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# Ester synthesis by lactic acid bacteria isolated from goat's and ewe's milk and cheeses

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ABSTRACT

The present work evaluates the ability of lactic acid bacteria isolated from goat's and ewe's milk and cheeses to synthesise short-chain fatty acid esters. In order to elucidate the mechanisms of ester synthesis involved, cell-free extracts were incubated in sodium phosphate buffer containing triglyceride plus ethanol (alcoholysis) and free fatty acid plus ethanol (esterification). After 24 h incubation at 37 °C esters were extracted and determined by gas chromatography. Strains evaluated were able to synthesise ethyl esters from 2 to 10 carbon atoms, mainly ethyl butanoate and ethyl hexanoate. A great variability amongst strains was observed. In general, higher ester-forming activities by esterification were detected. In enterococci strains the alcoholysis mechanism was also involved.

These strains could be useful as adjunct cultures for small ruminants' dairy products and would contribute to the development of fruity flavour notes. A deeper understanding of ester synthesis mechanisms involved would allow the control of flavour development.



## 1. Introduction

Goat's and ewe's milk cheeses produced in the northwest of Argentina are semi-hard varieties made from raw milk, without addition of starter cultures. These cheeses are greatly appreciated because of their particular organoleptic characteristics.

Lipolysis, the process of milk fat hydrolysis, plays a crucial role in the development of flavour in these cheeses (Freitas & Malcata, 1998; Macedo, Costa, & Malcata, 1996; Tavaria, Silva Ferreira, & Malcata, 2004). The major flavour compounds that are released during lipolysis are free fatty acids (FFA), which directly affect cheese flavour. FFA can also be transformed by microorganisms to other and often more potent flavour compounds, including methyl ketones, lactones, esters, secondary alcohols and aldehydes, which also directly affect flavour in different cheeses (Collins, McSweeney, & Wilkinson, 2003).

Esters are part of the aroma array of cheeses made from goats' and ewes' milk (e.g., Feta, Manchego, Serra da Estrela and Roncal), but the number and type of esters found vary between cheese varieties (Dahl, Tavaria, & Malcata, 2000; Horwood, Lloyd, & Stark, 1981; Le Quere, Pierre, Riaublanc, & Demaizieres, 1998; Martínez-Castro, Sanz, Amigo, Ramos, & Martín-Alvarez, 1991; Ortigosa, Torre, & Izco, 2001). Of the esters identified in these cheeses, the five ethyl esters of the straight-chain fatty acids of C2–C10 are most frequently found (Liu, Holland, & Crow, 2004). These esters, which are potent flavour compounds at less than 5 ppm, are important for development of the characteristic "fruity" type flavours such as ethyl butanoate and ethyl hexanoate (Moio & Addeo, 1998). Esters may also mask the impact of off-flavours (e.g., pungent, sharp) imparted by high levels of short-chain FFA. Excessive levels of ethyl esters of short-chain FFA (typically ethyl butanoate and ethyl hexanoate) cause a fruity flavour defect in some raw and pasteurised milks, and Cheddar cheese (Horwood, Stark, & Hull, 1987; Whitfield, Jensen, & Shaw, 2000).

Lactic acid bacteria (LAB) are used as starter or adjuncts cultures or are present as secondary microbial flora (non-starter LAB) in cheese fermentation (Crow, Curry, & Hayes, 2001). Indigenous LAB isolated from ewe's and goat's milk and artisanal cheeses manufactured in the provinces of northwest Argentina were identified as enterococci, lactococci, leuconostoc and lactobacilli. *Enterococcus faecium* and *Lactobacillus plantarum* were the most frequently isolated species from ewe's and goat's milk and cheeses (Medina, Katz, González, & Oliver, 2001; Oliszewski, González, & Pérez Chaia, 2006).

The contribution of cheese microflora to the formation of flavour compounds occurs *via* the esterase/lipase systems of lactic and propionic acid bacteria, non-starter LAB (NSLAB), surface microorganisms, yeasts and moulds (McSweeney & Sousa, 2000). Moreover, some volatile compounds arise from microbial amino acid catabolism (Tavaria & Malcata, 2003). We have previously demonstrated that LAB isolated from goat's and ewe's dairy prod-

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ucts present very complex intracellular esterolytic systems able to release C2–C6 fatty acids and thus they actively contribute to flavour development in these products. (Abeijón, Medina, Katz, & González, 2006; Abeijón Mukdsi et al., 2009; Katz, Medina, González, & Oliver, 2002; Oliszewski, Medina, González, & Pérez Chaia, 2007).

