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Evaluation of technological properties and selection of wild lactic acid bacteria for starter culture development

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ABSTRACT

Sixty-six lactic acid bacteria strains, isolated from traditional cheeses, were identified by MALDI-TOF technology, characterised through the evaluation of their enzymatic activities (acidifying and proteolytic capacities and carboxypeptidase, aminopeptidase, dipeptidase and esterase activities) and selected through a scoring system based on activity results in order to select wild strains of technological interest for cheese manufacturing. The strains were identified as *Lactococcus lactis* (9), *Leuconostoc citreum* (3), *Leuconostoc mesenteroides* (2), *Leuconostoc pseudomesenteroides* (1), *Levilactobacillus brevis* (7), *Lactiplantibacillus plantarum* (32), *Lactiplantibacillus paraplantarum* (7) and *Lacticaseibacillus paracasei* (5). In general, *Lactococcus lactis* strains showed the highest degree of acidifying activity, especially in the first hours of fermentation, and extracellular proteolytic activity. In contrast, intracellular activities, assayed from cell-free extracts, were higher in the lactobacilli strains. *L. paracasei* strains showed the highest level of aminopeptidase activity, while some *L. plantarum* strains obtained high values of dipeptidase activity. Carboxypeptidase activity was very low or undetectable in many strains, although in others the activity values were exceptionally high. Esterolytic activity was generally low, although *L. paracasei* strains showed higher activity on short-chain substrates. Finally, 11 strains were selected using the scoring system that could be used in the design of starter cultures and co-cultures.

1. Introduction

Lactic acid bacteria (LAB) are a very interesting microbial group because of their use as starter cultures to produce fermented products, especially in the dairy industry. In this sector, and especially in Europe, the starter culture market is experiencing a substantial growth in recent years (Tidona et al., 2020). Therefore, there is a continuous search for new candidate strains with specific technological characteristics for the design of new starter cultures. In this way, traditional foods, such as artisanal cheeses, constitute a reservoir of wild LAB that may be of interest to the dairy industry (Câmara et al., 2019). The first important aspect in the isolation and selection of autochthonous strains is the correct identification. Among the bacterial identification methods, the Maldi-Tof MS has established itself as an efficient and very reliable technique for the identification of food-borne micro-organisms, such as LAB. It is a high-throughput identification method based on whole cell protein analysis. Thus, compared to conventional, phenotypic or PCR-based identification, Maldi-Tof MS shows fast turnaround times, low sample volume requirements and modest reagent cost (Sandrin et al., 2013). The efficacy of this method has been tested in LAB by several authors (De Bruyne et al., 2011; Doan et al., 2012; Gantzias et al., 2020; Kanak & Yilmaz, 2019; Teramoto et al., 2007). These authors have demonstrated that Maldi-Tof MS is a valid alternative that can be used for the rapid identification of LAB isolated from fermented foods. Furthermore, it has the potential to replace conventional identification techniques based on genomic fingerprinting.

In cheese making, LAB play a key role because of their enzymatic activities that contribute significantly to the taste, texture, nutritional value and microbiological safety of the cheese (García-Cano et al., 2020; Pangallo et al., 2019). The role of LAB in cheese production has importance in cheese manufacturing, because LAB ferment the lactose of the milk, transformed it in lactic acid, that reduce de pH, and other metabolites as glucose and galactose which promote the formation of cheese flavor; and contribute the cheese ripening, where proteins are degraded in free amino acids (FAAs) and lipids are hydrolyzed to free fatty acids (FFAs) (Li et al., 2020; Meng et al., 2018). During ripening, proteolysis and lipolysis are two important processes in cheese flavor development (Karakas-Sen & Karakas, 2018; Medjoudj et al., 2018). In

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Table 1
Species, number of strains and strain identification of 66 wild lactic acid bacteria strains.

Species	No. of strains	Strains TAUL[a]
Lactococcus lactis	9	TAUL12, TAUL13, TAUL88, TAUL227, TAUL228, TAUL241, TAUL266, TAUL8000, TAUL9000
Leuconostoc citreum	3	TAUL1213, TAUL1226, TAUL1231
Leuconostoc mesenteroides	2	TAUL1341, TAUL1342
Leuconostoc pseudomesenteroides	1	TAUL1798
Levilactobacillus brevis	7	TAUL68, TAUL174o, TAUL174t, TAUL1262, TAUL1267, TAUL1270, TAUL1567
Lactiplantibacillus plantarum subsp. *plantarum*	32	TAUL67, TAUL180, TAUL185, TAUL188, TAUL189, TAUL191, TAUL238, TAUL1259, TAUL1337, TAUL1339, TAUL1368, TAUL1469, TAUL1521, TAUL1539, TAUL1569, TAUL1588, TAUL1641, TAUL1651, TAUL1660, TAUL1667, TAUL1672, TAUL1680, TAUL1689, TAUL1692, TAUL1694, TAUL1695, TAUL1699, TAUL1700, TAUL1746, TAUL1754, TAUL1760, TAUL1765
Lactiplantibacillus paraplantarum	7	TAUL1365, TAUL1399, TAUL1453, TAUL1454, TAUL1457, TAUL1464, TAUL1744
Lacticaseibacillus paracasei	5	TAUL1505, TAUL1508, TAUL1580, TAUL1583, TAUL1752

[a] TAUL: Tecnología de los Alimentos Universidad de León.

this regard, LAB possess genes encoding for a larger number of catabolic enzymes involved in lipolysis, proteolysis and bioconversions of FAAs (Câmara et al., 2019).

Different types of extracellular enzymes are involved in proteolysis, e.g. proteinase, encoded by *prtP* gene, that are able to cleave caseins into smaller peptides; as well as intracellular enzymes (PepP, PepN and PepX) which are involved in the degradation of these peptides in FAAs (Pangallo et al., 2019). These FAAs are first converted into α-ketoacids by the action of aminotransferases (Pangallo et al., 2019) and then these α-ketoacids participate in decarboxylation, deamination, transamination and desulphurization reactions (García-Cano et al., 2019) to form methylketones, aldehydes, ethyl esters, alcohols, sulfur compounds, carboxylic acids and aromatic hydrocarbons, which play a crucial role in determining cheese flavor (Li et al., 2020). In addition to contributing to cheese flavor, proteolysis modifies the texture, improves digestibility, decreases whey protein antigenicity and can generate bioactive peptides (García-Cano et al., 2019). On the other hand, although LAB generally have a weak lipolytic activity, in cheeses with long ripening times, lipolysis reactions release metabolites that even at low concentrations contribute to flavor development and serve as a substrate for other catabolic reactions (García-Cano et al., 2019, 2020).

Traditionally, the selection of LAB for inclusion in starter cultures was focused on strains with high acidification rate, phage resistance and a good salt tolerance (Tidona et al., 2020). However, this strategy decreases biodiversity and the possibility of finding new strains with enzymatic activities to develop and improve the sensory characteristics of cheeses (Câmara et al., 2019). In fact, there are fewer studies that deal in depth with the enzymatic characterization of LAB strains. Therefore, the aim of this study was to evaluate the enzymatic activity of wild LAB strains and to select strains with the best technological characteristics for the design of starter and adjunct cultures for cheese production.

2. Materials and methods

2.1. Strains, media and culture conditions

Table 1 shows the 66 wild LAB strains used in this study from the research group's collection of lactic acid bacteria strains. *Levilactobacillus*, *Lactiplantibacillus*, *Lacticaseibacillus* and *Leuconostoc* strains were grown in MRS broth (Oxoid, Basingstoke, UK) at 32 °C; while *Lactococcus* strains were grown in M17 broth (Difco, Becton Dickinson, Sparks, MD, USA) supplemented with 1% (w/v) glucose (M17-Glu) at 32 °C.

2.2. Strains identification by MALDI-TOF MS

Identification of the isolates was performed by the Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF). Spectra were acquired with the MALDI Biotyper system (Bruker Daltonik, Bremer, Germany) and compared with the reference database (Bruker Daltonik).

2.3. Acidifying activity

Acidifying activity of the strains was determined by measuring pH and titratable acidity after 4, 8 and 24 h of incubation at 32 °C. Tubes containing 50 mL of sterile skimmed milk (reconstituted at 10%) were inoculated in triplicate with a 1% overnight culture. For each time of measure, 9 mL of the milk culture were extracted and pH and titratable acidity were determined.

2.4. Proteolytic activity

Proteolytic activity of whole cells in milk was determined by using the O-phthaldialdehyde (OPA) spectrophotometric assay (Church et al., 1983). This test is based on the absorbance measure at 340 nm of the reaction of the free α-amino groups, released by hydrolysis of the casein after 24 h of incubation, with the OPA, in the presence of β-mercaptoethanol. The results were calculated from a calibration curve obtained from dilution of glycine in distilled water and were expressed in mmol Gly L^{-1} of milk.

