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 (200/200)

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 circumvent that, 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 PDO cheese and reported as generally recognized as safe, were screened. Most of them were well adapted to the cheesemaking process, showing a good acidification capacity and antimicrobial effect. The properties integration 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, 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.

KEYWORDS: Serpa PDO cheese; Lactic acid bacteria; Technological attributes; Autochthonous starter culture.

1. INTRODUCTION

Serpa is a Protected Designation of Origin (PDO) cheese, manufactured from ovine raw milk and vegetable coagulant (*Cynara cardunculus* L.), without pasteurization or other thermal process, or addition of any starter culture (Araújo-Rodrigues et al., 2020; Freitas & Malcata, 2000; Gonçalves Dos Santos et al., 2017; Gonçalves et al., 2018; Yeluri Jonnala et al., 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 et al., 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, Gómez-Ruiz, et al., 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 (Araújo-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 microorganisms in the complex

microbial community present in raw milk (Chourasia et al., 2021; Leroy & De Vuyst, 2004; Montel et al., 2014; Tavaria et al., 2006).

The use of autochthonous starter cultures in the artisanal cheesemaking 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 microorganisms over the raw or pasteurized milk microbiota, ensuring a favorable microenvironment for the technological process and optimizing 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 et al., 2015; Chourasia et al., 2021; Dolci et al., 2020).

Although the use of a starter culture is not permitted by the Portuguese legislation for PDO cheeses, a tailor-made starter culture may contribute to introduce in the market new products with sensory properties 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 et al., 2003; Leroy & De Vuyst, 2004; Nieto-Arribas et al., 2009; Schornsteiner et al., 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 (Câmara et al., 2019; Chourasia et al., 2021; Li et al., 2020; Nieto-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 protective performance of LAB isolated from Serpa cheese in order to select and develop an autochthonous starter culture for this traditional product. 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 *Lactobacillus* 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 ºC for 72 h. Regarding the extracellular proteolytic activity, 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 ºC 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 surrounding the colonies' growth.

2.2.2 RESISTANCE TO TEMPERATURE AND SALT

Growth in MRS broth at different NaCl concentrations and temperatures 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 ºC, at intervals of 1 h. Regarding the ability to grow at different temperatures, namely 4, 15, 30 and 45 ºC 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 modifications. The bacterial isolates were grown in MRS broth at 30 °C overnight 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 ºC, 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 ºC, 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 8000 × g for 5 min, before filtering through 0.22 μm filters (Thermo Fisher Scientific). The lactate isomeric types present in fermented broth were 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 allows 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), supplemented with 10% of supernatant filtered-sterilized from each LAB strain, was evaluated by following the microbial growth at 37 ºC in an automated turbidometer Bioscreen C Analysing System (Labsystems, Helsinki, Finland). Each LAB supernatant was obtained from an overnight culture at 37 ºC 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 pathogen 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

198 variance (ANOVA), at a degree of significance of α=0.05. Data were compared
199 statistically using ANOVA to understand the significance and confirm a normal
200 distribution of the data. Post-hoc multiple comparisons were carried out using Turkey's
201 test (α=0.05). A biplot principal component analysis (PCA) was performed in order to
202 have a global integration of technological and protective properties for autochthonous
203 starter culture selection.
204
205 **3. RESULTS AND DISCUSSION**
206 Ruiz-Moyano et al. (2019) evaluated the acid-tolerant properties of 116 LAB isolated
207 and identified from Serpa PDO cheese with 30 days of ripening (Gonçalves et al., 2018).
208 In the reported work, 20 of these microbial strains exhibited acid-tolerant properties and
209 some safety and probiotic attributes were investigated. In this work, the characterization
210 of 11 of these autochthonous strains, reported as GRAS by Ruiz-Moyano et al. (2019),
211 was performed in a technological and protective perspective. Some safety and probiotic
212 characteristics studied by Ruiz-Moyano et al. (2019) are summarized in Table 1.
213 Important technological and protective features for the cheesemaking process of these
214 autochthonous LAB strains were carried out in the present study to select promising
215 strains well adapted to the fermentation process, for the development of autochthonous
216 starter culture.
217
218 ***Lipolytic and extracellular proteolytic potential***
219 In Table 2, the extracellular proteolytic and lipolytic activities of the LAB under study
220 are presented. The results anticipated that only *Lb. casei* CA and *Lb. plantarum* PL2
221 strains did not exhibit extracellular proteolytic activity in PCA medium supplemented
222 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 microorganisms, 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 specific attributes of each cheese (Yilmaz et al., 2005).

