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Selection of Lactic Acid Bacteria Isolated from San Simón da Costa Cheese (PDO) in Order to Develop an Autochthonous Starter Culture

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Abstract

Traditional cheeses are an important reservoir of microbial diversity that can have important biotechnological applications, especially with a view to improving the characteristics unique to each type of cheese, and in this respect, starter cultures consisting of autochthonous lactic acid bacteria strains are of particular interest. In the present study, we investigated lactic acid bacteria population diversity in San Simón da Costa cheese (PDO, Galicia, Spain) and found a predominance of the genus *Lactobacillus*, which by the end of ripening accounted for 78% of the strains isolated in Rogosa agar, around 40% of those in M17 agar and about 10% of those in MSE agar. The main species of lactic acid bacteria identified were *Lactococcus lactis* subsp. *lactis*, *Lactobacillus casei* subsp. *casei*, *Lb. paracasei* subsp. *paracasei*, *Leuconostoc mesenteroides* and *Enterococcus faecalis*. Virtually all the strains studied from a technological point of view yielded more than or equal to 0.24 g 100 mL^{-1} lactic acid. *Lactococcus lactis* subsp. *lactis* (SS 194), *Lactobacillus paracasei* (SS 1695 and SS 1689) and *Enterococcus faecalis* (SS 1378 and SS 1449) strains exhibited the greatest proteolytic capacity. Based on the overall technological aptitude of the tested strains, we can propose starter cultures and co-cultures that include different combinations of previous strains with a view to manufacturing San Simón da Costa cheese from pasteurised milk.

Keywords

Cow Milk Cheese, Lactic Acid Bacteria, Enzymatic Activities, Autochthonous Starter

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1. Introduction

San Simón da Costa cheese is a protected designation of origin (PDO) cheese manufactured in Galicia (Spain). It is made in the region of A Terra Chá, which encompasses several municipalities in the province of Lugo (Galicia, Spain). San Simón da Costa is a matured cheese made with full fat cow's milk without colostrum or preservatives and with a balanced composition of fat and protein, in accordance with the characteristics of the breeds of origin and the season of the year, so that the final product has a 45% fat content in dry matter. It is a semi-hard, semi-elastic cheese with a few small eyes, which has a conical pear shape and a honey-coloured, waxy rind. The smell is slightly acid, although the smell of smoke predominates once matured because processing includes a stage of smoking.

A determining factor in the development of the textures and organoleptic characteristics of cheeses is the enzymatic potential of the strains used as starter cultures in cheese manufacture. Lactic cultures play a crucial role at the beginning of fermentation, developing acidity and favouring coagulation. In addition, they are involved in ripening and development of the aroma of the cheeses [1] [2]. Non-starter lactic acid bacteria (NSLAB) also have a strong influence on the development of the flavour, aroma and texture of cheeses and therefore, on their quality [3].

Some microorganisms present enzymatic activities that allow them to survive in the gastrointestinal tract and/or prevent colonisation of the same by potential pathogens, improving the digestibility of certain foods and/or modulating the immune system of the host [4] [5].

The study of the microbiota of artisanal cheeses makes possible to select autochthonous lactic acid bacteria for the production of starter cultures that present a good capacity for adaptation in fermented dairy products and which can be used at an industrial level to obtain cheeses presenting organoleptic characteristics similar to those of artisanal cheeses.

In previous research, we focused on *Enterobacteriaceae*, *Enterococcus* and *Micrococcus* groups isolated during the manufacture and ripening of San Simón da Costa cheese [6]-[8]. In the present study, we focused on the lactic acid bacteria population in San Simón da Costa cheese, with two objectives: to determine the most representative species which may exercise a predominant role in the ripening process of this cheese, and depending on their technological capacity, to select those strains best suited to form part of a starter or adjunct culture that could be used in the industrial manufacture of this cheese.

2. Material and Methods

2.1. Cheese Manufacture and Sampling

Four batches of cheese were manufactured for this study by cheese artisans according to the traditional manufacturing procedure described in García *et al.* (2001) [7].

