autolysis. The importance of this lies in the release of intracellular enzymes. Autolyzed cells have been shown to accelerate peptidase activity and reduce the bitterness of cheese (*Cogan and Beresford, 2002*; *Cheng et al., 2022*). Cell autolysis accelerates cheese ripening through the release of enzymes. Generally, there is no substantial difference in the autolytic ability of the tested strains at 30 °C and 37 °C. For *Lactiplantibacillus argentoratensis* L8G, autolysis rates of at least 22.58% were observed after four hours at 30 °C and 45.65% at 37 °C. The highest percentage decrease in OD at

30 °C was found for *Lactiplantibacillus argentoratensis* L8G (12.962 g / 100 ml) and the lowest for *Lacticaseibacillus paracasei* L13C (49.683%).

After 48 hrs, the lowest change in relative OD was 32.5% for *Lactiplantibacillus argentoratensis* L4C, while autolysis was 5.278% for *Lactiplantibacillus pentosus* L9G.

After 72 hrs, the autolysis of *Lactiplantibacillus argentoratensis* L3C was 20.649% and 4 g/100ml for *Lactiplantibacillus pentosus* L9G (*Figure 2*).

For one strain, *Lactiplantibacillus argentoratensis* L2C, at least 39.85% autolysis was detected at 37 °C after 4 hrs. The percentage decrease in the OD at 37 °C after 24 hrs for *Lactiplantibacillus argentoratensis* L3C was 50.943% and 9.444 g/100ml in the case of *Lactiplantibacillus pentosus* L9G. After 48 hrs, the highest decrease in OD was observed for *Lactiplantibacillus argentoratensis* L3C (22.594 g / 100 ml). The lowest reduction in OD was observed for *Lactiplantibacillus pentosus* L9G (4.086%).

After 72 hours, the autolysis ability, expressed as the change in OD, varied from 21.259% for *Lactiplantibacillus pentosus* L9G to 3.640% for *Lactiplantibacillus argentoratensis* L3C.

Regarding the growth ability at different temperatures, it can be noted that the bacterial strains grew slightly at 4 and 45 °C. At 15 °C, the OD values increased three times and four to five times at 37 °C compared to the initial OD. The highest growth was observed at 30 °C (data not shown). The results indicate that the tested cultures are mesophilic bacteria due to their strong growth at 30 °C.

Lactic acid bacteria produce different aroma compounds that contribute to the formation of the flavour and aroma of the product. The diacetyl concentration was the highest in the case of *Lacticaseibacillus paracasei* L13C and *Lactiplantibacillus plantarum* L7C. For the other bacterial strains, diacetyl production was moderate, as the positive result appeared after a few hours. LAB contribute to the development of specific organoleptic characteristics of certain cheeses by producing this aroma compound. The aroma production in Caciocavallo cheese by *Lactocaseibacillus plantarum* and *Lactocaseibacillus paracasei* is attributed to the low activity of aminopeptidase type N and cystathionine lyase (*Yavuz et al., 2021*).

These microorganisms are capable of converting non-carbohydrate compounds into aroma compounds, such as diacetyl and acetoin, through their metabolism (*Ruiz Rodríguez et al., 2017*). *Lazzi et al.* (2016) found that the intracellular enzymes released during the autolysis of LAB were mainly involved in the cheese aroma formation in some specific cheese varieties. Estherolytic and proteolytic activities enhance the flavour production of lactic acid bacteria (*Albayrak and Duran*, 2021).

The antibacterial activity of the bacterial strains was evaluated using the agar diffusion method against six Gram-positive and Gram-negative bacteria. The effects of the studied LAB supernatant are shown in *Table 2*.

When selecting starter cultures, one consideration is the antimicrobial activity of the bacterial strains. With this potential, LAB contribute to suppressing pathogenic or spoilage bacteria.

Table 2. Antimicrobial effect of lactic acid bacteria on Gram-positive and Gram-negative bacteria

