Technological properties of *Enterococcus faecium* isolated from ewe's milk and cheese with importance for flavour development

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Abstract: Eight *Enterococcus faecium* strains isolated from ewe milk and artisanal cheese from northwest Argentina were screened for biotechnological properties relevant to flavour development. The API ZYM test showed absence of proteases, presence of high amounts of peptidases, and high esterase–lipase activities. Low extracellular proteolytic activity was observed. Most strains produced diacetyl in milk, with *E. faecium* OvL 214 and OvL 254 being the best producers. Biomass and growth rate increased when citrate was added to the medium, suggesting that these strains could use citrate as a main energy source. After 24 h of incubation, citrate was completely consumed in complex medium supplemented with glucose and citrate. An average of 17% residual citrate was detected in complex media supplemented with citrate. For all strains, esterase activity was detected up to α-naphthyl-caproate. They hydrolyzed α-naphthyl derivatives of fatty acids in this order: C3 > C6 > C4 > C8 > C2. Post-electrophoretic detection of esterase activities revealed the presence of multiple esterases. Hydrolysis of tributiryn, tricaprylin, and milk fat was observed in cell-free extracts. *Enterococcus faecium* strains isolated from ewe milk and artisanal cheese from northwest Argentina present the metabolic potential to contribute to cheese flavour development.

Key words: flavour compounds, enterococci, ewe milk cheese.

Résumé : Huit souches de *Enterococcus faecium* isolées de lait de brebis et de fromage artisanal du nord-ouest de l'Argentine ont été criblées pour des propriétés biotechnologiques utiles au développement de la saveur. Le test API ZYM a démontré l'absence de protéases et la présence de quantités élevées de peptidases et d'activités estérase–lipase élevées. Une faible activité protéolytique extracellulaire fut observée. La plupart des souches ont produit du diacétyle dans le lait, les meilleures productrices étant *E. faecium* OvL 214 et OvL 254. La biomasse et le taux de croissance ont augmenté lorsque du citrate fut ajouté au milieu, ce qui indique que ces souches pourraient utiliser le citrate comme principale source d'énergie. Après 24 h d'incubation, le citrate fut complètement consommé dans un milieu complexe supplémenté avec du glucose et du citrate. Une moyenne de 17 % de citrate résiduel fut détectée dans un milieu complexe supplémenté avec du citrate. Une activité estérase fut détectée chez toutes les souches jusqu'au α-naphthyl-caproate. Elles ont hydrolysé des dérivés α-naphthyl d'acides gras dans cet ordre: C3 > C6 > C4 > C8 > C2. Une détection post-électrophorétique des activités estérase a révélé la présence de multiples estérases. L'hydrolyse de la tributyrine, de la tricapryline et du gras laitier fut observée dans des extraits acellulaires. Les souches d'*E. faecium* isolées de lait de brebis et de fromage artisanal du nord-ouest de l'Argentine ont présenté un potentiel métabolique pouvant contribuer au développement gustatif des fromages.

Mots clés : composés gustatifs, entérococques, fromage de lait de brebis.

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Introduction

The consumption of ewe's milk cheese in Argentina is continuously increasing and there is good incentive to expand the range and quality of these fermented dairy products.

The cheese produced in northwest Argentina, a semi-hard variety, is made from raw ewe's milk, without the addition of starter cultures. This cheese is ripened at 20 °C for 30 days. The artisanal procedures determine quality to be very variable, which makes diffusion of such products to national and international markets difficult.

Indigenous lactic acid bacteria found in these artisanal cheeses were previously identified as *Enterococcus* (59%) and *Lactobacillus* (41%). Enterococcal isolates were identified mostly as *Enterococcus faecium* (62%) and *E. faecalis* (38%) (Medina et al. 2001).

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Enterococci are Gram-positive bacteria and fit within the general definition of lactic acid bacteria (Franz et al. 1999). The presence of enterococci in dairy products has long been considered as an indicator of insufficient sanitary conditions during production and processing of milk. However, enterococci have a long history of safe use in food. Moreover, a considerable number of strains belonging to different species of the genus *Enterococcus* share interesting biotechnological traits, such as bacteriocin production and probiotic characteristics (Franz et al. 1999). They occur and grow in a wide variety of artisanal cheeses produced in southern Europe (Portugal, Spain, Italy, and Greece) from raw or pasteurized goat, ewe, water buffalo, or bovine milk (Litopoulou-Tzanetaki 1990; Litopoulou-Tzanetaki et al. 1993; Macedo et al. 1995; Villani and Coppola 1994; Centeno et al. 1996). The persistence and prevalence of enterococci during ripening has been attributed to their wide range of growth temperatures and their high tolerance to heat and salt (Sarantinopoulos et al. 2001).