Full fat untreated cow's milk was heated to approximately 30˚C, and the milk was then salted using 10 - 20 g salt per litre of milk. The milk was coagulated at 30˚C - 32˚C by adding 100 mL of commercial calf rennet (strength 1:10,000) for every 100 L of milk. Thirty min after the addition of rennet, the curd was cut to obtain pieces measuring 5 - 12 mm in diameter. After leaving the cut curds to rest for a period of 5 to 10 min, they were washed and the first manual pressing was performed, gently pressing the curds without removing them from the container until a well-drained homogeneous mass formed at the bottom of the container. Approximately 25% of the volume of the whey was removed and the pressed curd was scalded with approximately the same volume of hot water as the separated whey. When the curd was well-compacted, the whey was drained, and the curd was pre-pressed and transferred into conical moulds. Pressing was initially performed using a pressure of 1 Kg·cm^{-2}, which was increased by the same amount every hour for a total of 3 h. Then, the cheeses were removed from the moulds and scalded. The cheeses were ripened at a temperature of 10˚C - 12˚C and a relative humidity of 70% - 80%, for 45 - 60 days. The final step before sale was to smoke the cheese.

Samples were collected of milk, curd and cheese at 1, 2, 4 and 6 weeks of ripening. All samples were taken to the laboratory under refrigeration and analysed upon arrival.

2.2. Microbiological Analysis

Fifty g of milk, curd and core samples of the cheeses at different stages of ripening were collected aseptically and homogenised with 200 mL of a sterile 2% solution of tribasic sodium citrate at 40˚C - 45˚C for 2 min in a

Sorvall Homogeniser model 17,106. A 1/5 solution was obtained, which was used to prepare successive decimal solutions by mixing 10 mL of the stock solution with 90 mL of 0.1% sterile peptone, according to International Dairy Federation (IDF) Standard 122B (1992).

Different media were employed depending on their selectivity for enumeration and isolation of lactic acid bacteria. MSE agar (Biokar Diagnostics, Beavais, France), is one of the most frequently used media culture for count and isolation of *Leuconostoc*, after incubation at 22°C for 4 days [9]. It is a medium with a very high concentration of sucrose (10%) which promotes the presence of mucoid colonies by dextrane producing strains. In addition it contains sodium azide, a known inhibitor of Gram negative bacteria and partially inhibitor of the lactococci as well. The rich complex medium M17 agar (Biokar Diagnostics, Beavais, France) promotes the growth of *Lactococcus*, especially those with complex nutrient requirements, after incubation at 30°C for 18 to 24 h [10]. The medium includes among others ingredients lactose (5%) and sodium glycerophosphate (19%) which increases the buffering capacity. Rogosa agar (Oxoid, Basingstoke, UK) is an acidic medium (approximately 5.4) used for enumeration and isolation of *Lactobacillus*, after incubation at 30°C for 5 days [11]. This medium includes sodium acetate as one of the ingredients and small amounts of Mn, Mg and Fe which stimulate the growth of lactobacilli. With the M17 and MSE agar plates, the surfaces were inoculated with 0.1 mL of each solution, while with the Rogosa agar plates, the mass itself was inoculated with 1 mL, and after the agar had solidified, a cover of the same medium was added to generate conditions that favoured the growth of lactobacilli. Enumeration was performed on plates with between 30 and 300 colonies. The counts were expressed as colony forming units per gram or millilitre (cfu/g or mL).

2.3. Isolation and Identification of Strains

Nine strains were randomly isolated from the samples of milk, curd and cheese core at different stages of ripening, in the M17 agar, MSE agar and Rogosa agar, a total of 216 strains for each culture medium. The 648 strains were purified by means of growing alternate subcultures in MRS agar and broth [12], and then stored at −30°C with 20% glycerol. The strains were revived in MRS broth and subjected to the various tests described below, in order to identify them at genus level and subsequently at species level.

The tests performed for genus identification included Gram staining and observation of morphology, as well as the catalase test, production of CO_2 from glucose, deamination of amino acid L-arginine, growth at 10°C and 45°C and growth in the presence of 6.5% NaCl.

Species identification was performed by means of different tests depending on the genus in question [13] [14].

The tests performed for assignment to a *Lactococcus* species included growth at 45°C, 40°C and 10°C, the capacity for deamination of L-arginine, the capacity for production of diacetyl, the Voges-Proskauer test, growth in the presence of 4% and 6.5% NaCl and the capacity for fermentation of various sugars (arabinose, xylose, sucrose, trehalose, mannitol, salicin, raffinose, inulin, glycerol, sorbitol, rhamnose, ribose and maltose).

Tests for the identification of *Leuconostoc* species included the capacity to grow at 37°C, in the presence of 6.5% NaCl and in the presence of 10% ethanol, as well as the capacity for production of dextran and fermentation of sugars (arabinose, cellobiose, fructose, glucose, lactose, maltose, melibiose, sucrose, salicin, and trehalose).