LAB strain	*Bacillus cereus*	*Bacillus subtilis*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Pseudomonas fluorescens*	*Staphylococcus aureus*
	Inhibition zone diameter ± SD (mm)					
Lactiplantibacillus argentoratensis L1C	12.5 ± 0.5^{d}	16 ± 0^{cd}	10.5 ± 0.5^{cd}	10 ± 0^{a}	20.5 ± 0^{f}	11.5 ± 0.5^{bcd}
Lactiplantibacillus argentoratensis L2C	10.5 ± 0.5^{c}	17 ± 0^{d}	11 ± 0^{cde}	22.5 ± 2.5^{f}	20.5 ± 0.5^{f}	11.5 ± 0.5^{bcd}
Lactiplantibacillus argentoratensis L3C	7.5 ± 0.5 ^{ab}	15 ± 0^{c}	0 ± 0^{a}	18 ± 0^{cd}	19 ± 1^{def}	10.5 ± 0.5^{abc}
Lactiplantibacillus argentoratensis L4C	6.5 ± 0.5 ^{a}	15.5 ± 0.5^{c}	10 ± 1 ^{c}	16.5 ± 0.5 ^{bc}	17.5 ± 2.5^{bcd}	11.5 ± 0.5^{bcd}
Lactiplantibacillus pentosus L5C	7.5 ± 0.5^{ab}	15 ± 0^{c}	12.5 ± 1.5^{ef}	21 ± 1^{def}	16.5 ± 0.5^{bc}	11.5 ± 1.5^{bcd}
Lactiplantibacillus pentosus L6C	12 ± 0 ^{cd}	11 ± 0^{b}	10.5 ± 0.5^{cd}	10 ± 0^{a}	18 ± 1^{cde}	8.5 ± 0.5^{a}
Lactiplantibacillus plantarum L7C	7.75 ± 0.75^{ab}	15.5 ± 0.5^{c}	10 ± 0^{c}	17.5 ± 0.5^{c}	15.5 ± 0.5^{ab}	11.5 ± 0.5^{bcd}
Lactiplantibacillus argentoratensis L8G	8.25 ± 0.25^{ab}	12 ± 0.5 ^{b}	12 ± 1^{def}	18 ± 0^{cd}	14 ± 0^{a}	11 ± 1^{bcd}
Lactiplantibacillus pentosus L9G	6.5 ± 0.5^{a}	16 ± 0^{cd}	7 ± 0^{b}	15.5 ± 0.5^{bc}	20 ± 0^{ef}	10 ± 0^{ab}
Lactiplantibacillus pentosus L10G	8.5 ± 0.5^{b}	16 ± 0^{cd}	13.5 ± 0.5 ^{f}	21.5 ± 0.5^{ef}	18 ± 0^{cde}	13 ± 1^{d}
Lactiplantibacillus pentosus L11G	11 ± 1^{cd}	16 ± 0^{cd}	10.5 ± 0.5^{cd}	24 ± 0^{f}	25 ± 0^{g}	12 ± 1^{bcd}
Lactiplantibacillus pentosus L12C	12 ± 1^{cd}	13 ± 0.5^{b}	12.5 ± 0.5^{ef}	13.5 ± 1.5^{b}	20 ± 0^{ef}	12.5 ± 0.5^{cd}
Lacticaseibacillus paracasei L13C	10.5 ± 0.5^{c}	0 ± 0^{a}	10.5 ± 0.5^{cd}	18.5 ± 1.5^{cde}	20.5 ± 0^{f}	8.5 ± 0.5^{a}

Note: Means ± standard deviation with different letters are significantly different by Tukey's test ($p < 0.05$).

The antimicrobial activity is exerted by various mechanisms due to their metabolites including dissociated or undissociated organic acids, bacteriocins, etc. The growth-inhibitory activity is due to the termination of the transmembrane proton motive force (*Laslo et al.*, 2020). Based on the inhibition zone, a greater inhibitory effect against *Bacillus cereus* was observed in the case of the cell-free supernatant of *Lactiplantibacillus argentoratensis* L1C and the supernatant of two strains of *Lactiplantibacillus pentosus* L11G and *Lactiplantibacillus pentosus* L12C. *Bacillus subtilis* was strongly inhibited by the supernatant of *Lactiplantibacillus argentoratensis* L2C. The cell-free supernatant of four strains inhibited *Bacillus subtilis* at the same level. *Lactiplantibacillus pentosus* L10G exhibited a strong inhibitory effect against *E. coli*. Weak antibacterial effect was detected in the cases of *Lactiplantibacillus pentosus* L12C and *Lactiplantibacillus pentosus* L5C. The strongest antibacterial effect was observed against *Pseudomonas aeruginosa. Pseudomonas fluorescens and Ps. aeruginosa* were the most inhibited by the cell-free supernatant of *Lactiplantibacillus pentosus* L11G. The following bacteria were also found to have a strong antibacterial effect: *Lactiplantibacillus argentoratensis* L2C and *Lactiplantibacillus pentosus* L10G. The largest inhibition zone was observed in the case of *Lactiplantibacillus pentosus* L11G against *Ps. fluorescens*.