2.5. Preparation of cell free extracts and determination of protein concentration

Tubes with 50 mL of appropriate broth were inoculated at 1% and incubated during 16–18 h at 32 °C. After incubation, cultures were centrifuged at 7000×*g* for 10 min at 4 °C and the supernatants were discarded. Then, cells were resuspended in 5 mL of 50 mM of sodium phosphate buffer (pH 7) and centrifuged at 7000×*g* for 10 min at 4 °C. This step was repeated in three times. Pellets were dissolved in 4 mL of 50 mM TRIS-HCl buffer (pH 7.5), incubated at 32 °C for 1 h to release the cell wall-bound proteinases, and then centrifuged at 7000×*g* for 20 min. After centrifugation, pellets were dissolved in 5 mL of 50 mM TRIS-HCl buffer (pH 7.5) and distributed in sterile 2 mL microtubes that contained 0.3 g of 0.1 mm zircon beads. The cell suspensions were lysed by mechanic disrupt in a MiniBead Beatter (Biospec, Oklahoma, USA) into three intervals of 5 min of treatment with two breaks of 5 min in ice to maintain the temperature ≤0 °C. Finally, the cells were centrifuged at 11.000×*g* for 10 min at 0 °C and supernatants were filtered through a 0.22 μm cellulose membrane (Millipore, Burlington, MA, USA) to obtain the cell free extracts (CFE) which was stored frozen at −80 °C. The protein concentration in the CFE was determined according to the method of Lowry (Lowry et al., 1951).

2.6. Proteolytic activities of the CFE

The aminopeptidase activity (AP) of the CFE was determined by the

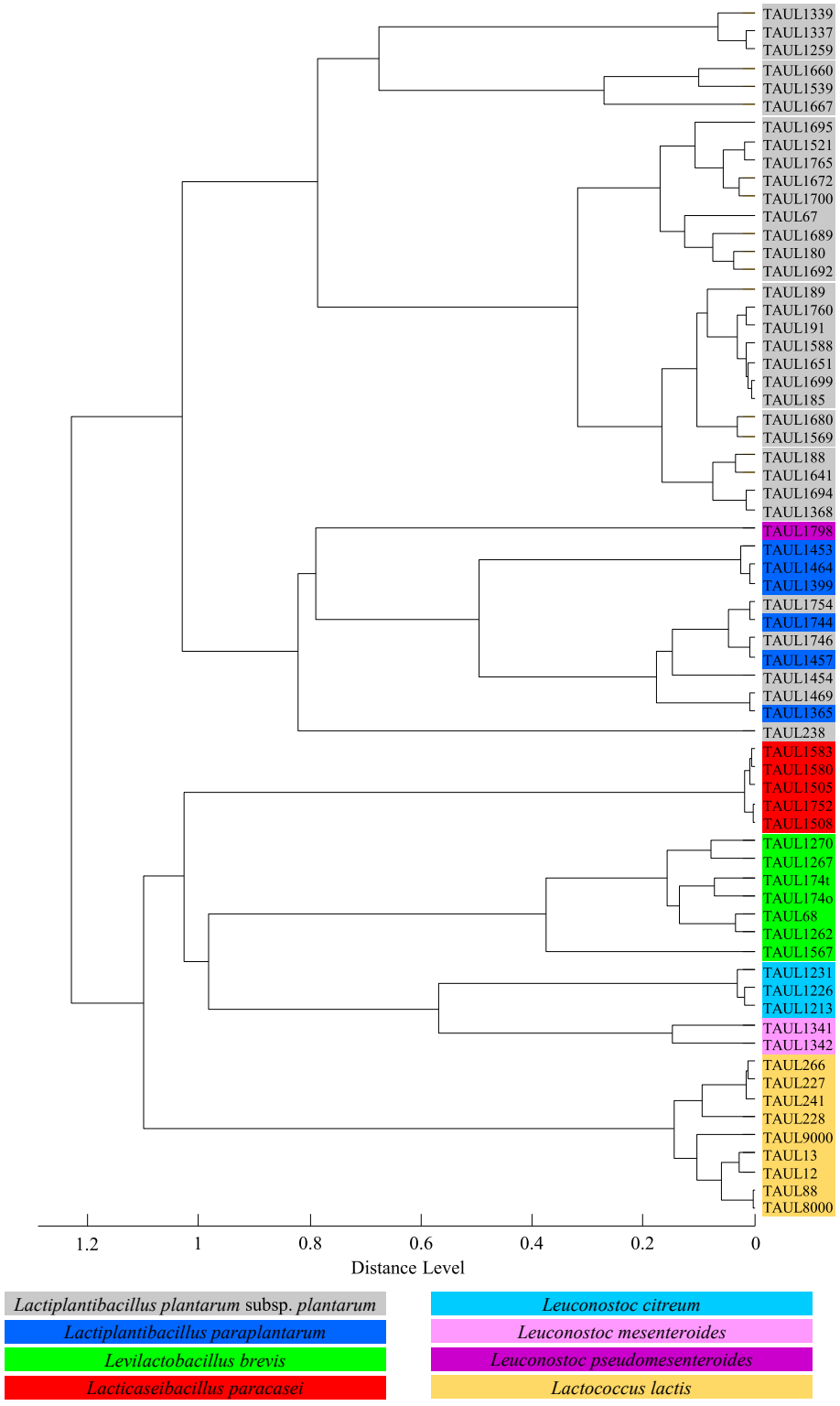

Fig. 1. Main spectrum (MSP) dendrogram of Matrix-Assisted Laser Desorption Ionization – Time of Flight (MALDI-TOF) mass spectral profiles generated by the MALDI Biotyper. Distance is displayed in relative units.

method described in Herreros et al. (2003) with some modifications. The method is based on the spectrophotometric measurement of the hydrolysis rates of p-nitroanilide substrates (Sigma-Aldrich, St. Louis, Missouri, USA): L-alanine *p*-nitroanilide, L-lysine *p*-nitroanilide, L-leucine *p*-nitroanilide and L-proline *p*-nitroanilide. The reaction mixture contained 190 μL of 1 mM of substrate in 50 mM TRIS-HCl buffer (pH 7.0) and 10 μL of CFE. Absorbance at 410 nm was measured on the samples in triplicate at 30 °C for a 30 min using a 96-well plate spectrophotometer (BioTek Synergy, Winooski, Vermont, USA). One unit of AP activity was defined as the amount of enzyme giving an absorbance increase of 0.001 units at 410 nm after incubation for 1 min. AP specific activity was expressed as the number of enzymatic activity units per mg of protein in the CFE per min.

Dipeptidase activity (DP) of the CFE was determined by a

Table 2
Acidification activity[a] and proteolytic activity[b] (average ± standard error) of whole cells of 66 wild lactic acid bacteria strains.

