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Screening of Lactic Acid Bacteria Isolated from Artisanally Produced *Alheira* Fermented Sausages as Potential Starter Cultures †

Ana Sofia Faria, Nathália Fernandes, Vasco Cadavez and Ursula Gonzales-Barron *

Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia 5300-253 Bragança, Portugal; anafaria@ipb.pt (A.S.F.); nathaliaraquelx@gmail.com (N.F.); vcadavez@ipb.pt (V.C.)

* Correspondence: ubarron@ipb.pt; Tel.: +351-273-303-325

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Abstract: In this work, a total of 335 lactic acid bacteria strains were isolated from 40 artisanally produced *alheira* sausages. Their antimicrobial activity was determined in vitro, and a subset of 63 promising isolates were subjected to further analysis to determine their in vitro acidifying and proteolytic activities as well as lactic acid production. Principal component analysis evidenced that, for both MRS and M17 isolates, some strains presented both great antimicrobial properties and high acidification activity, characteristics which may be helpful in designing a customised starter culture for application in *alheira* production.

Keywords: *alheira*; non-ready-to-eat sausage; lactic acid bacteria; pathogens; principal component analysis

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1. Introduction

Alheira is a long-standing traditional Portuguese, non-ready-to-eat fermented sausage. Its origin dates back to the 15th century, during the Portuguese Inquisition, when persecuted Jews, created a sausage made with poultry and game meats mixed with bread and spices. These sausages mimicked pork sausages traditionally consumed by Christians but forbidden by Jewish faith, allowing them to escape identification.

It became a popular regional dish amongst the population and nowadays *alheira* is traditionally produced with mix of pork, lard, and poultry meat, although newer formulations experimenting with game meat, cod fish, or even vegetarian options can be found in the market.

Meats are cooked, shredded, and mixed with salt, garlic, spices, and sliced bread soaked in the meats' leftover broth, to form a non-uniform paste. This paste is stuffed into natural casings and left to dry and mature at cold temperatures for 7–14 days. Fermentation occurs due to naturally present lactic acid bacteria (LAB), without the aid of any starter culture. Despite being a highly commercialised product in Portugal, recent microbiological surveys at regional producers have pointed out low-to-moderate prevalence of *Staphylococcus aureus* and sporadic presence of *Salmonella* spp. [1]. Thus, the objective of this study was to isolate LAB naturally present in *alheira* and screen their antimicrobial and technological potential for the subsequent development of a functional starter.

2. Materials and Methods

Forty *alheiras* originating from 8 different artisanal producers located in the Trás-os-Montes region were analysed [1]. Briefly, after dilution, 1mL 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. Four typical colonies were selected from MRS and M17 for purification and incubated at 30°C for 48 h in their respective media and confirmed by microscopy and biochemical tests [2]. A total of 335 presumptive LAB were preserved in MRS broth with 25% glycerol at −80°C until further testing.

2.1. Pathogenic Strains and Preparation of Cell Suspensions

Staphylococcus aureus subsp. *aureus* strain ATCC 6538 and *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain ATCC 43971, obtained from the Polytechnic Institute of Bragança stock collection, were used for all experiments. Each strain was cultured into Nutrient Agar slants. A loop of culture was inoculated in BHI broth and cultivated at 37°C overnight to obtain a cell suspension (~10^8 CFU/mL).