Homofermentative *Lactobacillus* species were identified by performing the following tests: growth at 45°C and the capacity for deamination of L-arginine and fermentation of sugars (lactose, arabinose, melibiose, sucrose, sorbitol, xylose, raffinose, rhamnose, inulin and sorbose). Heterofermentative *Lactobacillus* strains were identified according to growth at 15°C and 45°C and the capacity for deamination of L-arginine and fermentation of sugars (arabinose, cellobiose, lactose, melezitose, melibiose, raffinose, salicin, trehalose, xylose and mannose).

Tests for the identification of *Enterococcus* species were based on growth in the presence of 0.1% methylene blue, deamination of L-arginine, growth in the presence of 0.4% potassium tellurite, growth in KF agar, growth at 45°C and 50°C and fermentation of sugars (arabinose, melezitose, melibiose, sorbitol, rhamnose, raffinose, starch and sucrose).

2.4. Genetic Characterisation

Genetic characterisation was performed in order to confirm the identification obtained using classical methodology. DNA was extracted from the strains using the procedure described by O'Sullivan *et al.* (1993) [15] with some modifications. Polymerase chain reaction (PCR) amplification and DNA sequence analysis were performed following the methodology described by González *et al.* (2007) [16].

2.5. Technological Characterisation

The acidifying activity of the strains was determined in accordance with IDF Standard 306 (IDF, 1995). The strains were revived in MRS broth at 30˚C for 24 h. The microbial culture was inoculated in a proportion of 1 mL per 100 mL of 10% reconstituted skimmed milk.

Titratable acidity (TA) and pH were determined after 6, 12 and 24 h of incubation at 30˚C.

The proteolytic activity of the strains was determined by spectrophotometry using the O-phthaldialdehyde derivatisation method [17]. The results were expressed in mMGly·L^{-1} of milk.

The enzymatic activity of the strains was evaluated by means of the API-ZYM test (BioMérieux, Marcy-L'Étoile, France). *Leuconostoc* and *Lactobacillus* strains were incubated in MRS broth, and *Lactococcus* strains were incubated in Elliker broth for 16 h at 30˚C. The cultures were centrifuged at 7000 g for 15 min at 4˚C, and the sediment was resuspended in 2 mL phosphate buffer (50 mM, pH 7) until attaining an optical density of between 5 and 6 on the McFarland scale, and 65 μL was then inoculated in the API-ZYM strip microtubes. The API-ZYM strips were incubated at 37˚C for 4 h, and enzyme activity was scored from 0 to 5 by comparison of the colour developed at 5 min with the API-ZYM test colour reaction chart. The enzymatic activities examined included: alkaline phosphatase, esterase (C4), lipase esterase (C8), lipase (C14), leucinearylamidase, valine-arylamidase, cystine-arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, and N-acetyl-β-glucosaminidase.

3. Results and Discussion

3.1. Identification of the Lactic Acid Bacteria

From the strains isolated in the three culture media, 76.39% were identified as lactic acid bacteria at the genus and species level. A proportion of 28.48% were isolated in M17 agar, 33.74% in MSE agar and 37.78% in Rogosa agar. **Table 1** shows the genera isolated from M17 agar, MSE agar and Rogosa agar during the ripening of San Simón cheese.

Table 1. Changes in the genera isolated from M17 agar, MSE agar and Rogosa agar during the manufacture and ripening of San Simón cheese[a].

Medium	Genera	Milk		Curd		1		2		4		6	
		N° cepas	(%)	N° cepas	(%)	N° cepas	(%)	N° cepas	(%)	N° cepas	(%)	N° cepas	
M17	*Lactococcus*	---		2	5.6	1	2.8	1	2.8	1	2.8	3	8.3
	Lactobacillus	2	5.6	2	5.6	3	8.3	4	11.1	3	8.3	12	33.3
	Leuconostoc	---		---		2	5.6	---		1	2.8	1	2.8
	Enterococcus	---		5	13.9	2	5.6	9	25	6	16.7	11	30.6
MSE	*Lactococcus*	1	2.8	1	2.8	4	11.1	6	16.7	1	2.8	4	11.1
	Lactobacillus	3	8.3	1	2.8	1	2.8	2	5.6	1	2.8	2	5.6
	Leuconostoc	1	2.8	1	2.8	8	22.2	9	25	15	41.7	5	13.9
	Enterococcus	9	25	9	25	14	38.9	9	25	12	33.3	16	44.4
ROGOSA	*Lactococcus*	---		1	2.8	---		---		5	13.9	3	8.3
	Lactobacillus	22	61.1	16	44.4	24	66.6	19	52.8	18	50	28	77.8
	Leuconostoc	2	5.6	1	2.8	1	2.8	1	2.8	3	8.3	2	5.6
	Enterococcus	2	5.6	3	8.3	3	8.3	4	11.1	10	27.8	3	8.3

Ripening time (weeks) spans the data columns.