Esterases are defined as the enzymes that hydrolyse carboxyl ester linkages in water-soluble substrates (e.g., slightly water-soluble short-chain glycerides and aliphatic esters) in aqueous solutions. These enzymes can also synthesise esters under certain conditions (Bornscheuer, 2002). The mechanisms of ester synthesis are: esterification (reaction of an acid and an alcohol), alcoholysis (reaction of an ester and an alcohol), acidolysis (reaction of an acid and an alcohol) and transesterification (reaction of two esters) (Holland et al., 2005; Malcata, Reyes, Garcia, Hill, & Amundson, 1992).

Presumably, esterases from starter and NSLAB are responsible for release of short-chain FFA from milk fat at elevated water activity ($a_w$) and synthesis of short-chain ethyl esters as $a_w$ decreases with ripening (Holland et al., 2005). The equilibrium existing between these processes is dependent on the $a_w$, the enzymes present, pH, temperature and availability of substrates characteristic of each cheese variety (Ha & Lindsay, 1992; Moio & Addeo, 1998).

The acid or acyl CoA moieties of esters are formed from the action of the cheese microflora and their enzymes on lactose, lactate, lipids and proteins of cheese curd (Urbach, 1997). Ethanol in cheese is thought to result from the activity of obligatory heterofermentative lactobacilli and/or from yeasts (Chamba & Irlinger, 2004). We have previously detected ethanol production from citrate metabolism in goat's milk fermented by enterococci (Cabral, 2005).

Evidence is provided that esterases of LAB catalyse the synthesis of esters by esterification (Liu, Holland, & Crow, 1998) and alcoholysis (Liu, Holland, & Crow, 2003a; Liu, Baker, et al., 2004). It is not known whether esterases of LAB can catalyse acidolysis and transesterification.

Even though there are many reports concerning volatile compound formation in goat's and ewe's milk cheeses manufactured with starter LAB (Abeijón Mukdsi et al., 2009; Georgala et al., 2005; Randazzo, Pitino, De Luca, Scifò, & Caggia, 2008; Tavaria, Tavares, Silva-Ferreira, & Malcata, 2006), only a few studies on ester synthesis mechanisms by these bacteria have been carried out. Some reports about ester synthesis by lactobacilli, streptococci and lactococci have been made, but no information about enterococci is available.

The aim of the present work was to evaluate the ester synthesis ability of indigenous lactobacilli and enterococci strains from goat's and ewés dairy products, and to go deeper into the elucidation of the prevalent mechanisms of ester synthesis in aqueous medium.

## 2. Materials and methods

### 2.1. Microorganisms and growth conditions

Strains provided by Laboratorio de Ecofisiología Tecnológica of the Centro de Referencia para Lactobacilos (CERELA), were isolated from Argentinean goat's and ewe's milk and cheese (Medina et al., 2001; Oliszewski et al., 2006). *Lactobacillus fermentum* ETC1, *L. delbrueckii* subsp. *bulgaricus* ETC2, *L. rhamnosus* ETC14, *L. plantarum* ETC17, *L. casei* ETC19, *Enterococcus faecium* ETC124 and *E. faecium* ETC418 were isolated from goat's milk and cheeses. *Lactobacillus plantarum* Ov156, Ov161, Ov186, Ov236, *Enterococcus faecium* Ov157, Ov167, Ov178, Ov194, Ov242, Ov254, Ov426, Ov409 and *E. durans* Ov421 were isolated from ewe's milk and cheeses.

These strains were previously selected for their ability to produce flavour compounds from sugars, citrate and proteins, and for their esterase–lipase activities (Abeijón et al., 2006; Cabral, Abeijón Mukdsi, Medina, & González, 2007; Katz et al., 2002; Oliszewski et al., 2007). *Enterococcus* strains were previously tested for vancomycin/teicoplanin resistance and haemolysin and gelatinase production (Katz, 2005; Oliszewski, 2006).

All strains were frozen at −70 °C in MRS broth (Merck, Darmstadt, Germany) containing glycerol 20% (v/v), and grown in MRS broth for 16 h. Cultures were incubated at 37 °C.

### 2.2. Cell-free extract preparation

Cells cultured in 400 ml of MRS broth were harvested after 16 h by centrifugation at 10,000*g* for 10 min at 4 °C, washed twice with 100 mM sodium phosphate buffer, pH 7, and resuspended at 50% (w/v) of the same buffer. The cell suspensions were disrupted by three successive passes through a French pressure cell at 1000 psi (ThermoSpectronic, NY, USA). Cellular debris was removed by centrifugation (20,000*g* for 30 min at 4 °C) and the supernatant was used as cell-free extract (CFE).

### 2.3. Mechanisms of ester synthesis

#### 2.3.1. Synthesis of ethyl esters by alcoholysis

These assays were performed according to the protocol of Liu et al. (2003a) with some modifications.

