Lactic acid bacteria from artisanal raw goat milk cheeses: technological properties and antimicrobial potential

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Key words: dairy, proteolytic activity, acidifying activity, biopreservation, starter culture.

Contributions: VC, UGB, conceptualization, supervision, project administration, resources and funding acquisition; ASF, CR, UGB, methodology; NF, LC, CR, VC, UGB, software; BNS, UGB, validation, formal analysis and data curation; NF, LC, BNS, investigation; BNS, writing - original draft; UGB, writing - review and editing; JAT, UGB, visualization.

Conflict of interest: the authors declare that they have no competing interests, and all authors confirm accuracy.

Funding: the authors are grateful to the Foundation for Science and Technology (FCT, Portugal) for financial support through national funds FCT/MCTES (PIDDAC) to CIMO (UIDB/00690/2020 and UIDP/00690/2020) and SusTEC (LA/P/0007/2020). They are also grateful to the EU PRIMA program and FCT for funding the ArtiSaneFood project (PRIMA/0001/2018). This study was supported by FCT under the scope of the strategic funding of the UIDB/04469/2020 unit and BioTecNorte operation (NORTE-01-0145-FEDER-000004) funded by the European Regional Development Fund under the scope of Norte2020 – Programa Operacional Regional do Norte. B. N. Silva acknowledges the financial support provided by FCT through the Ph.D. grant SFRH/BD/137801/2018.

Availability of data and materials: data and materials are available from the corresponding author upon request.

Received: 1 July 2023.
Accepted: 11 Septmber 2023.
Early access: 27 October 2023

Italian Journal of Food Safety 2023; 12:11559
doi:10.4081/ijfs.2023.11559

Abstract

In cheese-making, a starter culture composed of adequately chosen lactic acid bacteria (LAB) may be suitable to ensure the rapid acidification of milk, improve textural and sensory characteristics, and avoid pathogen proliferation. In this work, 232 LAB isolates collected from artisanal goat's raw milk cheeses produced in Portugal were evaluated for their antimicrobial capacity (at 10 and 37°C), as well as their acidifying and proteolytic properties. Among the 232 isolates, at least 98% of those isolated in De Man-Rogosa-Sharpe (MRS) agar presented antagonism against *Listeria monocytogenes*, *Salmonella* Typhimurium, or *Staphylococcus aureus*, whereas less than 28.1% of M17-isolated LAB showed antagonism against these pathogens. M17-isolated LAB displayed better results than MRS ones in terms of acidifying capacity. As for the proteolytic assay, only 2 MRS isolates showed casein hydrolysis capacity. Principal component analyses and molecular characterization of a subset of selected isolates were conducted to identify those with promising capacities and to correlate the identified LAB genera and species with their antimicrobial, acidifying, and/or proteolytic properties. *Lactococcus* strains were associated with the highest acidifying capacity, whereas *Leuconostoc* and *Lacticaseibacillus* strains were more related to antimicrobial capacities. *Leuconostoc mesenteroides*, *Lactococcus lactis*, and *Lacticaseibacillus paracasei* were the predominant organisms found. The results of this work highlight various strains with pathogen inhibition capacity and suitable technological properties to be included in a customized starter culture. In future work, it is necessary to appropriately define the starter culture and implement it in the cheese-making process to evaluate if the *in-vitro* capacities are observable in a real food system.

Introduction

Lactic acid bacteria (LAB) are accountable for the cheese fermentation process, whether they are naturally present in milk or purposefully added (starter culture), since they produce organic acids (mainly lactic and acetic acids) that cause the rapid acidification of milk and consequently promote coagulation, curd firmness, and control of contaminants (Piraino *et al*., 2008; Ribeiro *et al*., 2014). The metabolic characteristics of LAB, such as the proteolytic capacity, can contribute to the development of pleasurable organoleptic properties (such as texture, aroma, and flavor compounds) (Castro *et al*., 2018; Silva *et al*., 2018), which are particularly important in artisanal cheeses.

Furthermore, LAB can also increase the safety of cheeses and act as biopreservative agents as a result of the production of

antimicrobial metabolites during fermentation, such as organic acids, hydrogen peroxide, diacetyl, fatty acids, reuterin, and bacteriocins (Margalho *et al*., 2021), and thus replace chemical preservatives that are used in dairy products, such as sorbic acid, sodium benzoate, calcium sorbate, potassium sorbate, and natamycin (Favaro *et al*., 2015; Mazdeh *et al*., 2017). However, not all LAB are suitable to be added to food products. LAB that belong to the genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and some *Streptococcus* have generally regarded as safe (GRAS) or qualified presumption of safety (QPS) status, which means that there is reasonable evidence that such microorganisms do not raise safety concerns and that their use in foods is approved by the US Food and Drug Agency or by the European Food Safety Authority (EFSA), respectively (EFSA, 2007). On the other hand, the *Enterococcus* genus and some *Streptococcus* species can be pathogenic and present virulence factors and resistance to a variety of antibiotics, which is the reason why they are not eligible for GRAS/QPS status and, therefore, may not be used in foods (EFSA, 2007). Considering that artisanal cheeses produced from raw goat milk may have poor microbial quality (Gonzales-Barron *et al*., 2017; Margalho *et al*., 2021), it was hypothesized that the addition of selected LAB strains with functional properties as a customized starter culture could be used as a control measure for the growth of *Staphylococcus aureus*. In that regard, the autochthonous microbiota of raw milk cheeses is complex and diverse, offering a wide range of species with antimicrobial and/or acidifying capacities (Morandi *et al*., 2019; Gonzales-Barron *et al*., 2020; Margalho *et al*., 2020; Araújo-Rodrigues *et al*., 2021; Coelho *et al*., 2022). It is possible that a single LAB strain is not capable of inhibiting a pathogen in milk or cheese. In the case of bacteriocinogenic LAB, for example, there may be various limiting factors, such as the level of bacteriocin expression, the low capacity for bacteriocin production in the food system, the interaction between bacteriocin and the food matrix, the antagonism of other bacteria toward the LAB strain, and the effect of the physicochemical parameters on the bacteriocin activity (Favaro *et al*., 2015). For that reason, a mixture of strains can be used to build the starter culture and to guarantee the desired antagonistic effect, it may be convenient to use strains with distinct capacities: acidifying LAB strains, for instance, also plays a key role in inhibiting pathogenic bacteria during cheese ripening by promoting an acidic environment (Gonzales-Barron *et al*., 2020), and can be combined with bacteriocinogenic LAB to enhance the antimicrobial power of the starter culture. Therefore, the first objective of this work was to collect and evaluate the antimicrobial, acidifying, and proteolytic capabilities of LAB isolated from artisanal Portuguese goat's raw milk cheeses. Then, using statistical analysis, the second objective was to select a subset of LAB isolates with the potential to be included in a customized starter culture and used in cheese manufacture and perform their molecular identification by *16S rRNA* sequencing.

