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Research Note

Esterolytic and Lipolytic Activities of Lactic Acid Bacteria Isolated from Ewe's Milk and Cheese

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ABSTRACT

In the present work, we report on the esterase and lipase activities of lactic acid bacteria representing the genera *Lactococcus, Leuconostoc, Lactobacillus,* and *Enterococcus* isolated from ewe's milk and cheeses. Esterase activity was studied using α- and β-naphthyl derivatives of 2 to 12 carbon atoms and postelectrophoretic detection. The lactic acid bacteria evaluated had intracellular esterase activities, which preferentially degraded the α- and β-naphthyl derivatives of 2 to 6 carbon atoms. By studying postelectrophoretic patterns, it was found that some strains presented more than one esterase. *Lactobacillus plantarum* O236 showed four enzymes that hydrolyze carboxyl ester linkages with different specificity. Lipase activity was studied in intracellular and extracellular fractions using tributyrin, tricaprylin, triolein, and milk fat as substrates. The intracellular and extracellular fractions of *Leuconostoc mesenteroides* O257, *Lactobacillus plantarum* O236, and *Lactobacillus acidophilus* O177 were able to hydrolyze tributyrin. *L. plantarum* O186, *L. acidophilus* O252, *Enterococcus faecium* O174 and O426, and *Enterococcus faecalis* Ov409 showed lipase activity associated with the intracellular fraction on tributyrin. *Lactococcus lactis* O233, *L. plantarum* O155, and *Lactobacillus casei* O190 did not hydrolyze triglycerides. Not all strains that showed esterase activity exhibited high activity on triglycerides. Esterase and lipase activities were species- and strain-specific. Wide variations in activity between strains highlight the need for selecting appropriate starters to produce enzyme-modified cheese as well as accelerated ripened cheese.

The proteolysis of milk proteins and the lipolysis of milk fat by lactic acid bacteria (LAB) constitute the main biochemical changes in cheese flavor development *(17, 22).* The enzymatic activities responsible for the hydrolysis of proteins and peptides have been studied extensively to determine both their physiological significance and their importance in cheese flavor *(22).* However, the lipolytic and esterolytic systems of LAB remain poorly characterized *(13, 14, 17).* Esterases from LAB, yeasts, and *Pseudomonas* spp. may be involved in the development of fruity flavors in foods *(2, 18, 25),* and pregastric lipases and esterases are essential to the development of typical flavor in Italian cheese *(12).* Microbial lipases and esterases may improve the quality or accelerate the maturation of cheeses, cured bacon, and fermented sausages *(23).* Essentially, nothing is known about the contribution of bacterial lipases and esterases to flavor formation in Cheddar, Dutch, and Swiss cheeses *(12).*

The production of ovine milk and cheese manufactured with ovine milk has been historically localized in Mediterranean countries *(3).* In Argentina, the consumption of fermented products from ewe's milk is continuously increasing. The cheese, a semihard variety, is produced in northwest Argentina and is made from raw ewe's milk, without the addition of starter culture. This cheese is ripened at 20°C for 30 days. The typical characteristics of the milk produced by the breed of sheep in northwest Argentina, the geoclimatic conditions of the region, the natural pastures, and the traditional (artisanal) techniques used to produce and ripen these cheeses may explain the unique sensory characteristics of this cheese.

Because of the general composition of ewe's milk fat *(1)* and the short ripening period (30 to 40 days for artisanal cheese), the lipid fraction and the primary products of its degradation, i.e., free volatile fatty acids, play an important role in the particular flavor characteristics of this cheese. Indigenous LAB in ewe's milk and the artisanal cheese isolated from the province of northwest Argentina were identified as enterococci, lactococci, leuconostocs, and lactobacilli *(26).* These microorganisms play an important role in cheese ripening.

Several authors have studied the free fatty acid (FFA) profiles of ewe's milk cheese and the changes in individual FFAs during cheese ripening *(6, 11, 29, 31).* However, there is no information about the contribution of lipases and esterases of LAB to the formation of cheese flavor manufactured with ovine milk. In this study, the esterase and lipase activities of LAB isolated from ewe's milk and cheese were investigated to determine the contribution of the esterolytic

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and lipolytic systems of these microorganisms to the flavor development of ewe's milk cheese.