Ester synthesis by alcoholysis was analysed in an assay mixture containing 100 mM sodium phosphate buffer (pH 7), 100 mM ethanol, 33 mM triglyceride (tributyrin or tricaproin) and CFE (ca. 1–3 mg/ml). The assay mixtures were incubated statically at 37 °C for 24 h. A 1 ml sample was removed and added to 2 ml diethyl ether. Extraction was performed by shaking vigorously for about 2 min, followed by centrifugation (1300*g* for 5 min). The top solvent layer was then transferred to a gas chromatography (GC) vial. Controls lacking substrates were included to check for endogenous ester production. Controls lacking CFE were also included to test for the non-enzymatic formation of esters. Both controls were incubated and processed as described above.

#### 2.3.2. Synthesis of ethyl esters by esterification

Ester synthesis by esterification was analysed in an assay mixture containing 100 mM sodium phosphate buffer (pH 7), 100 mM ethanol, 100 mM free fatty acid (butanoic acid or hexanoic acid) and CFE (ca. 1–3 mg/ml). The assay mixtures were incubated statically at 37 °C for 24 h. A 1 ml sample was removed and processed as described above. Controls mentioned above were also included.

### 2.4. Gas chromatography analysis

The following ethyl esters of short-chain fatty acids were determined with a gas chromatograph (Agilent 6890N, Agilent Technologies, CA, USA) equipped with a flame ionisation detector (FID): ethyl acetate (EtC2), ethyl butanoate (EtC4), ethyl hexanoate (EtC6), ethyl octanoate (EtC8), ethyl decanoate (EtC10). An HP-5 column (length 30 m, i.d. 0.32 mm, thickness 0.25 μm) (Hewlett-Packard, CA, USA) was used. The oven was temperature-programmed at 30 °C for 5 min, followed by increasing the temperature to 250 °C at 10 °C/min and held at 250 °C for 2 min. Sample injections of 1 μl were performed in split less mode. The injector and FID detector temperatures were 270 and 300 °C, respectively. Column flow rate was 1 ml/min using nitrogen as carrier gas. The FID output signal was recorded and processed using appropriate software (Agilent ChemStation Software, Agilent, CA, USA.)

Esters were quantified from the regression curve ($R^2 > 98\%$) of the corresponding standard (Sigma, MO, USA), using external standard calibration and the GC conditions described above. To check the recovery efficiency of each ester from the different assay mixtures, a known amount of standard solution was added to each mixture. Extraction and GC analysis were performed as described above. Correction factors were calculated and used to accordingly adjust ester concentration values.

A unit of ester-forming activity was defined as the amount of enzyme that formed 1 nmol of ester in 24 h. Specific ester-forming activity was defined as units per milligram of protein.

### 2.5. Protein determination

Protein concentrations were determined by the method of Bradford (1976), using a kit from Bio-Rad (CA, USA) and bovine serum albumin (Sigma, MO, USA) as standard.

### 2.6. Statistical analysis

All experiments were repeated twice. The results were submitted to analysis of variance (ANOVA). Tukey's test was performed for multiple mean comparisons ($P < 0.05$). Relations between strains and production level of each ester were studied by means of the Multiple Correspondence Analysis (MCA) method (Abdi & Valentin, 2007). For the MCA method, all quantitative data was transformed in categorical data. Results were expressed as production (ppm) and four categories (levels) were established according to minimal and maximal production level for each ester (Tables 1 and 2). Calculations and graphics were carried out with the Infostat Professional software 2004p1.

## 3. Results and discussion

### 3.1. Synthesis of ethyl esters

#### 3.1.1. Synthesis of ethyl esters by alcoholysis

Ester-synthesising activity by alcoholysis (transferase activity) was determined on CFE in assay mixtures containing triglyceride (tributyrin or tricaproin) plus ethanol. Results are shown in Table 3.

**Table 1**
Categories established according to ester production by alcoholysis.

| Level | Ester production (ppm) | | | | |
|---|---|---|---|---|---|
| | EtC2 | EtC4 | EtC6 | EtC8 | EtC10 |
| 1 | ⩽1.30 | ⩽3.10 | ⩽1.80 | ⩽0.023 | ⩽0.40 |
| 2 | ⩽2.60 | ⩽6.20 | ⩽3.60 | ⩽0.046 | ⩽0.80 |
| 3 | ⩽3.90 | ⩽9.30 | ⩽5.40 | ⩽0.069 | ⩽1.20 |
| 4 | ⩽5.20 | ⩽12.40 | ⩽7.20 | ⩽0.092 | ⩽1.60 |

EtC2, ethyl acetate; EtC4, ethyl butanoate; EtC6, ethyl hexanoate; EtC8, ethyl octanoate; EtC10, ethyl decanoate.