Materials and Methods

Bacterial strains

For each pathogen, a loop was taken from a fresh culture kept on nutrient agar slants and inoculated in brain heart infusion (BHI) broth (10 mL). Broth tubes were incubated for 16 hours at 37°C, following 2 consecutive inoculations, to reach approximately 10^8 CFU/mL. *Listeria monocytogenes* required pre-activation in 5 mL of BHI at 37°C for 16 hours.

Lactic acid bacteria isolation and confirmation

A total of 4 batches of artisanal goat's raw milk cheeses (n=20) were collected at the end of production from a regional factory located in Mirandela, Portugal, between November 2020 and March 2021. LAB were isolated from cheese samples as described by the International Organization for Standardization standard 15214:1998, with minor modifications (ISO, 1998). Briefly, after dilution, aliquots were incorporated in De Man-Rogosa-Sharpe (MRS) agar (selective medium for enumeration and isolation of lactobacilli) and M17 agar (non-selective medium for enumeration and isolation of lactococci) and overlayed with 1.2% bacteriological agar. Next, plates were incubated at 30°C for 48 hours, and after that, 8 typical colonies on MRS and M17 agar (each) were selected for purification and incubated at 30°C for another 48 hours in the respective media. Finally, to filter and refine LAB isolate selection, catalase (3% hydrogen peroxide) and Gram tests, as well as morphologic observation, were performed. Presumptively identified LAB cultures were maintained in MRS broth with 25% glycerol at -80°C.

Determination of antimicrobial, proteolytic and acidifying capacities of lactic acid bacteria

The antimicrobial ability was evaluated as described by Campagnollo *et al*. (2018), using the spot-on-lawn assay with a few modifications. Succinctly, each LAB isolate from the cryopreserved stock culture was reactivated in MRS broth (37°C, 24 hours) and spotted onto MRS (3 µL) or M17 (5 µL) agar plate surfaces, following incubation at 30°C for 16 hours. Different volumes were used to obtain similar and comparable colony sizes in both agars and to improve the accuracy of the measurements of colony sizes and inhibition zones produced. For example, spotting 5 µL in MRS agar resulted in colonies with such a diameter that numerous isolates produced inhibition zones that were too large and difficult to measure, whereas spotting 3 µL in M17 agar resulted in very small colony diameters that were difficult to measure with accuracy. Then, the plates were covered with 10 mL of BHI broth with 0.75% (w/v) bacteriological agar seeded with 1 mL of each bacterial strain (separately) at approximately 8 log CFU/mL. After pre-incubation at 4°C for 2 hours followed by incubation at 37°C for 16 hours, the inhibition zone diameters were measured with a caliper. A subset of LAB isolates presenting antimicrobial capacity at 37°C and that complied with the following criteria were also tested at 10°C for 10 days: distance between LAB colony limit and halo circumference greater than 8 mm for *Salmonella enterica* ser. Typhimurium and *L. monocytogenes*, or 5 mm for *S. aureus* for MRS agar; or greater than 0.5 mm for *S. aureus,* 3.5 mm for *S. enterica* ser. Typhimurium, or 6 mm for *L. monocytogenes* in the case of M17 agar. These criteria were defined after carefully reviewing the total set of results, aiming to select the isolates that produced the largest inhibition diameters for each pathogen/agar combination.

For the subset of LAB isolates presenting antimicrobial activity at 37°C, proteolytic activity and acidifying capacity were evaluated as described by Franciosi *et al*. (2009) and Durlu-Ozkaya *et al.* (2001), respectively, with a few modifications. From the cryopreserved stock, each isolate was reactivated separately in MRS or M17 broth overnight (30°C, 24 hours). Then, a loop of culture was placed in 10 mL of sterile reconstituted skim milk supplemented with yeast extract [0.3% (w/v)] and glucose [0.2% (w/v)] for 2 successive subcultures (30°C for 24 hours). Sterile reconstituted skim milk (100 mL) was then inoculated with 1 mL of the 24-hour activated culture. For the acidification profiling, pH changes were

determined using a pH meter (model HI5522, Hanna Instruments, Smithfield, RI, USA) equipped with a HI1131 glass (Hanna Instruments, Smithfield, RI, USA) penetration probe during incubation at 30°C during 8 hours (t=0, 2, 4, 6, 8 hours) and after 24 hours (Durlu-Ozkaya *et al*., 2001). For every strain, pH data was fitted to a decay curve to characterize acidification capacity (Faria *et al*., 2021). The following descriptors were extracted from the fitted curves: ΔpH_{02}: pH decrease between t=0 hours and t=2 hours; ΔpH_{06}: pH decrease between t=0 hours and t = 6 hours; ΔpH_{26}: pH decrease between t=2 hours and t=6 hours; and pH_6: pH at t=6 hours. LAB isolates were considered good acidifiers when they were able to reduce the medium pH below 5.3 after 6 hours at 30°C (Beresford *et al*., 2001).

For the determination of exocellular proteolytic activity, the 24-hour-activated cultures were spotted (3 µL) on the surface of milk agar [composed of 10% (w/v) skim milk powder and 2.5% (w/v) agar] and incubated at 35°C for 4 days (Franciosi *et al*., 2009). Proteolytic activity was checked as clear zones around each LAB colony, whose diameters were measured against the LAB colony diameter.

