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Comparison of the effects of two soy waste-based culture media on the technological properties of *Lacticaseibacillus paracasei* 90 as adjunct culture in miniature Cremoso cheese

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Research Article

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Abstract

We compared the effects of two waste-based culture media (M1 and M2) on the technological properties of *Lacticaseibacillus paracasei 90* (L90) for its application as a secondary culture in Cremoso cheese. The following parameters were studied at different ripening times: pH (7, 20, and 40 d), microbiological counts, carbohydrates and organic acids (7 and 40 d), moisture, fat, protein and volatile compounds (40 d). The viability and the metabolic performance of the strain in cheeses were also verified along ripening. Lactobacilli counts in experimental cheeses manufactured with L90 were ~8 log CFU/g at 40 d, meanwhile adventitious lactobacilli reached ~4 log CFU/g in the control cheese (made without L90). The levels of lactic acid, citric acid and pyruvic acid were similar among cheeses at 40 d, reaching levels of ~1433 mg/100 g, ~172 mg/100 g, and 7 mg/100 g, respectively. However, the concentration of lactic acid showed numerical differences between experimental and control cheeses. The cheeses made with the adjunct culture showed lower residual galactose (< 588 mg/100 g) in comparison with the control cheese (836 mg/100 g), highlighting the potential of L90 to play a bioprotective role. In the same vein, orotic and hippuric acids were metabolized at different degrees in cheeses made with L90, reaching levels of < 3.7 mg/100 g and < detection limit, respectively, at 40 d. In general, volatile compounds profiles were not negatively affected by the culture media used. On the contrary, the production of key aroma compounds (diacetyl and acetoin) was positively affected by the growth of L90 in these alternative media. The results demonstrated that the L90 strain could be used as an adjunct culture in Cremoso cheese, regardless of the culture media employed for its growth.

Consumers are more and more interested in fermented foods due to the increasing evidence of their benefits to human health (Şanlier *et al.*, 2019; Vinderola *et al.*, 2023; Gänzle *et al.*, 2024). At the same time, to meet the growing demand for fermented foods, new starter and secondary cultures are being developed by industries, especially for the dairy sector. For the last three decades, a large number of lactobacilli strains have been selected for different applications (flavour producers, texture enhancers, biopreservatives, probiotics etc.), and many of them have been isolated from cheese samples as non-starter lactic acid bacteria (NSLAB: Settanni and Moschetti, 2010; Gobbetti *et al.*, 2015). These strains, mostly mesophilic lactobacilli isolated from good-quality cheeses, are selected considering their metabolic activities, especially their ability to enhance flavour. For example, numerous strains were selected based on their aminotransferase and glutamate dehydrogenase profiles (Kieronczyk *et al.*, 2004; García-Cayuela *et al.*, 2012; Lee *et al.*, 2020; Mazhar *et al.*, 2020). These intracellular enzymes are involved in the initial steps of amino acid catabolism, playing a key role in the cheese aroma development. Another criterion for strain selection is associated with the ability to produce diacetyl and acetoin by citrate metabolism (citrate-positive strains: Skeie *et al.*, 2008; Beresford, 2022). Regardless of the selection criteria, it is crucial that the characteristics for which a strain is selected remain intact during its industrialization. The development of such cultures requires not only large amounts of biomass produced at industrial scale but also a convenient formulation that ensures long-term viability and the conservation of the metabolic activity during storage (Parente *et al.*, 2017).

Lacticaseibacillus paracasei 90 (L90) is an autochthonous strain isolated from good quality semi-hard cheese (Ugarte *et al.*, 2006) that has been well characterized when grown in commercial MRS. Distinguishing features are its potential for enhancing the flavour profile of cheese, which could be associated with its capacity to produce 2,3-butanedione (also known as diacetyl: Milesi *et al.*, 2010; Peralta *et al.*, 2016a, 2020), and its peptidolytic activity that contributes to the cheese ripening process (Peralta *et al.*, 2016b, 2023a). Furthermore, its

protective role against the growth and the undesirable metabolic activity of contaminant and adventitious microorganisms has been reported in Cremoso cheese (Peralta *et al.*, 2020; Giménez *et al.*, 2021). Aiming at the potential scaling up of L90, the production of its biomass was optimized in three different culture media based on waste derived from soy protein concentrate production (Beret *et al.*, 2021). In addition, both freeze-drying and spray-drying technologies were tested on L90 as preservation strategies. Peralta *et al.* (2017) reported that spray-drying was appropriate to produce a dehydrated adjunct culture for this strain. Recently, Peralta *et al.* (2023b) also reported the effects of freeze-drying and long-term storage on the viability and metabolic activity of L90 grown in the alternative media proposed by Beret *et al.* (2021). In this study, the metabolic potential of L90 as adjunct culture in a semi-hard cheese after its growth in glucose-supplemented residue-based culture medium was verified. Even though several studies have verified the good performance of L90 as a secondary culture in cheeses, in most of them the strain was grown in a commercial medium such as MRS broth. Therefore, information on its activity as secondary culture after growing in potential industrial media is scarce. In this context, this study aimed to compare the effects of two culture media based on the waste from soy industrialization, on the technological properties of L90 for its application as a secondary culture in Cremoso cheese, the variety most commonly consumed in Argentina. An advantage of this approach is that the use of such media may contribute to the circular economy in countries with large volumes of industrialization of this legume (Chua and Liu, 2019; Magnano *et al.*, 2024).