**Table 2**
Categories established according to ester production by esterification.

| Level | Ester production (ppm) | | | |
|---|---|---|---|---|
| | EtC4 | EtC6 | EtC8 | EtC10 |
| 1 | ⩽17 | ⩽36 | ⩽2 | ⩽0.10 |
| 2 | ⩽34 | ⩽72 | ⩽4 | ⩽0.20 |
| 3 | ⩽51 | ⩽108 | ⩽6 | ⩽0.30 |
| 4 | ⩽68 | ⩽144 | ⩽8 | ⩽0.40 |

EtC2, ethyl acetate; EtC4, ethyl butanoate; EtC6, ethyl hexanoate; EtC8, ethyl octanoate; EtC10, ethyl decanoate.

Strains evaluated in this work showed a great variability in their ability to synthesise ethyl esters by alcoholysis. Ethyl esters of C4 and C6 were detected when CFE were incubated in presence of tributyrin and ethanol. Amongst lactobacilli strains the highest EtC4-synthesising activity was observed in *L. casei* ETC19 and *L. plantarum* ETC17 (49.18 ± 6.28 and 41.13 ± 4.33 U/mg, respectively). Enterococci strains showed higher EtC4-forming activity than lactobacilli, displaying *E. faecium* Ov194 and *E. faecium* ETC124 the highest activities (105.56 ± 11.23 and 104.88 ± 15.04 U/mg, respectively). Amongst all of the strains evaluated, only *L. plantarum* Ov161 showed significant EtC6-synthesising activity (48.92 ± 5.66 U/mg). *E. faecium* Ov178 was the only strain displaying EtC8-forming activity (0.40 ± 0.15 U/mg). None of the strains was able to synthesise ethyl esters of C2 and C10.

In the assay mixture containing tricaproin and ethanol, EtC2-forming activity was detected in *L. plantarum* ETC17, *L. plantarum* Ov186, *E. faecium* Ov167 and *E. faecium* Ov157. Other ethyl ester-forming activities were significantly lower in evaluated strains. Only two strains were able to produce EtC4 and four strains synthesised EtC8 (Table 3). *E. faecium* Ov254 was the only strain displaying EtC6-forming activity (0.63 ± 0.22 U/mg). *L. plantarum* Ov186 showed the highest EtC10-forming activity (7.41 ± 1.03 U/mg).

Liu et al. (2003a) reported that dairy LAB can synthesise substantial amounts of EtC4 from tributyrin and ethanol in aqueous systems *via* a transferase reaction (alcoholysis) in which butyryl groups from tributyrin are transferred directly to ethanol. They also found LAB to vary in their ability to produce esters *via* alcoholysis with *S. thermophilus* and *L. fermentum* displaying the highest transferase activities amongst a range of LAB studied. It was demonstrated that the enzymes that catalyse ester biosynthesis *via* the transferase reaction are indeed esterases that display acyltransferase activities (Liu, Baker, et al., 2004).

The highest ester-forming activities observed in enterococci strains would indicate that alcoholysis would be the main ester synthesis mechanism in this genus. This fact could be explained by the high esterolytic activities of these strains, which would allow the release of fatty acids from triglycerides and their concomitant esterification with ethanol (Abeijón et al., 2006; Katz et al., 2002; Oliszewski et al., 2007).

Oliszewski et al. (2007) have reported that enterococci strains isolated from goat's milk and cheeses showed the highest esterolytic activities on α-naphthyl (α-NA) derivatives of fatty acids of 4 and 6 carbon atoms. These results are in agreement with Katz et al. (2002), who observed that enterococci strains isolated from ewes' milk and cheese had higher esterolytic activity on α-NA butyrate and caproate than lactobacilli strains. Tsakalidou et al. (1994) concluded that enterococci strains show significantly higher esterolytic activity than strains of most other genera of LAB.

Other authors have reported significant EtC4-synthesising activity in *Streptococcus thermophilus* than other LAB. This correlates with both the high esterolytic activity detected in *S. thermophilus* (Crow, Holland, Pritchard, & Coolbear, 1994) and the perceived sweet/fruity flavour notes associated with this thermophilic starter (Law, 1998). Nevertheless, some authors have observed that there is no correlation between ester-hydrolysing and synthesising activities (Gandolfi, Gaspari, Franzetti, & Molinari, 2000).