Molecular identification of lactic acid bacteria isolates by 16S rRNA sequencing

The cryopreserved isolates of a subset of 40 isolates with promising antimicrobial and technological properties were reactivated in MRS or M17 agar and incubated at 37°C for 48 hours. Isolated colonies were inoculated in 5 mL of MRS broth (Himedia, Einhausen, Germany) and incubated at 37°C for 24 hours. After incubation, 1.5 mL of culture was transferred to Eppendorf tubes and centrifuged at 10,000× g for 2 minutes; the process was repeated 2 times for each culture. The supernatant was discarded, and the pellet was kept at 4°C.

Genomic DNA was extracted from samples using a GF-1 bacterial DNA extraction kit (Vivantis, Shah Alam, Malaysia), with the optional RNA removal step. This optional step was not performed considering that, from our experience, RNA co-purification was not an issue in previous assays. The DNA concentration and purity were analyzed by measuring absorbance at 260 nm and 280 nm and using the 260/280 nm ratio. The primers used for amplification of the *16S rRNA* gene were 27f 5′-AGA GTT TGA TCC TGG CTC AG-3′ and 1492r 5′-CTA CGG CTA CCT TGT TAC GA-3′ (Hou *et al*., 2018). The polymerase chain reaction (PCR) cycle was 94°C for 2 minutes, followed by 30 cycles of 94°C for 10 seconds, 55°C for 20 seconds, and 72°C for 1 minute, using DFS-Taq DNA polymerase (Bioron Life Sciences, Römerberg, Germany). PCR products were visualized *via* electrophoresis on 1% (w/v) agarose gel, stained with ethidium bromide, purified with the GF-1 PCR clean-up kit (Vivantis, Shah Alam, Malaysia), and used as templates in the sequencing reactions. The quality of the amplicons was measured using the 260/280 nm ratio. For sequencing reactions, a BigDyeTM terminator v3.1 system was used, and for the purification of samples, a SAM/BigDyeXTerminatorTM bead solution was employed (ThermoFisher Scientific, Oeiras, Portugal). Capillary electrophoresis was carried out using a SeqStudio genetic analyzer (Applied Biosystems, Porto, Portugal).

The sequence data obtained were aligned with sequences from the National Center for Biotechnology Information *16S rRNA* database using the basic local alignment search tool (BLAST) algorithm. Finally, sequences with identities higher than 97% were accepted as the best matches for the LAB isolates.

Statistical analysis

Principal component analysis: De Man-Rogosa-Sharpe- versus M17-isolated lactic acid bacteria

Data were divided into 2 subsets, one for MRS-isolated LAB and another for M17-isolated LAB. Principal component analysis (PCA) of each subset was performed to evaluate the contribution of the antimicrobial, proteolytic, and acidifying properties to the discrimination of isolates. From the antimicrobial assays, only the data referring to *L. monocytogenes* and *S. aureus* inhibition were used (n=84), as these are the pathogens of greater concern (among the 3 tested) in cheese.

The function prcomp from the factoextra package was used in R software (version 3.6.2, R Foundation for Statistical Computing, Vienna, Austria), and from the 3-dimensional PCA, maps of the antimicrobial, acidifying, and proteolytic characteristics of cheeses were built by projecting sample scores onto the span of the principal components.

Principal component analysis: a subset of isolates with promising antimicrobial and technological properties

Considering the results of the previous PCA, a subset of 40 LAB isolates (20 MRS-isolated and 20 M17-isolated) with promising antimicrobial and technological properties was defined, and a second PCA was carried out to appraise the relationship between genus and species and the antimicrobial, proteolytic, and acidifying properties of the isolates. Again, the function prcomp from the factoextra package was used in R.

Results and Discussion

Antimicrobial, acidifying and proteolytic capacities of lactic acid bacteria isolates

In total, 97 isolates from MRS agar and 135 isolates from M17 agar were collected, composing a total of 232 LAB isolates. Antimicrobial testing at 37°C showed that 98%, 100%, and 100% of MRS-isolated LAB showed antagonism against *L. monocytogenes*, *S. aureus* and *S. enterica* ser. Typhimurium, respectively. On the other hand, only 13.3% and 28.1% of M17-isolated LAB presented antibacterial effects against *L. monocytogenes* and *S. enterica* ser. Typhimurium, respectively. No antagonism was detected against *S. aureus*.

After selecting isolates with antimicrobial activity at 37°C according to the specific criteria described in the "Materials and Methods" section, 84 isolates (58 MRS-isolated and 26 M17-isolated) were subjected to the spot-on-lawn assay at 10°C. This assessment showed that all 84 isolates kept their antimicrobial activity even at 10°C, which is an important ability considering that pathogens such as *L. monocytogenes*, *S. aureus*, and *S. enterica* ser. Typhimurium can grow or survive in a wide range of temperatures (Ministry for Primary Industries, 2011). The bactericidal effect of LAB is a consequence of various mechanisms, which include competition against pathogens for the available substrate, production of antimicrobial substances such as bacteriocins, and production of non-proteinaceous compounds such as H_2O_2 (Ribeiro *et al.*, 2014).

In terms of acidifying capacity, LAB isolated from M17 agar displayed better results than LAB isolated from MRS agar. In fact, 12 out of the 26 isolates (46%) obtained from M17 agar were able to promote a reduction of milk broth pH below 5.3 after 6 hours at 30°C. Conversely, no MRS isolates were capable of such a pH

reduction under these conditions. The results obtained by Campagnollo *et al*. (2018) also revealed a higher percentage (39%) of M17-isolated LAB with good acidifying activity (pH below 5.3 after 6 hours at 30°C), compared to only 15% of LAB from MRS agar. On the other hand, Cogan *et al*. (1997) reported that most of their *Lactococcus* isolates (grown in M17-agar) were poor acid producers, as only 8.3% of the 1582 isolates tested reduced the pH of reconstituted skim milk below 5.3.

