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Contribution of Lactic Acid Bacteria Esterases to the Release of Fatty Acids in Miniature Ewe's Milk Cheese Models

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The present work evaluates the contribution of esterase acivities of lactic acid bacteria isolated from ewe's dairy products to the release of free fatty acids (FFA) in ewe's milk cheese models. At 60 days of ripening, single-strain cheeses Ov 409 and Ov 421 showed high levels of total FFA (3075 and 2494.62 mg/kg, respectively). Cheeses Ov 227−Ov 409 and Ov 421−Ov 409 presented high percentages of short-chain fatty acids (SCFA). The highest levels of volatile free fatty acids (VFFA) were detected in cheeses Ov 409, Ov 421−Ov 409, and Ov 421−Ov 227. Studies on esterase activities showed that these strains hydrolyzed α-naphthyl derivatives of fatty acids from C2 to C6, mainly associated with the wall-membrane fraction. The results showed that the strains studied contributed to the release of FFA during ripening of ewe's milk cheese models. The increase of SCFA throughout ripening involves the action of esterases of starter strains.

KEYWORDS: Esterases; lipolysis; ewe's milk cheese; lactic acid bacteria

INTRODUCTION

In Argentina, the consumption of fermented products from ewe's milk is continuously increasing (*1*), and there is good incentive to expand the range and quality of these fermented dairy products.

The design of cultures with technological properties that respond to the physicochemical composition of milk and ripening conditions (temperature and humidity of the region) is necessary for artisanal cheese manufacture. Specific cultures for ewe's milk cheese are not available in Argentina, so manufacturers are forced to use bovine cultures. This situation makes it difficult to obtain products with optimal organoleptic characteristics. Constant quality and acceptable sensorial attributes are crucial for making products acceptable for consumers and competitive into markets.

The cheese produced in northwestern Argentina, a semihard variety, is made from raw ewe's milk, without the addition of starter cultures. The general composition of ewe's milk fat and the primary products of its degradation during ripening, that is, volatile free fatty acids, play important roles in the particular flavor characteristics of this cheese (*2*).

Indigenous lactic acid bacteria isolated from ewe's milk and artisanal cheese manufactured in the provinces of northwestern Argentina were identified as enterococci, lactococci, leuconostoc, and lactobacilli (*3*). *Enterococcus faecium* and *Lactobacillus plantarum* were the most frequently isolated species from ewe's milk and artisanal ewe's milk cheese from Argentina (*3*). These microorganisms are involved in the development of flavor during cheese ripening (*4, 5*).

Lipolysis, the process of milk fat hydrolysis, is the main event during ripening. The major flavor compounds that are released during lipolysis are free fatty acids (FFA), which directly affect cheese flavor. FFA can also be transformed by microorganisms to other and often more potent flavor compounds, including methyl ketones, lactones, esters, secondary alcohols, and aldehydes, which also directly affect flavor in different cheeses (*6*). Volatile fatty acids were found to be major contributors to the overall aroma of Serra da Estrela cheese (*7, 8*).

Lactic acid bacteria (LAB) are used as starter or adjuncts cultures or are present as secondary microbial flora (nonstarter LAB) in cheese fermentation (*9*). The contribution of cheese microflora to lipolysis occurs via the esterase/lipase systems of lactic and propionic acid bacteria, nonstarter LAB, surface microorganisms, yeasts, and molds (*10*). The role of esterases of LAB and nonstarter LAB (NSLAB) in lipolysis and in the transformation of FFA to other flavor compounds in dairy

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products is largely undefined. Presumably, esterases from starter and NSLAB are responsible for the release of short-chain fatty acids (SCFA) from milk fat at elevated a_w and the synthesis of short-chain ethyl esters as a_w decreases with ripening (*11*).

Several authors have studied the FFA profile of ewe's milk cheese and changes in individual FFA during cheese ripening (*12, 13*). However, there is little information about the contribution of esterases from LAB to the formation of flavor in cheese manufactured with ewe's milk.

In Picante cheese, manufactured from mixtures of ovine and caprine milk, the major microflora present throughout ripening are LAB, the most abundant species being enterococci (*E. faecium*, *E. faecalis*, and *E. durans*) and lactobacilli (*L. plantarum* and *L. casei*) (*14*−*16*).

The contribution of enterococci in matured cheeses to the development of good and acceptable flavor or not is still debated. Di Cagno et al. (*17*) reported the contribution of *L. plantarum* 2739, isolated from cheese, to lipolysis of a Caciotta cheese model system. Freitas et al. (*16*) reported that the action of *E. faecium* and *L. plantarum* appeared in Picante cheese throughout the whole 6 months of ripening and contributed to lipolysis of this cheese type.

In raw milk Cebreiro cheese, enterococci represent the dominant flora, and they should be included in starters for making Cebreiro cheese from pasteurized milk to reproduce the typical characteristics of traditional cheese (*18, 19*). These microorganisms have been used on numerous occasions to accelerate ripening and to improve the organoleptic characteristics of cheese, because these LAB present lipolytic activity (*2, 18, 19*).

The present work evaluates the contribution of enterococci and *L. plantarum* Ov 236 strains used as starter culture to the release of FFA in a ewe's milk cheese model system. To achieve this aim, we determined esterase activities of enterococci and lactobacilli strains and followed the release of FFA during the ripening of miniature ewe's milk cheeses elaborated under controlled microbiological conditions. At present there are only a few papers concerning the influence of esterase activity of lactic acid bacteria on FFA release in ewe's milk cheeses.

MATERIALS AND METHODS

Microorganisms and Growth Conditions. *Enterococcus faecium* Ov 409, *Enterococcus faecium* Ov 227, *Enterococcus durans* Ov 421, and *Lactobacillus plantarum* Ov 236, provided by Laboratorio de Ecofisiología Tecnológica of the Centro de Referencia para Lactobacilos (CERELA), were isolated from Argentinean ewe's milk and cheese (*3*). For enzymatic activity determinations, all bacteria were grown in MRS broth (Merck, Darmstadt, Germany). For cheesemaking, all strains were grown in 10% (w/v) skim milk powder. Cultures were incubated at 37 °C for 16 h.

