biology and life sciences forum

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Proceeding Paper

Technological Potential of Lactic Acid Bacteria Isolated from Portuguese Goat's Raw Milk Cheeses †

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† Presented at the 2nd International Electronic Conference on Foods, 15–30 October 2021; Available online: https://foods2021.sciforum.net/.

Abstract: In this work, a total of 97 MRS-grown and 135 M17-grown lactic acid bacteria strains were isolated from 20 goat's raw milk cheeses, and their antimicrobial, acidifying, and proteolytic activities were determined in vitro. Principal component analyses adjusted to a subset of 84 promising isolates evidenced that, for MRS isolates, antagonism against *Staphylococcus aureus* correlated well with higher acidification potential, whereas for M17 isolates, the antagonisms against *S. aureus* and *Listeria monocytogenes* were more correlated. The outcomes highlighted various strains with pathogen inhibition ability and satisfactory technological properties that may be useful for the development of a customised starter culture.

Keywords: antimicrobial activity; proteolytic activity; acidifying activity; biopreservation

Citation: Silva, B.N.; Faria, A.S.; Cadavez, V.; Teixeira, J.A.; Gonzales-Barron, U. Technological Potential of Lactic Acid Bacteria Isolated from Portuguese Goat's Raw Milk Cheeses. *Biol. Life Sci. Forum* **2021**, *6*, 9. https://doi.org/10.3390/Foods2021-11101

Academic Editor: Susana Casal

Published: 14 October 2021

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1. Introduction

Lactic acid bacteria (LAB) are responsible for the cheese fermentation process, but they can also increase the safety of cheeses by acting as biopreservatives, and promote desirable organoleptic properties (such as texture, aroma, and flavour compounds). In this sense, the objective of this work was to collect and assess the antimicrobial, acidifying, and proteolytic capacities of LAB strains from the microflora of artisanal Portuguese goat's raw milk cheeses for potential development of a customised starter culture and application in cheese manufacture.

2. Materials and Methods

2.1. Isolation and Confirmation of Lactic Acid Bacteria

Samples of four batches of artisanal goat's raw milk cheeses (n = 20) were collected in Mirandela, Portugal, between November 2020 and March 2021. LAB strains were isolated from cheese samples as described by the ISO standard 15214:1998 [1]. Briefly, after dilution, aliquots were plated in MRS agar (selective medium for enumeration and isolation of lactobacilli) and M17 agar (non-selective medium for enumeration and isolation of lactococci), overlayed with 1.2% bacteriological agar, and plates were incubated at 30 °C for 48 h. Then, eight typical colonies on MRS and M17 agar were selected for purification and incubated at 30 °C for another 48 h in the respective media. Lastly, to eliminate non-LAB isolates, catalase (3% hydrogen peroxide) and Gram tests, as well as morphologic observation, were performed. Cultures presumptively identified as LAB were maintained in MRS broth with 25% glycerol at −80 °C.

2.2. Bacterial Strains and Preparation of Cell Suspensions

Salmonella enterica subsp. *Enterica* serovar Typhimurium ATCC 43971, *Listeria monocytogenes* WDCM 00019, and *Staphylococcus aureus* ATCC 6538, obtained from the Polytechnic Institute of Bragança stock collection, were used for all experiments. A loop of culture kept on Nutrient Agar slants was inoculated separately in 10 mL of BHI broth. Broth tubes were incubated at 37 °C for 16 h, following two successive inoculations, to achieve a concentration of approximately 10^8 CFU/mL. *L. monocytogenes* required a pre-activation in 5 mL of BHI at 37 °C for 16 h.

2.3. Screening of LAB for Antimicrobial Capacity

Antimicrobial ability of selected LAB strains was determined by the spot-on-lawn assay as described by Campagnollo et al. [2], with some modifications.

Each LAB strain was cultured separately in MRS broth overnight (37 °C, 24 h), spotted onto the surface of MRS or M17 agar plates (3 µL for LAB strains isolated in MRS and 5 µL for M17 agar, respectively), following incubation at 37 °C for 16 h. Inoculated plates were covered with 10 mL of BHI soft agar (BHI broth with 0.75% bacteriological agar) seeded with 1 mL of each bacterial strain (tested separately). After incubating plates at 37 °C for 16 h (pre-incubation at 4 °C for 2 h), the diameter of the inhibition zones of each pathogen were measured with a calliper.