Species	Strain	Incubation time (h)				Proteolytic activity
		8 h		24 h		(at 24 h)
		pH	TA[c]	pH	TA	mmol Gly L^{-1}
Lactococcus lactis	TAUL12	5.94 ± 0.04 ^{a}	0.26 ± 0.01 ^{a}	4.78 ± 0.02 ^{a}	0.51 ± 0.01 ^{a}	0.021 ± 0.002 ^{a}
	TAUL13	5.87 ± 0.05 ^{a}	0.29 ± 0.01 ^{ab}	4.45 ± 0.05 ^{ab}	0.65 ± 0.00 ^{ab}	0.032 ± 0.016 ^{ab}
	TAUL88	5.70 ± 0.10 ^{ab}	0.32 ± 0.01 ^{ab}	4.64 ± 0.05 ^{ab}	0.56 ± 0.00 ^{ab}	0.086 ± 0.021 ^{ab}
	TAUL227	5.72 ± 0.03 ^{ab}	0.33 ± 0.00 ^{ab}	4.49 ± 0.01 ^{ab}	0.78 ± 0.01 ^{ab}	0.826 ± 0.051 ^{ab}
	TAUL228	5.72 ± 0.02 ^{ab}	0.30 ± 0.00 ^{ab}	4.41 ± 0.00 ^{ab}	0.73 ± 0.01 ^{ab}	1.003 ± 0.005 ^{b}
	TAUL241	5.61 ± 0.03 ^{ab}	0.34 ± 0.02 ^{ab}	4.77 ± 0.03 ^{a}	0.58 ± 0.02 ^{ab}	0.037 ± 0.016 ^{ab}
	TAUL266	5.47 ± 0.08 ^{ab}	0.45 ± 0.01 ^{ab}	4.26 ± 0.02 ^{ab}	0.85 ± 0.05 ^{b}	0.289 ± 0.031 ^{ab}
	TAUL8000	5.28 ± 0.08 ^{ab}	0.44 ± 0.03 ^{ab}	4.14 ± 0.02 ^{ab}	0.74 ± 0.03 ^{ab}	0.883 ± 0.034 ^{ab}
	TAUL9000	5.03 ± 0.07 ^{b}	0.54 ± 0.05 ^{b}	4.05 ± 0.01 ^{b}	0.84 ± 0.04 ^{b}	0.934 ± 0.045 ^{b}
Leuconostoc citreum	TAUL1213	6.44 ± 0.03	0.18 ± 0.01	6.37 ± 0.01 ^{ab}	0.19 ± 0.00	0.413 ± 0.034
	TAUL1226	6.45 ± 0.01	0.19 ± 0.00	6.35 ± 0.00 ^{b}	0.20 ± 0.01	0.383 ± 0.015
	TAUL1231	6.48 ± 0.01	0.18 ± 0.00	6.39 ± 0.00 ^{a}	0.20 ± 0.01	0.462 ± 0.020
Leuconostoc mesenteroides	TAUL1341	5.85 ± 0.26 ^{b}	0.32 ± 0.06 ^{b}	5.80 ± 0.05	0.31 ± 0.02	0.305 ± 0.022
	TAUL1342	6.32 ± 0.02 ^{a}	0.18 ± 0.01 ^{a}	5.65 ± 0.10	0.36 ± 0.02	0.287 ± 0.018
Leuconostoc pseudomesenteroides	TAUL1798	6.43 ± 0.02	0.19 ± 0.00	6.28 ± 0.02	0.21 ± 0.00	0.431 ± 0.037
Levilactobacillus brevis	TAUL68	6.47 ± 0.00 ^{ab}	0.19 ± 0.00 ^{b}	6.35 ± 0.00 ^{ab}	0.29 ± 0.00	0.174 ± 0.002 ^{a}
	TAUL174o	6.53 ± 0.00 ^{a}	0.18 ± 0.00 ^{ab}	6.50 ± 0.00 ^{a}	0.23 ± 0.00	1.428 ± 0.140 ^{ab}
	TAUL174t	6.35 ± 0.00 ^{ab}	0.18 ± 0.00 ^{ab}	6.33 ± 0.00 ^{ab}	0.21 ± 0.00	1.756 ± 0.058 ^{b}
	TAUL1262	6.20 ± 0.02 ^{b}	0.18 ± 0.00 ^{ab}	6.18 ± 0.01 ^{b}	0.23 ± 0.03	1.245 ± 0.384 ^{ab}
	TAUL1267	6.27 ± 0.04 ^{ab}	0.18 ± 0.01 ^{ab}	6.24 ± 0.02 ^{ab}	0.25 ± 0.01	0.327 ± 0.015 ^{ab}
	TAUL1270	6.49 ± 0.02 ^{ab}	0.21 ± 0.02 ^{b}	6.47 ± 0.03 ^{a}	0.21 ± 0.02	0.427 ± 0.020 ^{ab}
	TAUL1567	6.35 ± 0.02 ^{ab}	0.17 ± 0.01 ^{a}	6.35 ± 0.01 ^{ab}	0.22 ± 0.01	0.251 ± 0.002 ^{ab}
Lactiplantibacillus plantarum subsp. *plantarum*	TAUL67	6.31 ± 0.00 ^{ab}	0.23 ± 0.00 ^{ab}	5.21 ± 0.00 ^{ab}	0.63 ± 0.00 ^{c}	0.119 ± 0.007
	TAUL180	6.09 ± 0.05 ^{ab}	0.25 ± 0.01 ^{ab}	5.22 ± 0.01 ^{ab}	0.49 ± 0.01 ^{abc}	0.487 ± 0.037
	TAUL185	6.18 ± 0.01 ^{ab}	0.25 ± 0.01 ^{ab}	5.51 ± 0.03 ^{ab}	0.45 ± 0.02 ^{abc}	0.146 ± 0.031
	TAUL188	6.10 ± 0.00 ^{ab}	0.22 ± 0.00 ^{ab}	5.34 ± 0.03 ^{ab}	0.56 ± 0.01 ^{abc}	0.209 ± 0.033
	TAUL189	6.04 ± 0.02 ^{ab}	0.22 ± 0.01 ^{ab}	5.39 ± 0.07 ^{ab}	0.41 ± 0.04 ^{abc}	0.234 ± 0.011
	TAUL191	6.09 ± 0.03 ^{ab}	0.21 ± 0.01 ^{ab}	5.45 ± 0.09 ^{ab}	0.33 ± 0.00 ^{abc}	0.356 ± 0.045
	TAUL238	6.54 ± 0.01 ^{a}	0.17 ± 0.01 ^{a}	6.25 ± 0.05 ^{a}	0.21 ± 0.01 ^{a}	0.143 ± 0.017
	TAUL1259	5.96 ± 0.04 ^{ab}	0.20 ± 0.02 ^{ab}	4.99 ± 0.19 ^{ab}	0.44 ± 0.03 ^{abc}	0.376 ± 0.038
	TAUL1337	5.93 ± 0.02 ^{b}	0.26 ± 0.02 ^{b}	5.07 ± 0.09 ^{ab}	0.46 ± 0.01 ^{abc}	0.357 ± 0.007
	TAUL1339	5.94 ± 0.03 ^{b}	0.24 ± 0.01 ^{ab}	5.14 ± 0.05 ^{ab}	0.44 ± 0.02 ^{abc}	0.332 ± 0.000
	TAUL1368	5.96 ± 0.02 ^{ab}	0.27 ± 0.01 ^{b}	5.32 ± 0.02 ^{ab}	0.41 ± 0.01 ^{abc}	0.230 ± 0.123
	TAUL1469	6.30 ± 0.03 ^{ab}	0.19 ± 0.01 ^{ab}	5.40 ± 0.05 ^{ab}	0.35 ± 0.01 ^{abc}	0.266 ± 0.346
	TAUL1521	6.21 ± 0.04 ^{ab}	0.21 ± 0.01 ^{ab}	5.15 ± 0.04 ^{ab}	0.41 ± 0.00 ^{abc}	0.087 ± 0.037
	TAUL1539	6.18 ± 0.04 ^{ab}	0.22 ± 0.01 ^{ab}	5.13 ± 0.02 ^{ab}	0.45 ± 0.01 ^{abc}	0.256 ± 0.108
	TAUL1569	6.29 ± 0.02 ^{ab}	0.24 ± 0.00 ^{ab}	5.39 ± 0.03 ^{ab}	0.46 ± 0.01 ^{abc}	0.229 ± 0.033
	TAUL1588	6.27 ± 0.03 ^{ab}	0.20 ± 0.01 ^{ab}	4.93 ± 0.20 ^{ab}	0.42 ± 0.05 ^{abc}	0.230 ± 0.125
	TAUL1641	6.12 ± 0.00 ^{ab}	0.22 ± 0.01 ^{ab}	5.23 ± 0.02 ^{ab}	0.41 ± 0.01 ^{abc}	0.268 ± 0.019
	TAUL1651	6.23 ± 0.06 ^{ab}	0.22 ± 0.01 ^{ab}	4.78 ± 0.13 ^{b}	0.48 ± 0.01 ^{abc}	0.340 ± 0.028
	TAUL1660	6.34 ± 0.04 ^{ab}	0.19 ± 0.00 ^{ab}	5.60 ± 0.13 ^{ab}	0.25 ± 0.00 ^{ab}	0.051 ± 0.008
	TAUL1667	6.22 ± 0.02 ^{ab}	0.20 ± 0.00 ^{ab}	4.85 ± 0.10 ^{b}	0.42 ± 0.01 ^{abc}	0.237 ± 0.050
	TAUL1672	6.11 ± 0.02 ^{ab}	0.21 ± 0.00 ^{ab}	5.04 ± 0.05 ^{ab}	0.51 ± 0.00 ^{abc}	0.457 ± 0.026
	TAUL1680	6.24 ± 0.04 ^{ab}	0.23 ± 0.02 ^{ab}	5.18 ± 0.11 ^{ab}	0.50 ± 0.03 ^{abc}	0.260 ± 0.071
	TAUL1689	6.26 ± 0.03 ^{ab}	0.22 ± 0.01 ^{ab}	5.19 ± 0.05 ^{ab}	0.50 ± 0.01 ^{abc}	0.378 ± 0.153
	TAUL1692	6.23 ± 0.02 ^{ab}	0.20 ± 0.01 ^{ab}	5.13 ± 0.04 ^{ab}	0.52 ± 0.02 ^{abc}	0.469 ± 0.025
	TAUL1694	6.17 ± 0.03 ^{ab}	0.21 ± 0.01 ^{ab}	5.40 ± 0.11 ^{ab}	0.49 ± 0.04 ^{abc}	0.118 ± 0.064
	TAUL1695	6.28 ± 0.01 ^{ab}	0.21 ± 0.01 ^{ab}	5.20 ± 0.04 ^{ab}	0.60 ± 0.03 ^{bc}	0.233 ± 0.048
	TAUL1699	6.23 ± 0.02 ^{ab}	0.23 ± 0.01 ^{ab}	5.36 ± 0.01 ^{ab}	0.44 ± 0.01 ^{abc}	0.163 ± 0.098
	TAUL1700	6.25 ± 0.02 ^{ab}	0.20 ± 0.01 ^{ab}	5.39 ± 0.02 ^{ab}	0.45 ± 0.02 ^{abc}	0.054 ± 0.005
	TAUL1746	6.28 ± 0.03 ^{ab}	0.21 ± 0.01 ^{ab}	5.44 ± 0.06 ^{ab}	0.37 ± 0.01 ^{abc}	0.263 ± 0.015
	TAUL1754	6.34 ± 0.06 ^{ab}	0.20 ± 0.01 ^{ab}	5.47 ± 0.03 ^{ab}	0.41 ± 0.05 ^{abc}	0.264 ± 0.034
	TAUL1760	6.33 ± 0.01 ^{ab}	0.20 ± 0.01 ^{ab}	5.58 ± 0.04 ^{ab}	0.35 ± 0.01 ^{abc}	nd[d]
	TAUL1765	6.22 ± 0.06 ^{ab}	0.21 ± 0.01 ^{ab}	5.00 ± 0.02 ^{ab}	0.33 ± 0.03 ^{abc}	0.141 ± 0.083
Lactiplantibacillus paraplantarum	TAUL1365	6.08 ± 0.11 ^{b}	0.23 ± 0.01 ^{b}	5.25 ± 0.05 ^{b}	0.43 ± 0.01	0.253 ± 0.039
	TAUL1399	6.33 ± 0.03 ^{ab}	0.18 ± 0.01 ^{a}	5.72 ± 0.14 ^{a}	0.32 ± 0.02	0.081 ± 0.025
	TAUL1453	6.15 ± 0.03 ^{ab}	0.20 ± 0.01 ^{ab}	5.27 ± 0.02 ^{b}	0.42 ± 0.01	nd
	TAUL1454	6.35 ± 0.04 ^{a}	0.19 ± 0.01 ^{ab}	5.50 ± 0.01 ^{ab}	0.34 ± 0.01	nd
	TAUL1457	6.21 ± 0.02 ^{ab}	0.21 ± 0.01 ^{ab}	5.33 ± 0.02 ^{ab}	0.37 ± 0.01	0.309 ± 0.063
	TAUL1464	6.29 ± 0.03 ^{ab}	0.20 ± 0.01 ^{ab}	5.39 ± 0.04 ^{ab}	0.44 ± 0.17	0.057 ± 0.042
	TAUL1744	6.21 ± 0.02 ^{ab}	0.20 ± 0.01 ^{ab}	5.36 ± 0.06 ^{ab}	0.42 ± 0.04	0.337 ± 0.017
Lacticaseibacillus paracasei	TAUL1505	6.41 ± 0.03 ^{a}	0.21 ± 0.00 ^{ab}	5.37 ± 0.08 ^{a}	0.45 ± 0.02 ^{a}	0.018 ± 0.003
	TAUL1508	6.33 ± 0.01 ^{ab}	0.22 ± 0.00 ^{b}	5.05 ± 0.07 ^{ab}	0.50 ± 0.01 ^{ab}	nd
	TAUL1580	6.27 ± 0.02 ^{b}	0.21 ± 0.00 ^{ab}	4.88 ± 0.04 ^{b}	0.49 ± 0.02 ^{ab}	0.185 ± 0.085
	TAUL1583	6.29 ± 0.04 ^{ab}	0.20 ± 0.01 ^{ab}	4.94 ± 0.08 ^{ab}	0.46 ± 0.02 ^{ab}	0.166 ± 0.029
	TAUL1752	6.35 ± 0.03 ^{ab}	0.19 ± 0.01 ^{a}	5.05 ± 0.05 ^{ab}	0.60 ± 0.03 ^{b}	0.134 ± 0.065