In cheese-making, a prompt pH decline is essential to achieve adequate coagulation, curd firmness, and control of bacterial pathogen growth (Ribeiro *et al*., 2014; Campagnollo *et al*., 2018). In this sense, these 12 M17 LAB isolates revealed the potential to be used as starter and/or adjunct cultures to avoid defective fermentations. Nevertheless, LAB strains with poor acidifying capacity can still be included in a starter mixture if they present other technological properties that may assist cheese production (Ribeiro *et al*., 2014). The distinct acidifying capacities of MRS and M17-isolates may be linked to and explained by the genera of LAB being isolated in each agar medium, considering that MRS agar is a selective medium for lactobacilli, while M17 agar is a non-selective medium for lactococci. This was further explored through the molecular identification of a subset of LAB isolates.

As for the proteolytic capacity, only 2 MRS isolates (labeled isolate 16 and isolate 24 in Figure 1 and Table 1) presented transparent zones around the colonies. Isolate 16 showed a diameter of proteolytic activity of 1.94 mm, whereas isolate 24 presented a smaller halo of 1.45 mm in diameter around the LAB colony. The antimicrobial and acidifying properties of these isolates are presented in Table 1, along with the corresponding identified genus and species. Although only these 2 isolates showed transparent halos, other isolates also presented a zone around the LAB colony of less density than that of the milk agar but not totally transparent.

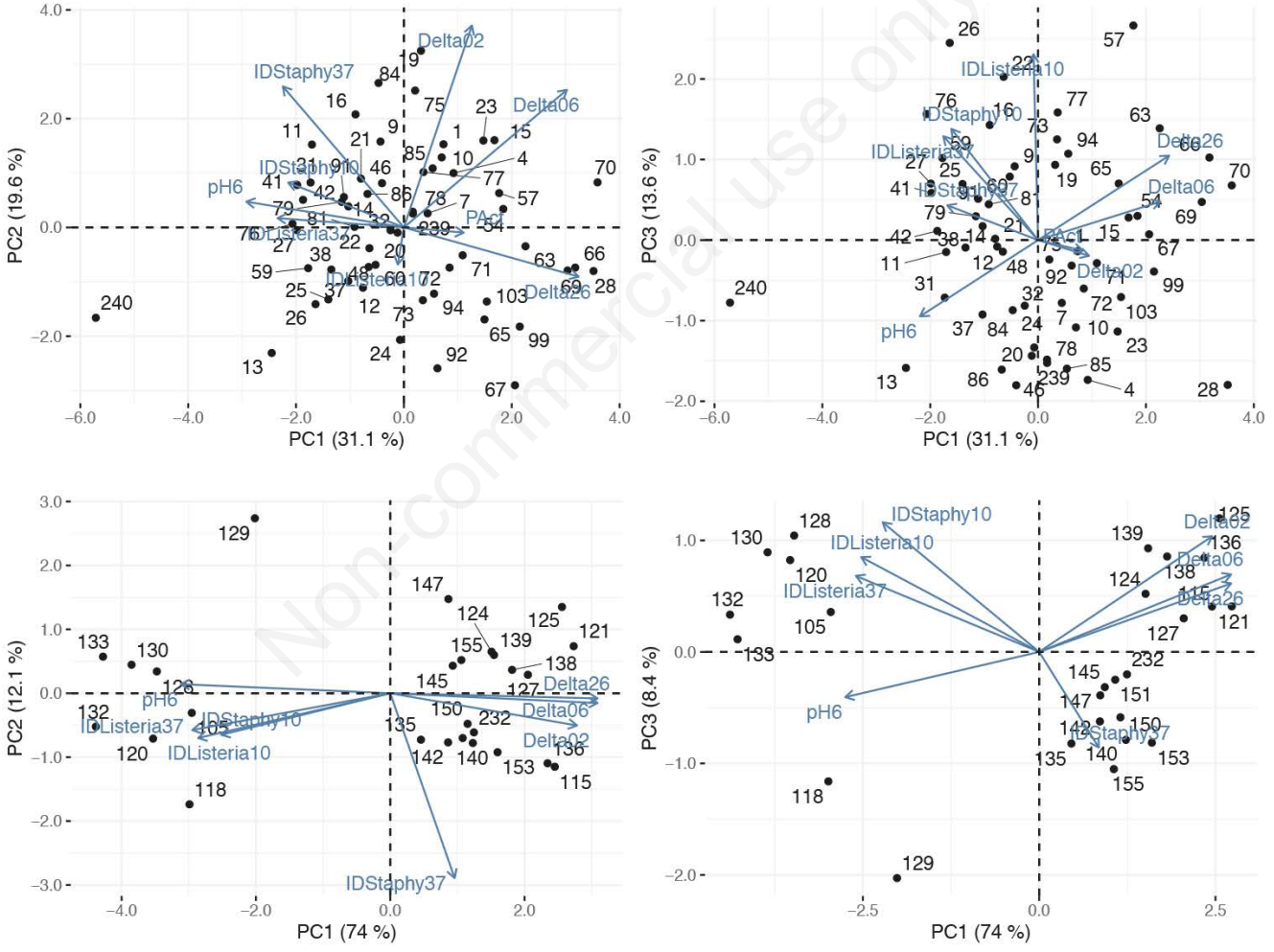

Figure 1. Maps of the first and second principal components (left) and the first and third principal components (right) of the tested technological properties of De Man-Rogosa-Sharpe-isolated lactic acid bacteria (top plots) and M17-isolated lactic acid bacteria (bottom plots). Delta02, Delta06, Delta26, pH decrease between t=0 hours and t=2 hours, t=0 hours and t=6 hours and t=2 hours and t=6 hours, respectively; pH6, pH value of milk broth after 6 hours at 30°C; IDListeria37 and IDListeria10, diameter of inhibition (mm) of *Listeria monocytogenes* tested at 37°C and 10°C, respectively; IDStaphy37 and IDStaphy10, diameter of inhibition (mm) of *Staphylococcus aureus* tested at 37°C and 10°C, respectively; PAct, diameter of proteolytic activity (mm); PC1, first principal component; PC2, second principal component; PC3, third principal component.

A clear zone around a colony is an indicator that proteolytic bacteria hydrolyze casein to form soluble nitrogenous compounds, and in the case of bacteria also producing acid from fermentable carbohydrates present in the medium, the clearer/more transparent the zone will be (HiMedia Laboratories, 2021). This may be the explanation for the 2 types of zones observed in this assay. In cheesemaking, casein hydrolysis is a determinant for texture and aroma development, as the released peptides can accelerate the latter (Campagnollo *et al*., 2018; Piraino *et al*., 2008). In this sense, these outcomes may suggest the potential of a few isolates to contribute to the improvement of cheese texture and aroma.

