Technological and protective performance of LAB isolated from Serpa PDO cheese: Towards selection and development of an autochthonous starter culture

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ABSTRACT
Serpa is an ovine raw milk cheese widely appreciated by the consumers. However, raw milk products may be seen with reservations in terms of safety or technological defects. To overcome that, the addition of an autochthonous starter culture may ensure the cheesemaking process optimization and microbiota dominance. In this work, the technological and protective performance of eleven lactic acid bacteria strains, isolated from Serpa Protected Designation of Origin cheese and reported as generally recognized as safe, were screened. The inte- gration of technological and protective properties studied in the PCA plot, coupled with the proteolytic and lipolytic analysis suggested that *Lb. plantarum* PL1 and PL4 strains may be the best candidates. These strains showed both proteolytic and lipolytic activities, a good acidification potential, low D-lactic acid production and were well adapted to the salt and temperatures used. PL1 strain also exhibited a higher antimicrobial effect against the pathogenic bacteria studied. Although *Lb. paracasei* strain showed lower acidification capacity, due to their technological and protective properties, it could be combined with other more acidifying strains. As future work, it is important to establish cheese model systems to complement this screening and implement an autochthonous starter culture.

1. Introduction

Serpa is a Protected Designation of Origin (PDO) cheese, manufac- tured from ovine raw milk and vegetable coagulant (*Cynara cardunculus* L.), without pasteurization or other thermal process, or addition of any starter culture (Araújo-Rodrigues, Tavaria, dos Santos, Alvarenga, & Pintado, 2020; Freitas & Malcata, 2000; Gonçalves Dos Santos, Benito, Cóordoba, Alvarenga, & Ruiz-Moyano Seco de Herrera, 2017; Gonçalves et al., 2018; Yeluri Jonnala, McSweeney, Sheehan, & Cotter, 2018). The PDO specifications require a minimum ripening period of 30 days, typically ranging the maturation period between 30 and 40 days before commercialization and consumption (Alvarenga, Silva, Garcia, & Sousa, 2008; Araújo-Rodrigues et al., 2020). Its high microbial diversity which is a consequence of the raw milk, ingredients and processing technology, allows the development of highly appreciated organoleptic attributes namely, a characteristic strong flavor coupled with a semi-soft and creamy texture (Araújo-Rodrigues et al., 2020; Gonçalves Dos Santos et al., 2017; Gonçalves et al., 2018; Roseiro, Goómez-Ruiz, García-Risco, & Molina, 2003). This traditional Portuguese cheese bears a strong economic and cultural impact due to its long-lasting cultural heritage, regarding the technological process and organoleptic properties (Araú- jo-Rodrigues et al., 2020; Freitas & Malcata, 2000).

Even though this unique sensorial profile is highly appreciated, the use of raw milk may be seen with reservations either in terms of food safety or in the development of technological drawbacks and defects. Several parameters may directly affect their final sensorial and safety characteristics including, the variations in the cheesemaking process, as well as in the physicochemical and microbiological milk composition. These factors contribute to variability and heterogeneity in the final product and may result in flavor, texture and safety shortcomings (Gonçalves Dos Santos et al., 2017; Montel et al., 2014; Silvetti et al., 2017). The safety risks associated with the consumption of raw milk cheeses are related to the possible presence of pathogenic microorgan- isms in the complex microbial community present in raw milk (Chourasia et al., 2021; Leroy & De Vuyst, 2004; Montel et al., 2014; Tavaria, Tavares, Silva-Ferreira, & Malcata, 2006).

The use of autochthonous starter cultures in the artisanal cheese-making process may overcome these problems, promoting consistent quality and safety (Câmara et al., 2019; Chourasia et al., 2021; Dolci et al., 2020; Leroy & De Vuyst, 2004; Li et al., 2020; Montel et al., 2014; Silvetti et al., 2017; Tavaria et al., 2006). An autochthonous starter culture consists of a single or a group of microbial strains well adapted to the technological process, isolated and selected from the original product. Its implementation implies a dominance of starter culture mi- croorganisms over the raw or pasteurized milk microbiota, ensuring a favorable microenvironment for the technological process and opti- mizing the fermentative process. This may allow the development of the typical and specific organoleptic properties of the autochthonous product and/or exalt some sensorial or functional features (Bassi, Puglisi, & Cocconcelli, 2015; Chourasia et al., 2021; Dolci et al., 2020).

Although the use of a starter culture is not permitted by the Portu- guese legislation for PDO cheeses, a tailor-made starter culture may contribute to introduce in the market new products with sensory prop- erties close to those of Serpa cheese, which could also be suitable for more restricted markets in terms of hygiene and safety regulations (Araújo-Rodrigues et al., 2020). In addition to overcoming the sensorial and safety issues, the use of autochthonous starter cultures would also maximize the resources of the small ruminant sector, helping traditional industries improving the use of raw milk (Silvetti et al., 2017). The raw material with an exceptional microbiological profile may continue to be directed to PDO production, while to the other, a value-added strategy may be adopted by using an autochthonous starter culture directly to raw or pasteurized milk and consequently, reproduce some of the original cheese typicality.

For autochthonous starter culture selection and development, the microbial strains should be generally recognized as safe (GRAS), well adapted to the technological conditions used during the cheesemaking process and improve the fermentative procedure (Chou, Edwards, Lue- decke, Bates, & Clark, 2003; Leroy & De Vuyst, 2004; Nieto-Arribas, Poveda, Sesena, Palop, & Cabezas, 2009; Schornsteiner, Mann, Bereuter, Wagner, & Schmitz-Esser, 2014). The capacity to produce acid is an extremely important attribute during cheese manufacture, being a typical feature of lactic acid bacteria (LAB). LAB also dominate the microflora during maturation and play important roles during this phase (Camara et al., 2019; Chourasia et al., 2021; Li et al., 2020; Nie- to-Arribas et al., 2009). Moreover, lipolytic and proteolytic activities are extremely relevant for the development of aroma, flavor and texture as well as the antimicrobial potential for final product safety (Chou et al., 2003; Leroy & De Vuyst, 2004; Li et al., 2020; Schornsteiner et al., 2014).

Therefore, this study aimed at screening the technological and pro- tective performance of LAB isolated from Serpa cheese in order to select and develop an autochthonous starter culture for this traditional prod- uct. From a set of 116 LAB strains belonging to *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Enterococcus* genera, previously isolated from Serpa cheese at the end of the ripening period (Gonçalves et al., 2018), 116 LAB were studied and 20 of these strains were selected as acid-tolerant strains and studied by Ruiz-Moyano et al. (Ruiz-Moyano et al., 2019).
The authors evaluated some safety and probiotic attributes of the autochthonous LAB strains (Ruiz-Moyano et al., 2019). In this study, 11 of those GRAS LAB were selected to evaluate their technological and protective potential for the development of an autochthonous starter culture for Serpa cheese.

2. Materials and methods

2.1. Bacterial isolates

Some safety and probiotic properties of LAB strains identified and isolated from Serpa cheese with 30 days of maturation (Gonçalves et al., 2018) were studied by Ruiz-Moyano et al. (2019). Through this screening of autochthonous LAB from Serpa PDO cheese, eleven *Lacto- bacillus* spp. strains were selected for the present study mainly based on safety aspects namely, antibiotic susceptibility and biogenic amine production as summarized in Table 1. All LAB under study have been kept as pure cultures in our institutional strain collection. The LAB strains were grown in triplicate in Man Rogosa-Sharpe broth (MRS; Biokar Diagnostics, Cedex, France) at 37 °C. Cell concentration was adjusted to ca. 10^8 colony-forming unit (CFU)/mL.

2.2. Technological characterization

2.2.1. Lipolytic and extracellular proteolytic activities

Lipolytic and extracellular proteolytic activities were evaluated as described by Câmara et al. (2018) and Ribeiro et al. (2013), respectively. For lipolytic activity evaluation, the LAB suspensions were streaked in Tributyrin agar (Merck, Darmstadt, Germany) plates in triplicate and incubated at 30 °C for 72 h. Regarding the extracellular proteolytic ac- tivity, LAB cultures were streaked in triplicate in plate count agar (PCA, Merck, Darmstadt, Germany) medium supplemented with 10% (v/v) of skim milk powder. The plates were also incubated at 30 °C for 72 h and, after this period, they were flooded with 1% HCl. The presence of lipolytic and proteolytic activities was detected by a clear zone sur- rounding the colonies' growth.

2.2.2. Resistance to temperature and salt

Growth in MRS broth at different NaCl concentrations and temper- atures was evaluated in duplicate by measuring optical density (OD 620 nm), using the method described by Ribeiro et al. (2013). Succinctly, in the first case, the ability of the strains to grow in MRS supplemented with 2, 6 and 10% (w/v) of NaCl was evaluated during 24 h at 30 °C, at intervals of 1 h. Regarding the ability to grow at different temperatures, namely 4, 15, 30 and 45 °C was also evaluated during 24 h at 1 h intervals.