Milk for Cheese Manufacture. Raw ewe's milk (20 L) from the Manchega breed was aseptically collected in glass jars, cooled (4 °C), and carried to the laboratory. The ewe's milk used in this study had the following composition: lactose, 4.9%; protein, 3.2%; fat, 7.6%; and pH, 6.6.

Protocol for the Manufacture of Miniature Cheese Models. Miniature cheeses were manufactured according to the protocols of Hynes et al. (*20*) with some modifications to fit our laboratory conditions (*2*). The process was carried out aseptically in a laminar air-flow unit (Clean Room). Sterile flasks were filled with 400 mL of raw milk to be pasteurized in a 65 °C thermostatic bath for 30 min. After cooling to 38 °C, each batch was inoculated with 1% (v/v) ($\sim 10^8$ cfu/mL) culture of different strains of lactic acid bacteria in sterilized reconstituted (10% w/v) skim milk powder. Eight different starter combinations were used for miniature cheese manufacture. On the basis of previous strain compatibility tests (*21*), the combinations used were the following: (A) *L. plantarum* Ov 236 (1% v/v); (B) *E. faecium* Ov 227 (1% v/v); (C) *E. durans* Ov 421 (1% v/v); (D) *E. faecium* Ov 409 (1% v/v); (E) *L. plantarum* Ov 236 and *E. faecium* Ov 409 (1% v/v, each); (F) *E. faecium* Ov 227 and *E. faecium* Ov 409 (1% v/v, each); (G) *E. durans* Ov 421 and *E. faecium* Ov 409 (1% v/v, each); (H) *E. durans* Ov 421 and *E. faecium* Ov 227 (1% v/v, each).

Inoculated milk was then incubated at 37 °C until the pH dropped to 6.4. After 30 min, sterile calcium chloride (0.1 g/L of milk) and microfiltered (0.22 μm, white GSWP, 25 mm, Millipore Corp., Bedford, MA) rennet powder from *Kluyveromyces lactis* (MAXIREN 150. 9000 mg/L, Gist-brocades, Delft, The Netherlands) were added.

Coagulation time was controlled by rocking and turning the bottles gently to test casein adhesion to their sides. Clotting took place in 25 min, after which the curd was cut in nut size cubes with a sterile knife and each flask was upturned 10 times. The mass was cooked for 5 min at 45 °C, and it was placed in sterile flasks with sterile tablespoons. The curd was first centrifuged at 1700*g* (Damon IEC B-20A centrifuge) for 10 min and then at 5000*g* for 20 min. Whey was discarded in both cases. The curd was laid in 90 mL sterile glass flasks and allowed to rest until reaching pH 5.2 at 25 °C. Sterile brine was added to each flask (330 g/L of NaCl, pH adjusted to 5.2 with lactic acid) and discarded after 5 min. Cheeses were matured inside the flasks at 12 °C and 80% relative humidity for 60 days. Eight miniature cheeses and a control cheese of approximately 120 g were manufactured from the same batch of milk.

Control cheese, without added culture, was manufactured according to the protocol described above. The pH was adjusted to 5.2 with lactic acid, and the cheese was incubated at conditions identical to those used for the experimental cheeses. Cheese manufacture was done in triplicate. Three samples (a cheese from each batch) were taken at 1, 15, 30, and 60 days of ripening for chemical, physical, and microbiological analyses.

Compositional Analysis. Cheeses were analyzed for fat according to the Gerber method (*22*) and for dry matter according to IDF Standard 4A (*23*). The pH of the cheeses was determined by pH-meter Metrohm 962.

Microbiological Analysis. For microbiological analysis, cheese samples (10 g) were dispersed in 90 mL of 2% (w/v) sodium citrate solution, homogenized for 2 min in a Stomacher (Laboratory Blender Stomacher model 400, Seward Medical, London, U.K.), diluted in peptone saline solution, and plated on specific media for viable counts. Microbiological counts were performed in duplicate after 1, 15, 30, and 60 days of ripening. Enterococci strains in experimental and control cheeses were determined on KF agar after incubation at 42 °C for 48 h. Lactobacilli strains in experimental and control cheeses were determined on MRS agar pH 5.5 after incubation at 30 °C for 5 days. Total mesophilic bacteria were enumerated on Plate Count Agar after incubation at 30 °C for 48 h.

Analysis of Volatile Free Fatty Acids (VFFA). VFFA content was determined as described by Kuzdzal-Savoie (*24*) modified by Katz (*2*). Cheese samples of 15 g were acidified with sulfuric acid prior to steam distillation and collection in 0.1 N NaOH. Salts were derivatized to methyl esters.

The results were expressed as milligrams of VFFA per 100 g of dry extract. The VFFA content was evaluated by gas chromatography. A Hewlett-Packard 6890 gas chromatograph with a flame ionization detector (FID) (Hewlett-Packard Co., Palo Alto, CA) and equipped with an INNOWAX capillary column (length, 30 m; i.d., 0.25 mm; film thickness, 0.25 μm) (J&W Scientific, Folsom, CA) was used. The oven was temperature-programmed at 120 °C for 1 min, followed by increasing the temperature to 200 at 10 °C/min, and held for 10 min. The injector and detector temperatures were 250 and 300 °C, respectively. Column flow rate was 1 mL/min using nitrogen as carrier gas. VFFA were identified and quantified by comparison with injections of known amounts of pure standards.