Reports on the influence of enterococci on cheese flavour are contradictory. High levels of contaminating enterococci are considered to lead to deterioration of sensory properties in some cheeses (López-Diáz et al. 1995). On the other hand, many authors claim that enterococci may have a desirable role in cheese production (Centeno et al. 1999). The benefical effect of enterococci in cheesemaking has been attributed to the production of typical cheese flavour compounds (Centeno et al. 1999).

Flavour development in cheese results from the metabolic activities of cheese bacteria by glycolysis, lipolysis, proteolysis, and citrate metabolism. To respond to the increasing demand for products with improved aroma characteristics, the use of bacterial strains for cheese ripening with enhanced flavour production is seen as promising.

Information is lacking about biochemical characteristics of *E. faecium* isolated from ewe milk or artisanal cheeses from Argentina. The aim of this work was to determine the biochemical properties of indigenous *E. faecium* strains present in ewe's milk and artisanal cheese, such as acidification ability, proteolytic and lipolytic activity, citrate utilization, and diacetyl production. The evaluation of these properties would aid in determining the contribution of such *E. faecium* strains to ewe milk cheese flavour development.

Materials and methods

Microorganisms and media

Enterococcus faecium strains used in this study were isolated from artisanal ewe milk cheese (OvQ 157, OvQ 167, OvQ 178, OvQ 194, and OvQ 426) and from ewe milk (OvL 214, OvL 242, and OvL 254) (Medina et al. 2001). All strains were tested for vancomycin and teicoplanin resistance, haemolysin production, and gelatinase production according to Andrighetto et al. (2001) and Eaton and Gasson (2001). They were all vancomycin and teicoplanin susceptible and did not produce haemolysin or gelatinase.

The strains frozen at –70 °C in Man–Rogosa–Sharpe (MRS) broth (De Man et al. 1960) containing glycerol 20% *v/v* were cultured in MRS broth (Merck, Darmstadt, Germany) at 37 °C for 12–24 h before carrying out the different assays.

Screening of enzymatic activities of whole cells by the API ZYM assay

The enzymatic activity of the strains was evaluated using the API ZYM system (BioMerieux, Marcy l'Etoile, France). The cells were inoculated at 0.1% *v/v* in 300 mL of MRS broth, incubated at 30 °C, and harvested at early stationary phase by centrifugation (7000*g* for 15 min at 4 °C). The cells were washed twice with phosphate buffer (50 mmol/L, pH 7.00) and resuspended at 25% *w/v*. Sixty-five microlitres of cell suspension were inoculated in each microtube of the API ZYM strip.

Acidifying activity

The acidifying activity of enterococci strains was evaluated by determination of milk pH variation and the amount of lactic acid produced (g/100 mL). The final pH of 10% *w/v* reconstituted skim milk powder was determined using an Altronix TP IX pH meter (Altronix Corporation, Brooklyn, New York, USA) after incubation of a 1% *v/v* inoculum for 6, 12, and 24 h at 37 °C. Acidity was determined by titration with 0.11 mol NaOH/L in the presence of phenolphthalein and was expressed as lactic acid produced (g/100 mL).

Proteolytic activity

Proteolytic activity of whole cells in milk was determined by the *o*-phthaldialdehyde spectrophotometric assay (Church et al. 1983). This test is based on the reaction of free α-amino groups released by hydrolysis of casein (after a 24 h incubation period in milk) with *o*-phthaldialdehyde in the presence of β-mercaptoethanol to form a complex that strongly absorbs at an optical density (OD) of 340 nm. A standard curve was prepared using glycine. The results were expressed as millimoles of glycine per litre of milk.

Diacetyl-acetoin production

The production of diacetyl-acetoin was colourimetrically determined as described by International Dairy Federation standard 149A (IDF 1997). Active cultures were inoculated at 1% *w/v* in 100 mL of 10% *w/v* reconstituted skim milk powder and incubated for 16 h. The production of diacetyl-acetoin was expressed as milligrams of diacetyl per litre of milk.

Growth and citrate utilization in complex medium LAPT

LAPT complex medium (De Figueroa et al. 1996) containing 15 g/L peptone, 10 g/L tryptone, 10 g/L yeast extract, and 1 mL/L Tween 80 (final pH 6.50) was used as basal culture medium. Cells were propagated in LAPTG (LAPT plus 10 g/L glucose). Growth was studied in LAPTc (LAPT plus 8 mmol/L sodium citrate), LAPTg (LAPT plus 5.5 mmol/L glucose) and LAPTg+c (LAPT plus 5.5 mmol/L glucose and 8 mmol/L sodium citrate). The basal culture medium was sterilized at 121 °C for 15 min. After autoclaving, sterile solutions of glucose and sodium citrate were added to reach the required concentrations of sugar and citrate. The media were inoculated at 2% *v/v* with a cell suspension from an overnight culture in LAPTG. Growth was measured spectrophotometrically at an OD of 540 nm using a Cecil 2021 spectrophotometer (Cecil Instruments, Cambridge, England). Growth rates were calculated by multiplying (by 2.303) the

slope of the regression equation of the linear portion of the curves relating log OD to time.