MATERIALS AND METHODS

Microorganisms and growth conditions. *Lactococcus lactis* biovar. *diacetylactis* O233, *Leuconostoc mesenteroides* subsp. *dextranicum* O257, *Lactobacillus plantarum* O236, *Lactobacillus acidophilus* O252, and *Enterococcus faecium* O242 were isolated from ewe's milk *(26). L. plantarum* O186, *L. plantarum* O155, *Lactobacillus casei* O190, *L. acidophilus* O177, *Enterococcus faecalis* Ov409, and *E. faecium* O426 and O174 were isolated from ewe's milk cheese *(26).* All bacteria were grown in deMan Rogosa Sharpe (Merck, Darmstadt, Germany) broth for 16 h. Cultures were incubated at 30°C (*Lactococcus* and *Leuconostoc*) or 37°C (*Lactobacillus* and *Enterococcus*).

Preparation of intracellular fraction. Cells were harvested after 16 h by centrifugation at 10,000 × *g* for 10 min at 4°C, washed twice with 50 mM sodium phosphate buffer, pH 7.0, and resuspended in 50% (wt/vol) of the same buffer. The suspension was disrupted by mixing with 1 to 2 g of glass beads N° 31/14 (diameter, 0.1 to 0.11 mm, B. Braun Biotech Int., Melsungen AG, Germany) using a cell disruptor (B. Braun). Five 1-min mixing sequences (speed, 6.5/s) were successively applied under a CO_2 atmosphere. Samples were cooled in ice for 5 min between each mixing sequence. Cellular debris was removed by centrifugation (20,000 × *g* for 30 min) at 4°C, and the supernatant was used as the intracellular fraction. 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., Bedford, Mass.).

Esterase activity. The esterase activity of microbial subcellular fractions was determined on α- and β-naphthyl derivatives (α-NA and β-NA) of C2 to C12 fatty acids (Sigma Chemical Co., St. Louis, Mo.) *(13).* The assay mixture contained 160 μl of 100 mM sodium phosphate buffer, pH 7.0, 20 μl of α- and β-NA substrate (10 mM in ethanol), and 100 μl of the intracellular or extracellular fraction. After incubation for 1 h at 37°C (*Lactobacillus* and *Enterococcus*) or 30°C (*Lactococcus* and *Leuconostoc*), the color was developed by adding 0.6 ml of Fast Garnet GBC (Sigma) preparation (5 mg/ml in 10% sodium dodecyl sulfate [SDS]) and further incubation at room temperature for 15 min. The absorbance was measured at 560 nm in a spectrophotometer (2021; CECIL, Cambridge, UK). A standard curve was prepared using α- or β-naphthol.

A unit of esterase activity was defined as the amount of enzyme that released 1 μmol of α- and β-naphthol per min. Specific esterase activity was defined as units per milligram of protein.

Postelectrophoretic detection of esterases. Intracellular fractions were subjected to polyacrylamide gel electrophoresis (PAGE) using 12% acrylamide gels without SDS in 25 mM Tris-0.19 M glycine buffer, pH 8.3, and 4% stacking gel *(21)* in a Mini-Protean II electrophoresis cell (Bio-Rad, Hercules, Calif.). The PAGE was conducted at 60 V for 2 h. Substrates used were the α- and β-NA derivatives of fatty acids of C2 to C12. Gels were incubated at 37°C in 0.1 M sodium phosphate buffer, pH 7.0, and contained the substrate (1% [wt/vol] in acetone) and 0.5 m/ml Fast Red TR (Sigma) *(16).* Activities were identified as colored bands on the gels.

Lipolytic activity. Tributyrin, tricaprylin, triolein, trilaurin (Sigma), and milk fat were used to determine the lipolytic activity by the agar diffusion assay *(30).* Agar plates contained 1.5% Agar-Agar ultra pure (Merck), 0.25% triglycerides, 0.02% sodium azide, and 100 mM sodium phosphate buffer, pH 7.0. Activity was observed as a detectable zone of hydrolysis after 48 h of incubation at 30°C (*Lactococcus* and *Leuconostoc*) or 37°C (*Lactobacillus* and *Enterococcus*).

Protein determination. Concentrations of proteins in enzyme solutions were determined by the method of Bradford *(4)* with bovine serum albumin (Sigma) as the standard.

Statistical analysis. Data of enzyme assays from three separate intracellular fractions were submitted to one-way analysis of variance by the Minitab Statis Program.