Analysis of Total Free Fatty Acids (FFA). Total FFA content was determined according to International Dairy Federation standard methods FIL-IDF (*25*) and the method of de la Fuente and Juárez (*26*). The results were expressed as milligrams of FFA per kilogram of cheese. A Hewlett-Packard 6890 gas chromatograph with a FID (Hewlett-Packard) and equipped with an INNOWAX capillary column

(length, 30 m; i.d., 0.25 mm; film thickness, 0.25 μm) (J&W Scientific) was used. The oven was temperature-programmed at 120 °C for 1 min, followed by increasing the temperature to 250 at 10 °C/min, and held for 20 min. The injector and detector temperatures were 250 and 300 °C, respectively. Column flow rate was 1 mL/min using nitrogen as carrier gas. FFA were identified and quantified by comparison with injections of known amounts of pure standards.

Cell-free Extract (CFE) Preparation. Cells were harvested after 16 h of incubation in MRS broth by centrifugation at 10000*g* for 10 min at 4 °C, washed twice with 50 mM sodium phosphate buffer, pH 7.0 and resuspended in 50% w/v of the same buffer. The suspension was disrupted by mixing with 1−2 g of glass beads 31/14 (diameter, 0.1−0.11 mm; B. Braun Biotech International) using a cell disruptor (B. Braun Melsungen AG). Five mixing sequences (speed, 6.5/s), each of 1 min duration, were successively applied. Samples were cooled in ice for 5 min between each mixing sequence. Cellular debris was removed by centrifugation (20000*g* for 30 min) at 4 °C, and supernatant was used as CFE. The extracellular fraction was obtained by means of the supernatant of the growth medium. The supernatant was sterilized by filtration (0.22 μm, white GSWP, 25 mm, Millipore Corp.).

Assay for Esterase Activity. The esterase activity of CFE was determined on α-naphthyl derivatives of fatty acids of 2−12 carbon atoms as substrate (Sigma, St. Louis, MO) (*27*). The assay mixture contained 160 μL of 100 mM sodium phosphate buffer, pH 7.0, 20 μL of α-naphthyl substrate (10 mM in ethanol), and 100 μL of CFE. After incubation for 1 h at 37 °C, color was developed by adding 0.6 mL of Fast Garnet GBC (Sigma) solution (5 mg/mL in 10% w/v SDS) and further incubation at room temperature for 15 min. The absorbance was measured at 560 nm in a spectrophotometer (CECIL 2021, Cambridge, U.K.). A standard curve was prepared using α-naphthol. A unit of esterase activity was defined as the amount of enzyme that released 1 μmol of α-naphthol per minute. Specific esterase activity was defined as units per milligram of protein.

Subcellular Fraction Preparation. Subcellular fractions were obtained according to the method described by Crow et al. (*28*) as modified by Katz (*2*).

Protein Determination. Protein concentrations were determined according to the method of Bradford (*29*), using bovine serum albumin (Sigma) as standard.

Statistical Analysis. Physicochemical and microbiological analyses were performed in triplicate. The results presented are means ± standard deviation. After the analyses of variance (ANOVA), Tukey's test was used to identify differences among cultures ($p < 0.05$). These analyses were carried out using statistical software (Minitab1.4, State College, PA). Relationships between starters and FFA release were studied by means of the principal component analysis method. Calculations and graphics were carried out with Infostat Professional software 2004p1.

RESULTS AND DISCUSSION

Cheese Compositional Characteristics. After 60 days of ripening, the mean values for the compositional parameters of experimental and control cheeses were 48−50% moisture, 49−52% dry matter, and 45−47% fat in dry matter. These compositional parameters of evaluated cheeses were in accordance with those of Dozet et al. (*30*), who reported values of 36−50% moisture and 50−53% fat in dry matter in ewe's milk cheese. In Tulum cheese, a ewe's and goat's milk cheese, 30−60% fat dry matter was observed after 3−6 months of ripening (*31*).

The pH values in all model cheeses ranged from 4.47 to 5.05 after 60 days of ripening. The minimum pH value was observed in the cheese made with *E. faecium* Ov 409 and *E. faecium* Ov 227. Similar results were reported by Kandarakis et al. (*32*) for Feta cheese of 60 days of ripening made with ewe's milk and recombinant rennet (*K. lactis*).

Cheese Microbiological Characteristics. All of the fresh curds (1 day of ripening) contained a cell count of starter cultures of ∼8.0−8.5 log cfu/g (**Figure 1**). Control cheese had a cell count of 3.5 log cfu/g, and it decreased to 1.4 log cfu/g at the end of ripening period (data not shown). At 60 days of ripening, in cheeses elaborated with enterococci strains was observed an increase of cell counts from ∼8.12 to ∼9.45 log cfu/g (∼1 log cycle), which is statistically significant. These results would indicate that enterococci strains remain viable in cheeses at 60 days of ripening. Freitas et al. (*16*) reported the presence of enterococci as the sole constituent of the microflora in Picante cheese ripened for 6 months. However, in cheeses elaborated with *L. plantarum* Ov 236 (Ov 236 and Ov 236−Ov 409), the cell count decreased from ∼8.10 to ∼7.26 log cfu/g (∼1 log cycle), indicating cell lysis. Di Cagno et al. (*17*) observed a decrease of cell counts of lactobacilli during ripening in a Caciotta cheese model system.