FFAs, peptides and FAAs resultant from both bioactivities are relevant organoleptic-related compounds, making these activities essential for starter culture selection and development. These molecules are also 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 Manchego cheese (Nieto-Arribas et al., 2009), Câmara 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 (Câmara 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 capacity

reported for LAB (McSweeney & Sousa, 2000). Regarding proteolytic 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.

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, et al., 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 procedure, milk coagulation of Serpa cheese occurs under a temperature of ca. 30 ºC and maturation between 8 and 13 ºC (Alvarenga et al., 2008; Araújo-Rodrigues et al., 2020; Roseiro, Andrew Wilbey, et al., 2003). The temperature resistance was tested by growing strains at 4, 15, 30 and 45 ºC, as depicted in Figure 1. Generally, the results indicated that the bacterial isolates possessed a higher growth at 30 ºC, being followed by growth at 15 ºC. However, different growth patterns were registered until the exponential phase according to the strain and temperature 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 reparation 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 ºC and maximum

at 30 ºC, 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 ºC but some differences at 45 ºC 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 ºC, these showed the capacity to adapt to this temperature and to display a growth rate similar to the growth presented at 15 ºC. The strain *Lb. pentosus* PE proved to be well adapted to this highest temperature, with growth similar to that registered at 30 ºC. The remaining bacterial isolates were not well adapted to temperature at 45 ºC and showed very low growth. The same was observed at 4 ºC, as well.

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 et al., 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 acidification 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 from 6.47-6.40 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 acidification 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), Ribeiro et al. (2013) and Câmara 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.

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 et al., 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 et al., 2006; Somers et al., 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 et al., 1990; Swearingen et al., 2004), which can be detrimental for consumer acceptance. Consequently, this technological property may be important for starter culture selection and the production of softer cheeses.

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

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

338

339 ***Protective potential***

340 In addition to the natural acidification occurring through cheesemaking and ripening
341 processes, some LAB strains also possess antimicrobial activity that may help to inhibit
342 contaminants, eliminating pathogenic microorganisms (Leroy & De Vuyst, 2004). The
343 antimicrobial activity of the 11 selected bacterial isolates against common foodborne
344 pathogens is presented in Table 5 and, in Figure 2 the corresponding growth inhibition
345 curves. None of the strains showed a high antimicrobial effect against *E. coli* CECT 4267
346 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 inhibition between 41-64%. Although is well known that LAB are more effective against Gram-positive pathogens, inhibitory activity towards Gram-negative bacteria such as *Salmonella* spp. has also been reported (Cálix-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 (Ołdak et al., 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 transmission of *L. monocytogenes* (Chourasia et al., 2021; Food & Authority, 2018; Verraes et al., 2015; West, 2008; Yoon et al., 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 et al., 2014; Margalho et al., 2020; Susana C. Ribeiro et al., 2016). The present study demonstrated that *Lb. plantarum* 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.

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 (Figure 3), the inhibitory capacity against all potential pathogen bacteria strains studied were used namely, the inhibition effect 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 (Figure 3). *Lb. plantarum* PL1 and PL2 strains stood out in terms of

397 combined antimicrobial effect against *L. monocytogenes* CECT 911, *L. innocua* CECT
398 910, *B. cereus* CECT 131 and *S. choleraesuis* CECT 4395, as suggested by Figure 3. As
399 also illustrated in the biplot, the strain *Lb. pentosus* (PE) is the strain that showed the
400 greatest antimicrobial activity against *E. coli* CECT 4267 strain. Figure 3 also indicate
401 that *Lb. paracasei* PC also stood out in terms of *S. aureus* CECT 976 antimicrobial
402 effects.