2.2.3. Acidifying activity

The acidification activity of LAB was monitored for 48 h in skim milk (Oxoid, Basingstoke, England), according to the methods described by Ribeiro et al. (2013)and Nieto-Arribas et al. (2009), with few modifi- cations. The bacterial isolates were grown in MRS broth at 30 °C over- night before testing. For acidification activity monitorization, a 1% (v/v) suspension of each strain was grown in 10 mL of skim milk and incubated at 30 °C, in triplicate. The pH value was measured at 0, 4, 8, 12, 24 and 48 h, using a pH meter.

2.2.4. Production of lactate D(-)- and L(+)-ISOMERS

In order to investigate the lactic acid production (D(—)- and L (+)-isomers), the LAB strains were grown in MRS modified broth (MRSMB) at 37 °C, during 24 h and under 10% CO_2. The MRSMB was formulated according to commercial MRS specifications, lacking glucose and sodium acetate as well as supplemented with 2 g L^{-1} lactose. Cell culture supernatants were obtained by media centrifugation at 8000x*g* for 5 min, before filtering through 0.22 Mm filters (Thermo Fisher Sci-entific). The lactate isomeric types present in fermented broth were

Table 1
Selected LAB strains isolated from Serpa cheese with 30 days-old. The probiotic attributes, biogenic amine production and antibiotic resistance are indicated by + when the activity was detected and - when the activity was not detected by Ruiz-Moyano et al. (2019).

Species	Strains	Number of isolates	Probiotic attributes	Biogenic amine production	Antibiotic resistance
Lb. paracasei	PC	1	-	-	-
Lb. casei	CA	1	+	-	-
Lb. plantarum	PL1	5	+	-	-
	PL2		+	-	-
	PL3		+	-	-
	PL4		+	-	-
	PL5		+	-	-
Lb. crustorum	CR	1	+	-	-
Lb. pentosus	PE	1	+	-	-
Lb. brevis	BR1	2	+	-	-
	BR2		+	-	-

determined by an enzymatic method as reported by Rulikowska et al. (2013), using kit K-DLATE assay (Megazyme Int., Wicklow, Ireland) according to the manufacturer's instructions. This enzymatic assay al-lows the quantification of both D(—)- and L(+)-lactic acid. Succinctly, K-DLATE assay is based on the measurement of UV absorbance at 340 nm of NADH produced when D(—)- and L(+)-lactic acid are oxidized to pyruvate by NAD, in the presence of D-lactate dehydrogenase (D-LDH) and L-lactate dehydrogenase (L-LDH), respectively. For all strains, three technical replicates were performed and evaluated.

2.3. Protective characterization

2.3.1. Antimicrobial activity

The LAB antimicrobial effect on some potential pathogenic bacteria present in strain collection (*Listeria monocytogenes* CECT 911, *L. monocytogenes* CECT 934, *L. innocua* CECT 910, *Bacillus cereus* CECT 131, *Staphylococcus aureus* CECT 976, *Salmonella choleraesuis* CECT 4395 and *Escherichia coli* CECT 4267) was investigated, according to the method described by Ruiz-Moyano et al. (2009). The ability of each pathogen to grow in Brain Heart Infusion (BHI) broth (Scharlab), sup- plemented with 10% of supernatant filtered-sterilized from each LAB strain, was evaluated by following the microbial growth at 37 °C in an automated turbidometer Bioscreen C Analysing System (Labsystems, Helsinki, Finland). Each LAB supernatant was obtained from an over- night culture at 37 °C under 10% CO_2 by centrifugation and pH adjustment at 7, to avoid acid inhibition. The percentage of inhibition was calculated with the formula:

$$Inhibition\ (\%) = \frac{OD\ (strain) - OD\ (assay)}{OD\ (strain)} \times 100$$

where OD (strain) corresponds to the pathogen strain growth optical density in the absence of LAB supernatant, and OD (assay) is the path- ogen strain growth optical density in LAB supernatant presence.

2.4. Statistic analysis

Statistical analysis was performed for acidifying activity, D-lactic production and antimicrobial activity, using the SPSS statistical package 26.0 via a one-way analysis of variance (ANOVA), at a degree of sig- nificance of A = 0.05. Data were compared statistically using ANOVA to understand the significance and confirm a normal distribution of the data. Post-hoc multiple comparisons were carried out using Turkey's test (a = 0.05). A biplot principal component analysis (PCA) was performed in order to have a global integration of technological and protective properties for autochthonous starter culture selection.

3. Results and discussion

Ruiz-Moyano et al. (2019) evaluated the acid-tolerant properties of 116 LAB isolated and identified from Serpa PDO cheese with 30 days of ripening (Gonçalves et al., 2018). In the reported work, 20 of these microbial strains exhibited acid-tolerant properties and some safety and probiotic attributes were investigated. In this work, the characterization of 11 of these autochthonous strains, reported as GRAS by Ruiz-Moyano et al. (2019), was performed in a technological and protective perspective. Some safety and probiotic characteristics studied by Ruiz-Moyano et al. (2019) are summarized in Table 1. Important tech- nological and protective features for the cheesemaking process of these autochthonous LAB strains were carried out in the present study to select promising strains well adapted to the fermentation process, for the development of autochthonous starter culture.

3.1. Lipolytic and extracellular proteolytic potential

In Table 2, the extracellular proteolytic and lipolytic activities of the LAB under study are presented. The results anticipated that only *Lb. casei* CA and *Lb. plantarum* PL2 strains did not exhibit extracellular proteolytic activity in PCA medium supplemented with skim milk. The production of free amino acids (FAAs) and peptides by peptidases of LAB and vegetable coagulant are essential for flavor development and intensity, contributing to the acceleration of the maturation process (Chourasia et al., 2021; Leroy & De Vuyst, 2004; Li et al., 2020).

Regarding lipolytic activity, only four bacterial isolates belonging to the species *Lb. paracasei* (PA) and *Lb. plantarum* (PL1, PL4 and PL5) demonstrated lipolytic potential in tributyrin agar (Table 2). Lipolysis is one of the most important reactions during cheese maturation, which occurs by the action of lipolytic enzymes present in milk and microor- ganisms, resulting in the production of free fatty acids (FFAs) and mono and diacylglycerides. FFAs play an essential role in flavor and aroma development and the differences in their proportions result in the spe- cific attributes of each cheese (Yilmaz, Ayar, & Akin, 2005).

FFAs, peptides and FAAs resultant from both bioactivities are rele- vant organoleptic-related compounds, making these activities essential for starter culture selection and development. These molecules are also

Table 2
Lipolytic and extracellular proteolytic activities of 11 LAB strains under study. Three experimental replicates were performed.

LAB strain	Lipolytic	Extracellular proteolytic
Lb. paracasei PC	+++	+++
Lb. casei CA		
Lb. plantarum PL1	+++	+++
Lb. plantarum PL2		
Lb. plantarum PL3		+++
Lb. plantarum PL4	+++	+++
Lb. plantarum PL5	+++	+++
Lb. crustorum CR		+++
Lb. pentosus PE		+++
Lb. brevis BR1		+++
Lb. brevis BR2		+++

+Activity detected; - Activity not detected.

precursors of other chemical groups involved in the sensorial attributes, such as methyl ketones, esters and thioesters (Leroy & De Vuyst, 2004; Yilmaz et al., 2005).

Previous works studied the lipolytic and proteolytic activities of *Lb. plantarum* and *Lb. paracasei subsp. paracasei* strains isolated from Man- chego cheese (Nieto-Arribas et al., 2009), Camara et al. (2019) also studied these activities of autochthonous LAB from Pico cheese (including *Lb. casei* and *Lb. paracasei* species). The results of both studies indicated that none of the strains showed lipolytic activity (Camara et al., 2019; Nieto-Arribas et al., 2009). The lipolytic and proteolytic activities of *Lactococcus lactis* and *Enterococcus faecalis* isolates were also studied and only one of the strains studied demonstrated both activities (Ribeiro et al., 2014). In the present study, only 4 strains demonstrated this activity in tributyrin agar, being aligned with the low lipolytic ca- pacity reported for LAB (McSweeney & Sousa, 2000). Regarding pro- teolytic activity, the results of Nieto-Arribas et al. (2009) showed that most of the *Lb. paracasei subsp. paracasei* and *Lb. plantarum* strains showed proteolytic activity, as also corroborated by the results of the present study.

3.2. Resistance to cheesemaking conditions

For starter culture application, it is also important that LAB are well adapted to all conditions applied during the cheesemaking process, namely the salt content and temperatures used during the manufacturing process. Serpa cheese possesses a content of NaCl in the moisture ranging from 3.61 to 4.43 g/100 g (Roseiro, Andrew Wilbey, & Barbosa, 2003). Accordingly, the growth of LAB was tested without NaCl addition and with NaCl concentrations of 2, 5 and 10% (w/w). The LAB strains were well adapted to all NaCl concentrations, during 24 h of growth (data not shown).