LAB strains that presented antimicrobial capacity at 37 °C according to the following criteria were also tested at 10 °C for 10 days: distance between halo circumference and LAB colony limit greater than 5 mm for *S. aureus*, or 8 mm for *L. monocytogenes* and *S. enterica* ser. Typhimurium—for MRS agar; or greater than 0.5 mm for *S. aureus*, 6 mm for *L. monocytogenes* or 3.5 mm for *S. enterica* ser. Typhimurium—for M17 agar.

2.4. Screening of LAB for Proteolytic and Acidifying Capacity

Proteolytic activity and acidifying capacity were evaluated according to the protocols of Franciosi et al. [3] and Durlu-Ozkaya et al. [4] respectively, for the subset of LAB strains presenting antimicrobial activity at 37 °C. Proteolytic activity was confirmed qualitatively as clear zones around each LAB colony. Acidifying capacity was assessed by measuring pH of the inoculated milk broth at 30 °C during 8 h (t = 0, 2, 4, 6, 8 h), and after 24 h. LAB strains are considered good acidifiers when able reduce the pH below 5.3 after 6 h at 30 °C [5].

2.5. Statistical Analysis

Data were separated into two subsets (one for MRS-isolated LAB; another for M17-isolated LAB) and each subset subjected to principal component analysis (PCA). From the antimicrobial assays, only the data referring to *L. monocytogenes* and *S. aureus* inhibition was used, as these are the pathogens of greater concern (among the three 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), where a varimax-rotated solution for three principal components was obtained. From the three-dimensional PCA, maps of antimicrobial, acidifying and proteolytic characteristics of cheeses were built from the projection of sample scores onto the span of the principal components.

3. Results and Discussion

3.1. Technological Properties of LAB Strains: Antimicrobial, Acidifying and Proteolytic Capacities

In total, 232 LAB strains (97 isolated in MRS agar and 135 isolated in M17 agar) were isolated. Antimicrobial tests at 37 °C revealed that 98%, 100% and 100% of LAB isolated in MRS agar presented antagonism against *L. monocytogenes*, *S. aureus* and *S. enterica* ser. Typhimurium, respectively. In contrast, only 13.3% and 28.1% of LAB isolated in M17 agar revealed antagonism against *L. monocytogenes* and *S. enterica* ser. Typhimurium, respectively (no antagonism was observed against *S. aureus*).

After selecting isolates with considerable antimicrobial activity at 37 °C, 84 strains (58 isolated in MRS agar and 26 isolated in M17 agar) were subjected to the spot-on-lawn assay at 10 °C. The results of this assessment revealed that all 84 strains from this subset maintained their antimicrobial activity even at 10 °C. The microbial inhibition offered by the isolated LAB may be a consequence of competition against pathogens for the available substrate, production of antimicrobial substances (for example, bacteriocins) and/or production of non-proteinaceous compounds such as H_2O_2 [6].

Regarding the acidifying capacity, LAB isolated from M17 agar presented better outcomes than LAB isolated from MRS agar. More specifically, 12 out of the 26 strains (46%) isolated in M17 agar were able to reduce the pH of milk broth below 5.3 after 6 h at 30 °C. This result indicates the potential of some strains to contribute to a rapid pH decrease, which is essential in cheese-making to achieve adequate coagulation, curd firmness, and control of bacterial pathogens growth, among other contaminants [2,6]. In this sense, these LAB strains demonstrate potential to be used as starter and/or adjunct cultures to avoid faulty fermentations. On the other hand, no strains isolated from MRS agar provided such a pH reduction under these conditions. Nevertheless, LAB strains with poor acidifying capacity can still be part of a starter mixture, if they possess other technological properties that may benefit cheese production [6].

Regarding the proteolytic capacity, only two strains isolated from MRS agar demonstrated irrefutable clear (transparent) zones around the colonies. However, various LAB also revealed a zone around the colony with less density than the milk agar, but not completely transparent. The clear zone surrounding the colonies is an indicator that proteolytic bacteria hydrolyse casein to form soluble nitrogenous compounds; more clear zones are seen on milk agar if the bacteria also produce acid from fermentable carbohydrates present in the medium [7]. This may explain the two types of zones observed in this assay. From the cheese-making perspective, casein hydrolysis is crucial for texture development, and the released peptides can also accelerate aroma development [2]. In this sense, the results obtained may suggest the potential, even if limited, of some strains to contribute to the improvement of cheese texture and aroma.

3.2. Principal Component Analysis

In each subset of MRS- and M17-isolated LAB, the contribution of the antimicrobial, acidifying and proteolytic attributes to the principal components can be evaluated in Tables 1 and 2, respectively, by their correlations with the three components extracted. Figures 1 and 2 represent the biplots of variables loadings and observation scores.