Principal component analysis: subsets of De Man-Rogosa-Sharpe- and M17-isolated lactic acid bacteria

In the subsets of LAB isolated in MRS and M17 agars, the contribution of the antimicrobial and acidifying attributes to the principal components can be evaluated in Table 2, through their correlations with the 3 principal components extracted [first principal component (PC1), second principal component (PC2) and third principal component (PC3)]. The communalities, *i.e*., the percentages of variance in an observed variable accounted for by the components, are also presented. In the subset of MRS-isolated LAB, the contribution of the proteolytic attribute to the principal components could also be evaluated. In the case of M17-isolated LAB, the contribution of such technological property could not be assessed as no isolate presented casein hydrolysis capacity. Figure 1 shows the biplots of variable loadings and observation scores.

From Table 2, analyzing the MRS subset, 64% of the variability in the 9 attributes was jointly explained by the 3 principal components. PC1 explained around 31% of the total variability and

Table 1. Antimicrobial, acidifying and proteolytic capacities of De Man-Rogosa-Sharpe- and M17-isolated lactic acid bacteria along with the corresponding identified genus and species.

Agar	Isolate	pH_6	ID Listeria37	ID Salmo37	ID Staphy37	ID Listeria10	ID Salmo10	ID Staphy10	PAct	Genus	Species
MRS	11	6.55	9.96	9.79	6.35	15.2	11.9	9.45	0	*Lacticaseibacillus*	*Lacticaseibacillus paracasei*
	16	6.48	9.33	12.1	7.88	16.8	10.6	14.5	1.94	*Lactobacillus*	*Lactobacillus plantarum*
	19	6.45	10.6	9.83	6.92	14.6	8.40	9.07	0	*Lacticaseibacillus*	*Lacticaseibacillus paracasei*
	21	6.49	9.17	10.1	6.77	15.6	7.69	6.85	0	*Lacticaseibacillus*	*Lacticaseibacillus paracasei*
	24	6.51	8.56	9.47	4.53	16.5	9.74	0.00	1.45	*Loigolactobacillus*	*Loigolactobacillus coryniformis*
	25	6.49	8.95	9.86	5.79	19.6	8.94	7.93	0	*Lacticaseibacillus*	*Lacticaseibacillus paracasei*
	28	6.46	4.84	8.91	3.60	12.0	6.82	2.32	0	*Leuconostoc*	*Leuconostoc mesenteroides*
	31	6.49	10.9	10.2	6.17	9.28	6.69	10.3	0	*Lacticaseibacillus*	*Lacticaseibacillus paracasei*
	57	6.40	9.18	7.72	5.35	25.0	8.98	5.44	0	*Lacticaseibacillus*	*Lacticaseibacillus paracasei*
	63	6.36	8.14	7.87	5.25	17.9	10.5	6.26	0	*Leuconostoc*	*Leuconostoc mesenteroides*
	65	6.40	8.92	6.66	4.50	18.6	9.83	1.95	0	*Leuconostoc*	*Leuconostoc mesenteroides*
	67	6.37	8.63	8.72	2.72	16.2	6.49	2.54	0	*Leuconostoc*	*Leuconostoc mesenteroides*
	69	6.36	8.95	8.01	4.07	14.7	6.96	2.36	0	*Leuconostoc*	*Leuconostoc mesenteroides*
	70	6.42	8.88	7.81	3.65	17.1	6.14	2.33	0	*Leuconostoc*	*Leuconostoc mesenteroides*
	73	6.46	10.5	7.63	4.19	15.9	7.19	9.75	0	*Leuconostoc*	*Leuconostoc mesenteroides*
	84	6.46	7.30	7.64	7.45	14.4	6.62	7.49	0	*Leuconostoc*	*Leuconostoc mesenteroides*
	92	6.49	7.01	8.25	4.21	17.4	5.60	4.79	0	*Leuconostoc*	*Leuconostoc mesenteroides*
	94	6.46	8.98	8.57	4.34	18.7	9.96	8.16	0	*Leuconostoc*	*Leuconostoc mesenteroides*
	99	6.47	5.36	8.39	3.65	16.7	4.70	6.71	0	*Leuconostoc*	*Leuconostoc mesenteroides*
	240	6.59	12.4	6.80	5.94	13.5	7.21	11.6	0	*Lacticaseibacillus*	*Lacticaseibacillus paracasei*
M17	105	6.12	6.91	0.00	0.38	13.7	5.65	3.21	0	*Enterococcus*	*Enterococcus faecalis*
	115	5.43	0.00	4.50	0.54	2.97	2.87	2.30	0	*Lactococcus*	*Lactococcus cremoris*
	118	6.19	8.28	3.67	0.59	12.7	5.51	2.57	0	*Enterococcus*	*Enterococcus faecalis*
	120	6.12	7.86	4.91	0.40	12.7	5.49	4.08	0	*Enterococcus*	*Enterococcus faecalis*
	121	5.28	0.00	4.14	0.35	1.34	5.38	1.90	0	*Lactococcus*	*Lactococcus lactis*
	124	5.42	0.74	3.95	0.34	1.85	5.47	2.60	0	*Lactococcus*	*Lactococcus lactis*
	125	5.28	0.00	4.22	0.25	2.23	3.24	2.20	0	*Lactococcus*	*Lactococcus cremoris*
	127	5.49	0.75	3.84	0.39	2.36	3.17	2.03	0	*Lactococcus*	*Lactococcus lactis*
	128	6.04	7.54	4.33	0.28	13.4	4.30	3.84	0	*Enterococcus*	*Enterococcus faecalis*
	132	6.26	7.70	3.91	0.39	12.4	9.37	4.25	0	*Enterococcus*	*Enterococcus faecalis*
	133	6.28	7.85	4.58	0.28	13.9	6.36	3.39	0	*Enterococcus*	*Enterococcus faecalis*
	135	5.69	0.00	0.00	0.53	2.36	4.17	2.88	0	*Lactococcus*	*Lactococcus cremoris*
	136	5.42	0.00	2.43	0.52	2.24	4.61	2.83	0	*Lactococcus*	*Lactococcus lactis*
	138	5.48	0.00	3.56	0.36	2.01	6.68	2.73	0	*Lactococcus*	*Lactococcus cremoris*
	140	5.52	0.00	4.06	0.54	2.22	5.62	2.42	0	*Lactococcus*	*Lactococcus lactis*
	150	5.55	0.38	3.28	0.50	2.00	3.78	2.48	0	*Lactococcus*	*Lactococcus lactis*
	151	5.53	0.34	4.12	0.51	1.81	4.42	2.87	0	*Lactococcus*	*Lactococcus lactis*
	153	5.50	0.53	2.88	0.56	1.68	4.48	2.18	0	*Lactococcus*	*Lactococcus lactis*
	155	5.58	0.38	3.80	0.41	1.73	4.47	1.87	0	*Lactococcus*	*Lactococcus lactis*
	232	5.51	0.00	0.00	0.50	2.38	5.84	2.75	0	*Lactococcus*	*Lactococcus lactis*