Concerning the temperatures used during the technological proced- ure, milk coagulation of Serpa cheese occurs under a temperature of ca. 30 °C and maturation between 8 and 13 °C (Alvarenga et al., 2008; Araújo-Rodrigues et al., 2020; Roseiro, Andrew Wilbey, & Barbosa, 2003). The temperature resistance was tested by growing strains at 4, 15, 30 and 45 °C, as depicted in Fig. 1. Generally, the results indicated that the bacterial isolates possessed a higher growth at 30 °C, being followed by growth at 15 °C. However, different growth patterns were registered until the exponential phase according to the strain and tem- perature used. The different lag phases mean that the bacterial cells require different periods to adapt to the new environmental conditions and reach the exponential phase. The lag phase may include the repa- ration of macromolecular damage accumulated during the stationary phase (Rolfe et al., 2012) and preparation for the upcoming exponential phase. All strains showed reduced growth at 4 °C and maximum at 30 °C, in accordance with Ribeiro et al. work (2013). Strains belonging to the *Lb. plantarum* species exhibited similar behavior at all temperatures tested, which was expected since these strains belonging to the same species, possessed more similarities at the genetic level. The results also indicated that *Lb. crustorum* strains had similar performances at 30, 15 and 4 °C but some differences at 45 °C growing conditions, where *Lb. crustorum* CR strain seems to adapt and grow better at this temperature. Although in the initial phase the strains *Lb. paracasei* PC, *Lb. crustorum* CR and *Lb. brevis* BR1 showed lower growth at 45 °C, these showed the capacity to adapt to this temperature and to display a growth rate similar to the growth presented at 15 °C. The strain *Lb. pentosus* PE proved to be well adapted to this highest temperature, with growth similar to that registered at 30 °C. The remaining bacterial isolates were not well adapted to temperature at 45 °C and showed very low growth. The same was observed at 4 °C, as well.

3.3. Decrease in pH

The decrease in pH value due to lactic acid production also plays an important role in cheese safety and maturation, inhibiting the growth of pathogens and undesired microorganisms, as well as contributing to the production of numerous organoleptic-related compounds (Alvarenga et al., 2008; Beresford, Fitzsimons, Brennan, & Cogan, 2001). The decrease in pH is also involved in a micelle demineralization effect (Ca^{2+} bonds) and subsequent dissociation, being extremely important for cheese softening (Alvarenga et al., 2008). In this context, the acidifica- tion activity of 11 LAB was evaluated in skim milk (Table 3).

The pH values suggested significant differences between bacterial strains during the incubation period and a decrease in pH between 6.47 and 6.40 until 3.65-4.60, after 24 h of incubation, and until 3.53-3.97, after 48 h of incubation. Although in all strains a high decrease in pH value was observed, the results indicated that *Lb. casei* CA, *Lb. plantarum* PL3 and PL4 and *Lb. pentosus* PE were the strains with a higher acidifi- cation capacity, while *Lb. paracasei* PC, *Lb. brevis* BR1 and BR2 strains showed a lower acidification activity but still a high acidifying capacity comparing with LAB strains studied by Nieto-Arribas et al. (2009),

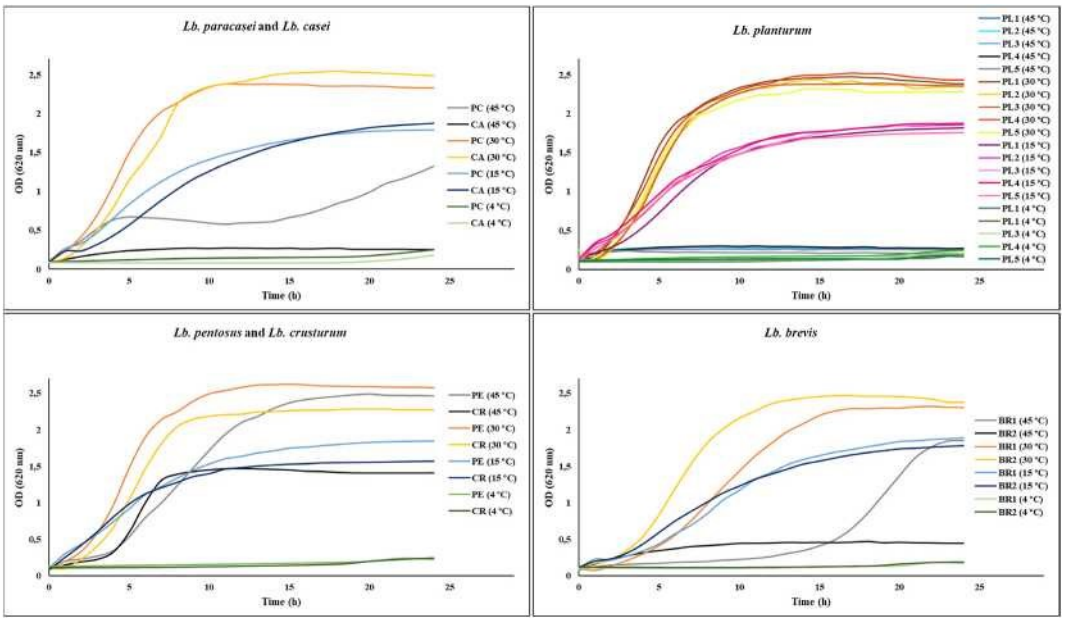

Fig. 1. Growth of selected LAB when inoculated at 4, 15, 30 and 45 · C during 24 h. Two experimental replicates were performed.

Table 3
pH **variation (mean ± standard deviation) in skim milk according to strain inoculation, during 48 h.** Three experimental replicates were performed. Values in bold corresponds to pH means below 5.50. Control sample corresponds to skim milk without strain inoculation.

LAB strain	0 h	4 h	8 h	12 h	24 h	48 h	A pH 24 h	A pH 48 h
Lb. paracasei PC	6.47 ± 0.01^{a}	5.55 ± 0.00^{a}	5.19 ± 0.01^{a}	**4.04 ± 0.04^{a}**	**4.60 ± 0.02^{a}**	**3.97 ± 0.01^{a}**	1.89	2.52
Lb. casei CA	6.47 ± 0.00^{a,b}	5.41 ± 0.02^{b,d}	4.87 ± 0.01^{b}	4.51 ± 0.01^{b}	**3.68 ± 0.06^{b}**	**3.53 ± 0.02^{b}**	2.79	2.94
Lb. plantarum PL1	6.49 ± 0.00^{b,c}	5.37 ± 0.00^{b}	4.70 ± 0.01^{c}	**4.34 ± 0.01^{c}**	**3.75 ± 0.00^{c}**	**3.66 ± 0.02^{c,d}**	2.72	2.81
Lb. plantarum PL2	6.48 ± 0.01^{a,b}	5.58 ± 0.02^{a,e}	5.32 ± 0.00^{d}	4.96 ± 0.02^{d}	**3.85 ± 0.03^{d}**	**3.71 ± 0.01^{d}**	2.64	2.78
Lb. plantarum PL3	6.49 ± 0.00^{a,b,c}	5.26 ± 0.01^{c}	4.52 ± 0.01^{e}	**4.17 ± 0.06^{a,h}**	**3.68 ± 0.00^{b}**	**3.54 ± 0.00^{b,e}**	2.80	2.94
Lb. plantarum PL4	6.49 ± 0.00^{b,c}	5.44 ± 0.02^{d}	4.84 ± 0.04^{b}	**4.31 ± 0.05^{c,h}**	**3.67 ± 0.01^{b}**	**3.54 ± 0.06^{b,e}**	2.82	2.95
Lb. plantarum PL5	6.48 ± 0.00^{a,b}	5.21 ± 0.18^{e}	**4.35 ± 0.06^{f}**	**4.14 ± 0.08^{a}**	**3.71 ± 0.02^{b,c}**	**3.60 ± 0.01^{c,e}**	2.77	2.88
Lb. crustorum CR	6.49 ± 0.00^{a,b,c}	5.64 ± 0.00^{c,f}	5.28 ± 0.01^{a,d}	5.07 ± 0.06^{d,e}	**4.19 ± 0.02^{e}**	**3.72 ± 0.04^{d}**	2.30	2.77
Lb. pentosus PE	6.47 ± 0.00^{a}	5.28 ± 0.01^{e}	**4.44 ± 0.01^{e,f}**	**4.08 ± 0.03^{a}**	**3.65 ± 0.02^{b}**	**3.56 ± 0.00^{b,e}**	2.75	2.91
Lb. brevis BR1	6.49 ± 0.00^{b,c}	5.72 ± 0.09^{g}	5.50 ± 0.07^{g}	5.42 ± 0.10^{g}	**4.42 ± 0.02^{g}**	**3.90 ± 0.04^{a,g}**	2.07	2.59
Lb. brevis BR2	6.49 ± 0.01^{b,c}	5.81 ± 0.02^{h}	5.43 ± 0.00^{g}	5.28 ± 0.01^{f,g}	**4.23 ± 0.02^{e}**	**3.86 ± 0.01^{f,g}**	2.26	2.63
Control	6.50 ± 0.00^{c}	6.51 ± 0.00^{i}	6.50 ± 0.00^{j}	6.51 ± 0.00^{i}	6.51 ± 0.00^{i}	6.51 ± 0.00^{h}	0.01	0.01

Means in the same column with different superscript letters differ significantly ($p < 0.05$).