A number of studies have demonstrated that water acts as a competitive inhibitor of lipase-catalysed esterification (Liu, Holland, et al., 2004), so ester synthesis by alcoholysis, in which fatty acid groups from acylglycerols are transferred to alcohols without direct involvement of water, would be the main mechanism involved in aqueous systems. The strains with high ester-forming activity by alcoholysis may be involved in the production

**Table 3**
Specific ester-synthesising activity[a] by alcoholysis detected in cell-free extracts of lactic acid bacteria isolated from goat's and ewe's milk and cheeses.

| Strains | Tributyrin + ethanol | | | | | Tricaproin + ethanol | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | EtC2 | EtC4 | EtC6 | EtC8 | EtC10 | EtC2 | EtC4 | EtC6 | EtC8 | EtC10 |
| *L. fermentum* ETC1 | n.d. | 25.82 ± 4.32$^{abc}$ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| *L. delbrueckii* subsp. *bulgaricus* ETC2 | n.d. | 11.21 ± 3.09$^{a}$ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| *L. rhamnosus* ETC14 | n.d. | 25.04 ± 5.19$^{abc}$ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 0.42 ± 0.10$^{b}$ | 0.16 ± 0.08$^{a}$ |
| *L. plantarum* ETC17 | n.d. | 41.13 ± 4.33$^{bc}$ | n.d. | n.d. | n.d. | 57.79 ± 9.25$^{b}$ | n.d. | n.d. | n.d. | n.d. |
| *L. casei* ETC19 | n.d. | 49.18 ± 6.28$^{c}$ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| *L. plantarum* Ov156 | n.d. | 16.65 ± 5.45$^{ab}$ | 1.94 ± 0.23$^{a}$ | n.d. | n.d. | n.d. | n.d. | n.d. | 0.12 ± 0.05$^{a}$ | 0.10 ± 0.02$^{a}$ |
| *L. plantarum* Ov161 | n.d. | 0.85 ± 0.12$^{a}$ | 48.92 ± 5.66$^{b}$ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| *L. plantarum* Ov186 | n.d. | 4.82 ± 0.98$^{a}$ | n.d. | n.d. | n.d. | 28.88 ± 6.21$^{ab}$ | n.d. | n.d. | n.d. | 7.41 ± 1.03$^{b}$ |
| *L. plantarum* Ov236 | n.d. | 25.49 ± 3.26$^{abc}$ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| *E. faecium* Ov157 | n.d. | 40.43 ± 3.94$^{bc}$ | n.d. | n.d. | n.d. | 2.90 ± 1.01$^{a}$ | n.d. | n.d. | n.d. | n.d. |
| *E. faecium* Ov167 | n.d. | 42.04 ± 5.05$^{bc}$ | n.d. | n.d. | n.d. | 35.69 ± 6.26$^{ab}$ | n.d. | n.d. | n.d. | 0.10 ± 0.04$^{a}$ |
| *E. faecium* Ov178 | n.d. | 24.76 ± 2.92$^{abc}$ | n.d. | 0.40 ± 0.15 | n.d. | n.d. | n.d. | n.d. | 0.53 ± 0.05$^{b}$ | n.d. |
| *E. faecium* Ov194 | n.d. | 105.56 ± 11.23$^{d}$ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| *E. durans* Ov421 | n.d. | 14.08 ± 2.36$^{ab}$ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 0.13 ± 0.02$^{a}$ |
| *E. faecium* Ov242 | n.d. | 29.68 ± 3.65$^{abc}$ | n.d. | n.d. | n.d. | n.d. | 13.73 ± 1.07$^{b}$ | n.d. | 0.42 ± 0.08$^{b}$ | n.d. |
| *E. faecium* Ov254 | n.d. | 53.39 ± 6.29$^{c}$ | n.d. | n.d. | n.d. | n.d. | n.d. | 0.63 ± 0.22 | n.d. | n.d. |
| *E. faecium* Ov426 | n.d. | 95.87 ± 7.11$^{d}$ | 0.09 ± 0.05$^{a}$ | n.d. | n.d. | n.d. | 2.59 ± 0.88$^{a}$ | n.d. | n.d. | n.d. |
| *E. faecium* Ov409 | n.d. | 98.02 ± 6.38$^{d}$ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| *E. faecium* ETC124 | n.d. | 104.88 ± 15.04$^{d}$ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| *E. faecium* ETC418 | n.d. | 24.62 ± 3.99$^{abc}$ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |

EtC2, ethyl acetate; EtC4, ethyl butanoate; EtC6, ethyl hexanoate; EtC8, ethyl octanoate; EtC10, ethyl decanoate.
n.d., Not detected.
[a] Results are expressed as U/mg of protein. Mean ± SD. Values in the same column with different superscript letters differ significantly ($P < 0.05$).

of these flavour-active compounds mainly in fermented milks, soft cheeses, and at the first stages of cheese ripening.

The multiple correspondence analysis (MCA) provides valuable information allowing the visualisation of the relations between strains and different production levels of each of the five ethyl esters analysed. The MCA of the results of ester synthesis by alcoholysis is shown in Fig. 1.

In the origin of the system (axes intersection delimiting the four quadrants) are situated all the strains having low levels of ester production (level 1). *L. plantarum* Ov186 differs significantly from the rest of the strains, being associated to the highest level of EtC10 (level 4) and intermediate level of EtC2 production (level 2) from tricaproin and ethanol. (Quadrant I).