MRS, De Man-Rogosa-Sharpe; pH6, fitted pH value of milk broth after 6 hours at 30°C; IDListeria37 and IDListeria10, diameter of inhibition (mm) of *Listeria monocytogenes* tested at 37°C and 10°C, respectively; IDStaphy37 and IDStaphy10, diameter of inhibition (mm) of *Staphylococcus aureus* tested at 37°C and 10°C, respectively; IDSalmo37 and IDSalmo10, diameter of inhibition (mm) of *Salmonella* Typhimurium tested at 37°C and 10°C, respectively; PAct, diameter of proteolytic activity (mm).

was highly correlated with the pH decrease of milk broth between t=2 hours and t=6 hours (ΔpH_{26}, R=0.48) and between 0 and 6 hours (ΔpH_{06}, R=0.45), and highly and inversely correlated with *S. aureus* inhibition at 10°C and 37°C (R=-0.32 and R=-0.33, respectively), with *L. monocytogenes* inhibition at 37°C (R=-0.34) and with milk broth pH value after 6 hours (R=-0.43); in contrast, it is not correlated with *L. monocytogenes* inhibition at 10°C (R=-0.02). Thus, PC1 indicates isolates with different inhibitory capacity against *S. aureus* (at 10°C and 37°C) and *L. monocytogenes* (at 37°C) and distinguishes the ability of isolates to promote a reduced pH value in milk broth after 2 hours at 30°C (Figure 1). PC2 explained approximately 20% of the data variability and presented high loadings on ΔpH_{02} (R=0.69) and ΔpH_{06} (R=0.47), 2 pH-related variables, as well as on *S. aureus* inhibition at 37°C (R=0.48). In this sense, dissimilarities across the PC2 axis (Figure 1) suggest LAB with distinct acidification profiles, particularly between t=0 hours and t=2 hours, and t=0 hours and t=6 hours, and with distinct antimicrobial capacity against *S. aureus* (at 37°C). PC3 explained around 14% of the total variability and was correlated with inhibition of *L. monocytogenes* at 10°C and 37°C (R=0.68 and R=0.38, respectively), and *S. aureus* inhibition at 10°C (R=0.41). Thus, PC3 reveals LAB isolates with distinctive antimicrobial capacities against these pathogens (Figure 1).

The properties of M17 isolates showed stronger relationships between variables, as higher total variability could be explained (95% in Table 2), compared to MRS isolates. PC1 explained a great portion (74%) of the total variability and was correlated with the pH decrease of milk broth (R=-0.40, R=0.36, R=0.40 and R=0.40 for pH_6, ΔpH_{02}, ΔpH_{06} and ΔpH_{26}, respectively), and negatively correlated with *L. monocytogenes* inhibition at 10°C and 37°C (R=-0.37 and R=-0.38, respectively) and *S. aureus* inhibition at 10°C (R=-0.32). For this reason, PC1 provides insight into the isolates' ability to lower the pH of milk broth after 6 hours and their acidification behavior between t=0 hours and t=6 hours, as well as their antibacterial capacities (Figure 1). PC2 and PC3 explained approximately 12% and 8% of the total variability, respectively. PC2 was highly and inversely correlated with *S. aureus* inhibition at 37°C (R=-0.92), whereas PC3 was well correlated with *S. aureus* inhibition at 10°C and 37°C (R=0.50 and R=-0.37, respectively) and with *L. monocytogenes* inhibition at 10°C (R=0.37) (Figure 1).

From Figure 1, looking at the plots of MRS-isolated LAB, clusters were not easily identified, which implies isolates of similar antimicrobial capacity and technological properties. On the other hand, 2 clusters of M17 isolates can be differentiated: one with greater acidification capacity and related to higher inhibition of *S. aureus* at 37°C, and another with better antimicrobial activity against *S. aureus* (at 10°C) and *L. monocytogenes* (at 10°C and 37°C).

The outcomes of this PCA may be useful in the design of a tailored starter culture, as the selection of a particular set of LAB isolates with desirable attributes (antimicrobial protection against pathogens and assistance in the development of texture and aroma/flavor compounds) to produce cheeses is facilitated.

Principal component analysis: a subset of isolates identified by 16S rRNA sequencing with promising antimicrobial and technological properties

Considering the results of the first PCA conducted, another subset of 40 LAB isolates (20 MRS-isolated and 20 M17-isolated) with promising technological properties was defined, and the molecular characterization of such isolates was performed with the goal of conducting a second PCA to appraise the relationship between genus and species and the antimicrobial, proteolytic, and acidifying properties of the isolates.

Table 2. Coefficients of correlation of the tested technological properties of De Man-Rogosa-Sharpe- and M17-isolated lactic acid bacteria, with the 3 components along with communalities and explained variances.