Materials and methods

Bacterial strain and experimental design

Lacticaseibacillus paracasei 90 (L90) was activated by two successive incubations in MRS broth (Biokar, France) at 37°C for 18 h. After that, L90 was grown in two culture media formulated with the aqueous by-product generated in the production of soy flour protein concentrates as per Beret *et al.* (2021). The media were labelled as M1 and M2. Both media were supplemented with yeast extract and minerals ($MgSO_4$ and $MnSO_4$), whilst only M1 was supplemented with glucose (online Supplementary Table S1). As a control, L90 was also grown in MRS, labelled as such. The cells obtained from M1, M2 and MRS were washed twice with 50 mM potassium phosphate buffer (pH = 7) and resuspended in a 10% (w/v) lactose solution. The suspensions were frozen at −80°C and then freeze-dried using a Martin Christ Alpha (1-4-LD Plus, Germany). The freeze-dried cultures were used as adjuncts in Cremoso miniature cheeses.

Cheesemaking

Miniature Cremoso cheeses (~200 g) were made in triplicate using the freeze-dried strain of L90 as secondary culture. Raw milk was obtained from a nearby dairy plant (Milkaut, Franck, Santa Fe) and was heat-treated by the batch method at 65°C for 30 min. Temperature was decreased at 37°C and the milk was transferred to four 2 L-vats which were kept at the same temperature. Calcium chloride was added to each vat (0.02% w/v) and afterwards the starter and adjunct culture were inoculated. Both control (C, without adjunct culture) and experimental (E, with the addition of adjunct culture) cheeses were manufactured using *Streptococcus thermophilus* (ST12, Chr. Hansen, Argentina) as starter culture at a level of 10^6 CFU/ml. Freeze-dried cultures obtained from M1, M2 and MRS were added in vats at a concentration of 10^6 CFU/ml to obtain the experimental cheeses E-M1, E-M2 and E-MRS, respectively. Rennet (ChyMax, Chr. Hansen, Argentina) was added to the cheese milk in a dose of 34 IMCU/l. When the curd reached the appropriate firmness, it was cut into cubes of approximately 0.5 cm^3 and softly stirred. Once the curd decanted to the bottom, the whey was drained and the curd was placed into micro-perforated moulds and incubated at 45°C until reaching a pH of 5.2–5.3. The pH was measured with a pHmeter (Orion Research Incorporated, USA) along the whole process. Finally, cheeses were individually plunged in sterile brine (20 g/l NaCl, pH 5.4, 5°C) for 1 h/kg. The cheeses were stored at 5°C for 3 d, vacuum packed, and stored again at 5°C to complete 40 d of ripening.

Gross composition and pH

Moisture, fat, and protein contents were analysed according to standardized methodologies (ISO, 2004, 2008, 2014) at 40 d of ripening. The pH of the cheeses was determined at 7, 20 and 40 d of ripening, according to Bradley *et al.* (1993).

Microbial counts

Microbial counts of total lactic acid bacteria (total LAB), mesophilic lactobacilli, coliforms, moulds and yeasts were performed in cheeses at 7 and 40 d of ripening according to Peralta *et al.* (2017). Total LAB were enumerated on skim milk agar. Lactobacilli, mainly mesophilic lactobacilli, were determined by enumeration on MRS-agar (Biokar, France) acidified at pH 5.4. Both cell counts were incubated for 48 h at 37°C under microaerobic conditions. Coliforms were enumerated on Bile Red Violet Lactose Agar (Biokar, France); the plates were incubated for 24 h at 30°C. Population of moulds and yeast were determined on yeast extract-glucose-chloramphenicol agar (Biokar, France), incubated for 7 d at 25°C. Results were expressed as log CFU/g of cheese.