Strains situated in the upper side of quadrant II are good producers of EtC4 from tributyrin and ethanol. *E. faecium* Ov409, Ov426, Ov194 and ETC124 showed the highest EtC4 production (level 4) when CFE were incubated in the presence of these substrates. Next to them are located some strains with level 2 production of this ester.

Strains situated in the quadrant III are good producers of ethyl esters from C2, 6 and 8. *L. plantarum* ETC17 and *E. faecium* Ov167 are associated with high production levels of EtC2 from tricaproin and ethanol (level 4 and 3, respectively). These strains are also associated with level 2 EtC4 production from tributyrin (Quadrant II). *L. plantarum* Ov161 is associated with level 4 EtC6 production. Strains associated with high EtC8 production in the medium containing tricaproin and ethanol are *E. faecium* Ov178, *E. faecium* Ov242 and *L. rhamnosus* ETC14 (level 4). *E. faecium* Ov178 is also related to level 4 EtC8 production from tributyrin and ethanol.

### 3.1.2. Synthesis of ethyl esters by esterification

Ester-synthesising activity by esterification was determined on CFE in assay mixtures containing free fatty acid (butanoic or hexanoic acid) plus ethanol. Results are shown in Table 4.

Large strain differences in the potential for ethyl ester formation amongst the LAB surveyed were observed. Liu et al. (1998) reported the formation of EtC4 from ethanol and butanoic acid by non-growing cells of several starter and non-starter LAB, which was both specific and strain dependent.

When CFE were incubated in an assay mixture containing butanoic acid and ethanol only EtC6-synthesising activity was observed. *L. casei* ETC19 was the only strain displaying EtC4-forming activity (88.54 ± 9.15 U/mg). In general lactobacilli strains showed higher ester-synthesising activities than enterococci when CFE were incubated with these substrates. Amongst lactobacilli strains *L. rhamnosus* ETC14 showed the highest EtC6-synthesising activity (679.20 ± 39.78 U/mg). *L. casei* ETC19 and *L. plantarum* Ov156 showed similar activities (210.12 ± 19.37 and 239.83 ± 31.20 U/mg, respectively). Only three strains of enterococci showed ester-synthesising ability: *E. faecium* ETC124, Ov178 and Ov194 (22.85 ± 2.88, 5.15 ± 1.44, 4.58 ± 0.89 U/mg of EtC6, respectively). None of the evaluated strains showed an ability to synthesise ethyl esters of C2, C8 and C10.

The fact that EtC6 was synthesised, whereas no EtC4 was formed in the presence of its immediate precursors (butanoic acid and ethanol) would indicate that other ester synthesis mechanism, besides esterification could be involved. Similar results were observed by Liu, Holland, and Crow (2003b), who reported that a commercial lipase produced EtC6 in addition to EtC4, when incubated in a cheese-based medium containing butanoic acid and ethanol. We reason that ester synthesis by acidolysis could be a tentative explanation. EtC6 can be formed by the reaction between the esters, formed by esterification between butanoic acid and ethanol, and the butanoic acid that remains in the medium. In addition, some authors suggested that cheese esters can be formed non-enzymatically (Liu, Holland, et al., 2004).

In the assay mixture containing hexanoic acid and ethanol, synthesising-activities of ethyl esters from C4 to C10 were observed, mainly EtC4 and EtC6. The highest EtC4-synthesising activities were observed in *E. faecium* ETC124 (569.46 ± 47.75 U/mg), *L. rhamnosus* ETC14 (470.43 ± 37.93 U/mg) and *L. plantarum* ETC17 (406.18 ± 42.33 U/mg).

*Lactobacillus fermentum* ETC1 displayed the highest EtC6-forming activity (991.38 ± 101.92 U/mg), whereas similar activities were observed in *L. rhamnosus* ETC14, *E. durans* Ov421 and *E. faecium* Ov242 (Table 4). Some strains showed EtC8 and EtC10-forming activities, being significantly lower than EtC4 and EtC6-synthesising activities (Table 4).