Residual citrate concentration was determined (Marier and Boulet 1958) in supernatants of LAPTc and LAPTc+g media after 24 h of incubation.

Preparation of cell-free extracts

Cells were harvested after 16 h by centrifugation at 10 000*g* for 10 min at 4 °C, washed twice with 50 mmol/L sodium phosphate buffer, pH 7.00, and resuspended at 50% *w/v*. The suspension was disrupted by adding 1–2 g of 0.10–0.11 mm diameter glass beads (No. 31/14; B. Braun Biotech International, Melsungen, Germany) and using a cell disruptor (B. Braun Biotech International). Five mixing sequences (speed, 6.5/s), each of 1 min duration, were successively applied. Samples were cooled on ice for 5 min between each mixing sequence. Cellular debris was removed by centrifugation (20 000*g* for 30 min at 4 °C) and each supernatant was used as a cell-free extract (CFE). The extracellular fraction (the culture medium supernatant) was sterilized by filtration (0.22 μm, white GSWP, 25 mm; Millipore Corporation, Bedford, Massachusetts, USA).

Esterase activity

The esterase activity of CFE was determined on α-naphthyl (α-NA) derivatives of C2–C12 fatty acids (Sigma-Aldrich, St. Louis, Missouri, USA) (Medina et al. 2004*a*). The assay mixture contained 160 μL of 100 mmol/L sodium phosphate buffer (pH 7.00), 20 μL of α-NA substrate (10 mmol/L in ethanol) and 100 μL of CFE. After incubation for 1 h at 37 °C, the colour was developed by adding 0.6 mL Fast Garnet GBC (Sigma-Aldrich) solution (5 mg/mL in 10% *w/v* sodium dodecyl sulfate) and incubating at room temperature for 15 min. The absorbance was measured at an OD of 560 nm. 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 min. Specific esterase activity was defined as units per milligram of protein.

Post-electrophoretic detection of esterases

The CFE was subjected to polyacrylamide gel electrophoresis using 12% *w/v* acrylamide gels without sodium dodecyl sulfate in 25 mmol/L Tris – 0.19 mol/L glycine buffer (pH 8.30) and 4% *w/v* stacking gel (Laemmli 1970) in a Mini Protean 3 electrophoresis cell (Bio-Rad, Hercules, California, USA). Polyacrylamide gel electrophoresis was conducted at 60 V for 2 h. Substrates used were α-NA derivates of C2–C12 fatty acids. Gels were incubated at 37 °C in 0.1 mol/L sodium phosphate buffer (pH 7.00), containing the substrate (1% *w/v* in acetone) and 0.5 mg/mL Fast Red TR (Sigma-Aldrich) (Medina et al. 2004*b*). Activities were identified by the appearance of coloured bands on the gels.

Lipolytic activity

Tributyrin, tricaprylin, triolein, trilaurin (all from Sigma-Aldrich), and milk fat were used to determine lipolytic activity by the agar diffusion assay (Medina et al. 2004*a*). Agar plates contained 1.5% *w/v* agar–agar ultra pure (Merck, Darmstadt, Germany), 0.25% *v/v* triglycerides, 0.02% *w/v* sodium azide, and 100 mmol/L sodium phosphate buffer (pH 7.00). Activity was observed as a detectable zone of hydrolysis after 48 h of incubation at 37 °C.

Protein determination

Protein concentrations were determined (Bradford 1976) using bovine serum albumin (Sigma-Aldrich) as the standard.

Statistical analysis

All experiments were repeated three times. The results were submitted to one-way ANOVA analysis of variance using Minitab statistical software (version 1.4; Minitab, State College, Pennsylvania, USA).

Results and discussion

Screening of enzymatic activities of whole cells by the API ZYM assay

The enzymatic activities of eight strains of *E. faecium* isolated from ewe milk and cheese, as evaluated by the semi-quantitative API ZYM system, are shown in Table 1.

Esterase (C4) activities were weak (5 nmol hydrolyzed substrate), with *E. faecium* OvQ 167 exhibiting the highest activity (20 nmol). Lipase (C14) activities were low or absent in all strains (0–5 nmol). However, esterase–lipase activities (C8) were high (30 nmol), with the exceptions of *E. faecium* OvQ 157 (5 nmol) and OvQ 167 (20 nmol). These enzymes would contribute to increase short chain fatty acid (C4–C8) concentration in cheese. In fact, we observed that short chain fatty acid concentration increased during ripening in ewe milk cheese manufactured with some of these enterococci strains as adjunct cultures (Katz et al. 2004). These fatty acids in appropriate quantities are responsible for piquancy flavours of cheese (McSweeney and Sousa 2000). The soapy defect caused by the accumulation of long chain fatty acids in many varieties of matured cheeses can be eliminated using strains with intermediate esterase and lipase activities (McSweeney and Sousa 2000).