^{abc} Values corresponding to different bacterial strains of the same species not showing a common superscript differ significantly ($p < 0.05$).
[a] Values presented are average ± SEM of three replicate evaluations for each bacterial strain.
[b] Proteolytic activity measured using the O-phthaldialdehide spectrophotometric assay and expressed as mmol Gly L^{-1} of milk.
[c] TA: total acidity (g 100 ml^{-1} lactic acid).
[d] nd: not detected.

Table 3
Aminopeptidase specific activity of crude cell-free extract of 66 wild lactic acid bacteria strains.

Specie	Ala-pNA			Arg-pNA			Leu-pNA			Lys-pNA			Pro-pNA		
	<100 [a]	100–250 [b]	>250 [c]	<100 [a]	100–250 [b]	>250 [c]	<100 [a]	100–250 [b]	>250 [c]	<100 [a]	100–250 [b]	>250 [c]	<100 [a]	100–250 [b]	>250 [a]
Lactococcus lactis Strains = 9	8	0	1	6	3	0	4	5	0	0	4	5	5	4	0
Leuconostoc citreum Strains = 3	3	0	0	1	2	0	1	2	0	0	3	0	1	2	0
Leuconsotoc mesenteroides Strains = 2	0	2	0	0	2	0	0	1	0	0	1	1	0	1	1
Leuconostoc pseudomesenteroides Strains = 1	1	0	0	1	0	0	1	0	0	1	0	0	1	0	0
Levilactobacillus brevis Strains = 7	3	4	0	0	4	3	1	2	4	0	4	3	7	0	0
Lactiplantibacillus plantarum Strains = 32	10	21	1	1	10	21	0	19	13	1	15	16	26	6	0
Lactiplantibacillus paraplantarum Strains = 7	2	4	1	0	3	4	0	3	4	0	5	2	6	1	0
Lacticaseibacillus paracasei Strains = 5	0	4	1	0	3	2	0	0	5	0	4	1	4	0	0

Aminopeptidase activity expressed as units of enzymatic activity mg^{-1} protein. One unit of aminopeptidase activity was the amount of enzyme giving an increase in absorbance of 0.001 at 410 nm in 1 min.
[a] < 100: nº of strains with aminopeptidase specific activity less than 100 units of enzymatic activity mg-1 protein of cell-free extract.
[b] 100-250: nº of strains with aminopeptidase specific activity between 100 and 250 units of enzymatic activity mg-1 protein of cell-free extract.
[c] > 250: nº of strains with aminopeptidase specific activity above 250 units of enzymatic activity mg-1 protein of cell-free extract.

modification of the cadmium-ninhydrin method (Doi et al., 1981; Herreros et al., 2003), using the following dipeptides as substrates: Leu-Leu, Tyr-Leu, Ala-Ala, Leu-Gly, and Phe-Ala (Sigma-Aldrich). One unit of enzymatic activity was defined as the amount of enzyme that produced an increase in absorbance of 0.1 units at 507 nm. The results were expressed as the number of enzymatic activity units per min per mg of protein in the CFE.

Carboxypeptidase activity (CP) of the CFE was measured by spectrophotometric method described in González et al. (2010) using N-carbobenzyloxy-L-Leu (Alfa Aesar, Karlsruhe, Germany). One unit of enzymatic activity was defined as the amount of enzyme that produced an increase in absorbance at 570 nm of 0.01 units. The results were expressed as enzymatic units per 15 min per mg of protein in the CFE.

2.7. Esterolytic activity of the CFE

The esterase activity of the CFE was determined by the method described in Herreros et al. (2004) using the following β-naphthyl substrates: β-naphthyl butyrate (C4), β-naphthyl caprylate (C8), β-naphthyl myristate (C14) and β-naphthyl stearate (C18) (Sigma-Aldrich). This method is based on the spectrophotometric measurement at room temperature of hydrolysis rates of β-naphthyl derivatives. The β-naphthol released by hydrolysis was estimated by measuring absorbance at 560 nm. Esterase activity was expressed as μmol of β-naphthol released per minute and per mg of protein.