Agar	Variable	PC1	PC2	PC3	Communalities
MRS	pH_6	-0.43	0.09	-0.28	2.50
	ΔpH_{02}	0.19	0.69	-0.06	1.25
	ΔpH_{06}	0.45	0.47	0.14	0.01
	ΔpH_{26}	0.48	-0.17	0.31	0.95
	ID *Listeria monocytogenes* 37°C	-0.34	0.03	0.38	1.15
	ID *Staphylococcus aureus* 37°C	-0.33	0.48	0.13	1.55
	ID *Listeria monocytogenes* 10°C	-0.02	-0.13	0.68	0.01
	ID *Staphylococcus aureus* 10°C	-0.32	0.15	0.41	0.49
	PAct	0.16	-0.02	-0.04	1.10
	Proportion variance	0.31	0.20	0.14	-
	Cumulative variance	0.31	0.51	0.64	-
M17	pH_6	-0.40	0.05	-0.17	1.31
	ΔpH_{02}	0.36	-0.16	0.45	2.40
	ΔpH_{06}	0.40	-0.05	0.30	0.72
	ΔpH_{26}	0.40	-0.03	0.26	0.06
	ID *Listeria monocytogenes* 37°C	-0.38	-0.18	0.29	1.43
	ID *Staphylococcus aureus* 37°C	0.12	-0.92	-0.37	1.44
	ID *Listeria monocytogenes* 10°C	-0.37	-0.22	0.37	0.16
	ID *Staphylococcus aureus* 10°C	-0.32	-0.21	0.50	0.48
	Proportion variance	0.74	0.12	0.08	-
	Cumulative variance	0.74	0.86	0.95	-

MRS, De Man-Rogosa-Sharpe; PC1, first component; PC2, second component; PC3, third component; pH_6, pH value of milk broth after 6 hours at 30°C; $\Delta pH_{02, 06}$ and $_{26}$, pH decrease between t=0 hours and t=2 hours, t=0 hours and t=6 hours and t=2 hours and t=6 hours, respectively; ID *Listeria monocytogenes* 37°C and 10°C, diameter of inhibition (mm) of *Listeria monocytogenes* tested at 37°C and 10°C, respectively; ID *Staphylococcus aureus* 37°C and 10°C, diameter of inhibition (mm) of *Staphylococcus aureus* tested at 37°C and 10°C, respectively.

The results of the pH_6 descriptor obtained from the fitted pH curve and of the antimicrobial and proteolytic assays of MRS- and M17-isolated LAB, along with the corresponding identified species obtained by *16S rRNA* sequencing, are presented in Table 1. Figure 2 shows the variable loadings and observation scores for each pathogen, and the ellipses group strains by species and genus.

Overall, according to the BLAST results from the *16S rRNA* sequencing, LAB from the genera *Lactococcus* and *Leuconostoc* were dominant (62.5%) among the subset of isolates with favorable technological capacities. *Lactococcus* and *Leuconostoc* were found in 35% and 27.5% of samples, respectively, whereas other bacteria of the genera *Lacticaseibacillus*, *Enterococcus, Loigolactobacillus,* and *Lactobacillus* were less frequent (17.5%, 15%, 2.5% and 2.5% of the samples, respectively). At the species level, *Leuconostoc mesenteroides* was the most abundant organism (27.5%), followed by *Lactococcus lactis* (25%), *Lacticaseibacillus paracasei* (17.5%), *Enterococcus faecalis* (15%), *Lactococcus cremoris* (10%) and *Lactobacillus plantarum* and *Loigolactobacillus coryniformis* (2.5% each). Despite the promising technological properties observed, isolates confirmed as *Enterococcus faecalis* could not be considered for incorporation into foods, as this species does not have QPS status for being among the leading causes of community- and hospital-acquired (nosocomial) infections (EFSA, 2007). In fact, the detection of this species in artisanal cheeses confirms its poor microbial quality, which motivated this study, as *Enterococcus faecalis* is present in the gastrointestinal tract of humans and animals.

Other researchers have also studied the composition of LAB communities in cheese and other dairy products. In their work, Cogan *et al.* (1997) studied 35 artisanal dairy foods (cheeses and fermented milk), collecting 4379 isolates that were classified as *Lactococcus*, *Enterococcus*, *Streptococcus thermophilus*, *Lactobacillus*, and *Leuconostoc*. More recently, another study evaluated the microflora of Caciocavallo cheese, which was composed mainly of *Lactobacillus paracasei* and *Lactococcus lactis* but also of *Lactobacillus delbrueckii*, *Lactobacillus fermentum*, *Enterococcus faecalis*, and *Lactobacillus rhamnosus* (Piraino *et al.* 2005). Gonçalves *et al*. (2018) investigated the bacterial community in a traditional Portuguese cheese (Serpa), and the predominating genera were *Lactococcus*, followed by *Lactobacillus* and *Leuconostoc*, while strains of the *Enterobacteriaceae* family were also detected. These results, much like our work, highlight the