Ribeiro et al. (2013) and Caémara et al. (2019).

Although acidifying activity is a key parameter for the selection and development of a starter culture, a less acidifying strain with other interesting technological properties can be combined with a more acidifying strain in a mixed starter culture.

3.4. D-lactic concentration

LAB ferment lactose into lactic acid, in amounts often reaching 1-2% in some fermented dairy products (Beresford et al., 2001; Marco et al., 2017). However, these may selectively produce L(+)-lactic or D(—)-lactic acid (Castillo Martinez et al., 2013; Garvie, 1980). Lactic acid isomers produced by lactobacilli are species-specific (Garmiene, Saikauskiene, & Kulikauskiene, 2005; Holzapfel, 2002). The isomer produced will depend on the presence of the specific enzyme NAD-LDH and its activity (D-HDL or L-HDL). In addition, D(—)-lactic acid can be obtained from L (+)-lactic acid through racemase catalyzation, but few microorganisms synthesize this enzyme (Garvie, 1980). Racemase positive *Lactobacilli* spp. have been associated with calcium lactate crystallization (CLC) in cheeses (Agarwal, Sharma, Swanson, Yuksel, & Clark, 2006; Somers, Johnson, & Wong, 2001). Since D(—)-lactate is less soluble than L (+)-lactate, their calcium salts tend to precipitate on the cheese surface as white crystalline deposits (Chou et al., 2003; Johnson, Riesterer, & Olson, 1990; Swearingen, Adams, & Lensmire, 2004), which can be detrimental for consumer acceptance. Consequently, this technological property may be important for starter culture selection and the pro- duction of softer cheeses.

The D(—)-lactate and L(+)-lactate concentrations produced by each strain are presented in Table 4 and the results showed that there are significant differences between lactate isomers production by LAB iso- lates in this study. The results in the culture medium with lactose as an energy source indicated that none of the strains produces stereo specifically D(—)-lactic acid. Although in very different proportions, all strains produced the two isomers.

Lb. paracasei PC and *Lb. casei* CA strains were the ones that produced the least amount of the D(—)-lactic acid isomer (<1 g/L or < 5%), the latter being one of the strains with the greatest acidifying capacity. *Lb. crustorum* CR and strains BR1 and BR2 belonging to *Lb. brevis* species produced low concentrations of this isomer (between 1 and 3.9 g/L or < 50%). *Lb. casei* strains were usually identified as weak producers of this isomer (Castillo Martinez et al., 2013; Garmiene et al., 2005; Holzapfel, 2002; Martín et al., 2005), as well as *Lb. brevis* (Castillo Martinez et al., 2013). All strains of *Lb. plantarum* produced the two isomers in close quantities with a slight predominance of the D(—)-lactic acid isomer (ranging between 50.3% and 71.4%). *Lb. plantarum* was among the species producing mixtures of both isomers (Castillo Martinez et al., 2013).

3.5. Protective potential

In addition to the natural acidification occurring through cheese- making and ripening processes, some LAB strains also possess antimi- crobial activity that may help to inhibit contaminants, eliminating pathogenic microorganisms (Leroy & De Vuyst, 2004). The antimicro- bial activity of the 11 selected bacterial isolates against common food- borne pathogens is presented in Table 5 and, in Fig. 2 the corresponding growth inhibition curves. None of the strains showed a high antimi- crobial effect against *E. coli* CECT 4267 growth, however, *Lb. pentosus* PE strain showed a significant higher antimicrobial effect against this pathogen, with approximately 15% of growth inhibition. Contrary, all strains showed moderated activity against *S. choleraesuis* CECT 4395 growth with some significant variations, with ranges of growth inhibi- tion between 41 and 64%. Although is well known that LAB are more effective against Gram-positive pathogens, inhibitory activity towards

Table 4

Lactate, D(—)- and L(+)-lactate isomers concentration (mean ± Standard deviation) of selected LAB. Three experimental replicates were performed.

Strain	Lactate (g L ^{1})	L(+)-lactic acid (g L-^{1})	%L(+)-lactic acid	D(—)-lactic acid (g L ^{1})	%D(—)-lactic acid
Lb. paracasei PC	7.31 ± 0.24^{d}	7.12 ± 0.23^{b}	97.40	0.19 ± 0.01^{c}	2.60
Lb. casei CA	11.10 ± 1.13^{c}	10.60 ± 1.15^{a}	95.53	0.49 ± 0.04^{c}	4.48
Lb. plantarum PL1	14.09 ± 0.41^{a,b}	6.19 ± 0.24^{b,c}	43.98	7.89 ± 0.35^{a,b}	56.02
Lb. plantarum PL2	15.93 ± 1.04^{a}	6.77 ± 0.67^{b}	42.45	9.16 ± 0.45^{a}	57.55
Lb. plantarum PL3	10.86 ± 0.18^{c}	4.82 ± 0.45^{c,d,e}	44.38	6.04 ± 0.39^{c}	55.62
Lb. plantarum PL4	11.89 ± 0.27^{b,c}	5.91 ± 0.23^{b,c,d}	49.68	5.98 ± 0.07^{c}	50.32
Lb. plantarum PL5	11.7 ± 0.73^{c}	3.35 ± 0.34^{e}	28.615	8.35 ± 0.64^{a,b}	71.39
Lb. crustorum CR	8.05 ± 0.26^{d}	6.88 ± 0.16^{b}	85.54	1.17 ± 0.23^{c}	14.46
Lb. pentosus PE	12.29 ± 0.60^{b,c}	4.68 ± 0.54^{c,d,e}	38.14	7.61 ± 0.73^{b}	61.86
Lb. brevis BR1	7.79 ± 0.21^{d}	3.98 ± 0.08^{e}	51.10	3.81 ± 0.16^{d}	48.90
Lb. brevis BR2	7.94 ± 0.35^{d}	4.11 ± 0.22^{d,e}	51.74	3.83 ± 0.14^{d}	48.26

Means with different superscript letters differ significantly ($p < 0.05$).

Table 5

Antimicrobial activity of selected LAB. Three experimental replicates were performed.

LAB strains	*L. monocytogenes* 911	*L. monocytogenes* 934	*L. innocua* 910	*B. cereus* 131	*S. aureus* 976	*S. choleraesuis* 4395	E. coli 4267
Lb. paracasei PC	8.25 ± 0.96^{c,d}	25.82 ± 3.62^{a,b}	4.95 ± 0.83^{c,d}	12.10 ± 0.96^{e}	17.68 ± 3.85^{a,b}	41.91 ± 6.50^{d}	0.41 ± 0.59^{c,d}
Lb. casei CA	15.67 ± 3.36^{b}	11.18 ± 2.96^{d,e,f}	17.73 ± 1.22^{b}	15.56 ± 1.43^{c,d}	12.28 ± 5.12^{b}	62.86 ± 5.81^{a,b}	0.20 ± 0.31^{c,d}
Lb. plantarum PL1	22.41 ± 2.84^{a}	24.61 ± 3.35^{a,b,c}	20.80 ± 6.66^{b}	20.33 ± 0.96^{a}	12.38 ± 1.93^{b}	64.17 ± 0.79^{a}	1.60 ± 0.43^{c}
Lb. plantarum PL2	23.39 ± 3.84^{a}	27.28 ± 2.73^{a}	26.93 ± 1.22^{a}	20.23 ± 1.55^{a}	11.85 ± 1.23^{b}	60.15 ± 7.25^{a,b}	1.07 ± 0.39^{c,d}
Lb. plantarum PL3	15.52 ± 2.44^{b}	21.07 ± 5.83^{a,b,c}	8.21 ± 1.53^{c}	15.36 ± 0,63^{c,d}	10.08 ± 1.95^{b,c}	45.92 ± 4.39^{c,d}	-1.15 ± 0.37^{e}
Lb. plantarum PL4	14.53 ± 2.24^{b,c}	17.50 ± 2.23^{b,c,d}	5.44 ± 1.07^{c,d}	13.41 ± 0.45^{d,e}	11.24 ± 0.92^{b}	51.18 ± 0.93^{b,c,d}	-0.08 ± 0.61^{d,e}
Lb. plantarum PL5	17.85 ± 0.55^{a,b}	15.67 ± 3.06^{c,d,e}	0.47 ± 0.39^{d,e}	9.27 ± 0.53^{f}	-0.23 ± 3.48^{d}	43.63 ± 2.73^{d}	8.72 ± 0.45^{b}
Lb. crustorum CR	7.25 ± 1.45^{d}	8.54 ± 2.11^{d,e,f}	4.28 ± 0.52^{c,d}	16.47 ± 1.67^{b,c}	11.27 ± 0.99^{b}	52.21 ± 0.96^{a,b,c,d}	8.91 ± 0.96^{b}
Lb. pentosus PE	4.90 ± 2.48^{d}	5.94 ± 1.31^{f}	-2.86 ± 0.40^{e}	8.21 ± 0.04^{f}	2.43 ± 3.24^{c,d}	44.28 ± 3.50^{d}	15.06 ± 0.51^{a}
Lb. brevis BR1	17.92 ± 0.36^{a,b}	7.08 ± 3.02^{e,f}	16.14 ± 1.99^{b}	17.02 ± 0.61^{b,c}	3.54 ± 0.95^{c,d}	57.59 ± 2.48^{a,b,c}	0.62 ± 0.39^{c,d}
Lb. brevis BR2	15.67 ± 3.36^{b}	11.18 ± 2.96^{d,e,f}	17.73 ± 1.22^{b}	15.56 ± 1.43^{c,d}	12.28 ± 5.12^{b}	62.86 ± 5.81^{a,b}	0.20 ± 0.31^{c,d}

Means with different superscript letters differ significantly ($p < 0.05$).