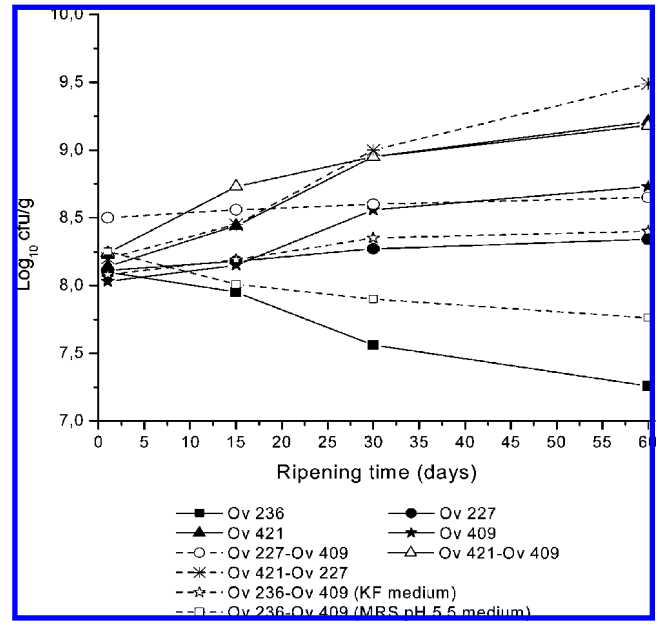

Figure 1. Cell counts (log cfu/g) of enterococci and *Lactobacillus plantarum* Ov 236 in experimental and control cheeses during ripening.

Table 1. Total FFA Concentrations in Miniature Ewe's Milk Cheeses[a]

	free fatty acid (mg/kg of cheese)		
miniature ewe's milk cheese	1 day	60 days	ratio FFA_{60}/FFA_1
control	1058.45 ± 33a	1078.32 ± 45a	1.01
Ov 236	1020.11 ± 24a	1334.06 ± 28b	1.30
Ov 227	1049.21 ± 19a	1635.35 ± 23d	1.50
Ov 421	1104.50 ± 67a	2494.62 ± 74c	2.26
Ov 409	1090.83 ± 43a	3075.00 ± 78e	2.82
Ov 236−Ov 409	1064.82 ± 55a	1846.94 ± 34f	1.70
Ov 227−Ov 409	1212.24 ± 48b	2460.85 ± 67c	2.03
Ov 421−Ov 409	1256.68 ± 34b	2814.96 ± 71e	2.24
Ov 421−Ov 227	1064.75 ± 27a	4457.73 ± 82g	4.18

[a] The data are mean values ± standard deviation of three independent determinations. Means in the same column with different letters are significantly different ($p < 0.05$).

Assessment of Lipolysis. Lipolysis in cheese can be caused by an indigenous milk lipase, by exogenously added mammalian pregastric esterases−lipases, and/or by microbial lipases (exogenously added or derived from microorganisms). Because milk lipase is thermally inactivated during pasteurization, the only source of lipolytic enzymes in the cheese model systems of this work are LAB used as starters.

Table 1 shows mean values and standard deviations for total FFA concentrations in cheese samples. Total FFA content in cheeses at 60 days of ripening varied considerably between 1334.06 and 4457.73 mg/kg. At 60 days of ripening, cheeses

made with single strains Ov 409 and Ov 421 showed higher levels of total FFA (3075 and 2494.62 mg/kg, respectively) than other single-strain cheeses. The ratio FFA_{60}/FFA_1 values for both Ov 409 and Ov 421 cheeses were 2.82 and 2.26, respectively. These results were similar to Picante cheese made with single strains of *E. faecium*, for which the FFA level at day 65 of ripening was approximately 3150 mg/kg (*16*). Cheese made with Ov 421−Ov 227 presented higher levels of total FFA (4457.73 mg/kg) than those made with these strains separately. This would indicate that strains Ov 421 and Ov 227 have a synergic effect on FFA release. The ratio FFA_{60}/FFA_1 for Ov 421-Ov 227 was 4.18. The average content of FFA of this cheese was considerably higher than that reported for Manchego cheese at 60 days of ripening (*33*). However, in Serra da Estrela cheese of 60 days of ripening a ratio of FFA_{60}/FFA_1 of 9.46 was observed (*7*).

A high content of total FFA was previously observed in similar cheeses made from ewe's raw milk such as Idiazábal, which can be attributed to the specific activity of the lipases present in the lamb rennet (*34, 35*). Control cheese did not show significant differences of $FFA_{60days/1day}$.

Figure 2 shows the ratio of short-chain (SCFA), medium-chain (MCFA), and long-chain (LCFA) fatty acids of single- or double-strain cheeses during ripening. In general, in single-strain cheeses at 60 days of ripening, the percentage of LCFA (C16−C18) decreased from ∼81 to ∼63%, whereas the percentage of MCFA (C10−C14) increased from ∼15 to ∼26% and that of SCFA (C4−C8) increased from ∼3.07 to ∼ 10%. Cheese made with *E. faecium* Ov 409 presented a higher percentage of SCFA (10.6%) at 60 days of ripening than other cheeses. In cheese made with *L. plantarum* Ov 236 was observed an increase of SCFA and MCFA at 30 days of ripening, but at 60 days was observed a decrease of SCFA, possibly due to their metabolism to other flavor compounds (*10*). *L. plantarum* Ov 236 would exert a strong lipolytic action until day 15 of ripening. The number of colony-forming units per gram of this strain decreased at day 60 (**Figure 1**). This fact would indicate cell lysis and the concominant release of enzymes that would transform SCFA into other flavor compounds. In a previous study we observed that *L. plantarum* Ov 236 synthesized ethyl butanoate and ethyl hexanoate from butyric and caproic acids and ethanol (*36*). Similar results were reported by Collins et al. (*37*) for lactococci in Cheddar cheese.

Cheeses made with two strains, Ov 227−Ov 409 and Ov 421−Ov 409, presented higher percentages of SCFA than other single- or two-strain cheeses (17 and 13%, respectively) (**Figure 2**).

The cheese made with combination of strains Ov 227−Ov 409 had a synergic effect on the release of SCFA, presenting higher levels of SCFA than single-strain cheeses Ov 227 and Ov 409, although cell counts during ripening were not significantly different (8.5−8.75 log cfu/g) (**Figure 1**).