High leucine arylamidase activity (≥40 nmol) was observed in all strains studied. El Din et al. (2002) reported that the enzymatic system of *E. faecium* strains presents higher activity on leucil-4-nitroanilide when compared to other 4-nitroanilide derivatives (arginine-, lysine-, alanine-, glycine-, and proline-4-nitroanilide). Valine and cystine arylamidase activities were generally lower (20–30 nmol) than leucine, although *E. faecium* OvL 214 and OvL 254 exhibited high valine arylamidase activities (≥40 nmol). Aminopeptidases could be an important tool in favouring the release of amino acids that would then contribute to the development of desirable flavours in cheese. These enzymes can also have a debittering effect during cheese ripening (El Soda et al. 1991).

Trypsin and α-chymotrypsin activities were very low or absent in all strains. Cheese starters with low protease and strong peptidase activities are also useful in reducing bitterness and improving body and textural defects (Davies and Law 1984).

Alkaline and acid phosphatase were high, reaching approximated values ≥40 nmol of hydrolyzed substrate in all strains. Acid phosphatase is an essential enzyme for the

Table 1. Enzymatic activity of whole cells of enterococci detected by API ZYM system.

	Enzyme activity																		
Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
OvQ 157	≥40	5	5	0	≥40	20	20	0	0	≥40	5	0	5	0	0	5	0	0	0
OvQ 167	≥40	20	20	0	≥40	20	20	5	5	≥40	10	0	0	0	0	5	0	0	0
OvQ 178	≥40	5	30	0	≥40	20	20	5	5	≥40	10	5	0	0	0	5	5	0	0
OvQ 194	≥40	5	30	5	≥40	20	20	5	5	≥40	10	0	0	0	0	5	0	0	0
OvQ 426	≥40	5	30	5	≥40	30	30	5	5	≥40	10	0	0	0	0	10	5	0	0
OvL 214	≥40	5	30	5	≥40	≥40	20	5	5	≥40	20	0	0	0	0	20	0	0	0
OvL 242	≥40	5	30	5	≥40	30	20	5	5	≥40	20	0	0	0	0	5	0	0	0
OvL 254	≥40	5	30	5	≥40	≥40	20	5	5	≥40	20	5	0	0	0	10	0	0	0

Note: Enzymatic activity (approximate values) is expressed as nanomoles of substrate hydrolyzed. Enzymes tested: 1, alkaline phosphatase; 2, esterase (C4); 3, esterase–lipase (C8); 4, lipase (C14); 5, leucine arylamidase; 6, valine arylamidase; 7, cystine arylamidase; 8, trypsin; 9, α-chymotrypsin; 10, acid phosphatase; 11, naphthol-AS-BI-phosphohydrolase; 12, α-galactosidase; 13, β-galactosidase; 14, β-glucuronidase; 15, α-glucosidase; 16, β-glucosidase; 17, *N*-acetyl-β-glucosaminidase; 18, α-mannosidase; 19, α-fucosidase.

hydrolysis of phosphopeptides prevalent in cheese ripening (Fox and McSweeney 1996).

Only *E. faecium* OvQ 157 showed β-galactosidase activity (5 nmol). The absence of such enzymatic activity may be because phospho-β-galactosidase – galactohydrolase is the predominant carbohydrase in enterococci (Devriese and Pot 1995). On the other hand, low α-galactosidase activity was observed for *E. faecium* OvQ 178 and OvL 254.

The absence of proteases (trypsin and chymotrypsin), the presence of high amounts of peptidases (leucine, valine, and cystine aminopeptidase), and low esterase–lipase (C4 and C8) activities are desirable traits of strains intended for use in accelerated ripening of cheese and production of cheese flavourings.

Acidifying activity

The acidifying activity of enterococci strains was evaluated by determination of milk pH variation and the amount of lactic acid produced (g/100 mL) (Table 2). The initial pH of milk was 6.70. After 6 h of incubation at 37 °C, pH decreased to 5.48 (OvL 242), 5.58 (OvL 254), and 5.66 (OvL 214). The strains isolated from cheese reduced pH to values of 5.93–6.02. Significant differences ($p < 0.05$) with respect to acidifying activity between strains isolated from cheese or milk were also observed after 12 and 24 h of incubation. After 24 h, *E. faecium* OvL 242 decreased milk pH to 4.51. The highest acidifying activities after 24 h incubation were displayed by strains OvL 242 (0.28 g lactic acid/100 mL), OvL 214 (0.26 g lactic acid/100 mL), and OvL 254 (0.24 g lactic acid/100 mL). *Enterococcus* strains examined in this study were poor acidifiers in milk and, therefore, are of little importance as starter organisms (Huggins and Sandine 1984). Sarantinopoulos et al. (2001) observed that *E. faecium* strains are poor acidifiers in milk. Other researchers also have reported poor acid production by enterococci in milk (Villani and Coppola 1994). Strains of *E. faecium* degrade lactose in ovine and caprine milk more slowly than *Lactobacillus paracasei*, which is itself a slow acidifier (Freitas et al. 1999).