Gram-negative bacteria such as *Salmonella* spp. has also been reported (Calix-Lara et al., 2014; Ferrari et al., 2016; Heredia-Castro et al., 2015).

Generally, concerning Gram-positive pathogens, the inhibition was not detectable, or weak antimicrobial effect, around 20%, was found. However, the results indicated that *Lb. plantarum* PL1 and PL2 were the strains that show a greater antimicrobial effect, with activity against the three *Listeria* spp. strains, *B. cereus* CECT 131 and *S. choleraesuis* CECT4395. Similar results were obtained by other authors for *L. plantarum* strains isolated from traditional cheeses (Oldak, Zielmska, Rzepkowska, & Kolozyn-Krajewska, 2017). In a recent study, *L. plantarum* isolated from artisanal cheeses and identified as the main bacteriocin-producing species, demonstrated antimicrobial activity against one or two distinct strains of *L. monocytogenes*. In a subsequent study, one of these strains was inoculated in micro cheeses, leading to a reduction of 2.52 log CFU/g in the count of *L. monocytogenes,* after 21 days of maturation (Margalho et al., 2020). Regarding the other strains under study, *Lb. plantarum* PL3 and PL4 as well as *Lb. casei* (CA) also exhibited a weak activity (around 20%) against the *L. monocytogenes* CECT 934, but still standing out from the other strains in terms of this antimicrobial effect.

The consumption of raw, soft or semi-soft cheeses with high moisture content, may be associated with the transmission of different infectious diseases, the greatest fears being those related to the possible trans- mission of *L. monocytogenes* (Chourasia et al., 2021; Food & Authority, 2018; Verraes et al., 2015; West, 2008; Yoon, Lee, & Choi, 2016). In Europe, positive results for sheep cheese were reported in Portugal, in 2017. Many studies have been focused on the control of *L. monocytogenes* in cheese, using protective LAB (Coelho, Silva, Ribeiro, Dapkevicius, & Rosa, 2014; Margalho et al., 2020; Susana C.; Ribeiro, O'Connor, Ross, Stanton, & Silva, 2016). The present study demonstrated that *Lb. plan- tarum* PL1 and PL2 were able to inhibit 5 of the 7 strains tested including the pathogenic *L. monocytogenes*, which, together with *Lb. paracasei* that exerts weak antimicrobial action on *S. aureus,* suggests the potential of these strains as potential biopreservatives in cheese.

3.6. Integration of properties for autochthonous starter culture selection

The integration of properties studied was carried out using the PCA methodology. Two analyses were carried out: (i) on the antimicrobial effect and (ii) on the technological parameters. In the bi-plot PCA of the antimicrobial effect (Fig. 3), the inhibitory capacity against all potential pathogen bacteria strains studied were used namely, the inhibition ef- fect on *L. monocytogenes* CECT 911, *L. monocytogenes* CECT 934, *L. innocua* CECT 910, *B. cereus* CECT 131, *S. aureus* CECT 976, *S. choleraesuis* CECT 4395 and *E. coli* CECT 4267. The similarity map defined by the first two principal components took into account 76.3% of the total variance. The first component (PC1) by itself condensed 56.8% and the second component (PC2) represented 19.6% of the total variance. The PC1 presented

negative correlations with *E. coli* CECT 4267 and positive correlations with *S. choleraesuis* CECT 4395, *L. monocytogenes* CECT 911, *B. cereus* CECT 131 and *L. innocua* CECT 910. The PC2 was negatively correlated to *S. aureus* CECT 976. The antimicrobial effect on *L. monocytogenes* CECT 934 was correlated simultaneously with the PC1 (positive values) and PC2 (negative values).

Some strains belonging to the same species were groups in the PCA plot namely, *Lb. brevis BR1* and *BR2 strains, Lb. plantarum* PL1 and PL2 strains as well as *Lb. plantarum* PL3 and PL4 (Fig. 3). *Lb. plantarum* PL1 and PL2 strains stood out in terms of combined antimicrobial effect against *L. monocytogenes* CECT 911, *L. innocua* CECT 910, *B. cereus* CECT 131 and *S. choleraesuis* CECT 4395, as suggested by Fig. 3. As also illustrated in the biplot, the strain *Lb. pentosus* (PE) is the strain that showed the greatest antimicrobial activity against *E. coli* CECT 4267 strain. Fig. 3 also indicate that *Lb. paracasei* PC also stood out in terms of *S. aureus* CECT 976 antimicrobial effects.

A PCA analysis of the technological attributes was also carried out on eight parameters namely, resistance to temperature and salt (growth at 30 and 15 ◦C during 24 h; and growth at 2 and 6% of NaCl during 24 h at 30 ◦C); the production of lactate (total g/L and D(—)-lactic acid%) and acidifying activity (pH at 30 ◦C after 8 h and after 24 h). The similarity map defined by the first two principal components took into account 72.1% of the total variance. The first component (PC1) by itself condensed 47.9% and the second component (PC2) represented 24.2% of the total variance. The PC1 presented negative correlations with growth at 30 ◦C and 15 ◦C, growth at 30 ◦C with 6% of NaCl and total lactate production (g/L) and positive correlations with pH values after 24 h of incubation. The PC2 was negatively correlated to the percentage of D(—)-lactic acid. The growth at 30 ◦C with 2% of NaCl was correlated simultaneously with the PC1 (negative values) and PC2 (positive values).

Strains belonging to the same species namely, *Lb. plantarum* PL1, PL2, PL3 and PL4 as well as *Lb. brevis* BR1 and BR2, were grouped in the PCA plot (Fig. 4). As we can see in the biplot, the strains *Lb. brevis* BR1 and BR2, *Lb. crustorum* CR and *Lb. paracasei* PC stood out in terms of pH values and, at the same time, were the ones with the least total lactate production (g/L), suggesting the lower acidification capacity and pro- duction of lactic acid of these strains. On the other side, *Lb. plantarum* PL5 revealed highest D-lactic (g/L) production. The results also indicated that *Lb. plantarum* PL1, PL2, PL3 and PL4, *Lb. pentosus* PE and *Lb. casei* CA were the strains that produced higher concentration of total lactate (g/L). The location in the PCA plot also suggested that *Lb. plantarum* PL1, PL2, PL3 and PL4, *Lb. pentosus* PE and *Lb. casei* CA were the strains with the more technological ability for Serpa cheesemaking process as well, owning to the higher acidification potential and the lower D-lactic pro- duction but also good resistances to temperature and salt (growth at 30 and 15 ◦C during 24 h; and growth at 2 and 6% of NaCl during 24 h at 30 ◦C).

Lb. casei CA was the one more adapted to the technological param- eters however, this strain did not exhibit proteolytic activity in PCA medium supplemented with skim milk (technological property not