The SCFA increased at least twice in all experimental cheeses. SCFA such as butanoic and hexanoic acids are potent flavor compounds at concentrations below 10 mg/mL, giving piquant flavor characteristics of ewe's milk cheeses (*38*). Esterases of LAB have the capacity to esterify fatty acids and ethanol. These acids, which are potent flavor compounds at <5 ppm, are important for the development of characteristic "fruity-type" flavors (*39*).

In Indiazábal cheese, made with raw ewe's milk, SCFA increased twice and MCFA remained constant during ripening at 90 and 180 days (*40*). Similarly, in Feta cheese, made with ewe's milk and calf rennet, the percentage of SCFA increased during ripening. These SCFA represented approximately 33% of the total FFA at 120 days. The percentage of MCFA decreased and that of LCFA remained constant throughout ripening (*41*).

The volatile free fatty acids (VFFA) present in cheese, mainly acetic, propionic, butyric, and caproic acids, are originated by microbial metabolism of sugar, citrate, proteins, and lipids of milk. These VFFA are responsible for organoleptic characteristics of dairy products. The content of VFFA in cheese is shown in **Table 2**. Among single-strain cheeses, the highest levels of VFFA were detected in cheese made with *E. faecium* Ov 409 after 60 days of ripening.

Higher contents in VFFA have also been reported by Centeno et al. (*19*) in cheese made with enterococci. Cheese made with *L. plantarum* Ov 236 showed higher levels of VFFA at 15 days of ripening rather than at 30 and 60 days. This fact would be due to the conversion of VFFA into other flavor compounds such as ethyl butanoate and ethyl hexanoate (*36*). Among cheeses made with a single enterococci strain, the lowest levels of VFFA at the end of ripening were found in the cheese elaborated with *E. durans* Ov 421.

In cheeses made with two strains, at 60 days of ripening, were observed higher levels of VFFA in cheeses made with Ov 421−Ov 409 and Ov 421−Ov 227. The ratio $VFFA_{60days/1day}$ was higher in cheeses made with both enterococci strains. In cheese made with Ov 236−Ov 409, the ratio $VFFA_{30days/1day}$ was 3.70, and this ratio decreased to 1.85 at day 60. Control cheese did not show significant differences of $VFFA_{60days/1day}$.

In all cheeses, acetic acid was the predominant VFFA and represented from 60 to 75% of VFFA. This acid is mainly formed from citrate fermentation (*2, 5*). Similar results were reported by Georgala et al. (*41*) for Feta cheese. In this cheese acetic acid contributed to the final flavor and was the major volatile fatty acid (30−50%). Although propionic acid was detected in all cheeses, a noticeable increase was not observed during ripening. Butyric acid was the second most abundant VFFA in all cheeses (14−28%). Caproic acid represented from 9 to 14% of total VFFA. Centeno et al. (*19*) reported that caproic acid was the second most abundant in cow's milk cheeses made with starter cultures containing *E. faecalis*.

Butyric and caproic acids would originate by microbial lipolytic activity on triglycerides of cheese. Butyric acid was found as a major FFA even in cheeses made from raw cow's milk (*42*). In this case, the high content of butyric acid has been attributed to the lipolytic activity of the lactic acid bacteria mainly composed by *Lactobacillus* and *Lactococcus* species.

In all experimental cheeses at 60 days of ripening the ratio between butyric and caproic acid concentration was low, varying from 1.17 to 2.27. These results would indicate that butyric acid originates from lipolysis. Similar results were reported by Chambra and Perreard (*43*), who observed a low concentration ratio of butyric and caproic acid in Emmental cheese (2.6).

In our study, the increase of SCFA and VFFA during 60 days of ripening indicates the contribution of the studied strains to the lipolysis of ewe's milk cheese. These specific starter cultures would present esterase activity, and released SCFA may significantly contribute to the characteristic aroma and intense flavor of ewe's milk cheese as reported by Katz (*2*).

The results of principal component analysis (PCA) shown in **Figure 3** explained 83% of the total variance of the data. The first principal component (PC1) explained 63% of the total variance of data and grouped all fatty acids and volatile fatty acids (acetic, propionic, butyric) to the right side of the plot, all of these compounds being clearly distinguished from each other. To this side of the plot are located the cheeses with higher