RESULTS AND DISCUSSION

Esterase activities. Esterase-specific activity was determined on the intracellular and extracellular fractions using α-NA and β-NA derivatives of fatty acids as substrates. Esterase activity was present in the cell extracts of all strains assayed (Table 1). *L. lactis* O233 and *L. mesenteroides* O257 showed the highest specific activity (38.29 to 59.87 U/mg) on α- and β-NA acetate. *L. lactis* O233 also hydrolyzed α- and β-NA butyrate, α- and β-NA propionate, and α- and β-NA caproate. Kamaly et al. *(20)* reported that both *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* could hydrolyze α- and β-NA butyrate but that β-NA caproate was hydrolyzed only by *L. lactis* subsp. *lactis.* Holland and Coolbear *(17)* purified a tributyrin esterase from *L. lactis* subsp. *cremoris* E8.

Of *Lactobacillus* strains, *L. casei* O190 had esterase activity on α- and β-NA acetate, α-NA propionate, and butyrate. No esterase activity was detected on β-NA propionate, butyrate, or α-NA caproate. *L. plantarum* strains O186, O236, and O155 had activity on the 2 to 6 carbon α- and β-NA derivatives. The highest specific activities were observed with C2 (acetate). *L. acidophilus* O252 and O177 had activity with C2, C3, C4, and C6 derivatives and had the highest activity with acetate. These results are according to Gobbetti et al. *(13),* who observed that thermophilic lactobacilli had higher esterase activity than mesophilic species.

E. faecium O426 showed the highest activity of all *Enterococcus* strains with C2, C3, C4, and C6 α-NA derivatives. *E. faecalis* Ov409 had high specific activity on α-NA propionate and butyrate.

None of the strains hydrolyzed α- or β-NA derivatives of fatty acids of C8, C10, or C12 carbon atoms. Preferential or exclusive hydrolysis of esters containing C2 to C6 of α- and β-NA fatty acid derivatives has been reported for other LAB systems *(9, 10, 13).*

Esterase activity was not observed with the extracellular fractions of the strains assayed. Several authors *(9, 10, 13, 23)* have reported that esterase activity is primarily intracellular.

Electrophoretic zymogram. The electrophoretic zymogram of strains studied is shown in Table 2. On the basis of electrophoretic mobility (R_f values), each strain gave an individual enzyme profile. *L. lactis* O233 presented two bands with esterase activity, E_1 and E_2, on α-NA acetate and propionate. E_1 also showed activity on α-NA butyrate.

TABLE 1. *Specific esterase activity in intracellular fractions of lactic acid bacteria*[a]

Source of enzyme	Substrate naphthyl derivative of fatty acid						
	α-C2	β-C2	α-C3	β-C3	α-C4	β-C4	α-C6
L. lactis biovar. *diacetylactis* O233	44.49 ± 1.50 A[b]	59.87 ± 1.56 A	2.67 ± 0.50 A	10.45 ± 0.56 A	1.82 ± 0.30 A	2.30 ± 0.70 AB	1.29 ± 0.10 A
L. mesenteroides subsp. *dextranicum* O257	53.82 ± 2.34 A	38.29 ± 3.56 B	12.68 ± 1.45 BD	2.77 ± 0.56 BD	2.45 ± 0.67 AB	3.13 ± 0.80 A	ND[c]
L. casei O190	26.68 ± 2.67 CF	25.23 ± 2.30 C	5.07 ± 0.56 E	ND	1.58 ± 0.13 A	ND	ND
L. plantarum O186	14.27 ± 1.09 D	9.52 ± 0.89 D	10.26 ± 0.80 B	3.66 ± 0.59 B	2.61 ± 0.12 B	0.69 ± 0.10 C	1.68 ± 0.46 A
L. plantarum O236	10.37 ± 0.43 E	11.90 ± 0.52 E	4.50 ± 0.32 E	5.88 ± 0.21 C	4.18 ± 0.14 C	1.2 ± 0.11 B	3.36 ± 0.73 B
L. plantarum O155	27.81 ± 1.09 C	12.13 ± 0.45 E	7.67 ± 0.78 F	3.73 ± 0.43 B	1.52 ± 0.23 A	0.45 ± 0.20 C	1.29 ± 0.67 A
L. acidophilus O252	47.35 ± 1.69 A	26.84 ± 1.20 F	10.37 ± 0.45 B	5.80 ± 0.20 C	4.28 ± 0.23 C	1.18 ± 0.1 AB	3.28 ± 0.34 B
L. acidophilus O177	33.18 ± 1.89 D	30.49 ± 1.34 G	21.60 ± 1.45 C	16.70 ± 1.07 E	2.30 ± 0.67 AB	2.45 ± 0.34 A	3.60 ± 0.45 B
E. faecium O242	2.03 ± 0.32 E	6.09 ± 0.56 H	2.67 ± 0.58 A	1.51 ± 0.81 D	5.40 ± 0.12 D	6.70 ± 0.42 D	ND
E. faecium O426	23.60 ± 1.45 F	3.91 ± 0.34 I	33.60 ± 1.68 D	18.54 ± 0.89 EF	34.38 ± 1.76 E	10.05 ± 0.76 E	26.06 ± 1.64 C
E. faecium O174	13.51 ± 1.04 D	23.96 ± 1.56 F	12.65 ± 1.40 B	ND	6.42 ± 0.45 F	7.89 ± 0.34 F	4.80 ± 0.23 D
E. faecalis Ov409	7.96 ± 0.23 H	8.05 ± 0.45 J	23.81 ± 0.78 C	20.45 ± 0.67 F	21.95 ± 0.56 G	19.80 ± 0.45 G	18.81 ± 0.34 E