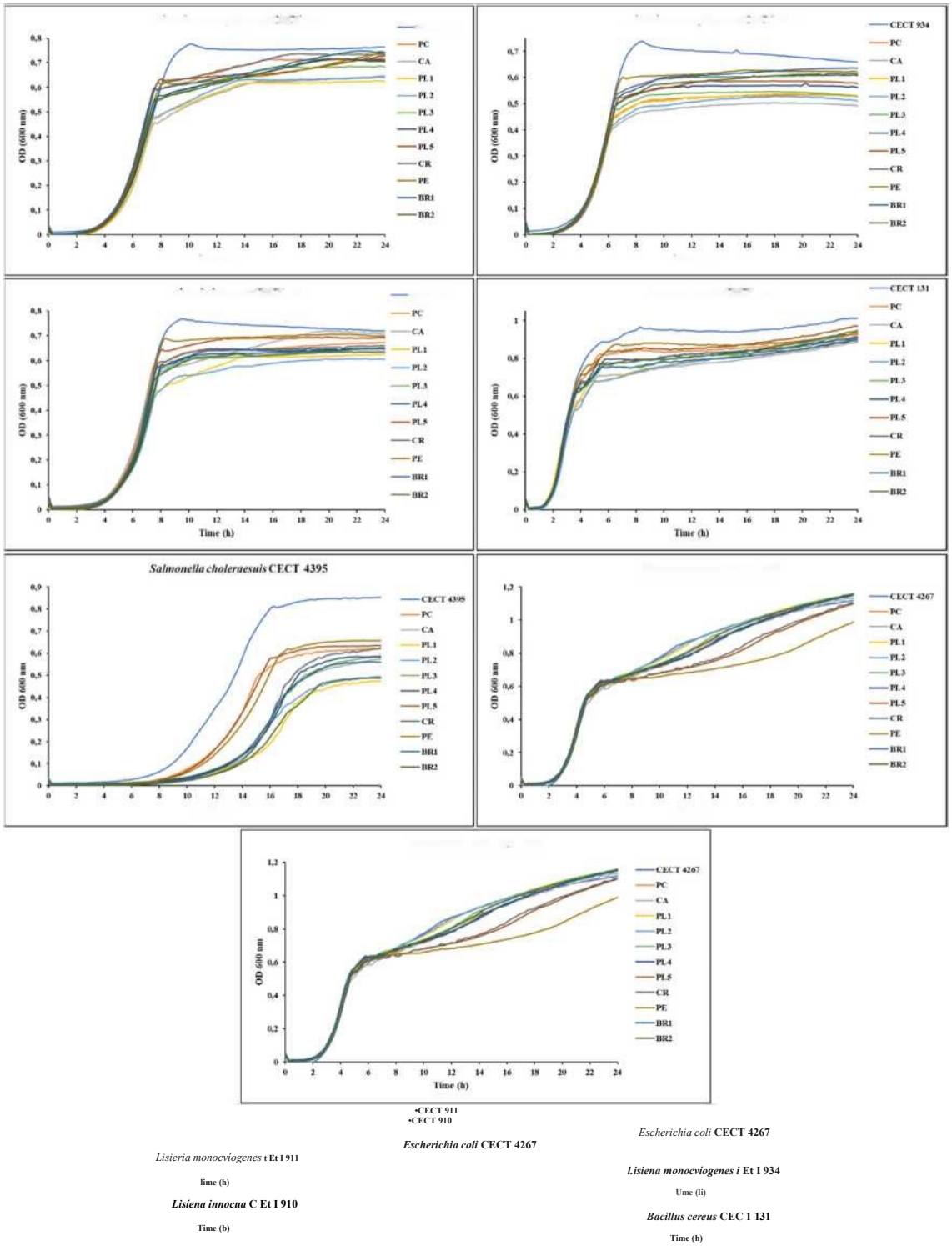

Fig. 2. Growth curves of *Listeria monocytogenes* CECT 911, *Listeria monocytogenes* CECT 934, *Listeria*. *innocua* CECT 910, *Bacillus cereus* CECT 131, *Staphylococcus aureus* CECT 976, *Salmonella choleraesuis* CECT 4395 and *Escherichia coli* CECT 4267 strains in BHI broth (Control: CECT_XXX) and in BHI broth **supplemented with 10% LAB supernatants.** Three experimental replicates were performed.

present in the PCA plot). The results also suggest that *Lb. plantarum* PL2, with a more central position in Fig. 4, did not show proteolytic activity. Regarding lipolytic activity, the results indicated that only four strains exhibited this activity in tributyrin agar namely, *Lb. plantarum* PL1, PL4 and PL5 as well as *Lb. paracasei* PC.

Accordingly, the bacterial strains *Lb. plantarum* PL1 and PL4 may be the best candidates for the autochthonous starter culture, since the re- sults indicated that these strains are well adapted to the technological conditions and, additionally, include both lipolytic and extracellular proteolytic activities, which are extremely relevant bioactivities for the cheesemaking process and autochthonous starter culture development. *Lb. plantarum* PL1 also showed antimicrobial effect against all *Listeria* spp. strains, *B. cereus* CECT 131 and *S. choleraesuis* CECT 4395. Although
Lb. paracasei strain (PC) was one of the strains that showed lower acidification activity, due to their proteolytic and lipolytic potential, this strain could be combined with other strain with more acidifying po- tential to develop an autochthonous starter culture. This strain also possesses higher antimicrobial activity

against *S. aureus* CECT 976. *Lb. plantarum* PL5 also showed proteolytic and lipolytic activities however, the results suggested that this strain produced higher amounts of D-lactic isomer that can be detrimental for the final product.

4. Conclusions

In this study, the technological and protective performance of 11 *Lactobacillus* spp. isolated from Serpa cheese was evaluated to select

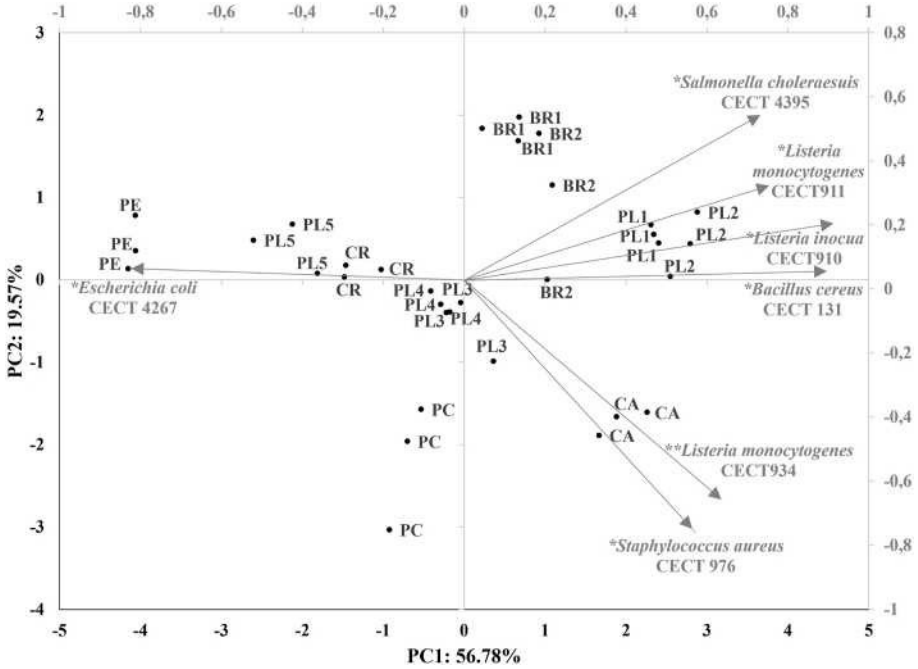

Fig. 3. Bi-plot principal component analysis for the antimicrobial effect: PC1 vs. PC2 projection of samples (n = 3). The most important variables for the definition of the two components are shown on the edge of each axis, indicating the direction in which the value of the parameter increases. In the plot the different LAB strains codes are represented. *Marked values were considered strongly (|r| > 0.7) correlated with the PC; **marked values were considered moderately (0.6 < |r| < 0.7) correlated simultaneous with the PC1 and PC2 following the classification used previously (Palma et al., 2009; Alvarenga et al., 2018).

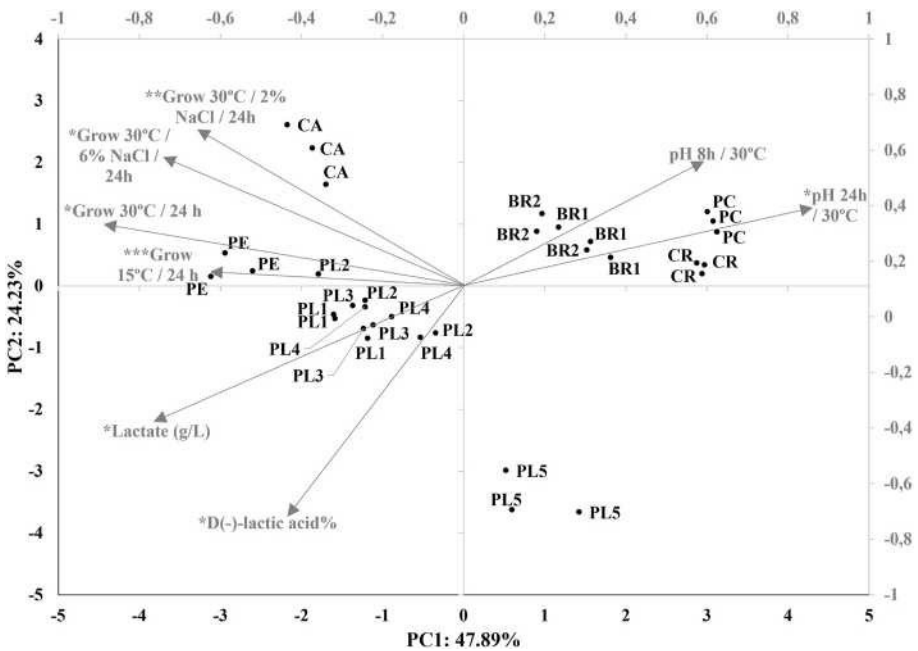

Fig. 4. Bi-plot principal component analysis for technological characterization: PC1 vs. PC2 pro-jection of samples (n = 3). The most important variables for the definition of the two components are shown on the edge of each axis, indicating the direc- tion in which the value of the parameter increases. In the plot the different LAB strains codes are repre- sented. *Marked values were considered strongly (|r| > 0.7) correlated with the PC; **marked values were considered moderately (0.6 < |r| < 0.7) correlated simultaneous with the PC1 and PC2; ***marked values were considered moderately (0.6 < |r| < 0.7) correlated with PC1 following the classification used previously (Palma et al., 2009; Alvarenga et al., 2018).

some LAB candidates for the development of autochthonous starter culture, which were previously reported as food GRAS by Ruiz-Moyano et al. (2019). The results indicated that most strains possessed a good or moderate acidification capacity, and these were well adapted to the cheesemaking conditions. The integration of technological properties in the PCA plot stands out *Lb. plantarum* PL1, PL2, PL3 and PL4, *Lb. pentosus* PE and *Lb. casei* CA as the most adapted

strains to the salt and temper- ature conditions as well as possessing the higher acidification potential and the lower D-lactic production.