Determination of organic acids and carbohydrates

The quantification of organic acids and carbohydrates in cheeses at 7 and 40 d of ripening was carried out by high-performance liquid chromatography (HPLC) as per Peralta *et al.* (2019). The cheese samples (5 g) were homogenized in 25 ml of 0.01 M H_2SO_4 using an Ultraturrax® homogenizer (3 cycles, 17 000 rpm, 1 min using model T25, IKA, Staufen, Germany), Afterwards, the suspensions were kept at 40°C in a water bath for 15 min and then centrifuged (10 000 ***g***, 30 min, 10°C). The supernatants were filtered through fast-flow filter paper and stored at −20°C until analysis. The samples were thawed at room temperature to perform the analysis, filtered again through 0.45 µm membranes (Millex, Millipore, São Paulo, Brazil), and injected into HPLC. The HPLC equipment (Perkin Elmer, USA) consisted of a quaternary pump, an on-line degasser, two on-line detectors, UV/vis and RI detectors and an analogic interphase connected to a computer provided with the software Chromera® for the acquisition and processing of data. UV detector was set at 210 nm for the quantification of organic acids while the RI detector, set at 35°C, was used for the determination of carbohydrates. The column employed was an *Aminex HPX-87H* (300 × 7.8 mm), and a guard column *Aminex Cation-H* (30 × 4.6 mm: Bio-Rad Laboratories, USA). The separation was

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performed isocratically at 0.6 ml/min with a mobile phase of H_2SO_4 0.01 M at a temperature of 65°C.

Analysis of volatile compounds

Volatile compounds were isolated and semi-quantified in the three replicates of the cheeses samples at 40 d of ripening by headspace solid-phase microextraction coupled with gas chromatography (SPME-GC) equipped with flame ionization detector (Agilent J&W, Agilent Technologies, USA) according to the conditions and procedure described in Giménez *et al.* (2023).The first identification of peaks was performed by comparing the retention time with those of authentic standards. The areas of these peaks were analysed using TotalChrom® software (Perkin Elmer, USA). In parallel, one replicate of each cheese was analysed by SPME-GC coupled to mass spectrometry (GCMS-QP2010, Ultra, Shimadzu) employing the same conditions as above, to confirm the tentative identification by standards. This second identification was performed by comparing the mass spectra of peaks with the mass spectra of libraries provided by the software. Only those peaks confirmed by GC-MS and tentatively detected with flame ionization are reported in this study.

Statistical analysis

The software Statgraphics Centurion XVI (StatPoint Technologies Inc., USA) was used, and one-way analysis of variance (ANOVA) and Tukey test ($P = 0.05$) were applied to compare the means of data obtained from cheese composition, pH, microbiological counts, carbohydrates and organic acids. Principal components analysis (PCA) was performed using the R corrplot package (www.r-project.org) for volatile compounds.

Results and discussion

Acidification kinetics, gross composition, and pH values

The pH of raw milk before the pasteurization treatment was 6.65, meanwhile the first pH measure of cheeses after moulding was between 6.15 and 6.20. Generally, the curd of this cheese variety has these levels of pH at the time of entry in the incubation stage. Activity of the starter culture, time in vat and milk composition are some of the factors that could impact on these values. Even though most of the acid production takes place in the stage of incubation at 45°C, lactic acid could also be produced by the metabolic activity of the starter during the stages of coagulating, cutting, draining, and moulding, where the vat temperature is kept at 37°C. In addition, but to a lesser extent, heat treatment can reduce the pH of milk. The acidification rates during the incubation at 45°C in the first hours of cheese-making are shown in Fig. 1. Regardless of the culture media used for L90 growth, the acidification was not affected by addition of the adjunct culture. The pH values gradually decreased to 5.10–5.30 after 150 min. Similar acidification kinetics for Cremoso cheese were previously reported by Briggiler-Marcó *et al.* (2007).

Regarding gross composition, no significant differences ($P > 0.05$) were found in moisture, fat and protein contents between experimental and control cheeses (Table 1); and these results were similar to those reported by other authors for Cremoso cheese (Briggiler-Marcó *et al.*, 2007; Milesi *et al.*, 2010; Peralta *et al.*, 2019). The changes in pH during cheese ripening are also shown in Table 1. After 7 and 20 d of ripening, pH remained

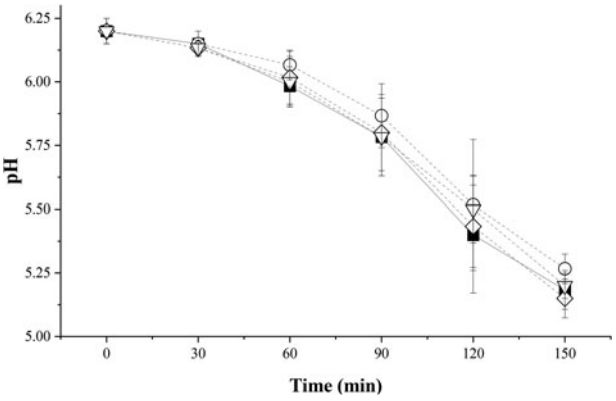

Figure 1. pH evolution during cheese-making of C (—■—), E-m1 (- -○- -), E-m2 (- -◇- -), and E-mrs (- -▽- -).