Intense antibacterial activity was also observed for *Lacticaseibacillus paracasei* L13C, *Lactiplantibacillus argentoratensis* L1C, and *Lactiplantibacillus argentoratensis* L2C. *Staphylococcus aureus* was weakly inhibited by the tested lactic acid bacteria. The highest level of inhibition was observed in *Lactiplantibacillus pentosus* L10G, with a slightly lower level observed in *Lactiplantibacillus pentosus* L12C. For this indicator bacterium, the antibacterial effect was weaker compared to the others. Similar results regarding the antibacterial efficacy of autochthonous lactic acid bacteria have also been described in the literature (*Araújo-Rodrigues et al.*, 2021; *Glieca et al.*, 2024).

LAB serve as biocontrol agents against potentially pathogenic bacteria. *L. plantarum* strains have been shown to have antimicrobial activity against Gram-negative and Gram-positive bacteria and moulds that can contaminate food and cause human diseases (*Russo et al.*, 2017; *Arena et al.*, 2016). Among our bacterial strains, three *Lactiplantibacillus pentosus* strains based on inhibition zone showed more substantial antibacterial effect against *E. coli* and mild effect against *Staphylococcus aureus* and *Bacillus cereus* compared to the results of 48 hrs (*Motahari et al.*, 2017).

Survival and stability to freeze-drying were performed for four bacterial species from the studied strains: *Lactiplantibacillus pentosus* L9G, *Lacticaseibacillus paracasei* L13C, *Lactiplantibacillus pentosus* L10G, and *Lactiplantibacillus argentoratensis* L2C.

Freeze-drying is a widely used method for the formulation of starter cultures due to its advantages in maintaining biological activity and convenient storage

at room temperature (*Chen et al.*, 2021). After freeze-drying, the cell number was $2.1 \cdot 10^{12}$ CFU/g in the case of *Lactiplantibacillus pentosus* L9G and $1.3 \cdot 10^{12}$ CFU/g in the case of *Lacticaseibacillus paracasei* L13C. In the case of *Lactiplantibacillus pentosus* L10G, $1.35 \cdot 10^{12}$ CFU/g was detected and in the case of *Lactiplantibacillus argentoratensis* L2C $6 \cdot 10^{12}$ CFU/g after freeze-drying. The results show that the viability of the four tested species was not affected during freeze-drying.

Multivariate analysis of technological LAB characteristics was performed using principal component analysis (PCA). PCA results showed that the first two components accounted for 89.01% of the total variance. The first component, which accounted for 76.391% of the total variance, had the highest eigenvalue of 2.241, and the second component accounted for 12.62 % of the total variance with an eigenvalue of 0.370.

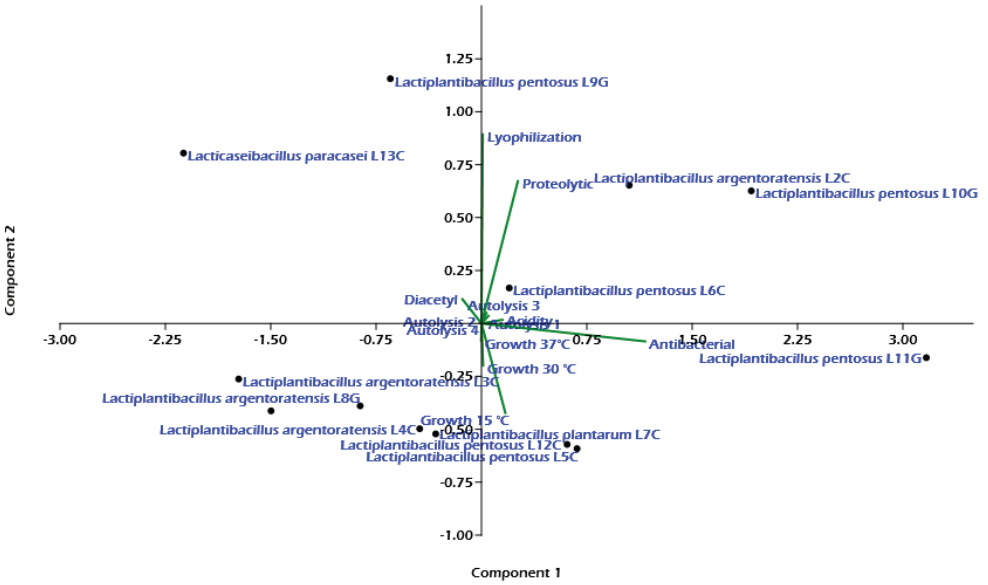

Figure 3. Principal component analysis biplot based on the technological properties of identified lactic acid bacteria