However, only four strains showed both extracellular proteolytic and lipolytic activities (*Lb. paracasei* PC and *Lb. plantarum* PL1, PL4 and PL5). Combining these bioactivities with the other technological properties, *Lb. plantarum* PL1 and PL4 could be the most promising strains for a starter culture development. *Lb. plantarum* PL1 also showed some anti- microbial effect, with activity against the three *Listeria* spp. *strains, B. cereus* CECT 131 and *S. choleraesuis* CECT 4395 growth. *Lb. paracasei* strain (PC) can also be tested in a mixed autochthonous starter culture, combined with a more acidifying strain. This strain also possesses higher antimicrobial activity against *S. aureus* CECT 976 strain. All strains showed moderated activity against *S. choleraesuis* CECT 4395 growth.

It is important to establish cheese model systems and tests to eval- uate these strains singly and in combination, understanding their impact in acidification, fatty acid and FAAs production as well as in other key parameters such as, aroma, texture and flavor. This additional screening will allow to select and implement an autochthonous starter culture in Serpa cheese and overcome quality and safety shortcomings,
maximizing the resources in the small ruminant sector.

CRediT authorship contribution statement

Helena Araújo-Rodrigues: Formal analysis, Investigation, Writing
- original draft. **Maria Teresa P.G. dos Santos:** Formal analysis, Investigation, Writing - review & editing. **Santiago Ruiz-Moyano:** Formal analysis, Investigation, Writing - review & editing. **Freni K. Tavaria:** Supervision, Writing - review & editing. **Antoónio P.L. Martins:** Supervision, Writing - review & editing. **Nuno Alvarenga:** Su- pervision, Formal analysis, Writing - review & editing. **Manuela E. Pintado:** Project administration, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare no conflict of interest regarding the publication of this Research paper.

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References

Agarwal, S., Sharma, K., Swanson, B. G., Yuksel, G.U., & Clark, S. (2006). Nonstarter lactic acid bacteria biofilms and calcium lactate crystals in cheddar cheese. *Journal of Dairy Science, 89*(5), 1452-1466. https://doi.org/10.3168/jds.S0022-0302(06)72213-5

Alvarenga, N., Silva, P., Garcia, J. R., & Sousa, I. (2008). Estimation of Serpa cheese ripening time using multiple linear regression (MLR) considering rheological, physical and chemical data. *Journal of Dairy Research, 75*(2), 233-239. https://doi.org/10.1017/S0022029908003191

Araújo-Rodrigues, H., Tavaria, F. K., dos Santos, M. T. P. G., Alvarenga, N., & Pintado, M. M. (2020). A review on microbiological and technological aspects of Serpa PDO cheese: An ovine raw milk cheese. *International Dairy Journal, 100*, 104561. https://doi.org/10.1016/j.idairyj.2019.104561

Bassi, D., Puglisi, E., & Cocconcelli, P. S. (2015). Comparing natural and selected starter cultures in meat and cheese fermentations. *Current Opinion in Food Science, 2*, 118-122. https://doi.org/10.1016/j.cofs.2015.03.002

Beresford, T. P., Fitzsimons, N. A., Brennan, N. L., & Cogan, T. M. (2001). Recent advances in cheese microbiology. *International Dairy Journal, 11*(4-7), 259-274. https://doi.org/10.1016/S0958-6946(01)00056-5

Caólix-Lara, T. F., Rajendran, M., Talcott, S. T., Smith, S. B., Miller, R. K., Castillo, A., et al. (2014). Inhibition of Escherichia coli O157:H7 and Salmonella enterica on spinach and identification of antimicrobial substances produced by a commercial Lactic Acid Bacteria food safety intervention. *Food Microbiology, 38*, 192-200. https://doi.org/10.1016/j.fm.2013.09.006

Caémara, S. P., Dapkevicius, A., Riquelme, C., Elias, R. B., Silva, C. C. G., Malcata, F. X., et al. (2019). Potential of lactic acid bacteria from Pico cheese for starter culture development. *Food Science and Technology International, 25*(4), 303-317. https://doi.org/10.1177/1082013218823129

Castillo Martinez, F. A., Balciunas, E. M., Salgado, J. M., Domínguez Gonzaólez, J. M., Converti, A., & Oliveira, R. P. de S. (2013). Lactic acid properties, applications and production: A review. *Trends in Food Science & Technology, 30*(1), 70-83. https://doi.org/10.1016/j.tifs.2012.11.007

Chou, Y. E., Edwards, C. G., Luedecke, L. O., Bates, M. P., & Clark, S. (2003). Nonstarter lactic acid bacteria and aging temperature affect calcium lactate crystallization in Cheddar cheese. *Journal of Dairy Science, 86*(8), 2516-2524. https://doi.org/10.3168/jds.S0022-0302(03)73846-6

Chourasia, R., Abedin, M. M., Chiring Phukon, L., Sahoo, D., Singh, S. P., & Rai, A. K. (2021). Biotechnological approaches for the production of designer cheese with improved functionality. *Comprehensive Reviews in Food Science and Food Safety, 20*(1), 960-979. https://doi.org/10.1111/1541-4337.12680

Coelho, M. C., Silva, C. C. G., Ribeiro, S. C., Dapkevicius, M. L. N. E., & Rosa, H. J. D. (2014). Control of Listeria monocytogenes in fresh cheese using protective lactic acid bacteria. *International Journal of Food Microbiology, 191*, 53-59. https://doi.org/ 10.1016/j.ijfoodmicro.2014.08.029

Dolci, P., Ferrocino, I., Giordano, M., Pramotton, R., Vernetti-Prot, L., Zenato, S., et al. (2020). Impact of Lactococcus lactis as starter culture on microbiota and metabolome profile of an Italian raw milk cheese. *International Dairy Journal, 110*, 104804. https://doi.org/10.1016/j.idairyj.2020.104804

Ferrari, I. da S., de Souza, J. V., Ramos, C. L., da Costa, M. M., Schwan, R. F., & Dias, F. S. (2016). Selection of autochthonous lactic acid bacteria from goat dairies and their addition to evaluate the inhibition of Salmonella typhi in artisanal cheese. *Food Microbiology, 60*, 29-38. https://doi.org/10.1016/j.fm.2016.06.014

Food, E., & Authority, S. (2018). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017. *EFSA Journal, 16*(12). https://doi.org/10.2903/j.efsa.2018.5500

Freitas, C., & Malcata, F. X. (2000). Microbiology and biochemistry of cheeses with appóelation d'origine protegóee and manufactured in the Iberian Peninsula from ovine and caprine milks. *Journal of Dairy Science, 83*(3), 584-602. https://doi.org/10.3168/jds.S0022-0302(00)74918-6

Garmiene, G., Saikauskiene, V., & Kulikauskiene, M. (2005). Lactic acid isomers in milk products. *Milchwissenschaft, 60*(3), 259-262.