[a]The percentage was calculated from the isolated strains in each sampling point and for the four batches (36 strains from each culture medium).

The proportion of lactic acid bacteria strains assigned to the genus *Lactococcus* was 9.29%. In this study, the M17 agar showed poor selectivity for the isolation of lactococci since only 3.75% of the strains isolated in this medium were assigned to this genus. The lactococci strains were identified as *Lactococcus lactis* subsp. *lactis* (28.26%), *L. lactis* subsp. *cremoris* (65.22%) and *L. lactis* subsp. *raffinolactis* (4.35%).

In most Spanish artisanal cheeses, *L. lactis* subsp. *lactis* constitutes the dominant species of the genus *Lactococcus*, whereas *L. cremoris* is only isolated on rare occasions [16] [18] [19].

Of the lactic acid bacteria strains, the 15.35% were identified as *Leuconostoc*. The MSE medium showed poor selectivity for the isolation of *Leuconostoc*. The strains of this genus were assigned to the species *Ln. paramesenteroides* (55.26%), *Ln. lactis* (17.10%), *Ln. mesenteroides* subsp. *mesenteroides* (6.58%), *Ln. cremoris* (3.95%) and *Ln. mesenteroides* subsp. *dextranicum* (1.31%).

A proportion of 47.07% of the lactic acid bacteria strains were assigned to the genus *Lactobacillus*. This genus was isolated in Rogosa agar (58.80%), in M17 agar (30.47%) and in MSE agar (10.73%). Therefore, the Rogosa agar showed high selectivity for the isolation of lactobacilli. Although homofermentative lactobacilli predominated (60.52%), heterofermentative lactobacilli were also identified (39.48%). López and Mayo (1997) [20] also detected a percentage of heterofermentative lactobacilli in studies conducted on several artisanal cheeses from Asturias.

The homofermentative lactobacilli strains isolated from San Simón cheese comprised *Lb. plantarum* (10.30% of the total lactobacilli), *Lb. casei* subsp. *casei* and *Lb. paracasei* subsp. *paracasei* (34.76%), *Lb. rhamnosus* (14.16%) and *Lb. paracasei* subsp. *tolerans* (1.29%), while the heterofermentative strains included *Lb. buchneri* (18.88%), *Lb. bifermentans* (16.31%) and *Lb. fermentum* (4.29%).

Lactobacilli form part of the non-starter lactic acid bacteria (NSLAB) that contribute to the development of the final flavour of ripened cheeses. It is of interest, therefore, to select lactobacilli strains with technological aptitudes that contribute to improving the intensity and quality of a cheese's flavour and texture. In this regard, it should be noted that heterofermentative lactobacilli are seldom included as co-cultures in assays conducted with non-starter strains since they may cause defects in the cheese [21].

Lastly, 28.28% of the strains isolated in the three culture media belonged to the genus *Enterococcus*. Of the enterococci, 70% were ascribed to the species *Enterococcus faecalis*. García *et al.* (2002) [8] ascribed between 63% and 79% of the strains from milk, curd and San Simón cheese core, isolated in KAA agar, to the genus *Enterococcus*, the dominant species again being *E. faecalis*. Similar results were obtained in other varieties of cheese made with milk from different species and presenting very different characteristics, such as Roquefort cheese [22], Manchego cheese [23], Armada cheese [6], Roncal and Idiazabal cheese [24] and Tenerife cheese [25].

This biodiversity of species, and in particular the presence of *Lb. paracasei*, *Lc. lactis*, *Ln. mesenteroides* and *E. faecalis*, has been also detected in a wide range of cheeses, and *Lb. paracasei* has also presented a notable phenotypic diversity [26].

We selected 20 strains of lactic acid bacteria that had been identified at species level by classical methods and obtained from different batches and sampling points, which were representative of the majority of the biochemical patterns found, in order to verify their assignment to species by genetic methods. **Figure 1** and **Figure 2** show the electrophoresis images of the PCR products of lactic acid bacteria strains. In all cases, a single DNA band, of 834 pb, was obtained, the result of the PCR amplification of DNA that codes for 16S ribosomal RNA. The corresponding band was sequenced and compared with sequences deposited in databases. For some strains, it was possible to confirm the identification using classical methods. Identification of strains using classical methodology and molecular techniques yielded 100% agreement for the strains *Lactococcus lactis* subsp. *lactis*, *Lactobacillus casei* subsp. *casei*, *Lactobacillus casei* subsp. *rhamnosus* and *Leuconostoc mesenteroides*. However, 7 strains assigned to the species *Lactobacillus casei* by classical methodology were identified as *Lactobacillus paracasei* by genetic techniques. Lastly, it should be noted that with regard to *Enterococcus faecalis,* assignment coincided by 67%.