Table 1. Gross composition (g/100 g) and pH evolution of cheeses during ripening

Parameters	C	E-M1	E-M2	E-MRS
Gross composition (40 d)				
Moisture (%)	53.0 ± 1.3[a]	54.3 ± 0.5[a]	53.1 ± 1.2[a]	54.1 ± 1.2[a]
Fat (%)	24.6 ± 0.5[a]	23.9 ± 0.5[a]	24.7 ± 0.4[a]	24.3 ± 1.1[a]
Protein (%)	18.7 ± 0.4[a]	17.9 ± 0.2[a]	18.4 ± 0.6[a]	18.0 ± 0.4[a]
pH evolution				
7 d	5.35 ± 0.13[a]	5.42 ± 0.15[a]	5.30 ± 0.09[a]	5.30 ± 0.09[a]
20 d	5.38 ± 0.02[a]	5.42 ± 0.10[a]	5.28 ± 0.13[a]	5.31 ± 0.12[a]
40 d	5.28 ± 0.14[a]	5.15 ± 0.05[a,b]	5.05 ± 0.05[b]	5.03 ± 0.08[b]

Values are means ± standard deviation of three cheese replicates. Different letters in a same row mean significant differences ($P < 0.05$).
Cheese labels: C: control; E-M1, E-M2, E-MRS: experimental cheeses with the addition of L90 grown in different culture media.
M1 = media supplemented with yeast extract and minerals.
M2 = media supplemented with glucose, yeast extract and minerals.
MRS = commercial MRS medium.

stable at values near 5.3 and 5.4, without significant differences among cheeses. At 40 d, cheeses E-M2 and E-MRS showed pH values of 5.0, which were significantly lower ($P < 0.05$) than pH in C (5.3) cheeses. Peralta *et al.* (2020) also reported a decline in pH values in Cremoso cheese by using a spray-dried culture of L90 grown in MRS. This reduction could be correlated with the higher galactose metabolism and lactic acid production in cheeses E-M2 and E-MRS (shown below). Other authors also found slightly lower pH values during ripening in cheeses made with *Lacticaseibacillus paracasei* as adjunct culture (Poveda *et al.*, 2014; Bancalari *et al.*, 2020).

Microbial counts

Microbial counts are shown in Fig. 2. Total LAB counts did not show significant differences among cheeses ($P > 0.05$) at either 7 or 40 d after manufacture. They were ~9 log CFU/g at the beginning of ripening and between 8.4 and 8.6 log UFC/g at the end. Lactobacilli counts in cheese E-M1 were ~7 log and ~8 log CFU/g at 7 and 40 d of ripening, respectively. Cheeses E-M2 and E-MRS held levels of ~8 log CFU/g from the beginning to

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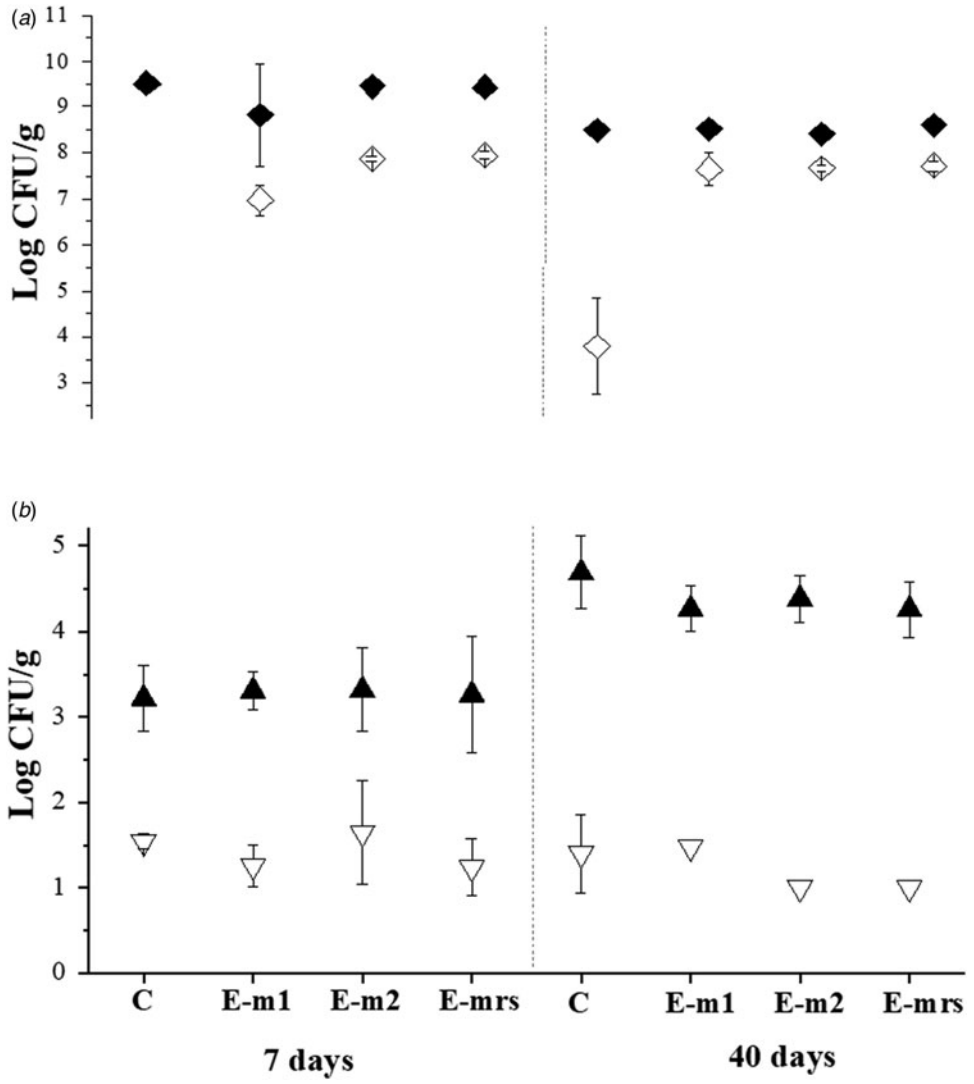