2.8. Statistical analysis

Cluster analysis was performed based on a comparison of strain-specific main spectra, created as described above. The dendrogram was constructed by the statistical toolbox of Matlab 7.1 (MathWorks Inc., Natick, MA, USA) integrated in the MALDI Biotyper 3.0 software. The parameter settings were: 'Distance Measure = Euclidean' and 'Linkage = Complete'. The linkage function is normalized according to the distance between 0 (perfect match) and 1000 (no match).

Statistical analysis of the experimental data was performed using SPSS v.25 (SPSS, Chicago, IL, USA). Because data had non-normal distribution, a Kruskal-Wallis test was performed to determine statistical differences ($p < 0.05$) between the strains of a single bacterial species with respect to the values of enzymatic activity. To analyze for differences between individual groups, post hoc Mann-Whitney test was used to analyze for differences between individual groups, and significant results were adjusted using the Bonferroni correction. The analysis of AP, CP, DP and esterase activities was performed by factor analysis with principal component extraction (FAEPC).

2.9. Ranking and scoring of strains

In order to simplify the large number of results obtained for the 66 strains studied, each enzyme activity of the strains, grouped by species, was given a score (S), according to the following parameters:

- S_{pH}: pH scores (at t = 8 and 24 h) of each strain were defined as follows: 1 (pH > 6.00), 2 (pH between 6.00 and 5.50), 3 (pH between 5.50 and 5.00), 4 (pH between 5.00 and 4.50) and 5 (pH between 4.50 and 4.00).
- S_{TA}: total acidity scores (at t = 8 and 24 h) of each strain were defined as follows: 1 (TA < 0.2 g 100 mL^{-1} lactic acid), 2 (TA between 0.20 and 0.29 g 100 mL^{-1} lactic acid), 3 (TA between 0.30 and 0.39 g 100 mL^{-1} lactic acid), 4 (TA between 0.40 and 0.49 g 100 mL^{-1} lactic acid), 5 (TA between 0.50 and 0.59 g 100 mL^{-1} lactic acid), 6 (TA between 0.60 and 0.69 g 100 mL^{-1} lactic acid), 7 (TA between 0.70 and 0.79 g 100 mL^{-1} lactic acid) and 8 (TA between 0.80 and 0.89 g 100 mL^{-1} lactic acid).
- S_{Prot}: extracellular proteolytic activity score of each strain was defined as follows: 2 = low proteolytic strains (<1 mmol Gly L^{-1}); 1

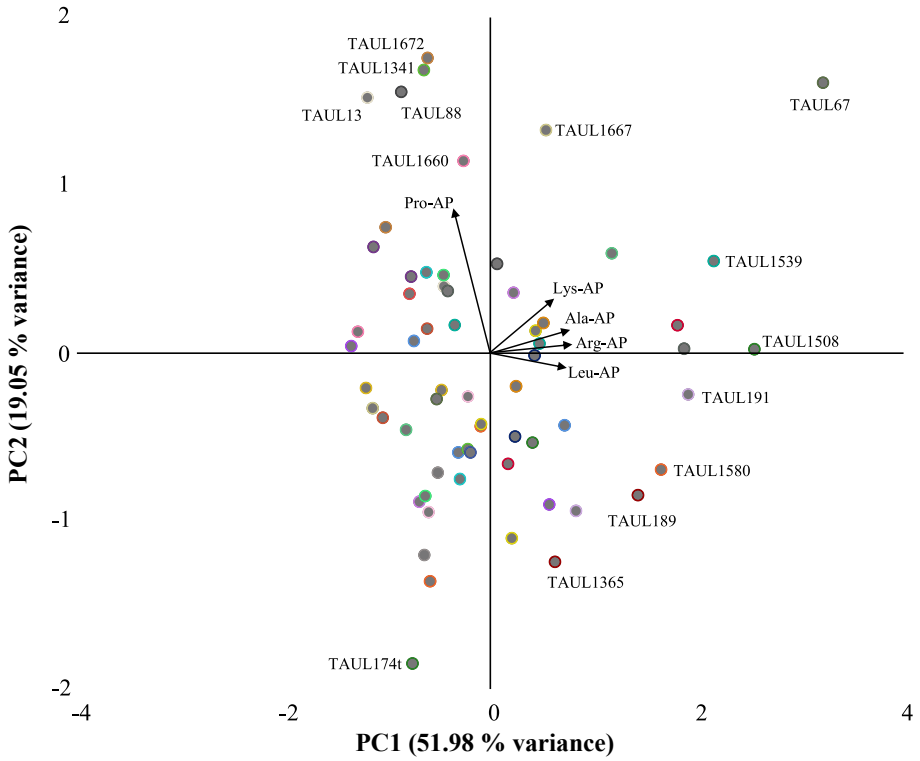

Fig. 2. Factor analysis with principal component extraction (FAEPC) of the aminopeptidase activities (Ala, Arg, Leu, Lys and Pro-aminopeptidase) by the 66 wild lactic acid bacteria.

= medium proteolytic strains (between 1 and 4 mmol Gly L^{-1}); 0 = very proteolytic strains (>4 mmol Gly L^{-1}).

- S_{Ala-AP}, S_{Arg-AP}, S_{Leu-AP}, S_{Lys-AP} and S_{Pro-AP}: AP activity scores were calculated for each aminoacid-pNA substrate as follows: 1 = low activity (<100 UE mg^{-1} protein); 2 = medium activity (between 100 and 250 UE mg^{-1} protein); 3 = high activity (>250 UE mg^{-1} protein).
- $S_{Ala-Ala}$, $S_{Leu-Gly}$, $S_{Leu-Leu}$, $S_{Phe-Ala}$ and $S_{Tyr-Leu}$: DP scores were calculated for each dipeptide as follows: 1 = low activity (<50 UE mg^{-1} protein); 2 = medium activity (between 50 and 200 UE mg^{-1} protein); 3 = high activity (>200 UE mg^{-1} protein).
- S_{Leu-CP}: CP score was defined as follows: 1 = low activity (<250 UE mg^{-1} protein); 2 = medium activity (between 250 and 500 UE mg^{-1} protein); 3 = high activity (>500 UE mg^{-1} protein).
- S_{C4}, S_{C8}, S_{C14} and S_{C18}: esterase activity scores were defined for each substrate as follows: 1 = low activity (<10 μmol β-naphthol min^{-1} mg^{-1}); 2 = medium activity (between 10 and 30 μmol β-naphthol min^{-1} mg^{-1}); 3 = high activity (>30 μmol β-naphthol min^{-1} mg^{-1}).

3. Results and discussion

3.1. Identification of LAB by MALDI-TOF/MS

The 66 isolates included in the study were identified to species level by MALDI-TOF. This methodology states that scores between 0,000 and 1699 indicate an unreliable identification, between 1700 and 1999 as probable genus identification, scores between 2000 and 2299 as a confident identification to genus level and a score between 2300 and 3000 indicates a reliable level for species identification. All strains tested scored between 2300 and 3000 and were ascribed to the following species: *Lactococcus lactis* (9), *Leuconostoc citreum* (3), *Leuconostoc mesenteroides* (2), *Leuconostoc pseudomesenteroides* (1), *Levilactobacillus brevis* (7), *Lactiplantibacillus plantarum* (32), *Lactiplantibacillus paraplantarum* (7) and *Lacticaseibacillus paracasei* (5). The high score values obtained made it possible to ascribe the strains to the species with high reliability. Although DNA techniques are considered more accurate for bacterial identification, the available literature indicates that identification of LAB strains by MALDI-TOF is becoming more widespread (Bujnakova & Strakova, 2017; Sánchez-Juanes et al., 2020).

From the dendrogram generated by MALDI Biotyper (Fig. 1), the proximity between the different isolates and species can be observed according to their mass spectra. The distances obtained reflect the proximity between the identified isolates and suggest which of these could be clones of the same strain. MALDI-TOF MS has been widely used for identification to genus and species level, its utility for use down to the strain level presents greater complexity because strains of the same species tend to be very similar in terms of genotype and phenotype and typing requires genetic techniques for determination (Ashfaq et al., 2022; Gantzias et al., 2020).

3.2. Acidifying activity

The main function of LAB as starter cultures in cheese making is the production of lactic acid from milk lactose for pH reduction, which improves the subsequent coagulation of the milk and contributes to textural changes and control of microorganisms in the cheese (Karakas-Sen & Karakas, 2018). The strains of the eight species included in the study differed in their acidifying activity, especially after 8 and 24 h of incubation in milk (Table 2). *Lc. lactis* strains showed the highest acidifying capacity. After 8 h of incubation, the average pH value was 5.59 and the total acidity value was 0.36 g 100 mL^{-1} lactic acid; while after 24 h of incubation, the pH and total acidity values were 4.44 and 0.69 g 100 mL^{-1} lactic acid, respectively. Significant differences ($p < 0.05$) were found between the strains after 8 h of incubation, at which time the strain TAUL9000 had the lowest pH value (5.03) and the highest acidity value (0.54 g 100 mL^{-1} lactic acid). After 24 h, all strains performed similarly, although again strain TAUL9000 recorded the lowest pH value (4.05); while in terms of total acidity, strains TAUL266 and TAUL9000 recorded the highest values (0.85 and 0.84 g 100 mL^{-1} lactic acid, respectively). The strains of the genus *Leuconostoc* showed a

Table 4
Dipeptidase specific activities of crude cell-free extract of 66 wild lactic acid bacteria strains.