Artisanal cheeses are an important reservoir of phenotypic and genotypic microbial diversity that can have important applications in biotechnology [27]-[29]. In fact, there is a growing interest in gaining a better knowledge of the microbiota of cheeses made with raw milk because of the need to find starter cultures that could complement or renew existing ones [30] [31].

3.2. Technological Aptitude and Enzymatic Characteristics of the Lactic Acid Bacteria

As reported above, 20 strains of lactic acid bacteria whose identification had been confirmed by molecular tech-
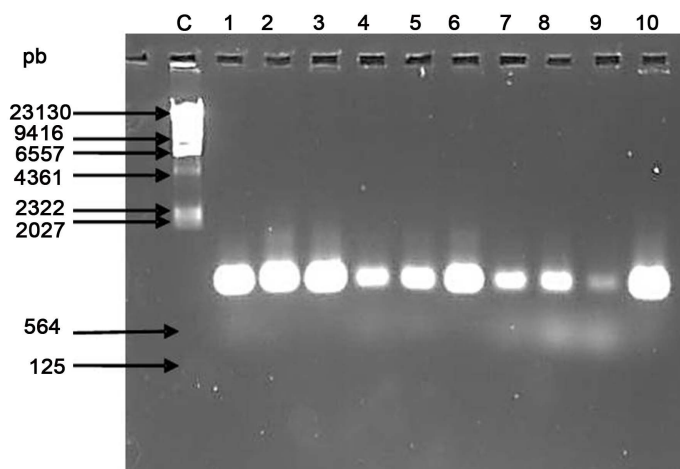
Figure 1. Electrophoresis of PCR products obtained with the 8 and 806 primers. C: Control. DNA of Lambda phage digested with *Hind* III. Order of 1 - 10: lactic acid strains isolated from artisanal San Simón cheese: *Lactobacillus plantarum* (SS 1239), *Lactococcus lactis* subsp. *lactis* (SS 13), *Enterococcus faecalis* (SS 26), *Lactococcus lactis* subsp. *lactis* (SS 193), *Lactococcus lactis* subsp. *lactis* (SS 194), *Leuconostoc mesenteroides* (SS 1437), strain not assigned to a genus (SS 1439), *Lactococcus lactis* subsp. *lactis* (SS 2379), *Enterococcus sulphureus* (SS 163), *Leuconostoc pseudomesenteroides* (SS 2068).
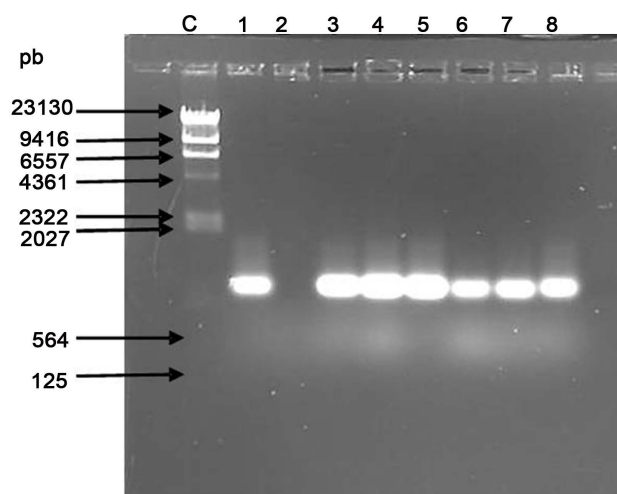

Figure 2. Electrophoresis of PCR products obtained with the 8-806 primer. C: Control. DNA of Lambda phage digested with *Hind* III. Order of 1-8: *Lactobacillus casei* (SS 1615), negative control without DNA, *Lactobacillus plantarum* (SS 1594), *Lactobacillus casei* (SS 263), *Enterococcus raffinosus* (SS 1293), *Lactobacillus casei* (SS 1614), *Lactococcus lactis* subsp. *lactis* (SS 2363), *Enterococcus* spp. (SS 164).

niques were selected in order to perform a technological aptitude study. The strains selected for study were as follows: *Lactococcus lactis* subsp. *lactis* (3 strains), *Lactobacillus casei* subsp. *casei* (3 strains), *Lb. paracasei* (7 strains), *Lb. casei* subsp. *rhamnosus* (1 strain), *Leuconostoc mesenteroides* (3 strains) and *Enterococcus faecalis* (3 strains).