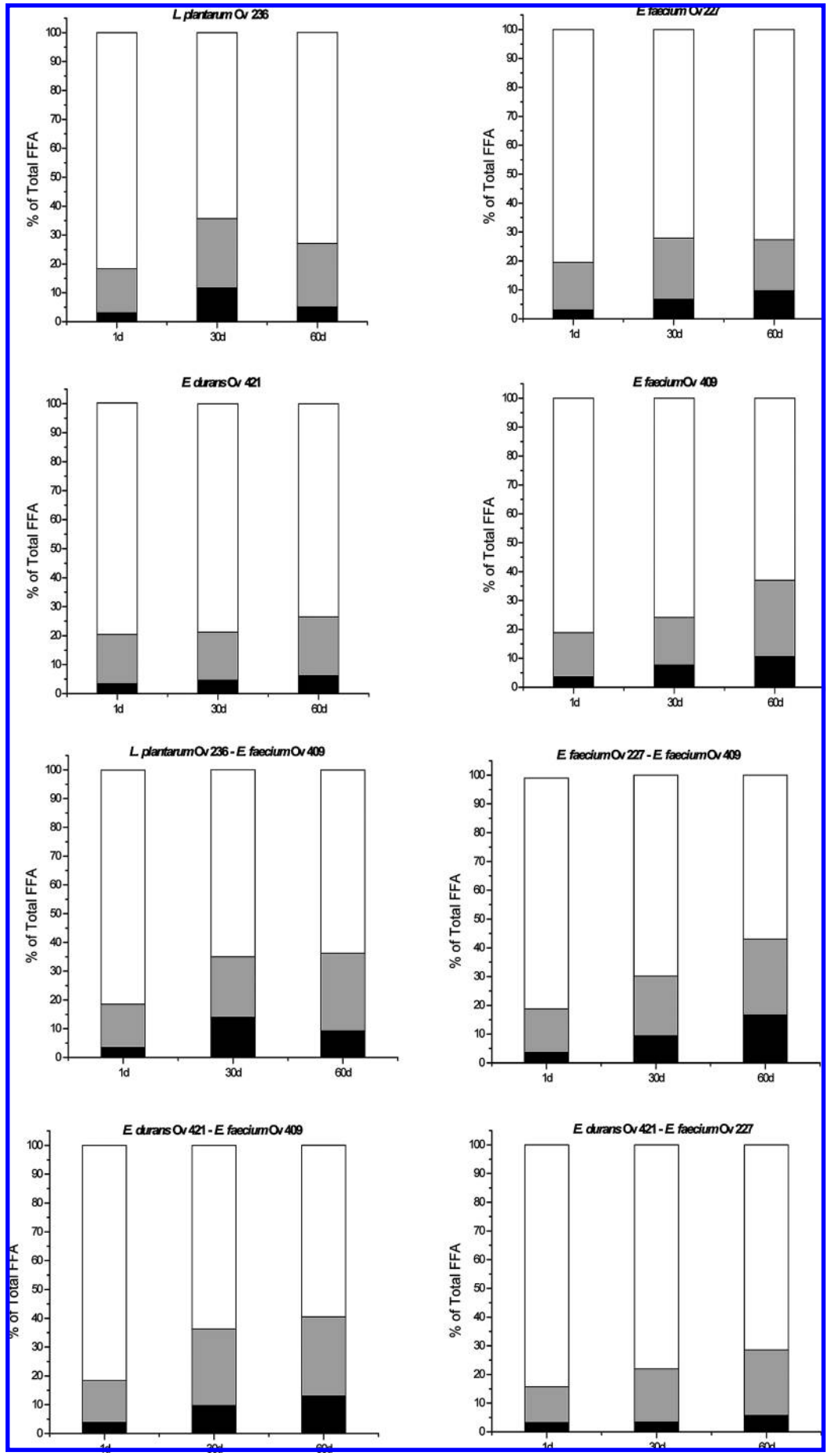

Figure 2. Variation of short-chain (black bars; SCFA, C4−C8), medium-chain (gray bars; MCFA, C10−C14), and long-chain (white bars; LCFA, C16−C18) fatty acid levels during ripening of single- or double-strain ewe's milk cheeses.

Table 2. Volatile Free Fatty Acid Contents during Ripening of Miniature Ewe's Milk Cheeses[a]

		volatile free fatty acid (mg/100 g dry extract)					
miniature ewe's milk cheese	day of ripening	acetic acid	propionic acid	butyric acid	caproic acid	total VFFA	ratio $VFFA_{60}/VFFA_1$
control	1	44.36 ± 7.12	0.75 ± 0.69	8.27 ± 0.75	6.60 ± 0.92	61.98	
	60	49.16 ± 9.00	0.80 ± 0.11	9.50 ± 1.02	7.02 ± 0.84	68.53	
Ov 236	1	45.92 ± 7.26	0.74 ± 0.30	9.94 ± 0.90	7.36 ± 0.45	63.96	1.30
	15	115.57 ± 9.71	1.06 ± 0.40	30.72 ± 1.55	19.59 ± 0.97	167.01	
	30	89.31 ± 5.20	1.42 ± 0.35	26.04 ± 1.45	19.96 ± 0.83	136.73	
	60	64.56 ± 3.74	1.51 ± 0.43	11.26 ± 1.54	6.08 ± 0.21	83.41	
Ov 227	1	43.91 ± 7.53	0.73 ± 0.20	10.47 ± 1.07	7.18 ± 0.80	62.29	3.05
	15	117.66 ± 8.37	0.73 ± 0.20	10.79 ± 0.93	7.55 ± 0.75	136.04	
	30	135.35 ± 10.60	0.91 ± 0.12	12.99 ± 1.18	9.43 ± 0.90	158.59	
	60	142.34 ± 3.51	0.94 ± 0.28	25.63 ± 1.25	21.29 ± 1.12	190.20	
Ov 421	1	47.01 ± 6.05	0.73 ± 0.15	10.21 ± 1.12	7.44 ± 0.87	65.39	1.81
	15	79.80 ± 7.51	0.80 ± 0.20	11.43 ± 1.09	8.30 ± 0.91	100.33	
	30	81.45 ± 3.57	1.30 ± 0.30	12.89 ± 1.13	9.27 ± 0.83	104.91	
	60	83.52 ± 4.01	1.72 ± 0.25	22.60 ± 1.15	10.83 ± 0.93	118.67	
Ov 409	1	45.10 ± 6.11	0.48 ± 0.31	8.72 ± 1.20	7.98 ± 0.78	64.51	4.24
	15	123.67 ± 9.70	0.73 ± 0.12	13.45 ± 1.17	8.15 ± 0.59	146.03	
	30	156.43 ± 3.84	0.94 ± 0.20	26.79 ± 1.28	15.78 ± 0.67	199.94	
	60	199.03 ± 5.53	1.03 ± 0.23	42.45 ± 1.45	31.16 ± 0.93	273.67	
Ov 236−Ov 409	1	43.48 ± 6.19	0.79 ± 0.18	9.27 ± 1.32	6.86 ± 0.93	60.40	1.85
	15	79.09 ± 8.02	0.93 ± 0.23	16.14 ± 1.45	6.94 ± 0.87	103.15	
	30	156.95 ± 12.60	0.99 ± 0.24	40.97 ± 1.25	24.64 ± 1.25	223.50	
	60	81.86 ± 10.27	1.32 ± 0.31	18.39 ± 1.21	10.24 ± 1.09	111.81	
Ov 227−Ov 409	1	49.17 ± 9.10	0.76 ± 0.10	9.85 ± 1.20	49.17 ± 9.10	67.50	2.47
	15	129.96 ± 12.51	0.98 ± 0.25	12.87 ± 1.09	129.96 ± 12.52	151.76	
	30	61.55 ± 5.06	1.16 ± 0.28	23.85 ± 1.50	61.55 ± 5.04	105.01	
	60	98.32 ± 9.20	1.55 ± 0.43	46.69 ± 1.45	98.32 ±9.23	167.17	
Ov 421−Ov 409	1	42.73 ± 5.83	0.93 ± 0.25	11.50 ± 1.25	7.40 ± 0.75	62.56	3.92
	15	nd	1.13 ± 0.12	15.63 ± 1.30	10.23 ± 0.55	26.99	
	30	130.33 ± 11.20	1.22 ± 0.25	24.78 ± 1.48	17.32 ± 0.63	173.65	
	60	183.97 ± 14.55	0.81 ± 0.13	32.69 ± 1.61	27.94 ± 0.92	245.41	
Ov 421−Ov 227	1	47.72 ± 6.92	0.66 ± 0.20	9.18 ± 1.02	7.78 ± 0.73	65.34	3.58
	15	65.64 ± 7.38	0.85 ± 0.15	11.42 ± 1.40	8.13 ± 0.69	86.04	
	30	109.92 ± 9.70	1.19 ± 0.25	24.58 ± 1.37	17.21 ± 0.97	152.9	
	60	156.06 ± 13.88	1.12 ± 0.17	43.31 ± 1.55	33.70 ± 1.21	234.19	