Specie	Ala-Ala			Leu-Gly			Leu-Leu			Phe-Ala			Tyr-Leu		
	<50 [a]	50–200 [b]	>200 [c]	<50 [a]	50–200 [b]	>200 [c]	<50 [a]	50–200 [b]	>200 [c]	<50 [a]	50–200 [b]	>200 [c]	<50 [a]	50–200 [b]	>200 [c]
Lactococcus lactis strains = 9	1	6	2	2	7	0	7	2	0	3	5	1	4	4	0
Leuconostoc citreum strains = 3	3	0	0	3	0	0	3	0	0	3	0	0	3	0	0
Leuconostoc mesenteroides strains = 2	2	0	0	2	0	0	2	0	0	2	0	0	2	0	0
Leuconostoc pseudomesenteroides strains = 1	1	0	0	1	0	0	1	0	0	1	0	0	0	0	0
Levilactobacillus brevis strains = 7	4	2	1	6	1	0	6	1	0	3	1	3	7	0	0
Lactiplantibacillus plantarum strains = 32	25	5	2	19	9	4	13	12	5	12	15	5	19	10	2
Lactiplantibacillus paraplantarum strains = 7	7	0	0	6	1	0	4	3	0	5	2	0	6	0	1
Lacticaseibacillus paracasei strains = 5	5	0	0	5	0	0	4	1	0	5	0	0	4	0	0

Dipeptidase specific activity expressed as units of enzymatic activity mg^{-1} protein. One unit of dipeptidase activity was the amount of enzyme giving an absorbance increase of 0.01 units at 505 nm in 15 min.
[a] < 50: nº of strains with dipeptidase specific activity less than 50 units of enzymatic activity mg^{-1} protein of cell-free extract.
[b] 50–200: nº of strains with dipeptidase specific activity between 50 and 200 units of enzymatic activity mg^{-1} protein of cell-free extract.
[c] 200: nº of strains with dipeptidase specific activity above 200 units of enzymatic activity mg^{-1} protein of cell-free extract.

significantly ($p < 0.05$) lower acidification capacity than *Lc. lactis*. Among them, the *Ln. mesenteroides* strains TAUL1341 and TAUL1342 showed significant differences ($p < 0.05$) with respect to the other strains, showing a better acidifying capacity after 24 h of incubation, with an average pH value of 5.73 and a total acidity of 0.34 g mL^{-1} lactic acid. The acidification developed by *L. brevis* strains was significantly lower ($p < 0.05$) than that produced by most strains of *L. plantarum*, *L. paraplantarum* and *L. paracasei* after 24 h. Among the strains of the same species, *L. plantarum* strains TAUL67, TAUL1651, TAUL1667 and *L. paracasei* strains TAUL1580 and TAUL1752 showed significant differences ($p < 0.05$) in pH and/or total acidity values.

The results obtained are similar to those obtained in other studies on acidifying activity in wild LAB strains (Câmara et al., 2019; González et al., 2010; Herreros et al., 2003). Acidification capacity is one of the most important starter culture characteristics. In cheese, the LAB starter culture must reduce the pH of the milk to below 5.3 in the first 6 h of incubation at 30 °C (Câmara et al., 2019; Dias et al., 2019). Among the strains studied, only two strains of *Lc. lactis* (TAUL8000 and TAUL9000) managed to reduce the pH below this value after 8 h of incubation. The rest of the *Lc. lactis* strains did not reach this value after 8 h of incubation, although after 24 h of incubation, the pH value was similar in all of them. The strains belonging to the genera *Levilactobacillus*, *Lactiplantibacillus*, *Lacticaseibacillus* and *Leuconostoc* showed the lowest acidification values. These results were to be expected, because of their lower capacity to metabolize lactose (Carafa et al., 2015; Meng et al., 2018). These results are in agreement with those obtained by other authors (Meng et al., 2018; Monteagudo-Mera et al., 2011). Sometimes the final acidification of *Lactiplantibacillus* or *Lacticaseibacillus* strains can be higher than that obtained by lactococci, which can be problematic in cheese making as it prolongs the acidification period and high acidification by NSLAB could lead to sensory defects in the cheese (Meng et al., 2018). In our case, the values after 24 h of acidification were high, but did not exceed those obtained by lactococci strains, so they would not imply a technological problem.

3.3. Proteolytic activity

Results of proteolytic activity, determined using the O-pthaldialdehyde (OPA) spectrophotometric assay, are shown in Table 2. The proteolytic activity of LAB observed was similar ($p > 0.05$) between groups, with low activity (<1 mmol Gly L^{-1} milk) predominating. However, some *Lc. lactis* and *L. brevis* strains showed significant differences ($p < 0.05$) with the rest of the strains of the same species, showing moderate proteolysis values (from 1.1 to 4 mmol Gly L^{-1} milk). On the one hand, *Lc. lactis* strains TAUL227, TAUL228, TAUL8000 and TAUL9000 reported the highest activity values, close to 1 mmol Gly L^{-1} milk. On the other hand, *L. brevis* strains TAUL174o, TAUL174t and TAUL1262 showed the highest activity values of all LAB included in the study (1.428, 1.756 and 1.245 mmol Gly L^{-1} milk, respectively).

Proteolytic activity is an essential property for starter cultures and NSLABs as the hydrolysis of caseins into peptides and FAA contributes to aroma formation and flavour formation in cheese ripening (Dias et al., 2019). The results obtained showed lower proteolytic activity values than those obtained in other investigations (González et al., 2010; Herreros et al., 2003), especially in lactococci strains. However, the medium-low proteolytic activity, especially in NSLABs, is interesting because high proteolytic activity is not always the most desirable characteristic for a strain to be used in a non-starter culture. In fact, excessive proteolysis can lead to uncontrolled production of bitter peptides and other undesirable compounds, or even excessive hydrolysis of casein, resulting in an excessively soft end product (Meng et al., 2018).

3.4. Proteolytic activities of the CFE

The intracellular proteolytic activities of LAB are of high importance

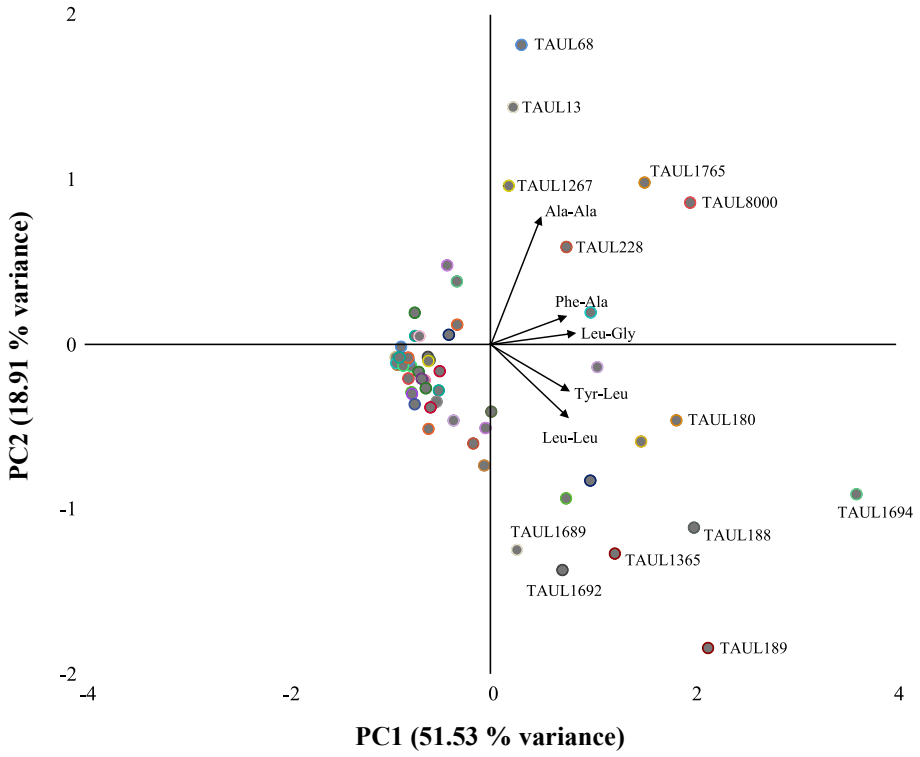

Fig. 3. Factor analysis with principal component extraction (FAEPC) of the dipeptidase activities (Ala-Ala, Leu-Gly, Leu-Leu, Tyr-Leu and Phe-Ala dipeptidase) by the 66 wild lactic acid bacteria.