Table 1. Coefficients of correlation of the tested technological properties of MRS-isolated LAB, with the three varimax-rotated factors (PC1, PC2, PC3) along with communalities and explained variances.

Variable	PC1	PC2	PC3	Communalities
pH_6	**−0.73**	−0.37	0.29	1.8
ΔpH_{02}	−0.18	**0.95**	0.07	1.1
ΔpH_{06}	0.27	**0.88**	−0.17	1.3
ΔpH_{26}	0.65	−0.12	−0.35	1.6
ID Listeria 37 °C	0.16	−0.24	**0.77**	1.3
ID Staphy 37 °C	−0.33	0.13	**0.70**	1.5
ID Listeria 10 °C	**0.76**	0.23	0.22	1.4
ID Staphy 10 °C	0.27	0.02	**−0.77**	1.2
PAct	−0.52	0.17	0.16	1.4
Proportion variance	0.23	0.22	0.22	-
Cumulative variance	0.23	0.46	0.68	-

pH_6: pH value of milk broth after 6 h at 30 °C; $\Delta pH_{02,\ 06}$ and $_{26}$: pH decrease between t = 0 h and t = 2 h, t = 0 h and t = 6 h and t = 2 h and t = 6 h, respectively; ID Listeria 37 °C and 10 °C: diameter of inhibition of *L. monocytogenes* tested at 37 °C and 10 °C, respectively; ID Staphy 37 °C and 10 °C: diameter of inhibition of *S. aureus* tested at 37 °C and 10 °C, respectively; PAct: proteolytic activity. Bold values indicate the highest coefficients of correlation for each factor (PC1, PC2 and PC3).

Table 2. Coefficients of correlation of the tested technological properties of M17-isolated LAB, with the three varimax-rotated factors (PC1, PC2, PC3) along with communalities and explained variances.

Variable	PC1	PC2	PC3	Communalities
pH_6	**0.91**	0.09	−0.34	1.3
ΔpH_{02}	−0.69	−0.60	−0.19	2.1
ΔpH_{06}	**−0.91**	0.05	0.34	1.3
ΔpH_{26}	−0.66	0.43	0.52	2.7
ID Listeria 37 °C	**0.89**	−0.28	−0.06	1.2
ID Staphy 37 °C	−0.10	0.03	**0.94**	1.0
ID Listeria 10 °C	**0.91**	−0.35	−0.05	1.3
ID Staphy 10 °C	**0.83**	−0.07	−0.02	1.0
PAct	−0.25	**0.88**	0.01	1.2
Proportion variance	0.55	0.17	0.16	-
Cumulative variance	0.55	0.72	0.88	-

pH_6: pH value of milk broth after 6 h at 30 °C; $\Delta pH_{02,\,06}$ and $_{26}$: pH decrease between t = 0 h and t = 2 h, t = 0 h and t = 6 h and t = 2 h and t = 6 h, respectively; ID Listeria 37 °C and 10 °C: diameter of inhibition of *L. monocytogenes* tested at 37 °C and 10 °C, respectively; ID Staphy 37 °C and 10 °C: diameter of inhibition of *S. aureus* tested at 37 °C and 10 °C, respectively; PAct: proteolytic activity. Bold values indicate the highest coefficients of correlation for each factor (PC1, PC2 and PC3).