2.2. Screening of LAB for Antimicrobial Capacity

Antimicrobial capacity of selected LAB strains was determined by deferred-antagonism assay according to Campagnollo et al. [3], with minor adjustments. Each LAB strain was cultured separately from stock into 10 mL MRS broth (37 °C, 24 h), spotted (2–5 μL) onto the surface of MRS or M17 agar plates (into same media of isolation, respectively), following overnight incubation at 30°C, in duplicate. Incubated plates were overlayed with 10 mL of BHI broth +0.75% bacteriological agar, seeded with 1ml of *S. aureus* or *S. typhimurium* cell suspension (~10^8 CFU/mL), pre-incubated at 4 °C for 2 h, and incubated overnight at 37 °C after. The plates were examined for zones of inhibition in the pathogen cell lawn and the inhibition diameter (ID) was measured against LAB colony diameter. A subset of 63 LAB strains (MRS, $n = 43$; M17, $n = 20$) with the strongest antimicrobial capacity were also tested at 10 °C (during 10 days) because the maturation process of *alheira* takes places commonly at this temperature

2.3. Screening of LAB for Acidifying Capacity and Proteolytic Activity

Acidifying and proteolytic activity were assayed as described by Durlu-Ozkaya et al. [4] and Franciosi et al. [5], respectively, with slight modifications.

For the acidification profiling, the selected subset of LAB stock strains were initially grown in 10 mL of MRS broth (30 °C, 24 h) and then in sterile reconstituted skim milk (10% w/v) supplemented with yeast extract (0.3% w/v) and glucose (0.2% w/v) for two successive subcultures (30 °C, 24 h). Sterile reconstituted skim milk (100 mL) was inoculated with 1mL of an activated culture and incubated at 30°C for 24h. pH was measured every 2 h for 8 h (t = 0, 2, 4, 6, 8 h), and then at 24 h post inoculation. For every strain, pH data was fitted to a decay curve in order to characterise acidification capacity. The following descriptors were extracted from the fitted curves: ΔpH_{03}: pH decrease between t= 0 h and t= 3 h; ΔpH_{06}: pH decrease between t= 0 h and t= 6 h; ΔpH_{36}: pH decrease between t= 3 h and t= 6 h; and pH_6: pH at t=6 h.

For determination of lactic acid concentration [LAC], selected LAB stock strains were initially cultured in 10 mL of MRS broth (30 °C, 24 h). A loop of the inoculated broth was streak plated into MRS or M17 agar (depending on media of origin) and incubated at 30 °C for 48 h. Two or more well isolated colonies were diluted in 5 mL sterile saline solution and vortexed to homogenate the suspension, to achieve a density of approximately 0.5 McFarland. Absorbance at 625 nm was confirmed using a spectrophotometer, to ensure a value between 0.08 and 0.13. A tube containing 4 mL of MRS broth was inoculated with 100 μL of the adjusted suspension and incubated at 30 °C for 4 h. 1 mL of the broth was

centrifuged at 13.000 rpm for 5 minutes, and 10 μL of the supernatant was diluted into 500 μL sterile distilled water. This suspension was then used for L-Lactic acid quantification, according to kit instructions (NZYTech, Portugal).

Proteolytic activity (PAct) was determined by spotting 3 μL of the second skim milk LAB subculture onto the surface of reconstituted skim milk (3% w/v) agar (1.5% w/v), in duplicate, and incubated at 35 C for 4 days. Proteolytic activity appeared as a clear zone around the colonies, and the diameter of these halos was measured against LAB colony diameter.

2.4. Statistical Analysis

To summarize the information resulting from all technological properties (ID Salmonella 37 °C; ID Staphy 37 °C; ID Salmonella 10 °C; ID Staphy 10 °C; PAct; pH_6; ΔpH_{03}; ΔpH_{06}; ΔpH_{36}; [LAC]), and their interrelationships, the data were divided by MRS- and M17-grown LAB and subjected to separate principal component analysis (PCA). The function *prcomp()* and the library *factoextra* were utilized in the 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 the tested technological properties of MRS and M17-grown lactic acid bacteria isolated from *alheiras* were built from the projection of sample scores onto the map of the principal components.

3. Results

The contribution of the technological properties of MRS and M17-grown LAB to the principal components can be evaluated by their correlations with the three components extracted (Table 1 and Table 2, respectively). These correlations are also represented in the maps depicted in Figure 1 and Figure 2, correspondingly.