Garvie, E. I. (1980). Bacterial lactate dehydrogenases. *Microbiological Reviews, 44*(1), 106-139. https://doi.org/10.1128/mmbr.44.1.106-139.1980

Gonçalves Dos Santos, M. T. P., Benito, M. J., Cóordoba, M. de G., Alvarenga, N., & Ruiz- Moyano Seco de Herrera, S. (2017). Yeast community in traditional Portuguese Serpa cheese by culture-dependent and -independent DNA approaches. *International Journal of Food Microbiology, 262*(July), 63-70. https://doi.org/10.1016/j. ijfoodmicro.2017.09.013

Gonçalves, M. T. P., Benito, M. J., Coórdoba, M. de G., Egas, C., Merchóan, A. V., Galvaón, A. I., et al. (2018). Bacterial communities in Serpa cheese by culture dependent techniques, 16S rRNA gene sequencing and high-throughput sequencing analysis. *Journal of Food Science, 83*(5), 1333-1341. https://doi.org/10.1111/1750-3841.14141

Heredia-Castro, P. Y., Meóndez-Romero, J. I., Hernóandez-Mendoza, A., Acedo-Feólix, E., Gonzaólez-Coórdova, A. F., & Vallejo-Cordoba, B. (2015). Antimicrobial activity and partial characterization of bacteriocin-like inhibitory substances produced by Lactobacillus spp. isolated from artisanal Mexican cheese. *Journal of Dairy Science, 98*(12), 8285-8293. https://doi.org/10.3168/jds.2015-10104

Holzapfel, W. H. (2002). Appropriate starter culture technologies for small-scale fermentation in developing countries. *International Journal of Food Microbiology, 75* (3), 197-212. https://doi.org/10.1016/S0168-1605(01)00707-3

Johnson, M. E., Riesterer, B. A., & Olson, N. F. (1990). Influence of nonstarter bacteria on calcium lactate crystallization on the surface of cheddar cheese. *Journal of Dairy Science, 73*(5), 1145-1149. https://doi.org/10.3168/jds.S0022-0302(90)78775-9

Leroy, F., & De Vuyst, L. (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science & Technology, 15*(2), 67-78. https://doi.org/10.1016/j.tifs.2003.09.004

Li, J., Huang, Q., Zheng, X., Ge, Z., Lin, K., Zhang, D., et al. (2020). Investigation of the lactic acid bacteria in kazak cheese and their contributions to cheese fermentation. *Frontiers in Microbiology, 11*(March), 1-13. https://doi.org/10.3389/ fmicb.2020.00228

Marco, M. L., Heeney, D., Binda, S., Cifelli, C. J., Cotter, P. D., Foligneó, B., et al. (2017). Health benefits of fermented foods: Microbiota and beyond. *Current Opinion in Biotechnology, 44*, 94-102. https://doi.org/10.1016/j.copbio.2016.11.010

Margalho, L. P., Feliciano, M. D. E., Silva, C. E., Abreu, J. S., Piran, M. V. F., & Sant'Ana, A. S. (2020). Brazilian artisanal cheeses are rich and diverse sources of nonstarter lactic acid bacteria regarding technological, biopreservative, and safety properties—insights through multivariate analysis. *Journal of Dairy Science, 103*(9), 7908-7926. https://doi.org/10.3168/jds.2020-18194

Martín, R., Olivares, M., Marín, M. L., Fernaóndez, L., Xaus, J., & Rodríguez, J. M. (2005). Probiotic potential of 3 lactobacilli strains isolated from breast milk. *Journal of Human Lactation, 21*(1), 8-17. https://doi.org/10.1177/0890334404272393

McSweeney, P. L. H., & Sousa, M. J. (2000). Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review. *Le Lait, 80*(3), 293-324. https://doi.org/10.1051/lait:2000127

Montel, M. C., Buchin, S., Mallet, A., Delbes-Paus, C., Vuitton, D. A., Desmasures, N., et al. (2014). Traditional cheeses: Rich and diverse microbiota with associated benefits. *International Journal of Food Microbiology, 177*, 136-154. https://doi.org/ 10.1016/j.ijfoodmicro.2014.02.019

Nieto-Arribas, P., Poveda, J. M., Sesena, S., Palop, L., & Cabezas, L. (2009). Technological characterization of Lactobacillus isolates from traditional Manchego cheese for potential use as adjunct starter cultures. *Food Control, 20*(12), 1092-1098. https://doi.org/10.1016/j.foodcont.2009.03.001

Oldak, A., Zielinska, D., Rzepkowska, A., & Kolozyn-Krajewska, D. (2017). Comparison of antibacterial activity of Lactobacillus plantarum strains isolated from two different kinds of regional cheeses from Poland: Oscypek and korycinski cheese. *BioMed Research International*, 1-10. https://doi.org/10.1155/2017/6820369. *2017*.

Ribeiro, S. C., Coelho, M. C., Todorov, S. D., Franco, B. D. G. M., Dapkevicius, M. L. E., & Silva, C. C. G. (2014). Technological properties of bacteriocin-producing lactic acid bacteria isolated from Pico cheese an artisanal cow's milk cheese. *Journal of Applied Microbiology, 116*(3), 573-585. https://doi.org/10.1111/jam.12388

Ribeiro, S. C., O'Connor, P. M., Ross, R. P., Stanton, C., & Silva, C. C. G. (2016). An anti- listerial Lactococcus lactis strain isolated from Azorean Pico cheese produces lacticin 481. *International Dairy Journal, 63*, 18-28. https://doi.org/10.1016/j. idairyj.2016.07.017

Rolfe, M. D., Rice, C. J., Lucchini, S., Pin, C., Thompson, A., Cameron, A. D. S., et al. (2012). Lag phase is a distinct growth phase that prepares bacteria for exponential growth and involves transient metal accumulation. *Journal of Bacteriology, 194*(3), 686-701. https://doi.org/10.1128/JB.06112-11

Roseiro, L. B., Andrew Wilbey, R., & Barbosa, M. (2003). Serpa Cheese: Technological, biochemical and microbiological characterisation of a PDO Ewe's milk cheese coagulated with Cynara cardunculus L. *Le Lait, 83*(6), 469-481. https://doi.org/ 10.1051/lait:2003026

Roseiro, L. B., Gómez-Ruiz, J. A., García-Risco, M., & Molina, E. (2003). Article. *Le Lait, 83*(4), 343-350. https://doi.org/10.1051/lait:2003017

Ruiz-Moyano, S., Gonçalves dos Santos, M. T. P., Galvóan, A. I., Merchóan, A. V., Gonzaólez, E., Coórdoba, M. de G., et al. (2019). Screening of autochthonous lactic acid bacteria strains from artisanal soft cheese: Probiotic characteristics and prebiotic metabolism. *Lebensmittel-Wissenschaft & Technologie, 114*(May), 108388. https://doi. org/10.1016/j.lwt.2019.108388

Rulikowska, A., Kilcawley, K. N., Doolan, I. A., Alonso-Gomez, M., Nongonierma, A. B., Hannon, J. A., et al. (2013). The impact of reduced sodium chloride content on Cheddar cheese quality. *International Dairy Journal, 28*(2), 45-55. https://doi.org/ 10.1016/j.idairyj.2012.08.007

Schornsteiner, E., Mann, E., Bereuter, O., Wagner, M., & Schmitz-Esser, S. (2014). Cultivation-independent analysis of microbial communities on Austrian raw milk hard cheese rinds. *International Journal of Food Microbiology, 180*, 88-97. https://doi. org/10.1016/j.ijfoodmicro.2014.04.010

Silvetti, T., Capra, E., Morandi, S., Cremonesi, P., Decimo, M., Gavazzi, F., et al. (2017). Microbial population profile during ripening of Protected Designation of Origin (PDO) Silter cheese, produced with and without autochthonous starter culture. *Lebensmittel-Wissenschaft & Technologie, 84*, 821-831. https://doi.org/10.1016/j. lwt.2017.06.022

Somers, E. B., Johnson, M. E., & Wong, A. C. L. (2001). Biofilm formation and contamination of cheese by nonstarter lactic acid bacteria in the dairy environment. *Journal of Dairy Science, 84*(9), 1926-1936. https://doi.org/10.3168/jds.S0022- 0302(01)74634-6

Swearingen, P. A., Adams, D. E., & Lensmire, T. L. (2004). Factors affecting calcium lactate and liquid expulsion defects in Cheddar cheese. *Journal of Dairy Science, 87* (3), 574-582. https://doi.org/10.3168/jds.S0022-0302(04)73199-9

Tavaria, F. K., Tavares, T. G., Silva-Ferreira, A. C., & Malcata, F. X. (2006). Contribution of coagulant and native microflora to the volatile-free fatty acid profile of an artisanal cheese. *International Dairy Journal, 16*(8), 886-894. https://doi.org/ 10.1016/j.idairyj.2005.08.004

Verraes, C., Vlaemynck, G., Van Weyenberg, S., De Zutter, L., Daube, G., Sindic, M., et al. (2015). A review of the microbiological hazards of dairy products made from raw milk. *International Dairy Journal, 50*, 32-44. https://doi.org/10.1016/j. idairyj.2015.05.011

West, H. G. (2008). Food fears and raw-milk cheese. *Appetite, 51*(1), 25-29. https://doi. org/10.1016/j.appet.2008.02.004

Yeluri Jonnala, B. R., McSweeney, P. L. H., Sheehan, J. J., & Cotter, P. D. (2018). Sequencing of the cheese microbiome and its relevance to industry. *Frontiers in Microbiology, 9*(MAY). https://doi.org/10.3389/fmicb.2018.01020

Yilmaz, G., Ayar, A., & Akin, N. (2005). The effect of microbial lipase on the lipolysis during the ripening of Tulum cheese. *Journal of Food Engineering, 69*(3), 269-274. https://doi.org/10.1016/j.jfoodeng.2004.08.017

Yoon, Y., Lee, S., & Choi, K.-H. (2016). Microbial benefits and risks of raw milk cheese. *Food Control, 63*, 201-215. https://doi.org/10.1016/j.foodcont.2015.11.013

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