Figure 2. Microbiological counts in cheeses C, E-m1, E-m2, and E-mrs after 7 and 40 d of ripening. (a) Total lactic acid bacteria (♦) and lactobacilli (◊); (b) coliforms (▽) and moulds and yeasts (▲).

the end of the ripening. Adventitious lactobacilli were below the detection limit in C at 7 d (<2 log CFU/g), while they increased during ripening to reach ~4 log CFU/g at 40 d of ripening. The high levels of mesophilic lactobacilli found in the experimental cheeses inoculated with L90 in comparison with the control group, together with the macroscopic characteristics of the colonies and the microscopic morphology of the cells, suggest that the microorganisms detected in these cheeses could correspond to the strain used. Coliforms were detected at levels around 1–2 log CFU/g along the ripening period. Moulds and yeasts reached ~3 and 4 log CFU/g at 7 and 40 d, respectively. For these two microbial groups, no significant differences were observed among treatments.

Starter cultures follow a pattern during ripening which strongly depends on the type of cheese. In Cremoso cheese, their levels usually remain high (>10^8 CFUg) from the beginning to the end of ripening (Milesi *et al.*, 2010; Peralta *et al.*, 2019). Lactobacilli counts in cheeses E-M1, E-M2 and E-MRS were consistent with previous results obtained for L90 when the strain was grown in MRS broth and applied as fresh or spray-dried culture in Cremoso cheese (Peralta *et al.*, 2017, 2019). Considering previous results and the ones obtained in this work, it could be suggested that the growth media and the freeze-drying process did not affect the viability of L90 in Cremoso cheese. Finally, and contrary to the results reported by Milesi *et al.* (2010), the adjunct culture did not significantly affect the development of coliforms, moulds and yeasts. This was probably due to differences in the hygienic conditions of manufacture and raw milk quality (Martin *et al.*, 2021).

Organic acid and carbohydrate analysis

Lactose and galactose concentrations in cheeses at 7 and 40 d of ripening are shown in Table 2. At 7 d, no significant differences were found for lactose among cheeses, while at 40 d, lactose levels in E-M1 and E-MRS were significantly higher ($P < 0.05$) than C. Regarding galactose, its concentration did not show significant differences among the cheeses at 7 d of ripening, while lower levels were observed in all the cheeses with adjunct culture at 40 d; this difference was significant ($P < 0.05$) between C and the treatments E-M2 and E-MRS. In general, *S. thermophilus* strains do not have the ability to metabolize galactose, therefore, only the glucose moiety of lactose is fermented to produce lactic

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Table 2. Concentration of organic acids and carbohydrates (mg/100 g) in cheeses at 7 and 40 d of ripening