Table 1. Coefficients of correlation of the tested technological properties of MRS-grown lactic acid bacteria isolated from Portuguese *alheira* sausage, with the three Varimax-rotated factors (PC1, PC2, PC3) along with communalities and explained variances.

Variable	PC1	PC2	PC3	Communalities
ID Salmonella 37 °C	0.36	0.32	0.59	1.9
ID Staphy 37 °C	**0.60**	0.21	−0.44	1.9
ID Salmonella 10 °C	0.05	**0.85**	0.16	1.1
ID Staphy 10 °C	−0.04	**0.90**	−0.03	1.0
PAct	0.16	0.09	**0.64**	1.2
pH_6	**−0.97**	−0.02	−0.16	1.1
ΔpH_{03}	0.37	0.46	0.38	2.9
ΔpH_{06}	**0.96**	0.10	0.19	1.1
ΔpH_{36}	**0.98**	−0.02	0.11	1.0
[LAC]	0.11	0.01	**−0.76**	1.0
Proportion Variance	0.39	0.20	0.14	-
Cumulative Variance	**0.39**	**0.59**	**0.73**	-

ID Salmonella 37 °C and 10 °C: diameter of inhibition of *Salmonella* 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; pH_6: pH value of milk broth after 6 h; $\Delta pH_{03, 06}$ and $_{36}$: pH decrease between t= 0 h and t= 3 h, t= 0 h and t= 6 h, and t= 3 h and t= 6 h, respectively.

For MRS-grown strains, a total of 73% of the variability in the 10 quality attributes was jointly explained by the three principal components. The first component (PC1) explained 39% of the total variability and was highly correlated with ΔpH_{36} (R = 0.98), ΔpH_{06} (R = 0.96), more weakly correlated with inhibition of *S. aureus* at 37 °C (R = 0.60), and

highly and inversely correlated with pH_6 (R = −0.97). In this case, PC1 appears to characterize strains with high acidification capability. In addition, the survival of *S. aureus* at 37 °C is directly affected by an efficient drop in pH, seemingly explaining the weaker correlation of *S. aureus* with PC1 (Figure 1a). The second component (PC2) explained 20% of the data variability and was highly correlated with inhibition of both *S. aureus* (R = 0.90) and *Salmonella* (R = 0.85) at 10 °C, thus characterizing strains with marked antimicrobial activity at refrigeration temperatures (Figure 1a). As for PC3, which explained 14% of the total variability, it presented a correlation with PAct (R = 0.64) and a highly but inversely correlation to [LAC] (R = −0.76). In this sense, PC3 allows the differentiation between strains with good proteolytic activity and lactic acid production (Figure 1b).

Table 2. Coefficients of correlation of the tested technological properties * of M17-grown lactic acid bacteria isolated from Portuguese *alheira* sausage, with the three Varimax-rotated factors (PC1, PC2, PC3) along with communalities and explained variances.