A rapid decrease in pH during the initial steps of cheese preparation is of crucial importance in cheese manufacture, since it is essential for coagulation and prevention or reduction of adventitious microflora growth (Sarantinopoulos et al. 2001). However, enterococci may still be useful as adjunct cultures for cheese fermentation.

Proteolytic activity

Proteolytic activity was 0.13 mmol glycine / L milk, 0.69 mmol glycine / L milk, and 0.83 mmol glycine / L milk for strains OvQ 157, OvL 242, and OvQ 178, respectively, (Table 3). There were significant differences ($p < 0.05$) with respect to proteolytic activity among these strains. The other strains showed lower activity (≤0.01 mmol glycine / L milk). Sarantinopoulos et al. (2001) also reported low extracellular proteolytic activity in *E. faecium* strains. Our results agreed with those from the API ZYM test, where low trypsin and chymotrypsin activities and high peptidase activities were observed (Table 1).

The degradation of casein plays an important role in the development of texture in cheese. In addition, some peptides contribute to the development of flavour, whereas other

Table 2. Acidifying activity of *Enterococcus faecium* strains.

	Acidifying activity after incubation for:					
	6 h		12 h		24 h	
Strain	pH	Titratable acidity (g/100 mL lactic acid)	pH	Titratable acidity (g/100 mL lactic acid)	pH	Titratable acidity (g/100 mL lactic acid)
OvQ 157	5.97	0.10±0.05a	5.69	0.13±0.07a	5.33	0.16±0.06a
OvQ 167	5.95	0.11±0.06a	5.66	0.14±0.08a	5.35	0.18±0.09a
OvQ 178	5.93	0.11±0.04a	5.61	0.14±0.06a	5.08	0.20±0.04b
OvQ 194	5.97	0.12±0.03b	5.69	0.14±0.07a	5.21	0.18±0.08a
OvQ 426	6.02	0.10±0.04a	5.72	0.14±0.08a	5.30	0.19±0.09a
OvL 214	5.66	0.13±0.07bc	5.16	0.18±0.05b	4.73	0.26±0.05c
OvL 242	5.48	0.15±0.06c	4.88	0.22±0.06c	4.51	0.28±0.04d
OvL 254	5.58	0.13±0.06bc	5.02	0.21±0.07c	4.61	0.24±0.08c

Note: Values presented are means of three replicate evaluations for each bacterial strain. Mean ± SD. Values in the same column with different letters differ significantly ($p < 0.05$).

Table 3. Proteolytic activity and diacetyl production of *Enterococcus faecium* strains.

Strain	Proteolytic activity (mmol glycine / L milk)	Diacetyl production (mmol glycine / L milk)
OvQ 157	0.13±0.08a	0.55±0.10a
OvQ 167	≤0.01	ND
OvQ 178	0.83±0.10b	0.60±0.12a
OvQ 194	≤0.01	0.69±0.09a
OvQ 426	≤0.01	1.02±0.14b
OvL 214	≤0.01	2.13±0.16c
OvL 242	0.69±0.09c	1.15±0.13b
OvL 254	≤0.01	2.25±0.15c

Note: Values presented are means of three replicate evaluations for each bacterial strain. Mean ± SD. Values in the same column with different letters differ significantly ($p < 0.05$). ND, not detected.

undesirable bitter tasting peptides can lead to off-flavour formation. Bacterial cell wall associated proteases and intracellular peptidases released after cell lysis in the curd are considered to play an important role in casein hydrolysis during cheese preparation (Wilkinson et al. 1994).

Diacetyl-acetoin production

The majority of enterococci strains examined produced diacetyl in ewe milk as shown in Table 3. The highest production was observed for *E. faecium* OvL 214 and OvL 254. *Enterococcus faecium* OvQ 167 did not produce diacetyl.

Diacetyl and acetoin have very distinct aroma properties and significantly influence the quality of fermented foods (Hugenholtz 1993). For instance, diacetyl determines the aromatic properties of fresh cheese, fermented milk, cream, and butter (De Figueroa et al. 1998).

The amount of diacetyl produced depends on the ability of strains to metabolize citrate present in milk (Divies et al. 1994). Centeno et al. (1999) observed higher concentrations of diacetyl and acetoin in Cebreiro cheese manufactured with enterococci as adjunct starters.

Citrate metabolism

The effect of citrate on growth rate was determined in LAPT complex medium supplemented with citrate (LAPTc), glucose in limiting concentration (LAPTg), and glucose plus citrate (LAPTg+c) (Table 4).