[a] Units per milligram of protein. Mean ± standard deviation.

[b] Means with different letters in the same column are significantly different ($P < 0.05$).

[c] ND, not detected.

When α-NA caproate and laurate were used as substrates, the strain did not give any reaction on the gel. Similar results were reported in lactococci by Tsakalidou et al. *(32)*.

L. mesenteroides subsp. *dextranicum* O257 presented one band with activity for α-NA acetate, propionate, and caproate. Vafopoulou-Mastrojiannaki et al. *(33)* reported that *L. mesenteroides* subsp. *dextranicum* $G_{1.11}$ presented only one very weak activity band for α-NA acetate.

L. acidophilus O252 and O177, *L. casei* O190, and *L. plantarum* O186 and O155 presented only one band with esterase activity on α-NA acetate and a different specificity for the other substrate (Table 2). *L. plantarum* O236 presented four bands with esterase activity on α-NA acetate. E_1 and E_4 bands also had activity on α-NA propionate and butyrate. This strain presented a very complex intracellular esterolytic system, producing four enzymes that hydrolyze carboxyl ester linkages with different specificity. Earlier reports *(9, 10, 14, 23)* have shown that lactobacilli possess more than one intracellular esterase. Multiple esterases were also found in lactococci *(20, 32)* and *Propionibacterium* spp. *(7, 8)*.

In *E. faecalis* Ov409, three bands were detected with esterase activity on α-NA acetate and caproate. None of the enterococci strains showed activity against α-NA butyrate (Table 2). However, esterase-specific activity for α-NA butyrate was shown for intracellular fractions of all enterococci strains (Table 1). Similar results were observed in other strains for different substrates (Tables 1 and 2). An explanation of this is that the substrate α-NA derivative of fatty acids may have problems of accessibility through the net of the electrophoresis gel *(28)*.

None of the strains evaluated showed activity on the caprylate, caprate, or laurate derivatives. Our results show that esterases from the LAB strains that were tested preferentially released short-chain fatty acids. These results were similar to those found for lactococci *(20, 32)*, lactobacilli *(9, 10)*, and leuconostocs *(33)*.

Lipase activity. Lipase activity was studied in intracellular and extracellular fractions with tributyrin, tricaprylin, and triolein as substrates. Intracellular and extracellular fractions of *L. mesenteroides* O257, *L. plantarum* O236, and *L. acidophilus* O177 were able to hydrolyze tributyrin.

L. plantarum O186, *L. acidophilus* O252, *E. faecium* O174 and O426, and *E. faecalis* Ov409 showed lipase activity associated with the intracellular fraction on tributyrin. *L. lactis* O233, *L. plantarum* O155, and *L. casei* O190 did not hydrolyze triglycerides. Meyers et al. *(27)* observed lipase activity associated with the intracellular fraction in the genera *Streptococcus, Lactococcus,* and *Lactobacillus;* the majority of strains with lipase activity were *Lactococcus* spp. These authors did not observe lipase activity in the extracellular fraction. In this study, neither the intracellular nor the extracellular fraction of *L. lactis* O233 hydrolyzed tributyrin.