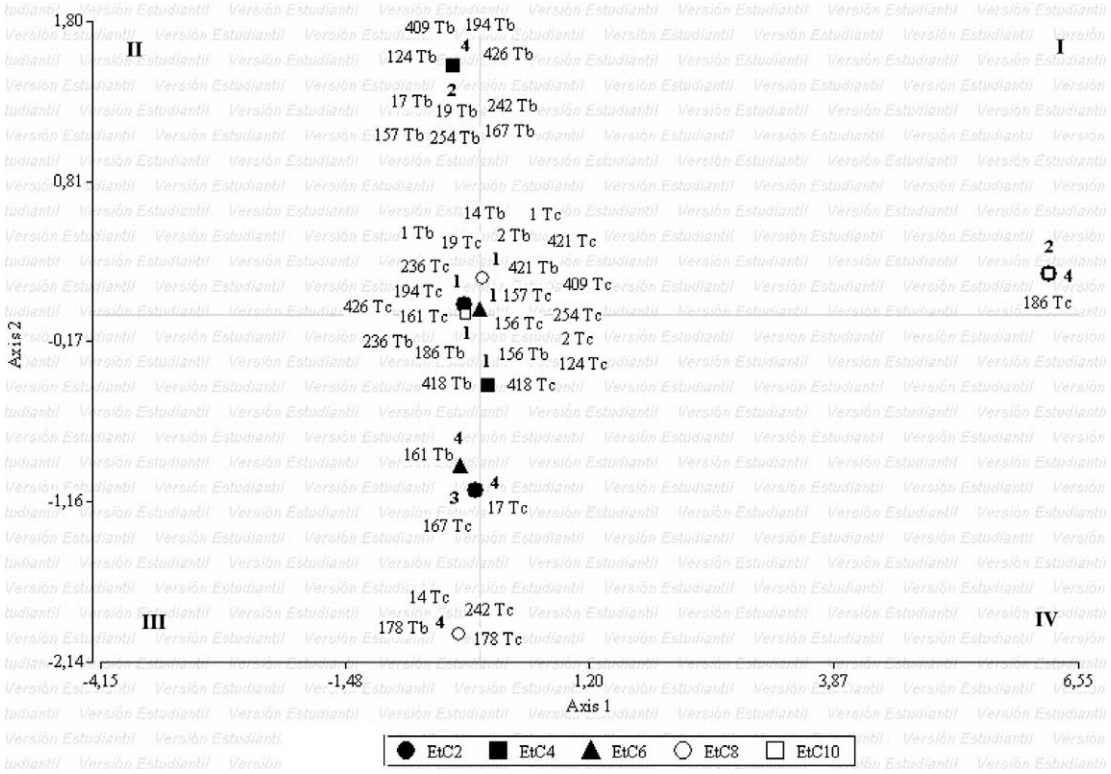


**Fig. 1.** Biplot obtained by Multiple Correspondence Analysis of ethyl esters of short-chain fatty acids produced by the mechanism of alcoholysis, expressed in ppm. Points are coded by strain (1, *L. fermentum* ETC1; 2, *L. delbrueckii* subsp. *bulgaricus* ETC2; 14, *L. rhamnosus* ETC14; 17, *L. plantarum* ETC17; 19, *L. casei* ETC19; 124, *E. faecium* ETC124; 418, *E. faecium* ETC418; 156, *L. plantarum* Ov156; 161, *L. plantarum* Ov161; 186, *L. plantarum* Ov186; 236, *L. plantarum* Ov236; 157, *E. faecium* Ov157; 167, Ov167; 178, Ov178; 194, Ov194; 242, Ov242; 254, Ov254; 426, Ov426; 409, Ov0409; 421, *E. durans* Ov421) and substrate (Tb, tributyrin; Tc, tricaproin). EtC2, ethyl acetate; EtC4, ethyl butanoate; EtC6, ethyl hexanoate; EtC8, ethyl octanoate; EtC10, ethyl decanoate. The position of some points was slightly modified to avoid overlapping of the labels.

**Table 4**
Specific ester-synthesising activity[a] by esterification detected in cell-free extracts of lactic acid bacteria isolated from goat's and ewe's milk and cheeses.