According to final OD values, biomass increased when citrate was added to LAPT and LAPTg media. No significant differences ($p < 0.05$) were observed in the final OD among strains. The increase in biomass can be attributed to the production of acetate, which stimulates the growth by entering biosynthetic pathways, in particular for lipid formation and (or) by formation of extra ATP (Sarantinopoulos et al. 2003).

The growth rate increased when citrate was added to LAPT and LAPTg media. These results suggested that these strains could use citrate as the main energy source. The final pH values in media containing citrate were higher than in LAPT and LAPTg (Table 4). Citrate utilization with production of carbon dioxide during the initial breakdown of citrate and (or) during the formation of acetyl-CoA from pyruvate may contribute to this increase of media pH (Sarantinopoulos et al. 2001).

Citrate was completely consumed in LAPTg+c medium after 24 h of incubation, whereas in LAPTc medium an average of 17% residual citrate was detected (Table 5). No significant differences ($p < 0.05$) were observed with respect to citrate consumed by these strains after 24 h of incubation.

Citrate utilization is an important technological characteristic of some starter lactic acid bacteria. Citrate in milk is metabolized by many species of lactic acid bacteria into flavour compounds, such as diacetyl, acetoin, and 2,3-butanediol (Hugenholtz 1993).

Table 4. Effect of citrate on growth.

	LAPT			LAPTc			LAPTg			LAPTg+c		
Strain	OD	Growth rate (per h)	pH	OD	Growth rate (per h)	pH	OD	Growth rate (per h)	pH	OD	Growth rate (per h)	pH
OvQ 157	0.67±0.02a	0.161±0.050a	6.55	0.83±0.03a	0.299±0.120a	7.35	0.95±0.03a	0.345±0.100a	5.25	1.20±0.09a	0.415±0.100a	5.33
OvQ 167	0.72±0.02a	0.184±0.070b	6.62	0.90±0.02b	0.368±0.100ab	6.85	0.98±0.02a	0.415±0.110ab	5.21	1.10±0.05a	0.483±0.090b	5.49
OvQ 178	0.68±0.04a	0.207±0.120b	6.59	0.93±0.04b	0.391±0.110b	6.82	0.98±0.04a	0.437±0.140b	5.11	1.10±0.08a	0.530±0.110c	5.38
OvQ 194	0.70±0.02a	0.115±0.040c	6.62	0.83±0.04a	0.276±0.120a	6.75	0.90±0.02b	0.322±0.120a	5.14	1.10±0.07a	0.461±0.120b	5.51
OvQ 426	0.68±0.03a	0.138±0.080d	6.70	0.82±0.04a	0.322±0.090a	6.80	0.94±0.02b	0.391±0.110a	5.09	1.05±0.08a	0.483±0.080b	5.56
OvL 214	0.75±0.03a	0.138±0.040d	6.70	0.90±0.05b	0.276±0.100a	6.85	0.95±0.03a	0.345±0.100a	5.14	1.10±0.07a	0.415±0.060a	5.39
OvL 242	0.69±0.02a	0.161±0.060a	6.64	0.80±0.02a	0.253±0.080a	6.79	0.90±0.02b	0.322±0.080c	5.07	1.10±0.08a	0.507±0.120b	5.49
OvL 254	0.67±0.03a	0.092±0.020c	6.67	0.80±0.04a	0.230±0.090a	6.75	0.93±0.03b	0.276±0.070c	5.16	1.05±0.07a	0.437±0.090a	5.41

Note: Values presented are means of two replicate evaluations for each bacterial strain. Mean ± SD. Values in the same column with different letters differ significantly ($p < 0.05$). OD, final optical density (540 nm) after 24 h of incubation. pH values are final after 24 h of incubation. LAPT, contains 15 g/L peptone, 10 g/L tryptone, 10 g/L yeast extract, and 1 mL/L Tween 80. LAPTc, LAPT plus 8 mmol/L sodium citrate. LAPTg, LAPT plus 5.5 mmol/L glucose. LAPTg+c, LAPT plus 5.5 mmol/L glucose and 8 mmol/L sodium citrate.

Table 5. Citrate consumption after 24 h of incubation in complex media.

	Citrate consumed (mmol/L)	
Strain	LAPTc	LAPTg+c
OvQ 157	6.40±0.12	7.95±0.10
OvQ 167	7.05±0.09	8.00±0.09
OvQ 178	6.95±0.13	7.95±0.11
OvQ 194	6.35±0.16	7.96±0.14
OvQ 426	6.29±0.11	8.00±0.12
OvL 214	7.35±0.17	8.00±0.06
OvL 242	6.25±0.12	8.00±0.13
OvL 254	6.55±0.11	8.00±0.08

Note: Values presented are means of two replicate evaluations for each bacterial strain. Mean ± SD. LAPTc, LAPT plus 8 mmol/L sodium citrate. LAPTg+c, LAPT plus 5.5 mmol/L glucose and 8 mmol/L sodium citrate.