Not all strains that showed esterase activity exhibited activity on triglycerides (Tables 1 and 3). Differences in the activities on naphthol esters and triglycerides would indicate the presence of separate esterase and lipase enzymes

TABLE 2. *Esterase electrophoretic patterns*[a] *of intracellular fractions of lactic acid bacteria*

Source of enzyme	Bands	R_f	Intensity of esterase activity[b] with substrate			
			α-Acetate	α-Propionate	α-Butyrate	α-Caproate
L. lactis biovar. *diacetylactis* O233	E1	0.6	+++	++	+	−
	E2	0.88	++	+	−	−
L. mesenteroides subsp. *dextranicum* O257	E1	0.40	++++	W	−	++
L. casei O190	E1	0.28	+	−	−	−
L. plantarum O186	E1	0.3	++++	+	W	−
L. plantarum O236	E1	0.40	++++	++	++	−
	E2	0.48	++	W	−	−
	E3	0.70	++	−	−	−
	E4	0.80	++++	++	++	−
L. plantarum O155	E1	0.49	++	−	++	+
L. acidophilus O252	E1	0.35	+++	++	+	−
L. acidophilus O177	E1	0.23	+	−	−	−
	E2	0.50	++++	+	−	−
E. faecium O242	E1	0.4	++	+	−	−
	E2	0.8	++	+	−	−
E. faecium O426	E1	0.20	++	−	−	−
	E2	0.66	++	++	−	−
	E3	0.57	++	−	−	−
E. faecium O174	E1	0.28	++	+	−	−
E. faecalis Ov409	E1	0.28	++	W	−	++
	E2	0.40	++	−	−	++
	E3	0.47	++	−	−	++

[a] Substrate used: α-naphtyl derivatives of acetate, propionate, butyrate, and caproate.
[b] Activity was expressed from W (weak) to ++++: from a very thin to a very deeply colored band. A minus sign denotes the absence of activity.

in LAB *(13)*. Lipases hydrolyze esters in triglycerides to release glycerol and FFAs, and they are able to act on emulsions of triglycerides at the lipid–water interface *(19)*. By contrast, esterases hydrolyze esters in solution and may also hydrolyze tri-, and especially di-, monoglycerides containing short-chain fatty acids. The enzymes examined in this study could be defined as esterases, because they strongly hydrolyzed water-soluble α- and β-NA esters derivative of C2 to C6 fatty acids. Some strains were able to hydrolyze tributyrin but were unable to hydrolyze trilauril and triolein (which are essentially insoluble in an aqueous system). Even some hydrolysis of milk fat was observed (Table 3).

Chávarri et al. *(6)* quantified the major FFAs during the ripening of Idiazabal cheese. Despite variations in the concentrations and proportions of FFAs, such cheeses contained high levels of C4 to C10 FFAs and acetic and propionic acids, which contribute to the cheese flavor. In previous work *(26),* we reported that *Enterococcus* spp. and *L. plantarum* were the major LAB found in ewe's milk and cheese. These strains presented esterase activities that may

TABLE 3. *Esterase-lipase activity on triglycerides and buttermilk*[a]

Source of enzyme	Tributyrin		Buttermilk	
	Intracellular	Extracellular	Intracellular	Extracellular
L. lactis biovar. *diacetylactis* O233	−	−	−	−
L. mesenteroides subsp. *dextranicum* O257	+	++	−	+
L. plantarum O186	+	−	−	−
L. plantarum O236	+	++	+	+
L. plantarum O155	−	−	−	−
L. casei O190	−	−	−	−
L. acidophilus O252	++	−	+	−
L. acidophilus O177	+	+	−	+
E. faecium O242	++	++	+	−
E. faecium O426	++	−	+	−
E. faecium O174	+	−	−	−
E. faecalis Ov409	+	−	+	−

[a] +, lipase activity detected; −, lipase activity not detected.

release C2 to C6 fatty acids during ripening and therefore actively contribute to the flavor development of ewe's milk cheese. Short-chain fatty acids such as butanoic and hexanoic acids are potent flavor compounds at concentrations below 10 mg/ml *(5)*. Esterases and lipases have the capacity for both hydrolyzing and synthesizing esters by the esterification of fatty acids and ethanol *(24)*. These esters, which are potent flavor compounds at less than 5 ppm, are important for the development of characteristic "fruity"-type flavors such as ethylbutyrate and ethylhexanoate *(15, 24)*.

The LAB strains evaluated in this work showed different esterase and lipase activity, which were species- and strain-specific. Wide variations in activity between strains highlight the need for selecting appropriate starters. Esterase activities of lactobacilli and enterococci under conditions simulating cheese ripening are in progress to incorporate the esterase system directly into milk to produce enzyme-modified cheese as well as accelerated ripened cheese.

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