One of the most important properties of the lactic acid bacteria that are used as starters in cheese making is their initial capacity to acidify milk [32]. In fact, in order for a lactic strain to be considered a good candidate for inclusion as a starter, it should produce sufficient acid to reduce the pH of the milk to values lower than 5.3 after 6 h of incubation in milk at 30°C [33]. Various authors agree that the difference between strains with a fast or slow acidifying capacity is that with the former, acidity increases within 6 h by up to at least 30°C [34]-[36].

The acidifying capacity of the 20 strains of lactic acid bacteria tested was determined after 6, 12 and 24 h of incubation at 30°C. The results are shown in **Table 2**.

Of the total number of strains tested, only 5 had the capacity to reduce milk pH to values lower than 6 after 6 hours of incubation at 30°C, and 8 strains yielded a titratable acidity in milk equal to or greater than 30°C, so in general can be considered slightly acidifying strains. These results are consistent with those previously obtained by other authors for other traditional cheeses [37] [38]. In general, the acidifying capacity of the strains belonging to *Lactococcus lactis* species was similar or slightly superior to that of the *Lactobacillus* and *Leuconostoc* strains, although there were variations in acidity production between strains of these species. These differences became more marked over the course of incubation, with values ranging from 0.39 to 0.85 g 100 mL^{-1} lactic acid, depending on the strain. The final acidification produced by some strains of lactobacilli and enterococci was higher than that yielded by *Lactococcus* and *Leuconostoc* strains, reaching values of around 0.75 - 0.85 g 100 mL^{-1} lactic acid at 24 h of incubation, a behaviour which was also observed by Herreros *et al.* (2003) [39].

The results obtained for the proteolytic activity of the 20 selected strains are shown in **Table 3**. Both *Leuconostoc* and *Lactococcus* strains presented low proteolytic activity in general, with the former presenting the lowest activity, yielding values that did not exceed 0.2 mMGly·L^{-1}, while values for the *Lactococcus* strains were below 0.5 mMGly·L^{-1}, with the exception of one *Lactococcus lactis* strain that presented values of 0.80 mMGly·L^{-1}. These values differed between strains but were generally slightly lower than those detected by various authors for other strains of *Lactococcus* isolated from traditional cheeses of different origins [37] [39] [40], although they were similar to those detected by Mayo *et al.* (1990) [41]. The majority of the *Lactobacillus* strains presented a proteolytic activity of between 0.1 and 0.5 mMGly·L^{-1}, and one *Lb. paracasei* strain yielded values of around 1.6 mMGly·L^{-1}. In the case of the *Enterococcus* strains, two of the three strains tested released high concentrations of amino groups (1.3 and 1.8 mMGly·L^{-1}) in comparison with the rest of the strains. In the later stages of ripening, NSLAB population contributes to proteolysis and development of the aroma of the cheeses.

Table 2. Acidifying activity of the 20 strains of lactic acid bacteria isolated from San Simón cheese.

Strain		Incubation time (h)					
		6 h		12 h		24 h	
		pH	Titratable acidiy[a]	pH	Titratable acidiy[a]	pH	Titratable acidiy[a]
Lactococcus lactis	SS 193	6.2	0.32	5.6	0.44	4.84	0.64
	SS 194	5.64	0.26	5.25	0.4	4.71	0.58
	SS 287	5.7	0.32	5.25	0.38	5.03	0.52
Lactobacillus casei sub sp. *casei*	SS 1614	6.23	0.31	5.63	0.41	5.06	0.59
	SS 1615	6.16	0.24	5.59	0.29	4.52	0.85
	SS 263	6.29	0.31	5.99	0.42	4.86	0.67
	SS 1644	6.08	0.26	5.29	0.39	4.9	0.74
	SS 1661	6.08	0.29	5.77	0.3	5.15	0.51
	SS 1695	6.21	0.2	5.66	0.28	5.07	0.55
Lactobacillus casei sub sp. *paracasei*	SS 1689	6.15	0.27	5.89	0.29	5.78	0.39
	SS 1770	6.12	0.21	5.66	0.4	4.5	0.75
	SS 1778	6.11	0.19	5.64	0.35	4.34	0.84
	SS 1785	6.10	0.3	5.72	0.3	4.32	0.85
Lactobacillus casei sub sp. *rhamnosus*	SS 1684	5.83	0.24	5.38	0.3	5.26	0.39
Leuconostoc	SS 1435	5.7	0.3	5.51	0.33	5.18	0.43
	SS 1437	6.13	0.31	5.56	0.4	5.39	0.46
	SS 1464	6.12	0.28	5.83	0.33	5.62	0.4
Enterococcus	SS 1378	5.88	0.33	5.02	0.48	4.55	0.8
	SS 191	6.08	0.27	5.4	0.56	4.77	0.85
	SS 1449	6.18	0.25	5.68	0.3	5.44	0.4

[a]Titratable acidity expressed as g 100 mL^{-1} lactic acid.