403 A PCA analysis of the technological attributes was also carried out on eight
404 parameters namely, resistance to temperature and salt (growth at 30 and 15 ºC during 24
405 h; and growth at 2 and 6% of NaCl during 24h at 30 ºC); the production of lactate (total
406 g/ L and D(-)-lactic acid%) and acidifying activity (pH at 30 ºC after 8 h and after 24 h).
407 The similarity map defined by the first two principal components took into account 72.1%
408 of the total variance. The first component (PC1) by itself condensed 47.9% and the second
409 component (PC2) represented 24.2% of the total variance. The PC1 presented negative
410 correlations with growth at 30 ºC and 15 ºC, growth at 30 ºC with 6% of NaCl and total
411 lactate production (g/ L) and positive correlations with pH values after 24 h of incubation.
412 The PC2 was negatively correlated to the percentage of D(-)-lactic acid. The growth at
413 30 ºC with 2% of NaCl was correlated simultaneously with the PC1 (negative values) and
414 PC2 (positive values).

415 Strains belonging to the same species namely, *Lb. plantarum* PL1, PL2, PL3 and PL4
416 as well as *Lb. brevis* BR1 and BR2, were grouped in the PCA plot (Figure 4). As we can
417 see in the biplot, the strains *Lb. brevis* BR1 and BR2, *Lb. crustorum* CR and *Lb. paracasei*
418 PC stood out in terms of pH values and, at the same time, were the ones with the least
419 total lactate production (g/ L), suggesting the lower acidification capacity and production
420 of lactic acid of these strains. On the other side, *Lb. plantarum* PL5 revealed highest D-
421 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 production but also good resistances to temperature and salt (growth at 30 and 15 ºC during 24 h; and growth at 2 and 6% of NaCl during 24 h at 30 ºC).

Lb. casei CA was the one more adapted to the technological parameters however, this strain did not exhibit proteolytic activity in PCA medium supplemented with skim milk (technological property not present in the PCA plot). The results also suggest that *Lb. plantarum* PL2, with a more central position in Figure 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 results 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 potential to develop an autochthonous starter culture. This strain also possesses higher antimicrobial activity against *S. aureus* CECT 976. *Lb. plantarum* PL5 also showed

447 proteolytic and lipolytic activities however, the results suggested that this strain produced
448 higher amounts of D-lactic isomer that can be detrimental for the final product.
449
450 **4. CONCLUSIONS**
451 In this study, the technological and protective performance of 11 *Lactobacillus* spp.
452 isolated from Serpa cheese was evaluated to select some LAB candidates for the
453 development of autochthonous starter culture, which were previously reported as food
454 GRAS by Ruiz-Moyano et al. (2019). The results indicated that most strains possessed a
455 good or moderate acidification capacity, and these were well adapted to the cheesemaking
456 conditions. The integration of technological properties in the PCA plot stands out *Lb.*
457 *plantarum* PL1, PL2, PL3 and PL4, *Lb. pentosus* PE and *Lb. casei* CA as the most adapted
458 strains to the salt and temperature conditions as well as possessing the higher acidification
459 potential and the lower D-lactic production.
460 However, only four strains showed both extracellular proteolytic and lipolytic
461 activities (*Lb. paracasei* PC and *Lb. plantarum* PL1, PL4 and PL5). Combining these
462 bioactivities with the other technological properties, *Lb. plantarum* PL1 and PL4 could
463 be the most promising strains for a starter culture development. *Lb. plantarum* PL1 also
464 showed some antimicrobial effect, with activity against the three *Listeria spp. strains, B.*
465 *cereus* CECT 131 and *S. choleraesuis* CECT 4395 growth. *Lb. paracasei* strain (PC) can
466 also be tested in a mixed autochthonous starter culture, combined with a more acidifying
467 strain. This strain also possesses higher antimicrobial activity against *S. aureus* CECT
468 976 strain. All strains showed moderated activity against *S. choleraesuis* CECT 4395
469 growth.
470 It is important to establish cheese model systems and tests to evaluate these strains
471 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.

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