Ripening time	Compound	C	E-M1	E-M2	E-MRS
	Organic acids				
7 d	Citric acid	175 ± 8.0^{a}	173 ± 3.1^{a}	174 ± 10^{a}	165 ± 7.5^{a}
	Orotic acid	4.09 ± 0.24^{a}	3.99 ± 0.33^{a}	4.13 ± 0.25^{a}	3.92 ± 0.18^{a}
	Pyruvic acid	5.87 ± 0.95^{a}	5.93 ± 0.85^{a}	6.81 ± 0.39^{a}	5.27 ± 0.44^{a}
	Lactic acid	1060 ± 228^{a}	913 ± 218^{a}	1092 ± 50.7^{a}	973 ± 132^{a}
	Hippuric acid	1.55 ± 0.30^{a}	0.78 ± 0.05^{b}	0.69 ± 0.11^{b}	0.54 ± 0.14^{b}
7 d	*Carbohydrates*				
	Lactose	549 ± 77^{a}	671 ± 59^{a}	622 ± 156^{a}	630 ± 36^{a}
	Galactose	749 ± 123^{a}	692 ± 142^{a}	763 ± 14.6^{a}	697 ± 92^{a}
	Organic acids				
40 d	Citric acid	176 ± 12^{a}	172 ± 1.46^{a}	172 ± 26^{a}	168 ± 18^{a}
	Orotic acid	4.20 ± 0.37^{a}	3.73 ± 0.22^{a,b}	3.51 ± 0.10^{b}	3.42 ± 0.20^{b}
	Pyruvic acid	6.83 ± 0.11^{a}	5.82 ± 1.23^{a}	6.93 ± 2.05^{a}	8.45 ± 1.64^{a}
	Lactic acid	1263 ± 130^{a}	1437 ± 71^{a}	1525 ± 133^{a}	1506 ± 211^{a}
	Hippuric acid	1.55 ± 0.10	ND	ND	ND
40 d	*Carbohydrates*				
	Lactose	415 ± 6.4^{b}	618 ± 45^{a}	530 ± 83^{a,b}	586 ± 44^{a}
	Galactose	836 ± 86^{a}	588 ± 153^{a,b}	537 ± 54^{b}	489 ± 136^{b}

Values are means ± standard deviation of three cheese replicates. Different letters in a same row mean significant differences ($P < 0.05$)
Cheese labels: C: control; E-M1, E-M2, E-MRS: experimental cheeses with the addition of L90 grown in different culture media.
M1: media supplemented with yeast extract and minerals.
M2: media supplemented with glucose, yeast extract and minerals.
MRS: commercial MRS medium.
ND, not detected (concentration below detection limit).

acid (St-Gelais *et al.*, 2009; Parente *et al.*, 2017). Probably for this reason the galactose concentration in C cheeses increased during ripening. In this respect, St-Gelais *et al.* (2009), who compared the consumption of this carbohydrate in Cheddar-type cheeses made with different starter cultures, observed an accumulation of galactose in cheeses for which *S. thermophilus* was employed as starter. Similarly, Peralta *et al.* (2019) did not observe galactose consumption in Cremoso cheese made with a commercial strain of *S. thermophilus* as starter culture. However, it is important to underline that galactose-positive *S. thermophilus* strains have been reported (Tidona *et al.*, 2020).

In contrast, in experimental cheeses made with L90, galactose was partially consumed during ripening. Peralta *et al.* (2020) found similar results, ie a reduction in galactose concentration during the ripening of Cremoso cheese made with the addition of L90 grown in MRS. In addition, the authors noted that this ability was increased by a cold chain interruption during cheese ripening. The ability to use residual sugars (galactose, lactose, etc) during ripening may prevent the potential spoilage of the product, because of the reduction of the levels of the energy sources for NSLAB (Hutkins and Ponne, 1991; Ortakci *et al.*, 2015; Blaya *et al.*, 2018). For example, Green *et al.* (2021) reported that the use of galactose-positive secondary cultures avoids the gas production by an obligatory heterofermentative lactobacilli such as *Paucilactobacillus wasatchensis* WDC04.

The analyses of organic acids in Cremoso cheese samples were useful for understanding the potential of L90 as a secondary culture after its growth in these alternative media. Regarding acids, five organic acids were quantified in cheeses at 7 and 40 d of ripening (Table 2). Lactic acid was produced in similar concentrations in all cheeses at both ripening times studied, reaching levels between 1200 and 1500 mg/100 g at 40 d. Although the differences were not significant, a clear tendency was perceived at 40 d of ripening since lactic acid levels in cheeses with adjunct culture were numerically higher compared to those in control cheese. The lactic acid in Cremoso cheese is mainly produced by the activity of *Streptococcus thermophilus* in the first hours of cheese making. Additionally, the galactose metabolism of cheese microbiota contributes to the synthesis of lactic acid. Lactic acid levels similar to those obtained in this study improve the taste and create an unfavourable environment for the growth of spoilage and pathogenic microorganisms in Cremoso cheese. However, to avoid an undesirable high concentration of this acid, as it may produce undesirable flavour notes and textures, it is important to analyse its level when lactobacilli strains are added as secondary cultures.

Levels of citric and pyruvic acids were ~172 and 7 mg/100 g, respectively, and no differences ($P > 0.05$) were observed among cheeses. It is interesting to study the citrate metabolism in Cremoso cheese not only because of its potential contribution to the aroma compounds, but also because of its contribution to the growth of heterofermentative NSLAB and concomitantly undesirable eye formation. Regarding pyruvic acid, it is an intermediate compound in the metabolic pathways of many LAB, including L90.