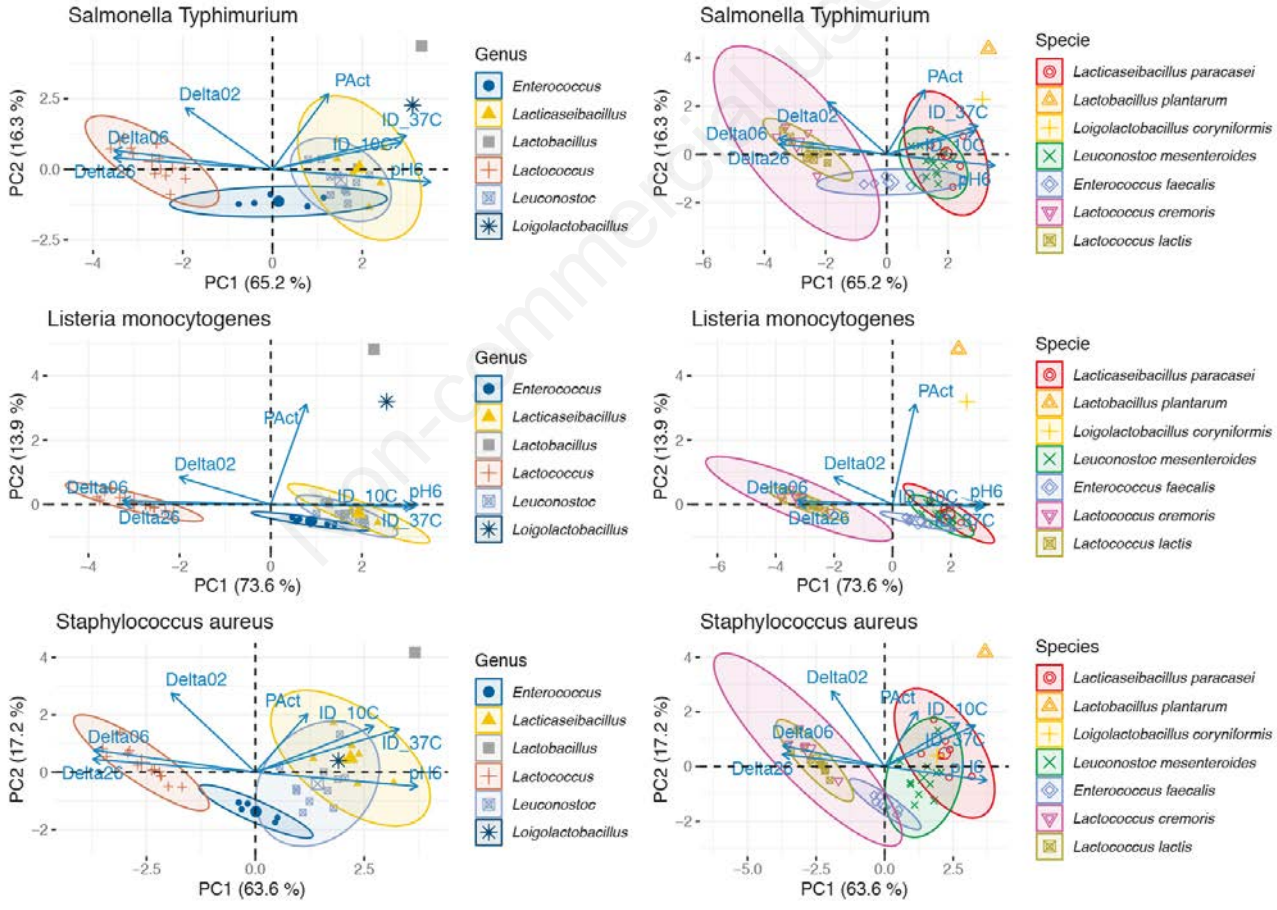

Figure 2. Maps of the first and second principal components of the tested properties of the subset of lactic acid bacteria. For each pathogen: ID_10C and ID_37C, diameter of inhibition (mm) tested at 10°C and 37°C, respectively. Delta02, Delta06, Delta26, pH decrease between t=0 hours and t=2 hours, t=0 hours and t=6 hours and t=2 hours and t=6 hours, respectively; pH6, pH value of milk broth after 6 hours at 30°C; PAct, diameter of proteolytic activity (mm); PC1, first principal component; PC2, second principal component; PC3, third principal component.

existence of a highly diversified LAB microflora in cheeses.

Contrasting the results from Table 1 and analyzing the biplots in Figure 2, it is evident that strains belonging to the *Lactococcus* genus (M17-isolated) have the highest acidifying capacity, regardless of the species (*Lactococcus cremoris* and *Lactococcus lactis*), as suggested by the high correlation with the Delta06, and Delta26 variables and the negative correlation with the pH_6 variable. Among the *Lactococcus* strains, isolate 121 (*Lactococcus lactis*) and isolate 125 (*Lactococcus cremoris*) promote the greatest milk broth acidification, as evidenced by the lowest estimated pH value after 6 hours among all isolates (5.28). On the other hand, the genera *Leuconostoc* and *Lacticaseibacillus* (both MRS-isolated) (Table 1 and Figure 2) displayed important antimicrobial capacities, regardless of the temperature and pathogen tested, as suggested by the high correlation with the ID_37C and ID_10C variables.

In 2 of the biplots (those for *S.* Typhimurium and *S. aureus*), the genus *Enterococcus* (M17-isolated) did not reveal an explicit correlation with a particular property, considering the position of its strains close to the plot origin. However, the biplot for *L. monocytogenes* suggests a greater association with antimicrobial capacities, as strains are clustered in the direction of the ID_37C and ID_10C arrows, yet away from the Delta02, Delta06 and Delta26 variables. This implies that *Enterococcus* strains present a higher antimicrobial effect against *L. monocytogenes* than against *S.* Typhimurium or *S. aureus*, which can be confirmed by examining the inhibition diameters in Table 1.

Lactobacillus and *Loigolactobacillus* genera (both MRS-isolated) were composed of only one strain each (*Lactobacillus plantarum* and *Loigolactobacillus coryniformis*), so no ellipses could be modeled. Therefore, no conclusion could be drawn regarding the capacities correlated with each of these genera. Nevertheless, the biplots indicate that both strains were associated with high proteolytic and antimicrobial activities, considering their placement along the horizontal axis. Since these 2 isolates (numbers 16 and 24) were the only ones collected that presented proteolytic capacity, as mentioned before, one may wonder if these genera are associated with such technological properties.

Conclusions

The detection of *Enterococcus faecalis* in artisanal goat's milk cheeses confirmed the importance of this study, whose main concern was related to the poor microbial quality of this product.

The genetic analysis of the isolates collected in this work showed a diverse lactic acid-producing community, with various strains presenting antimicrobial activity against different pathogens as well as acidifying and proteolytic capacities. The principal component analysis allowed for the grouping of isolates based on valuable characteristics for cheese production and enabled the correlation of the identified LAB genera with their properties (antagonistic, acidifying, and proteolytic). Overall, *Leuconostoc mesenteroides*, *Lactococcus lactis* and *Lacticaseibacillus paracasei* were the predominant organisms found in the subset of 40 LAB with promising antimicrobial and acidifying properties selected from the initial 232 isolates collected. The results of this work suggest that the application of selected indigenous LAB as a customized starter culture may be suitable for pathogen growth prevention (biopreservation potential) and to contribute to the proper acidification of milk during the cheese production process, thus promoting a stable microbiological environment and consequently improving the safety of this product.

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