Further research is necessary to understand citrate metabolism regulation by glucose and lactose in these microorganisms.

Esterase activity

Specific esterase activity was determined on CFEs and extracellular fractions using α-NA derivatives of fatty acids of C2–C12 as substrates (Table 6). Esterase activity was detected in the CFE of all strains assayed, hydrolyzing α-NA derivatives of C2–C8 fatty acids (Table 6). The highest mean specific activities were observed for α-NA propionate (4.02 ± 1.30 U/mg), butyrate (2.35 ± 0.79 U/mg), and caproate (2.70 ± 0.67 U/mg). A lower esterase activity was obtained for α-NA-acetate (1.51 ± 1.24 U/mg) and α-NA-caprylate (1.82 ± 0.60 U/mg). None of the strains hydrolyzed α-NA derivatives of C10 and C12 fatty acids. *Enterococcus faecium* strains tested in this work hydrolyzed α-NA derivatives of fatty acids in the following order: C3 > C6 > C4 > C8 > C2.

Enterococcus faecium strains are active on 4- and 2-nitrophenyl derivatives of fatty acids containing up to 10 carbon atoms (El Din et al. 2002). Similar observations were made by Tsakalidou et al. (1994) and Katz et al. (2002). Preferential hydrolysis of ester bonds of derivates of C2–C6 fatty acids have been reported for other lactic acid bacteria (Gobbetti et al. 1996; Katz et al. 2002). Presently, significant differences ($p < 0.05$) were observed with respect to specific esterase activity among these strains. Mean esterase activities were also statistically different ($p < 0.05$) (Table 6).

Esterase activity was not observed in extracellular fractions, consistent with reports that esterase activity is primarily intracellular (El Soda et al. 1986; Gobbetti et al. 1996; Katz et al. 2002).

Post-electrophoretic detection of esterase activities (Table 7) revealed the presence of more than a single esterase, which is a common trait among lactic acid bacteria (Sarantinopoulos et al. 2001; Katz et al. 2002; Tsakalidou et al. 1992). Based on electrophoretic mobility (R_f values), each strain gave an individual enzyme profile. For all strains, activity was detected up to α-NA-caproate. *Enterococcus faecium* OvQ 426, OvL 214, and OvL 242 presented four active esterolytic bands. *Enterococcus faecium* OvQ 194 showed two bands of esterase activity. In the other strains three bands per strain were observed. Sarantinopoulos et al.

Table 6. Specific esterase activity in cell-free extracts of *Enterococcus faecium* strains.

	Substrate				
Strain	Acetate	Propionate	Butyrate	Caproate	Caprylate
OvQ 157	0.79±0.27a	3.36±0.83a	2.46±0.42a	3.12±0.58a	2.89±0.61a
OvQ 167	0.35±0.10b	2.28±0.16b	1.75±0.17b	2.27±0.29b	1.54±0.10b
OvQ 178	3.68±1.11c	5.42±0.13c	3.62±0.12c	2.41±0.55b	1.62±0.67c
OvQ 194	1.58±0.33d	5.55±0.04c	2.09±0.31a	3.14±0.28a	1.67±0.31c
OvQ 426	0.61±0.17a	5.16±0.29c	3.18±0.29c	2.66±0.14b	2.53±0.34d
OvL 214	3.17±0.59e	4.42±0.43d	1.57+0.02d	3.95±0.26c	1.05±0.33e
OvL 242	0.92±0.29af	3.53±0.71a	1.51±0.02d	2.27±0.30b	1.46±0.48b
OvL 254	1.00±0.05f	2.50±0.14b	1.83±0.35b	1.84±0.15d	1.83±0.03c
Mean activity	1.51±1.24A	4.02±1.30B	2.35±0.79C	2.70±0.67C	1.82±0.60A

Note: Values presented are means of three replicate evaluations for each bacterial strain. Mean ± SD. Values in the same column with different lowercase letters differ significantly ($p < 0.05$). Values in the same row with different capital letters differ significantly ($p < 0.05$). Substrates used are α-naphthyl derivatives of acetate, propionate, butyrate, caproate, and caproate.

Table 7. Esterase electrophoretic patterns of cell-free extracts of *Enterococcus faecium* strains.