[a] The data are mean values ± standard deviation of three independent determinations. nd, not determined.

average values of the represented acids, whereas to the left are those with lower average values.

The second principal component (PC2) explained 20% of the total variance of data, and it is positively related to LCFA (mainly 18:2, 18:3) and with propionic acid. All LCFA contribute relatively the same to data variability, whereas propionic acid has a relatively lower significance than the other variables. PC2 is negatively related to SCFA and MCFA. The SCFA are closely related among them and with acetic acid.

According to this plot, relationships between cultures used for cheese manufacture and the type and amount of released fatty acids could be drawn: Of single-strain cheeses, Ov 409 deviated distinctly from the rest of the single-strain cheeses, having higher concentrations of SCFA. Cheese elaborated with Ov 236, Ov 421, and Ov 236−Ov 409 presented low levels of fatty acids.

The cheeses elaborated with two strains, Ov 421−Ov 409 and Ov 227−Ov 409, were similar, presenting high levels of SCFA. The cheese made with Ov 421−Ov 227 presented higher levels of all fatty acids than other cheeses. This cheese deviated significantly from single-strain cheeses Ov 421 and Ov 227, suggesting a synergic effect on FFA release among strains.

Esterase Activities. Specific esterase activity was determined on CFE using α-naphthyl (α-NA) derivatives of fatty acids of C2−C12 as substrates. Esterase activity was detected in the CFE of all strains assayed, hydrolyzing α-NA derivatives of C2−C6 fatty acids (**Table 3**). *E. durans* Ov 421 presented also esterase activity on α-NA derivatives of C8 (α-naphthyl caprylate). *E. faecium* Ov 409 presented higher specific esterase activity than other strains. This strain showed the highest specific activity on α-NA propionate, butyrate, and caproate (23.81, 21.90, and 18.8 units/mg, respectively). *L. plantarum* Ov 236 presented the highest specific activity on α-NA acetate (10.30 units/mg).

None of the strains hydrolyzed α-naphthyl derivatives of fatty acids of C10 or C12. Preferential hydrolysis of esters containing C2−C6 of α-NA fatty acid derivatives has been reported for other lactic acid bacteria (*44*).

To determine the subcellular distribution of esterase, specific esterase activity was determined in the extracellular fraction (supernatant), the intracellular fraction, and the cell surface-associated fraction (**Table 4**). None of the strains presented specific esterase activity in the extracellular fraction. The subcellular distribution of specific esterase activity in enterococci strains studied showed that a significant proportion of this activity was located in the cell surface. However, specific esterase activity of *L. plantarum* Ov 236 was mainly intracellular. Lysis of this strain would result in the release of their