Orotic and hippuric acids, which are found naturally in milk, were consumed at different degrees in cheeses. For orotic acid,

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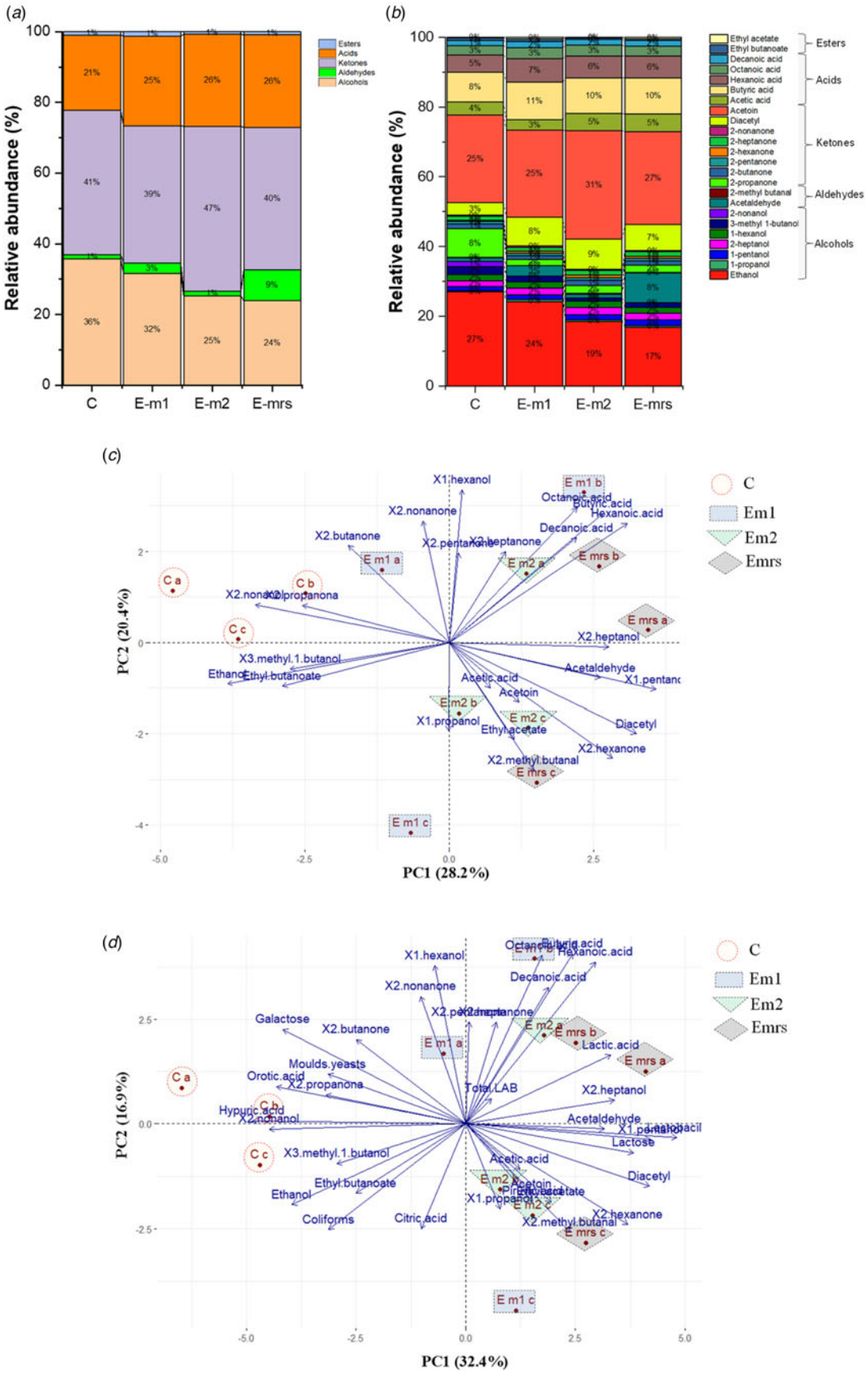

Figure 3. Volatile compounds in cheeses C, E-m1, E-m2, and E-mrs after 40 d of ripening. (a) The relative abundance of volatile compounds showing the different chemical classes. (b) The relative abundance of each volatile compound. (c) Scores and loading biplot of the PCA of the twenty-four volatile compounds. (d) Scores and loading biplot of the PCA including data from volatile compounds, carbohydrates, organic acids, and microbiological counts.

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there were no significant differences among cheeses at 7 d, while different levels appeared at 40 d ($P < 0.05$). In all E cheeses there was a decrease in this acid at the end of ripening, which did not occur in control cheeses. Hippuric acid was partially and totally consumed at 7 and 40 d, respectively, in cheeses with adjunct culture, while its levels remained unchanged in C cheeses during ripening. Both orotic acid and hippuric acid are part of the non-protein nitrogen of cow's milk (Fox *et al.*, 2015). In particular, orotic acid serves as an intermediate in the synthesis of pyrimidine nucleotides (O'Callaghan *et al.*, 2021). The ability to metabolize orotic acid has been reported in LAB (Haggerty *et al.*, 1984; Fernandez-Garcia and McGregor, 1994). In this respect, this study unveiled the L90 capacity to partially metabolize orotic acid. Regarding hippuric acid, the capacity of L90 to metabolize it could be associated with antimicrobial properties as it is a precursor of benzoic acid (Sieber *et al.*, 1995). In a previous study, this ability was also reported in L90 cells when grown in MRS (Giménez *et al.*, 2021). In a nutshell, the potential metabolic activity of L90 was maintained after growing in the alternative media.