			Substrate			
Strain	Band	R_f	Acetate	Propionate	Butyrate	Caproate
OvQ 157	E1	0.30	+	++	+	–
	E2	0.60	+	++	W	+
	E3	0.88	–	–	+	–
OvQ 167	E1	0.34	+	++	–	+++
	E2	0.70	+	++	++	+
	E3	0.90	–	–	+	W
OvQ 178	E1	0.38	+	–	–	+
	E2	0.46	+	+	W	++
	E3	0.58	++	++++	+++	++++
OvQ 194	E1	0.50	+	+	+	++++
	E2	0.75	++	++++	++++	++++
OvQ 426	E1	0.38	+	+	–	–
	E2	0.46	+	+	+	–
	E3	0.58	+	+++	+++	++
	E4	0.62	+	–	–	–
OvL 214	E1	0.34	W	–	–	–
	E2	0.61	+	++++	+++	+
	E3	0.64	++++	++++	++	W
	E4	0.88	+	W	W	+
OvL 242	E1	0.32	+	+++	–	++++
	E2	0.66	W	+++	W	–
	E3	0.72	–	–	–	+
	E4	0.88	+	–	–	W
OvL 254	E1	0.43	+++	+++	+++	++++
	E2	0.47	W	+++	+++	++
	E3	0.84	++++	W	W	W

Note: Activity was expressed from W (weak) to ++++, indicating a very thin to a very deep coloured band was produced. –, absence of activity. Substrates used are α-naphthyl derivatives of acetate, propionate, butyrate, and caproate.

(2001) reported that *E. faecium* were more esterolytic than *E. faecalis* and *E. durans*.

Lactic acid bacteria esterases release short chain fatty acids during cheese ripening, contributing to flavour development in cheddar, Dutch, and Swiss cheeses (Fox and Stepaniak 1993). The strains evaluated in this work presented esterase activities that released α-NA derivatives of C2–C8 fatty acids and, therefore, would improve the organoleptic characteristics of ewe's milk cheese.

Lipase activity

Lipase activity was studied in CFEs and extracellular fractions with tributyrin,tricaprilyn, triolein, trilaurin, and milk fat as substrates. The CFEs and extracellular fractions of

Table 8. Lipolytic activity on triglycerides.

	Tributyrin		Tricaprylin		Milk fat	
Strain	CFE	EF	CFE	EF	CFE	EF
OvQ 157	+	–	–	–	+	–
OvQ 167	+	–	–	–	+	–
OvQ 178	+	–	–	–	+	–
OvQ 194	+	–	–	–	–	–
OvQ 426	+	+	+	–	+	–
OvL 214	+	+	+	+	+	–
OvL 242	+	+	+	–	–	–
OvL 254	+	–	–	–	+	–

Note: +, lipase activity detected; –, lipase activity not detected. CFE, cell-free extract; EF, extracellular fraction.

E. faecium OvQ 426, OvL 214, and OvL 242 were able to hydrolyze tributyrin. In the other strains only hydrolysis of tributyrin by CFEs was observed. Meyers et al. (1996) reported lipase activity associated with CFEs in the genera *Streptococcus*, *Lactococcus*, and *Lactobacillus.* These authors did not observe lipase activity in the extracellular fraction.

The strains assayed did not hydrolyze triolein or trilaurin. CFEs of *E. faecium* OvQ 426 and OvL 242, and both the CFE and extracellular fraction of OvL 214 hydrolyzed tricaprilyn. Hydrolysis of milk fat by the CFEs of OvQ 157, OvQ 167, OvQ 178, OvQ 426, OvL 214, and OvL 254 strains was observed.

Sarantinopoulos et al. (2001) reported that all enterococci strains hydrolyze tributyrin (C4) to tristearin (C18) to a decreasing extent. They also reported that *E. faecalis* strains were the most lipolytic, followed in turn by *E. durans* and *E. faecium* strains.

Milk fat hydrolysis during cheese manufacture and ripening occurs because of endogenous milk lipase, lipolytic enzymes of starter and non-starter bacteria, lipases from psychrotrophic bacteria, and depending on the cheese variety, exogenous enzyme preparations. Released fatty acids contribute to cheese flavour (Collins et al. 2003).

According to the results obtained in the present study, all the enterococci strains examined hydrolyze tributyrin and present esterase activity on synthetic substrates, from α-NA-acetate to caprylate. It is generally accepted that lactic acid bacteria, and thus enterococci, are only weakly lipolytic (El Soda et al. 1995). However, limited and often contradictory information exists on the lipolytic activity of enterococci. Tsakalidou et al. (1994) concluded that enterococci strains show significantly higher esterolytic activity than strains of most other genera of lactic acid bacteria. Macedo and Malcasa (1997) reported that an *E. faecium* strain isolated from 35-day-old Serra cheese hydrolyzed milk fat to a greater extent than a *Lactococcus lactis* subsp. *lactis* strain.

The strains evaluated in this study exhibit low milk acidifying ability, low extracellular proteolytic activity, and high peptidolytic activities. They present esterolytic activity and are able to hydrolyze milk fat. The strains utilize citrate as their main energy source and produce diacetyl in milk. These strains could be useful as adjunct cultures in cheese manufacture. Finally, the present work shows that *E. faecium* strains isolated from ewe milk and artisanal cheese from northwest Argentina present the metabolic potential to contribute to cheese flavour development.

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