Table 3. Proteolytic activity of the 20 strains of lactic acid bacteria isolated from San Simón cheese.

Strain		Proteolytic activity[a]
Lactococcus lactis	SS 193	0.135 ± 0.010
	SS 194	0.804 ± 0.060
	SS 287	0.398 ± 0.020
Lactobacillus casei sub sp. *casei*	SS 1614	0.505 ± 0.018
	SS 1615	0.182 ± 0.012
	SS 263	0.051 ± 0.010
	SS 1644	0.135 ± 0.008
	SS 1661	0.060 ± 0.006
	SS 1695	0.637 ± 0.040
Lactobacillus casei sub sp. *paracasei*	SS 1689	1.605 ± 0.005
	SS 1770	0.470 ± 0.006
	SS 1778	0.290 ± 0.003
	SS 1785	0.398 ± 0.002
Lactobacillus casei sub sp. *rhamnosus*	SS 1684	0.111 ± 0.001
	SS 1435	0.099 ± 0.001
Leuconostoc	SS 1437	0.195 ± 0.003
	SS 1464	0.132 ± 0.005
	SS 1378	1.832 ± 0.006
Enterococcus	SS 191	0.374 ± 0.001
	SS 1449	1.320 ± 0.002

[a]Proteolytic activity expressed as mMGly·L^{-1} of milk.

While some authors have related the proteolytic and acidifying activity of some lactic acid bacteria such as the lactococci [34] [41] [42], no such relationship was observed in the present study, and appeared rather as the characteristic properties of each strain.

The enzymatic activities of the 20 strains assessed by means of the API-ZYM system are shown in **Table 4**.

None of the microorganisms presented activities for the following enzymes: trypsin, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Similar results have been reported for strains isolated from Arzúa-Ulloa cheese [43] and other artisanal cheeses [44]. In addition, the absence of some of these activities, for example β-glucuronidase, can be considered as being of a probiotic nature, since this enzyme increases the production of toxic and/or carcinogenic compounds [45]. A-chymotrypsin and trypsin activity showed low or null values. Bearing in mind that these may be involved in pathogenic processes, their absence is interesting from a probiotic point of view [46].

With respect to activity on the rest of the carbohydrates, most of the strains of the *Lactobacillus* and *Leuconostoc* genera showed high α-glucosidase activity, but this was not detected in *Lactococcus lactis* strains and only minimal activity was observed in *Enterococcus faecalis* strains. This enzyme participates in the hydrolysis of the α (1 - 4) glucans, which cannot be degraded by human glucosidases; thus, the lactobacilli that showed this activity could be of probiotic interest. At the same time, it catalyses the final steps of carbohydrate digestion and therefore its absence slows the assimilation of carbohydrates ingested in the diet, and is therefore useful for obese or diabetic patients. In terms of β-galactosidase activity, some strains of lactobacilli showed high activity which could be related to the capacity to reduce lactose intolerance and stimulation of the growth of bifidobacteria in the intestine [44].

Table 4. Enzymatic activity[a] (API-ZYM) of lactic acid bacteria isolated from San Simón cheese.