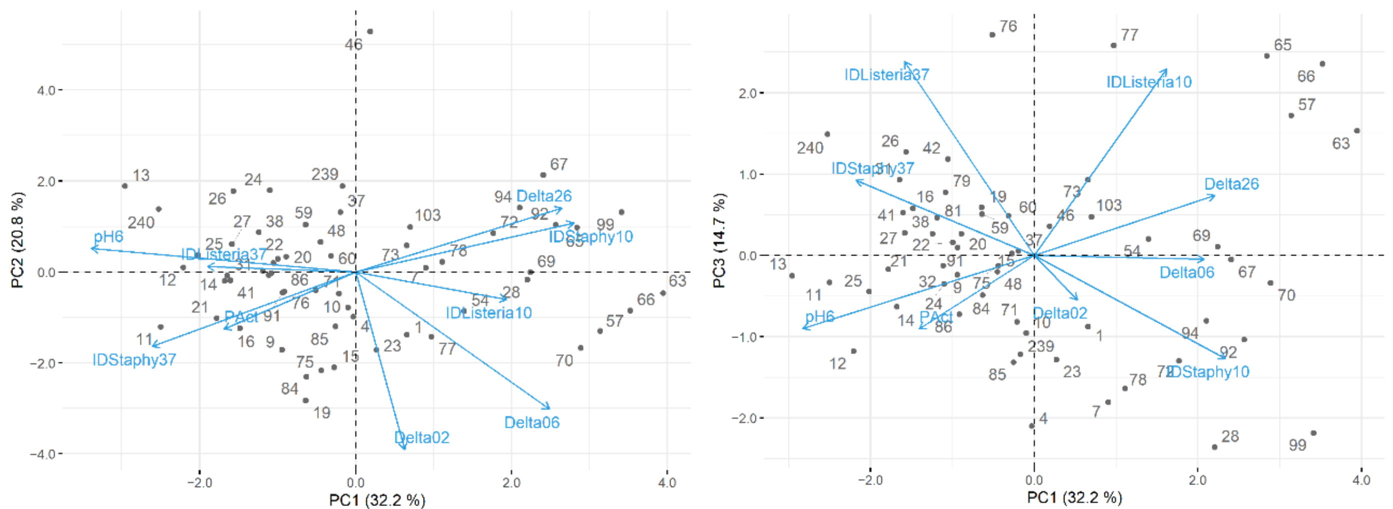

Figure 1. Maps of the first and second principal components (**left**) and the first and third principal components (**right**) of the tested technological properties of MRS-isolated LAB.

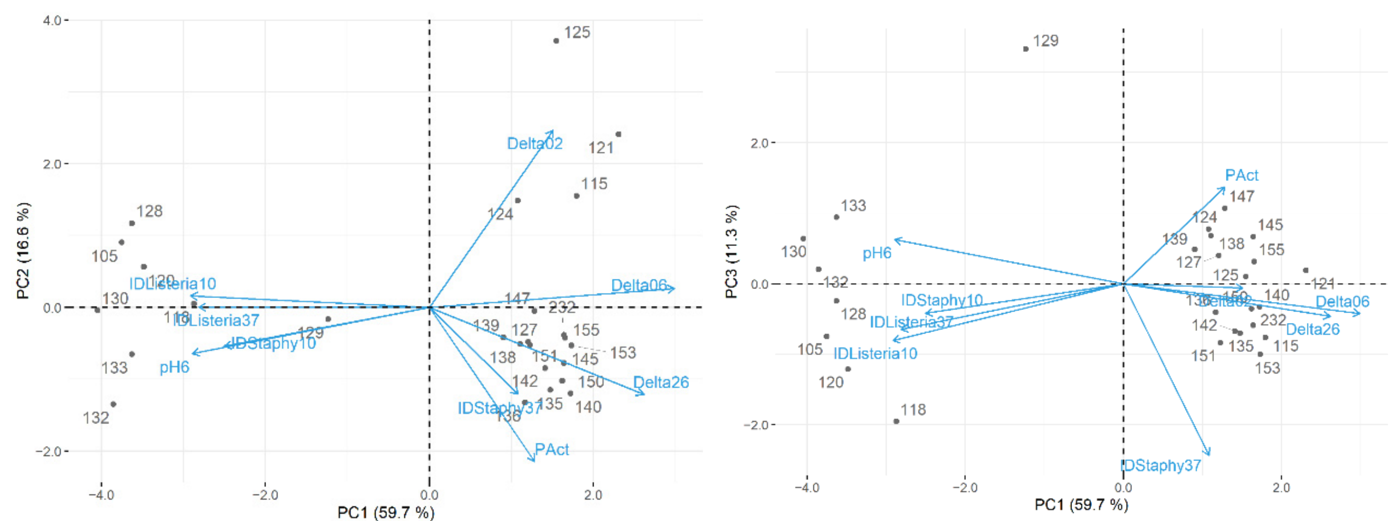

Figure 2. Maps of the first and second principal components (**left**) and the first and third principal components (**right**) of the tested technological properties of M17-isolated LAB.

From Table 1 (MRS subset), a total of 68% of the variability in the nine attributes was jointly explained by the three principal components. The first component (PC1) explained 23% of the total variability and was highly correlated with *L. monocytogenes* inhibition at 10 °C (R = 0.76), and highly and inversely correlated with milk broth pH value after 6 h (R = −0.73); in contrast, it was weakly correlated with inhibition of *L. monocytogenes* at 37 °C (R = 0.16). In this sense, PC1 indicates isolates with different inhibitory capacity against *L. monocytogenes* at 10 °C and 37 °C and distinguishes the ability of isolates to promote a reduced pH value in milk broth after 6 h at 30 °C (Figure 1).