Table 5
Carboxypeptidase and esterase specific activities of crude cell-free extract of 66 wild lactic acid bacteria strains.

Specie	Carboxypeptidase			Naphtyl-butyrate (C4)			Naphtyl-caprylate (C8)			Naphtyl-mirystate (C14)			Naphtyl-esterate (C18)		
	<250 [a]	250–500 [b]	>500 [c]	<10 [d]	10–30 [e]	>30 [f]	<10 [d]	10–30 [e]	>30 [f]	<10 [d]	10–30 [e]	>30 [f]	<10 [d]	10–30 [e]	>30 [f]
*Lactococcus lactis*strains = 9	5	1	3	7	2	0	4	4	1	0	9	0	4	3	0
Leuconostoc citreum strains = 3	2	0	0	0	0	3	0	3	0	3	0	0	2	0	0
Leuconostoc mesenteroides strains = 2	0	0	0	2	0	0	2	0	0	0	2	0	1	0	0
Leuconostoc pseudomesenteroides strains = 1	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0
Levilactobacillus brevis strains = 7	0	1	5	3	1	3	0	2	5	1	6	0	6	0	0
Lactiplantibacillus plantarum strains = 32	14	6	10	31	0	1	30	1	1	15	16	1	15	0	0
Lactiplantibacillus paraplantarum strains = 7	1	3	2	7	0	0	7	0	0	1	6	0	0	0	0
*Lacticaseibacillus paracasei*strains = 5	2	1	1	0	0	5	0	0	5	3	2	0	1	0	0

Carboxypeptidase activity expressed as units of enzymatic activity mg^{-1} protein. One unit of carboxypeptidase activity was the amount of enzyme giving an absorbance increase of 0.01 units at 570 nm in 1 min.

[a] < 250: nº of strains with carboxypeptidase specific activity less than 250 units of enzymatic activity mg^{-1} protein of cell-free extract.

[b] 250–500: nº of strains with carboxypeptidase specific activity between 250 and 500 units of enzymatic activity mg^{-1} protein of cell-free extract.

[c] > 500: nº of strains with carboxypeptidase specific activity above 500 units of enzymatic activity mg^{-1} protein of cell-free extract.

[d] < 10: nº of strains with esterase activity less than 10 μmol of β-naphthol released per min mg^{-1} protein.

[e] 10–30: nº of strains with esterase activity between 10 and 30 μmol of β-naphthol released per min mg^{-1} protein.

[f] 30: nº of strains with esterase activity above 30 μmol of β-naphthol released per min mg^{-1} protein.

in the development of flavor during cheese ripening (Câmara et al., 2019). In this sense, peptides derived from casein hydrolysis are degraded by the action of peptidases with different specificities. Among the LAB endopeptidases, aminopeptidases are the first enzymes to act on oligopeptides, releasing amino acids that can contribute directly or indirectly to flavor development during cheese ripening (Ianni et al., 2020).

Table 3 shows the results obtained for the AP activity of CFEs of the strains tested using p-nitroanilide substrates (Ala-pNA, Lys-pNA, Leu-pNA and Pro-pNA), organized in ranges of activity: low (<100 UE mg^{-1}

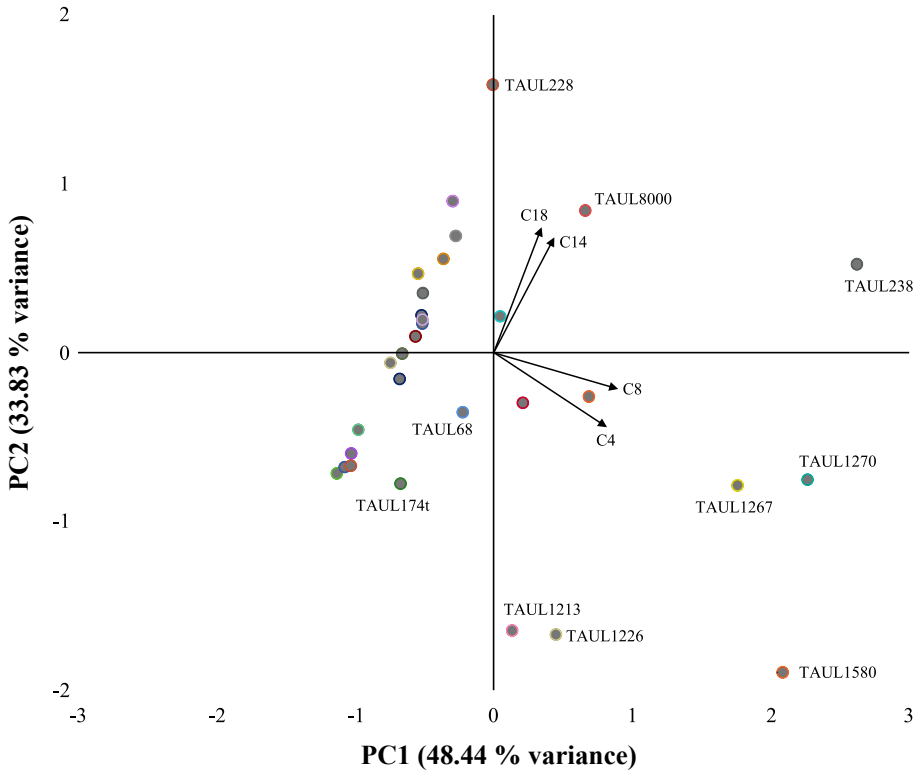

Fig. 4. Factor analysis with principal component extraction (FAEPC) of the esterolytic activities (butyrate (C4), caprylate (C8), myristate (C14) and stearate (C18) esterase) by the 66 wild lactic acid bacteria.

protein), medium (100–250 UE mg^{-1} protein) or high (>250 UE mg^{-1} protein). Significant differences were found between strains of different species and between strains of the same species for each of the substrates tested. In addition, the activity values for the Arg-pNA and Leu-pNA substrates were generally higher than for the other substrates, especially for Pro-pNA, where the lowest values were recorded. The highest value recorded for the substrate Ala-pNA was recorded by *L. paracasei* strain TAUL1508 (522.04 UE). In the case of Arg-pNA, *L. plantarum* strain TAUL188 recorded the highest activity with 983.47 UE. For Leu-pNA, *L. paracasei* strain TAUL1580 recorded the highest activity (880.09 UE), while for Lys-pNA it was *L. plantarum* strain TAUL67 that showed the highest activity (1836.69 UE). Finally, the *Ln. mesenteroides* strain TAUL1342 recorded an activity of 418.96 UE for Pro-pNA. To observe the AP activities of the strains as a whole, an FAEPC was performed (Fig. 2). It can be seen how certain strains stand out from the rest, especially the *L. plantarum* TAUL67 strain, which recorded very high activity values for almost all the substrates analyzed. Compared to the activities reported by other authors (Carafa et al., 2015; González et al., 2010), activity was detected in almost all strains, although most strains showed low activity values (<100 UE).

On the other hand, different DP activities were studied and the activity results for each of the dipeptides tested are shown in Table 4, where the strains were again grouped into activity ranges. For each of the substrates, significant differences were found between the different LAB strains tested. In general, the highest dipeptidase activity values were recorded by different *L. plantarum* strains: Ala-Ala (TAUL238 = 381.55 UE), Leu-Gly (TAUL180 = 379.07 UE), Leu-Leu (TAUL1694 = 334.90 UE), Tyr-Leu (TAUL1694 = 320.56 UE); except for Phe-Ala dipeptide, where the highest activity value was recorded by *Lc. lactis* strain TAUL8000 (602.45 UE). To observe the DP activities of the strains as a whole, an FAEPC was performed (Fig. 3). It is observed that several *Lc. lactis* strains (TAUL13, TAUL266 and TAUL8000), *L. brevis* strains TAUL174o and TAUL1267, as well as several *L. plantarum* strains (TAUL68, TAUL180, TAUL189, TAUL238, TAUL1694 and TAUL1765) stood out from the rest. This separation from the rest is because their activity values were generally high for all DP activities.