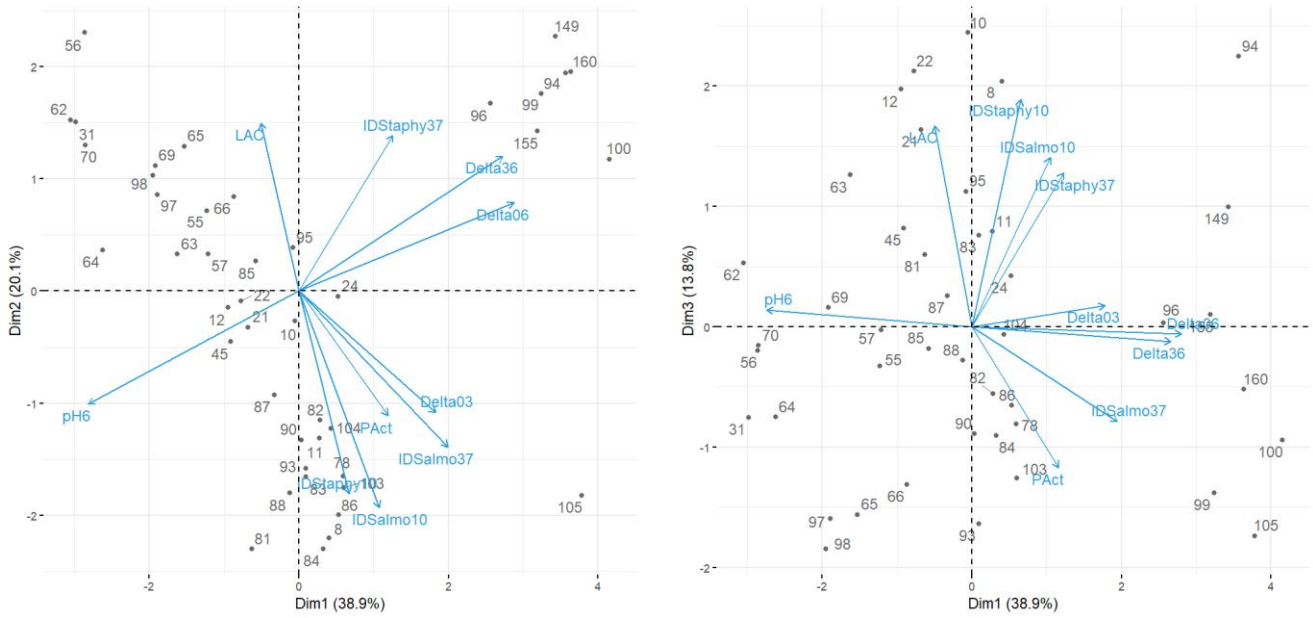
Variable	PC1	PC2	PC3	Communalities
ID Salmonella 37 °C	0.11	0.80	0.00	1.0
ID Salmonella 10 °C	0.01	**0.66**	0.20	1.2
ID Staphy 10 °C	−0.11	−0.01	**0.88**	1.0
pH_6	**−0.98**	−0.08	0.07	1.0
ΔpH_{03}	0.55	0.54	0.41	2.8
ΔpH_{06}	**0.98**	0.21	0.01	1.1
ΔpH_{36}	**0.96**	0.03	−0.15	1.1
[LAC]	−0.12	**−0.65**	0.24	1.4
Proportion Variance	0.44	0.20	0.12	-
Cumulative Variance	**0.44**	**0.64**	**0.76**	-

ID Salmonella 37 °C and 10 °C: diameter of inhibition of *Salmonella* 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; pH_6: pH value of milk broth after 6h; $\Delta pH_{03, 06}$ and $_{36}$: pH decrease between t= 0 h and t= 3 h, t= 0 h and t= 6 h, and t= 3 h and t= 6 h, respectively. (*) ID Staphy 37 °C and PAct were removed from the analysis since their values were zero.

From Table 2 (M17-grown subset), a total of 76% of the variability was explained by the three principal components. PC1 explained 44% of the total variability and was highly correlated with ΔpH_{06} (R = 0.98), ΔpH_{36} (R = 0.96), and highly but inversely correlated with pH_6 (R = -0.98), characterising strains with higher acidification activity (Figure 2a). PC2 justified 20% of data variability, correlating positively with inhibition of *Salmonella* at 10°C (R = 0.66), and negatively with lactic acid production (R = −0.65). PC2 appears to differentiate between strains with high lactic acid production and strains with *Salmonella* antimicrobial activity at 10 °C (Figure 2a). As for PC3, which explains 12% of variability, it is highly correlated with antimicrobial inhibition of *S. aureus* at 10°C (R = 0.88) (Figure 2b).