The second component (PC2) explained 22% of the data variability and presented high loadings on two pH-related variables: ΔpH_{02} (R = 0.95) and ΔpH_{06} (R = 0.88). For this reason, dissimilarities across the PC2 axis (Figure 1) indicate LAB with distinct acidification profiles, specifically between t = 0 h and t = 2 h (ΔpH_{02}), and t = 0 h and t = 6 h (ΔpH_{06}).

Finally, the third component (PC3) explained another 22% of the total variability and was highly correlated with inhibition of *L. monocytogenes* and *S. aureus* at 37 °C (R = 0.77 and R = 0.70, respectively), and highly and inversely correlated with inhibition of *S. aureus* at 10 °C (R = −0.77). Thus, PC3 reveals LAB strains with distinctive antimicrobial capacities, namely against *L. monocytogenes* and *S. aureus* at 37 °C, and *S. aureus* at 10 °C (Figure 1).

The properties of M17 strains presented stronger relationships between variables than MRS ones, since higher total variability could be explained (88% in Table 2). PC1 explained most (55%) of the total variability and was highly correlated with antimicrobial inhibition (R = 0.91 and R = 0.83 for *L. monocytogenes* and *S. aureus*, respectively, at 10 °C; R = 0.89 for *L. monocytogenes* at 37 °C), and the pH decrease of milk broth (R = 0.91 and R = −0.91 for pH_6 and ΔpH_{06}, respectively), respectively. For this reason, PC1 suggests isolates with varying inhibitory power against *L. monocytogenes* at 10 °C and 37 °C, and against *S. aureus* at 10 °C; it also provides insight on the isolates capacity to reduce the pH of milk broth after 6 h and their acidification profile between t = 0 h and t = 6 h (Figure 2).

PC2 and PC3 explained 17% and 16% of the total variability, respectively. The first was highly correlated with proteolytic activity (R = 0.88), whereas the second was well correlated with antimicrobial inhibition of *S. aureus* at 37 °C (R = 0.94) (Figure 2).

Further analysing the figures produced, from MRS isolates (Figure 1), clusters were not easily identified, hence suggesting strains of similar antimicrobial capacity and technological characteristics. However, from Figure 2, two clusters of M17 strains can be distinguished: one with greater acidification and proteolytic capacities and related to higher *S. aureus* inhibition 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 joint in-vitro information of the LAB strains will be very helpful in selecting a particular set with desirable characteristics to produce cheeses. More specifically, this PCA may assist in the design of a tailored starter culture that can offer antimicrobial protection against pathogens and assist in the development of pleasing aroma and flavour compounds in the product.

4. Conclusions

This study showed that the grouping of isolates by principal component analysis may be useful for strain selection based on advantageous characteristics for cheese production. In fact, the outcomes obtained indicate that application of indigenous LAB selected in this work, as a customised starter culture, may help prevent pathogen growth (biopreservation potential) and contribute to the development of texture and attractive organoleptic properties in cheeses.

Author Contributions: Conceptualization, V.C., J.A.T. and U.G.-B.; methodology, A.S.F. and U.G.-B.; software, B.N.S. and U.G.-B.; validation, B.N.S. and U.G.-B.; formal analysis, B.N.S. and U.G.-B.; investigation, B.N.S. and A.S.F.; resources, J.A.T., V.C. and U.G.-B.; data curation, B.N.S. and U.G.-B.; writing—original draft, B.N.S.; writing—review and editing, A.S.F. and U.G.-B.; visualization, B.N.S. and U.G.-B.; supervision, V.C., J.A.T. and U.G.-B.; project administration, V.C., J.A.T. and U.G.-B.;

funding acquisition, V.C., J.A.T. and U.G.-B. All authors have read and agreed to the published version of the manuscript.

Funding: The authors are grateful to the EU PRIMA program and the Portuguese Foundation for Science and Technology (FCT) for funding the ArtiSaneFood project (PRIMA/0001/2018) and for financial support through national funds FCT/MCTES to CIMO (UIDB/00690/2020). 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.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Summary data available upon request.

Acknowledgments: Silva acknowledges the financial support provided by FCT through the Ph.D. grant SFRH/BD/137801/2018. Gonzales-Barron acknowledges the national funding by FCT, P.I., through the Institutional Scientific Employment Program contract.

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

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