Volatile compounds profile

Twenty-four volatile compounds, including alcohols, aldehydes, ketones, acids and esters were identified in cheese samples at 40 d of ripening. Figure 3a illustrates the relative abundance of them in terms of the different chemical classes. The proportions were different among cheeses, especially between C and E cheeses. Furthermore, it is clearly seen that E-M2 and E-MRS presented a more similar pattern among them in comparison to C and E-M1, especially in the level of alcohols. The differences could be related to the different compositions of M1, M2 and MRS. It is well known that the activities of microorganisms could be affected by nutrients in their growth media (Jensen and Ardö, 2010; Zhong *et al.*, 2018). Pevious studies of L90 on different media showed differences in its enzymatic activity (Beret *et al.*, 2021; Peralta *et al.*, 2023b). Figure 3b illustrates the relative abundance of each volatile compound. Although the levels of some volatile compounds seem different among E cheeses (eg levels of ethanol in E-MRS and E-M2 lower than E-M1), it is interesting to highlight that the levels of both diacetyl and acetoin were higher in the experimental cheeses, regardless of the culture media used to grow L90.

PCA was applied to analyse the variability among the volatile profiles of the samples (Fig. 3c). The first and the second component explained 48.6% of the total variability. A clear separation between control and experimental cheeses, mainly explained by PC1, can be seen in the graph. Control cheeses, on the left side of PC1, were associated with 2-nonanone, 2 butanone, 2-propanone, 2-nonanol, 3-methyl-1-butanol, ethanol and ethyl butanoate. On the right side of PC1, cheeses E-M2 and E-MRS were associated with 2-pentanone, 2-heptanone, hexanoic acid, 2-heptanol, acetaldehyde, 1-pentanol, 2-hexanone, acetic acid, acetoin, diacetyl and 2-methyl-butanal, and between these two clusters, cheeses E-M1 were located. Furthermore, a PCA was also applied to analyse the levels of volatile compounds, organic acids, carbohydrates and microbial counts jointly (Fig. 3d). The first two principal components (PC) explained 49.3% of the total variation. Control cheeses were grouped on the left side of PC1, clearly separated from cheeses with L90, which were grouped together on the right side of PC1. Experimental cheeses were associated with several acids (decanoic, octanoic, hexanoic, butyric, pyruvic and lactic), diacetyl, acetoin, acetaldehyde, lactose, lactobacilli and streptococci, among others. Control cheeses, by conrast, were correlated with the variables galactose, moulds and yeasts, coliforms, hippuric and orotic acids, ethanol, butanone and propanone, among others.

From the results obtained for volatiles compounds, it is interesting to highlight that the growth media did not significantly affect the capacity of L90 to produce diacetyl and acetoin. Diacetyl and acetoin are volatile compounds that provide cream and butter aromas, which are of interest in certain cheese varieties such as Cremoso cheese. Their productions are mainly linked to the metabolism of citrate and some amino acids (Le Bars and Yvon, 2007; Beresford, 2022). In our work, the production of these volatile compounds could be attributed to the catabolism of some amino acids, explained by transamination, because the citrate remained at similar levels during ripening. In particular, aspartic acid catabolism is one of the metabolic pathways for the production of diacetyl and acetoin (Le Bars and Yvon, 2007). Peralta *et al.* (2016a) studied the aspartate aminotransferase activity of L90, having linked this metabolic pathway with the production of diacetyl and acetoin in cheeses with this adjunct culture. In addition, the ability of this strain to produce these aromatic compounds had been demonstrated in previous studies for fresh and spray-dried cultures, both in cheeses and cheese models (Peralta *et al.*, 2017, 2022). In the present work, it has been shown that the growth media did not significantly impact on the ability of L90 to produce these characteristic flavour compounds in Cremoso cheese, demonstrating its versatility to preserve its technological properties when grown in more economical media, suitable to be applied at industrial scale.

In conclusion, the use of soy waste-based culture media did not negatively affect the performance of L90 as adjunct culture in Cremoso miniature cheeses. The ability of this strain to metabolize galactose could be helpful to control adventitious NSLAB that may cause undesired organoleptic defects in cheese. Relevant aroma compounds, such as diacetyl and acetoin, were found in all the cheeses made with L90 as adjunct culture, regardless of the culture media used for its growth. In addition, this work is in line with a circular economy philosophy by proposing the use of waste derived from the local food industry for the production of adjunct cultures.

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