LAB whose characteristics highly correlate cluster together. On the right side in quadrant I, there are three LAB *Lactiplantibacillus pentosus* L10G, *Lactiplantibacillus argentoratensis* L2C, and *Lactiplantibacillus pentosus* L6C, and in the right quadrant IV there are *Lactiplantibacillus pentosus* L5C, *Lactiplantibacillus pentosus* L12C, and *Lactiplantibacillus pentosus* 11G. In left side in quadrant II , there are *Lactiplantibacillus pentosus* L13C and *Lactiplantibacillus pentosus* L9G, the rest of the characterized bacteria being grouped in the left quadrant III. With regard to the safety of the starter culture, the antibiotic resistance

is important. LAB can have multiple antibiotic resistance, which phenomenon occurs naturally or is genetically acquired (*Guo et al.*, 2017). Antibiotic resistance is species- and strain-dependent. Antibiotic susceptibility was highly dependent on the isolated strain. Based on the phenotypic antibiotic resistance determination, it can be concluded that among the most promising lactic acid bacteria, *Lacticaseibacillus paracasei* L13C was susceptible to penicillin, ampicillin, and clindamycin. The minimum inhibitory concentration (MIC) value was 1 μg/mL in three cases. For the other six antibiotics, the MIC was 128 or greater than 128 μg/ml. *Lactiplantibacillus pentosus* L10G was susceptible to penicillin – the MIC value was 1 μg/mL; for ampicillin: MIC = 1 μg/mL, clindamycin: MIC = 2 μg/mL, tetracycline: MIC = 32 μg/mL, and kanamycin: MIC = 64 μg/mL. *Lactiplantibacillus argentoratensis* L2C was susceptible to penicillin: MIC = 8 μg/mL, ampicillin: MIC = 1 μg/mL, clindamycin: MIC = 2 μg/mL, and tetracycline: MIC = 32 μg/mL. The MIC for streptomycin, kanamycin, erythromycin, chloramphenicol, and gentamicin was greater than 128 μg/ml. The identified bacterial strains have MIC > 128 μg/mL for streptomycin, erytromicin, chloramphenicol, gentamicin, and tetracycline. Our results are similar, with minor differences, for the bacteria described by *Radulović et al.* (2010) and *Zarzecka et al.* (2022).

It has been shown that the genes for resistance to erythromycin, tetracycline, and chloramphenicol are often found on mobile genetic elements in lactic acid bacteria. The possibility of gene transfer by lactic acid bacteria is a concern, and antibiotic resistance of starter or adjunct cultures is undesirable (*Dušková et al.*, 2020; *Zarzecka et al.*, 2022). Further research is needed to determine the presence of antibiotic resistance genes.

Table 3. Minimum inhibitory concentrations (MIC μg/mL) of the LAB strains tested for nine different antibiotics

LAB	**Antibiotics μg/mL**								
	Penicillin	**Streptomycin**	**Ampicillin**	**Kanamycin**	**Clindamycin**	**Erythromycin**	**Chloramphenicol**	**Gentamicin**	**Tetracycline**
Lacticaseibacillus paracasei L13C	1	128	1	128	1	> 128	> 128	> 128	> 128
Lactiplantibacillus pentosus L10G	1	> 128	1	64	2	> 128	> 128	> 128	32
Lactiplantibacillus argentoratensis L2C	8	> 128	1	> 128	2	> 128	> 128	> 128	32

4. Conclusions

Naturally fermented traditional cheeses are promising sources for the selection of novel starter cultures. The autochthonous microflora of cow's and goat's milk cheeses produced in our region without commercial starter cultures includes the *Lactiplantibacillus* and *Lacticaseibacillus* species. The results showed that the majority of the strains exhibited proteolytic, antibacterial, and autolysis activity. The lactic acid bacteria tested were moderate acid producers, grew well at different temperatures, and showed stability to the freeze-drying process.

Based on this study, *Lactiplantibacillus pentosus* L10G, *Lactiplantibacillus plantarum* L7C, *Lacticaseibacillus paracasei* L13C, and *Lactiplantibacillus argentoratensis* L2C with good technological properties were found to be promising candidates for the development of cheese or fermented dairy products after further safety assessment as antibiotic-resistant genes.

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