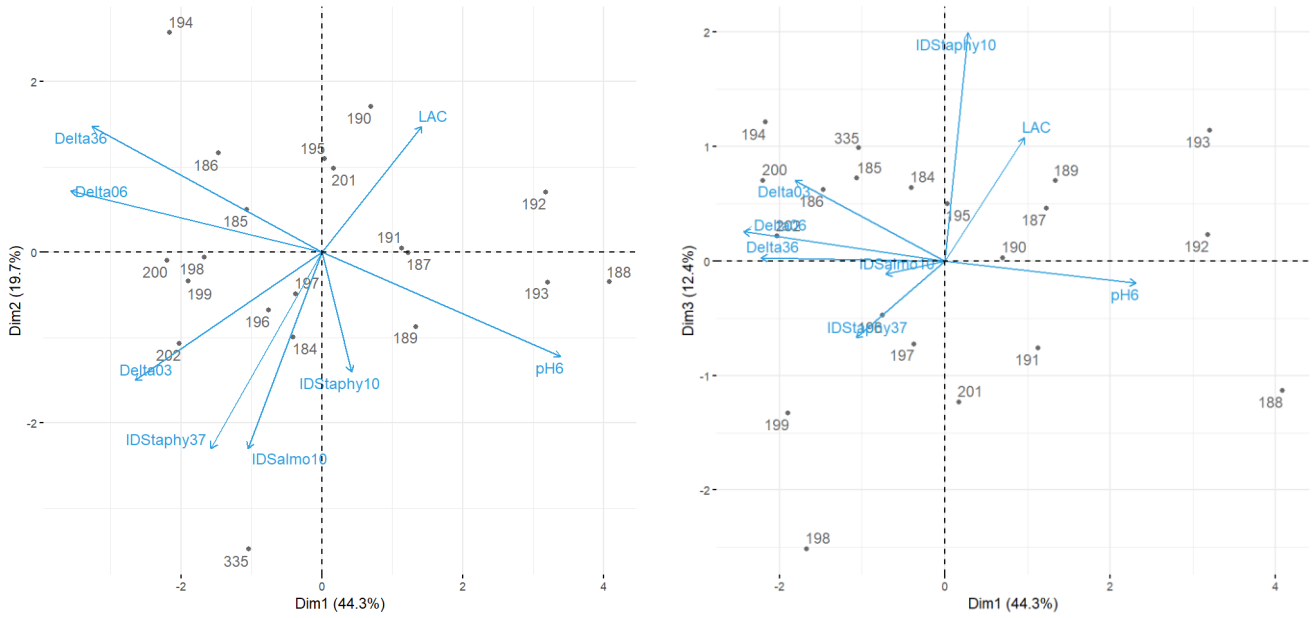
A closer analysis of the PCA maps generated for MRS-grown isolates (Figure 1) allows for a quick identification of three clusters: one with higher acidification capacity, related to greater *S. aureus* inhibition at 37 °C; a second cluster with better overall antimicrobial activity and linked to higher proteolytic activity; and a third cluster of strains with more rapid production of lactic acid (Figure 1a). Additionally, one standalone MRS strain is highlighted by each map (#105 and #94), for presenting both greater acidification and antimicrobial potential (Figure 1a,b).

From M17 isolates (Figure 2), no clustering was identified. However, two strains, #194 and #335 (Figure 2a) outstood for their highest acidification ability and pathogenic inhibition.



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

Figure 1. Maps of the first and second principal components (**a**) and the first and third principal components (**b**) of the tested technological properties of MRS-grown lactic acid bacteria isolated from *alheira* sausage produced in Northeastern Portugal.



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

Figure 2. Maps of the first and second principal components (**a**) and the first and third principal components (**b**) of the tested technological properties of M17-grown lactic acid bacteria isolated from *alheira* sausage produced in Northeastern Portugal.

4. Conclusions

This study showed that grouping of isolates by principal component analysis might be very useful towards the selection of strains with desirable qualities for *alheira* production, specifically in developing a tailored started culture that provides protection against foodborne pathogens, while conferring pleasing organoleptic properties of *alheira*. In-situ essays are ongoing.

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