Strains		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Lactococus lactis	SS 193	-	5 - 10	10	-	20	-	5	-	5	10	5	-	-	-	-	-	-	-	-
	SS 194	-	5	10	-	40	5	5	-	5	10	5	-	-	-	-	-	-	-	-
	SS 287	-	<5	<5	-	30	-	-	-	-	40	5	-	-	-	-	-	-	-	-
Lactobacillus casei subsp. *casei*	SS 1614	-	5 - 10	10	-	40	10 - 20	-	-	<5	5	10	-	-	-	20	-	-	-	-
	SS 1615	-	10 - 20	20	-	40	40	20	-	-	5	20	-	-	-	20	-	-	-	-
	SS 263	-	20	30	-	40	40	-	-	-	10	20	-	10	-	40	-	-	-	-
Lactobacillus casei sub sp. *paracasei*	SS 1644	-	10	10	-	>40	>40	5	-	5	20	40	-	20	-	>40	5	-	-	-
	SS 1661	-	20	20	-	≥40	≥40	5	-	5	20	30	-	30	-	≥40	5	-	-	-
	SS 1695	-	10	10	-	>40	>40	5	-	5	20	30	-	20	-	30	-	-	-	
	SS 1689	-	10 - 20	10	-	5	-	-	-	-	5	5	-	-	-	-	-	-	-	-
	SS 1770	-	10 - 20	10 - 20	-	40	20	10	-	5	10	20	-	<5	-	10	-	-	-	-
	SS 1778	-	20	10	<5	40	40	5	-	-	5 - 10	20	-	<5	-	30 - 40	-	-	-	-
	SS 1785	-	10 - 20	10	-	40	40	-	-	-	5	20	-	-	-	40	-	-	-	-
Lb. casei sub sp. *rhamnosus*	SS 1684	-	10	10	-	30	-	-	-	-	20	30 - 40	-	10 - 20	-	40	-	-	-	-
Leuconostoc	SS 1435	-	10 - 20	5 - 10	-	5	-	-	-	-	5 - 10	20	10	10 - 20	-	40	-	-	-	-
	SS 1437	-	10	10	-	20	-	-	-	-	5	10	-	-	-	-	-	-	-	-
	SS 1464		-	-	-	5	-	-	-	-	20	-	20	5	-	30 - 40	-	-	-	-
Enteroccocus	SS 1378	-	20	20	-	30	-	5	-	-	5	-	-	5	-	-	-	-	-	-
	SS 191	<5	20	5	-	30	5	10	-	20	20	5	-	5	-	5	-	-	-	-
	SS 1449	-	20	10	-	-	-	0 - 5	-	5	5	5	-	-	-	-	<5	-	-	-

[a]Enzimatic activity (approximate values) expressed as nmol of substrate hydrolysed. [b]Enzimes tested: 1. Alkaline phosphatase; 2. Esterase (C4); 3. Esterase lipase (C8); 4. Lipase (C14); 5. Leucinearilamidasa; 6. Valina arilamidase. 7. Cistina arylamidasa; 8. Tripsin; 9. α-Quimotrypsin; 10. Acid-phosphatase; 11. Naphthol-AS-BI-phosphohydrolase; 12. α-galactosidase; 13. β-galactosidase; 14. β-glucuronidase; 15. α-glucosidase; 16. β-gluco-sidase; 17. N-acetyl-β-glucosaminidase; 18. α-mannosidase; 19. α-fucosidase.

No alkaline phosphatase activity was detected in any of the strains tested, what appears to be common in the case of lactic acid bacteria [39] [47] [48]. In contrast, acid phosphatase activity, an enzyme necessary for hydrolysis of phosphopeptides, attained very variable values ranging from 5 to 20 nmol of substrate hydrolysed, with the exception of one *Lactococcuslactis* strain which yielded values of 40 nmol. The high phosphatase activity exhibited by lactic acid bacteria strains may have an important application in the degradation of phytate.

Esterase and esterase-lipase activities were different depending on the groups and strains tested; in general, lactococci strains presented the lowest values while *Enterococcus faecalis* strains presented the highest values. None of the strains except for one showed any activity on long chain fatty acids, which confirms the fact that lactic acid bacteria show a preference for short-chain substrates.

Leucineaminopeptidase activity was detected in all strains, and elevated activity (in the order of 40 nmol of hydrolysed substrate) was observed in the majority of the *Lactobacillus casei* strains, with levels comparable to those obtained by other authors. A high valineaminopeptidase activity was also observed in these strains, which was not detected in the rest of the tested strains. The presence of aminopeptidase activity is desirable since it can help generate the characteristic aroma of cheese and decrease bitterness by hydrolysing the peptides formed during cheese ripening.

4. Conclusion

In conclusion, some of the lactic acid bacteria strains isolated from San Simón da Costa cheese may contribute to the acidification of milk and curd and to the formation of aromas and flavours as a consequence of their proteolytic and esterase-lipase activities. Two *Lactococcus lactis* subsp. *lactis* strains (SS 193 and SS 1437) could be selected to constitute a starter culture based on their acidifying capacity. One *L. lactis* subsp. *lactis* strain (SS 194) and two *Lb. paracasei* strains (SS 1689 and 1695 SS) were notable for their proteolytic capacity, and could be used as co-cultures. The proteolytic activity of *Enterococcus* strains 1378 SS and SS 1449 is also noteworthy.

Acknowledgements

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