The presence of CP activity in the strains is also important during the hydrolysis of peptides generated from the hydrolysis of caseins, although it is an atypical activity in LAB (González et al., 2010). The results for the specific dipeptidase and CP activities of CFEs of LAB strains are shown in Table 5. Low activity values (<250 UE) or no activity was recorded for most strains. The detection of LAB strains with CP activity are very rarely and references to it are scarce (González et al., 2010; Herreros et al., 2003). Comparing the results obtained between the strains studied *Lc. lactis* and *Leuconostoc* strains showed, with some exceptions, lower activity than did the rest of the strains. However, some strains of *L. plantarum* and *L. brevis* showed high activity values. In the case of *L. brevis*, strain TAUL68 recorded an activity of 1967.62 UE; in the case of *L. plantarum*, strain TAUL185 reported an activity of 1126.37 UE.

In summary, the results obtained for the different endopeptidase assayed revealed strains with high values for certain enzyme activities and others that presented high activity values for AP, CP and DP activities. The combination of proteolytic strains with strains that have high endopeptidase activities is of great interest in the design of starter cultures for cheese, because the joint activity of peptidases and proteases is essential to achieve the desired level of proteolysis in cheeses (Câmara et al., 2019; Carafa et al., 2015). During the long periods of cheese ripening, these enzymes release FAAs that are subsequently catabolized into other compounds (for example, aldehydes, ketones, alcohol and low molecular weight sulfur compounds) that define the sensory properties of the cheese (Meng et al., 2018).

3.5. Esterolytic activity

The esterolytic activity of the CFEs is shown in Table 5. Although there were significant differences between the strains of the different species studied, in general the levels of activity obtained were low or not

Table 6
Lactic acid bacteria strains selected through the designed scoring system and their aminopeptidase, dipeptidase, carboxypeptidase and esterase activity values (average of three replicates).

Strain	TS[a]	Ala-AP[b]	Arg-AP[b]	Leu-AP[b]	Lys-AP[b]	Pro-AP[b]	Ala-Ala[c]	Leu-Gly[c]	Leu-Leu[c]	Phe-Ala[c]	Tyr-Leu[c]	Leu-CP[d]	C4[e]	C8[e]	C14[e]	C18[e]
Lactococcus lactis																
TAUL8000	54	268.47	134.24	223.73	536.94	223.73	90.21	182.02	81.08	602.45	114.91	1082.84	15.62	22.76	27.43	3.19
TAUL9000	50	74.24	148.48	148.48	346.46	98.99	102.45	184.71	86.42	135.42	122.35	153.43	11.89	15.62	17.01	3.21
Leuconostoc mesenteroides																
TAUL1341	31	105.58	237.55	184.76	211.15	158.37	3.80	4.43	40.23	7.29	0.95	nd	2.60	2.48	10.83	nd
Levilactobacillus brevis																
TAUL1270	37	113.82	170.72	199.18	312.99	85.36	18.10	13.66	11.95	33.80	5.122	802.39	43.30	64.95	19.93	2.81
Lactiplantibacillus plantarum																
TAUL67	39	242.05	840.04	469.85	1836.69	42.71	19.14	4.96	35.37	56.38	14.35	486.94	2.23	6.35	15.05	0.55
TAUL180	41	106.11	257.69	197.05	682.11	45.47	59.30	379.07	219.55	179.53	44.75	322.87	2.49	8.12	22.96	0.52
TAUL188	45	234.16	983.47	312.21	593.21	46.83	53.95	195.38	228.92	297.29	171.03	608.82	2.66	5.87	18.81	1.16
TAUL1694	46	151.15	673.32	398.50	604.62	82.45	201.17	286.26	334.90	304.73	320.56	878.07	1.80	2.11	8.80	0.36
TAUL1765	40	155.94	396.95	283.54	496.19	70.88	111.43	288.19	121.81	349.26	11.23	238.17	1.86	3.38	6.30	0.24
Lactiplantibacillus paraplantarum TAUL1365	40	132.75	398.25	360.32	379.29	18.96	39.83	137.45	134.50	101.50	226.21	449.46	2.18	4.41	15.38	nd
Lacticaseibacillus paracasei TAUL1508	41	116.07	219.25	464.29	206.35	nd	5.57	10.52	34.20	19.50	38.85	3.87	34.71	54.70	10.50	nd

[a] TS: Total Score calculated by summing $S_{pH8} + S_{pH24} + S_{TA8} + S_{TA24} + S_{Prot} + S_{Ala\text{-}AP} + S_{Arg\text{-}AP} + S_{Leu\text{-}AP} + S_{Lys\text{-}AP} + S_{Pro\text{-}AP} + S_{Ala\text{-}Ala} + S_{Leu\text{-}Gly} + S_{Leu\text{-}Leu} + S_{Phe\text{-}Ala} + S_{Tyr\text{-}Leu} + S_{Leu\text{-}CP} + S_{C4} + S_{C8} + S_{C14} + S_{C18}$.
[b] Aminopeptidase activity expressed as units of enzymatic activity mg^{-1} protein. One unit of aminopeptidase activity was the amount of enzyme giving an increase in absorbance of 0.001 at 410 nm in 1 min.
[c] Dipeptidase specific activity expressed as units of enzymatic activity mg^{-1} protein. One unit of dipeptidase activity was the amount of enzyme giving an absorbance increase of 0.01 units at 505 nm in 15 min.
[d] Carboxypeptidase activity expressed as units of enzymatic activity mg^{-1} protein. One unit of carboxypeptidase activity was the amount of enzyme giving an absorbance increase of 0.01 units at 570 nm in 1 min.
[e] Esterase specific activity expressed as μmol of β-naphthol released per 10 min mg^{-1} protein of cell-free extract.

detectable. However, it was observed that the activity was higher on short-chain substrates (C4 and C8), compared to the activity on long-chain substrates (C14 and C18). The highest levels of C4 esterase activity were found in *L. paracasei* strain TAUL1583 (48.82 μmol of β-naphthol). The highest C8 and C14 activities were recorded by *L. plantarum* strain TAUL238 (76.37 and 30.66 μmol of β-naphthol, respectively). Finally, *Lc. lactis* strain TAUL88 reported the highest C16 activity with a value of 19.54 μmol of β-naphthol released per min mg^{-1} protein. Despite the low levels of lipolytic activity in starter strains, they may be important in the development of cheese aroma. This is because of the low detection threshold of the compounds produced by the lipolytic action and the long ripening process required for some cheeses. Similar results to those obtained have already been observed by other authors who have reported a higher activity of LAB strains on short-chain fatty acids (Câmara et al., 2019; González et al., 2010).

To observe the esterase activities of the strains as a whole, an FAEPC was performed (Fig. 4). After analysis, some strains were found to have different results from the rest: *Lc. lactis* strains TAUL88, TAUL227, TAUL228 and TAUL8000; *L. brevis* strains TAUL1262, TAUL1267 and TAUL1270; *L. plantarum* strain TAUL238. This separation from the rest is because of their activity values were generally high for all esterase activities.

3.6. LAB-strains selection

Each strain was ranked according to the total score (TS), which was obtained by adding the score obtained from the acidifying activity (pH value at 8 and 24 h and TA at 8 and 24 h) plus the score obtained from the extracellular proteolytic activity and the scores obtained from the intracellular activities (aminopeptidases, carboxypeptidases, dipeptidases and esterases). The selection of the best strains was done by comparing the TS obtained between strains of the same species, except for the strains of the three *Leuconostoc* species that were compared with each other. In this way, a total of 11 strains with the best TS of each species were selected (Table 6), which could be candidates for inclusion as starter cultures or NSLAB.

4. Conclusion

LAB strains isolated from fermented foods, such as traditional cheeses, constitute an important reservoir of strains for the design of new starter and adjunct cultures that contribute not only to the acidification of milk, but also to the formation of flavors as a consequence of their proteolytic and lipolytic activities. The identification of a large number of strains, as well as their characterization through the study of a wide variety of enzymatic activities and the design of a selection model allowed the selection of a total of 11 strains from the different species studied. Some of these strains could be combined in mixed cultures and further evaluated in cheese production and could be the target of further studies. Among the selected strains, *Lc. lactis* strains TAUL8000 and TAUL9000 stand out for their acidifying capacity and proteolytic activity. The rest of the selected strains stand out for several of their intracellular activities, which are determinant in the production of flavor compounds during cheese ripening, and could be used as adjunct cultures to complement the activities present in the starter culture.

CRediT authorship contribution statement

D. Abarquero: Investigation, Formal analysis, Methodology, Data curation, Writing – original draft. **E. Renes:** Conceptualization. **P. Combarros-Fuertes:** Investigation. **J.M. Fresno:** Conceptualization, Supervision. **M.E. Tornadijo:** Conceptualization, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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