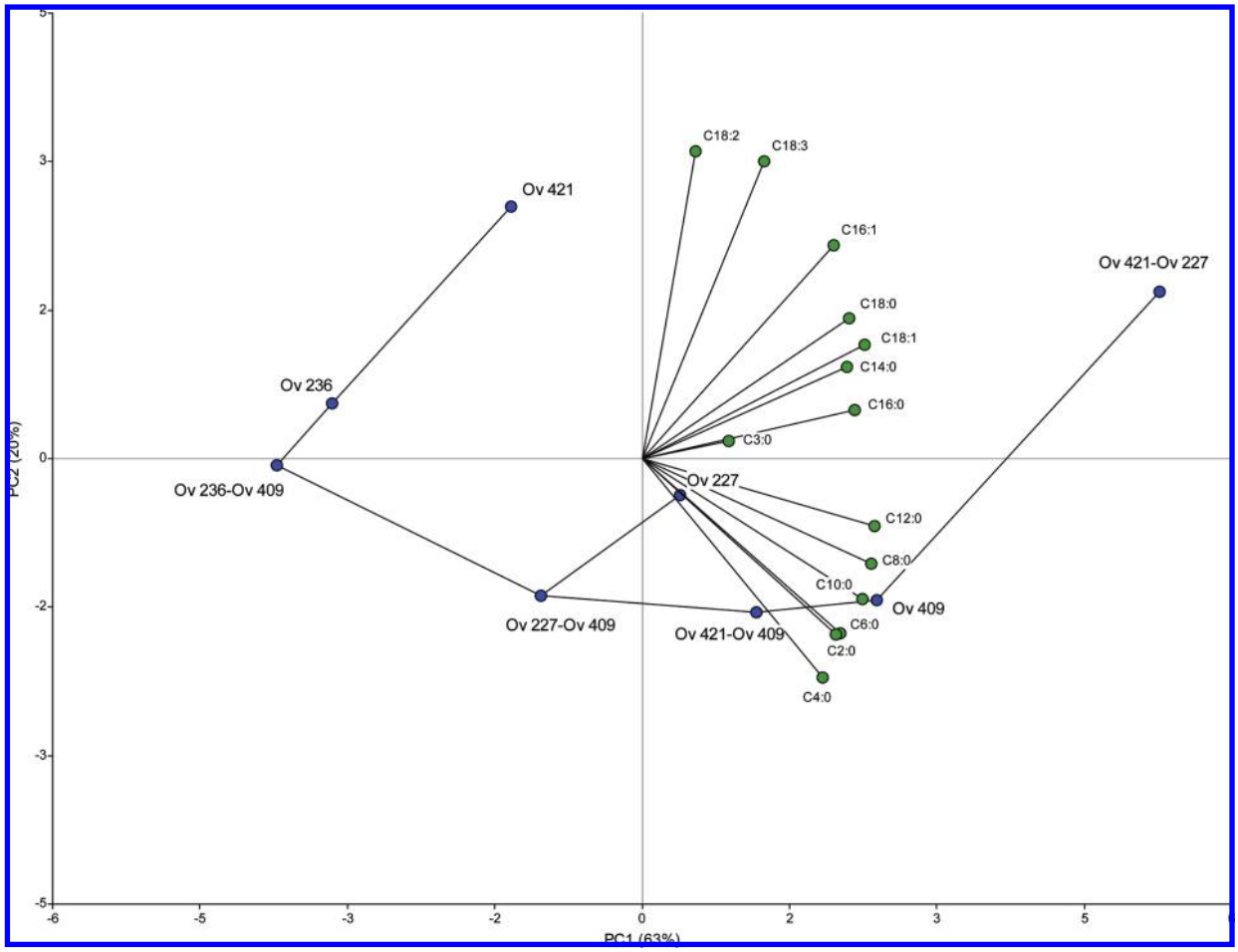

Figure 3. Biplot obtained by principal component analysis of fatty acids released in single- and double-strain miniature ewe's milk cheeses at 60 days of ripening. C2:0, acetic acid; C3:0, propionic acid; C4:0, butyric acid; C6:0, caproic acid; C8:0, caprylic acid; C10:0, capric acid; C12:0, lauric acid; C14:0, myristic acid; C16:0, palmitic acid; C16:1, palmitoleic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3, linolenic acid.

Table 3. Specific Esterase Activity (Units per Milligram of Protein)[a] in Cell-free Extracts of Lactic Acid Bacteria

	substrate α-naphthyl derivative				
source of enzyme	acetate	propionate	butyrate	caproate	caprylate
L. plantarum Ov 236	10.30 ± 0.41a	4.52 ± 0.30a	4.13 ± 0.12a	3.41 ± 0.72a	nd
E. faecium Ov 227	5.42 ± 0.25b	3.14 ± 1.05a	7.05 ± 0.82b	9.90 ± 0.20b	nd
E. durans Ov 421	3.20 ± 0.60c	4.40 ± 1.40a	1.52 ± 0.25c	3.90 ± 0.32c	1.00 ± 0.30
E. faecium Ov 409	8.01 ± 0.22b	23.81 ± 0.81b	21.90 ± 0.51d	18.80 ± 0.30d	nd

[a] The data are mean values ± standard deviation of three independent determinations. Values in the same column with different letters differ significantly ($p < 0.05$). nd, not detected.

Table 4. Specific Esterase Activity (Units per Milligram of Protein)[a] in Subcellular Fractions

	specific esterase activity in different subcellular fractions		
source of enzyme	extracellular activity	intracellular activity	cell surface-associated activity
L. plantarum Ov 236	nd	25.82 ± 0.27a	1.45 ± 0.21a
E. faecium Ov 227	nd	5.21 ± 0.34b	20.43 ± 0.34b
E. durans Ov 421	nd	0.49 ± 0.04c	12.60 ± 0.34c
E. faecium Ov 409	nd	7.34 ± 0.56b	27.20 ± 0.97d

[a] The data are mean values ± standard deviation of three independent determinations. Values in the same column with different letters differ significantly ($p < 0.05$). nd, not detected.

intracellular esterase in cheese paste, inducing an increase in the lipolysis of cheese manufactured with *L. plantarum* at 15 days of ripening (**Figure 1**; **Table 2**).

Gobbetti et al. (*45*) reported intracellular esterase activity for *L. plantarum* 2739 and did not detect cell surface-associated activity. Other lactic acid bacteria (lactococci and lactobacilli) presented intracellular and cell surface-associated esterase activity (*28*, *45*, *46*). In enterococci strains esterase activity detected in the wall-membrane fraction was significantly higher than in the intracellular fraction, suggesting that these enzymes would be strongly associated with cell envelopes.

The results show that the strains studied contributed to the release of FFA during ripening of miniature ewe's milk cheese models. The increase of SCFA throughout ripening involves the action of esterases located in the wall-membrane fraction of starter strains used in cheese manufacture. The miniature ewe's milk cheese models constituted a system useful to study strain contribution to flavor development. The highest esterase activities detected in *E. faecium* Ov 409 are in accordance with the highest VFFA levels present in cheeses elaborated with this strain.

On the basis of our findings, esterases of starter LAB would have a potential role in ewe's milk cheese flavor development, due to their ability to mediate FFA release during ripening. A deeper understanding of esterase activity of strains would be a promising way to select strains as tailor-made starter cultures for ewe's milk cheese manufacture. This is of great relevance because it would allow cheese manufacturers to control the formation of flavor compounds, obtaining products of appropriate sensorial attributes and constant quality.

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