| Strains | Butanoic acid + ethanol | | | | | Hexanoic acid + ethanol | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | EtC2 | EtC4 | EtC6 | EtC8 | EtC10 | EtC2 | EtC4 | EtC6 | EtC8 | EtC10 |
| *L. fermentum* ETC1 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | $267.17 \pm 39.25^{efg}$ | $991.38 \pm 101.92^{f}$ | $2.01 \pm 0.08^{a}$ | $1.78 \pm 0.55^{b}$ |
| *L. delbrueckii* subsp. *bulgaricus* ETC2 | n.d. | n.d. | $3.36 \pm 1.02^{a}$ | n.d. | n.d. | n.d. | $52.81 \pm 7.03^{abc}$ | $198.59 \pm 22.30^{abc}$ | $1.12 \pm 0.05^{a}$ | $0.41 \pm 0.10^{ab}$ |
| *L. rhamnosus* ETC14 | n.d. | n.d. | $679.20 \pm 39.78^{c}$ | n.d. | n.d. | n.d. | $470.43 \pm 37.93^{hi}$ | $605.63 \pm 74.32^{e}$ | $10.32 \pm 2.05^{a}$ | n.d. |
| *L. plantarum* ETC17 | n.d. | n.d. | $45.01 \pm 5.75^{a}$ | n.d. | n.d. | n.d. | $406.18 \pm 42.33^{gh}$ | n.d. | $44.78 \pm 6.21^{c}$ | n.d. |
| *L. casei* ETC19 | n.d. | 88.54 ± 9.15 | $210.12 \pm 19.37^{b}$ | n.d. | n.d. | n.d. | n.d. | $316.71 \pm 25.09^{bcd}$ | $0.30 \pm 0.10^{a}$ | $0.02 \pm 0.01^{a}$ |
| *L. plantarum* Ov156 | n.d. | n.d. | $239.83 \pm 31.20^{b}$ | n.d. | n.d. | n.d. | $202.98 \pm 31.09^{def}$ | $553.86 \pm 39.44^{de}$ | $4.42 \pm 0.88^{a}$ | $0.98 \pm 0.17^{ab}$ |
| *L. plantarum* Ov161 | n.d. | n.d. | $2.03 \pm 0.90^{a}$ | n.d. | n.d. | n.d. | $29.83 \pm 1.92^{a}$ | $112.54 \pm 15.60^{ab}$ | $0.31 \pm 0.12^{a}$ | n.d. |
| *L. plantarum* Ov186 | n.d. | n.d. | $2.89 \pm 1.23^{a}$ | n.d. | n.d. | n.d. | $18.73 \pm 2.50^{a}$ | $1.64 \pm 0.40^{a}$ | n.d. | n.d. |
| *L. plantarum* Ov236 | n.d. | n.d. | $11.43 \pm 0.78^{a}$ | n.d. | n.d. | n.d. | n.d. | $443.86 \pm 33.07^{cde}$ | $7.03 \pm 1.22^{a}$ | $0.77 \pm 0.20^{ab}$ |
| *E. faecium* Ov157 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | $32.85 \pm 4.29^{ab}$ | n.d. | n.d. | n.d. |
| *E. faecium* Ov167 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | $9.25 \pm 1.52^{a}$ | n.d. | n.d. | n.d. |
| *E. faecium* Ov178 | n.d. | n.d. | $5.15 \pm 1.44^{a}$ | n.d. | n.d. | n.d. | $50.79 \pm 8.58^{abc}$ | $104.35 \pm 12.84^{ab}$ | $0.11 \pm 0.05^{a}$ | n.d. |
| *E. faecium* Ov194 | n.d. | n.d. | $4.58 \pm 0.89^{a}$ | n.d. | n.d. | n.d. | $138.76 \pm 21.15^{abcde}$ | $433.68 \pm 60.21^{cde}$ | $3.00 \pm 0,45^{a}$ | n.d. |
| *E. durans* Ov421 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | $106.12 \pm 9.75^{abcd}$ | $622.42 \pm 57.94^{e}$ | $4.76 \pm 1.10^{a}$ | $0.02 \pm 0.01^{a}$ |
| *E. faecium* Ov242 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | $689.89 \pm 44.26^{e}$ | $3.52 \pm 0.22^{a}$ | n.d. |
| *E. faecium* Ov254 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | $215.52 \pm 28.34^{def}$ | n.d. | $2.65 \pm 0.45^{a}$ | n.d. |
| *E. faecium* Ov426 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | $346.60 \pm 29.12^{fgh}$ | n.d. | $33.43 \pm 2.89^{b}$ | n.d. |
| *E. faecium* Ov409 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | $181.24 \pm 23.65^{cde}$ | n.d. | $3.63 \pm 0.29^{a}$ | n.d. |
| *E. faecium* ETC124 | n.d. | n.d. | $22.85 \pm 2.88^{a}$ | n.d. | n.d. | n.d. | $569.46 \pm 47.75^{i}$ | $280.21 \pm 30.05^{bc}$ | n.d. | n.d. |
| *E. faecium* ETC418 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | $178.03 \pm 19.45^{bcde}$ | n.d. | $7.39 \pm 1.03^{a}$ | $1.54 \pm 0.29^{b}$ |

EtC2, ethyl acetate; EtC4, ethyl butanoate; EtC6, ethyl hexanoate; EtC8, ethyl octanoate; EtC10, ethyl decanoate.
n.d., Not detected.
[a] Results are expressed as U/mg of protein. Mean ± SD. Values in the same column with different superscript letters differ significantly ($P < 0.05$).

The MCA of the results of ester synthesis by esterification is shown in Fig. 2. Strains with low ester production levels are concentrated in the origin of the system. All of the strains are related to the lowest level of ester production in the assay mixture containing butanoic acid and ethanol, except for *L. rhamnosus* ETC14 which is related to level 3 production of EtC6. This strain is also related to level 3 production of this ester from hexanoic acid and ethanol (Quadrant IV). Very close situated in the same quadrant are

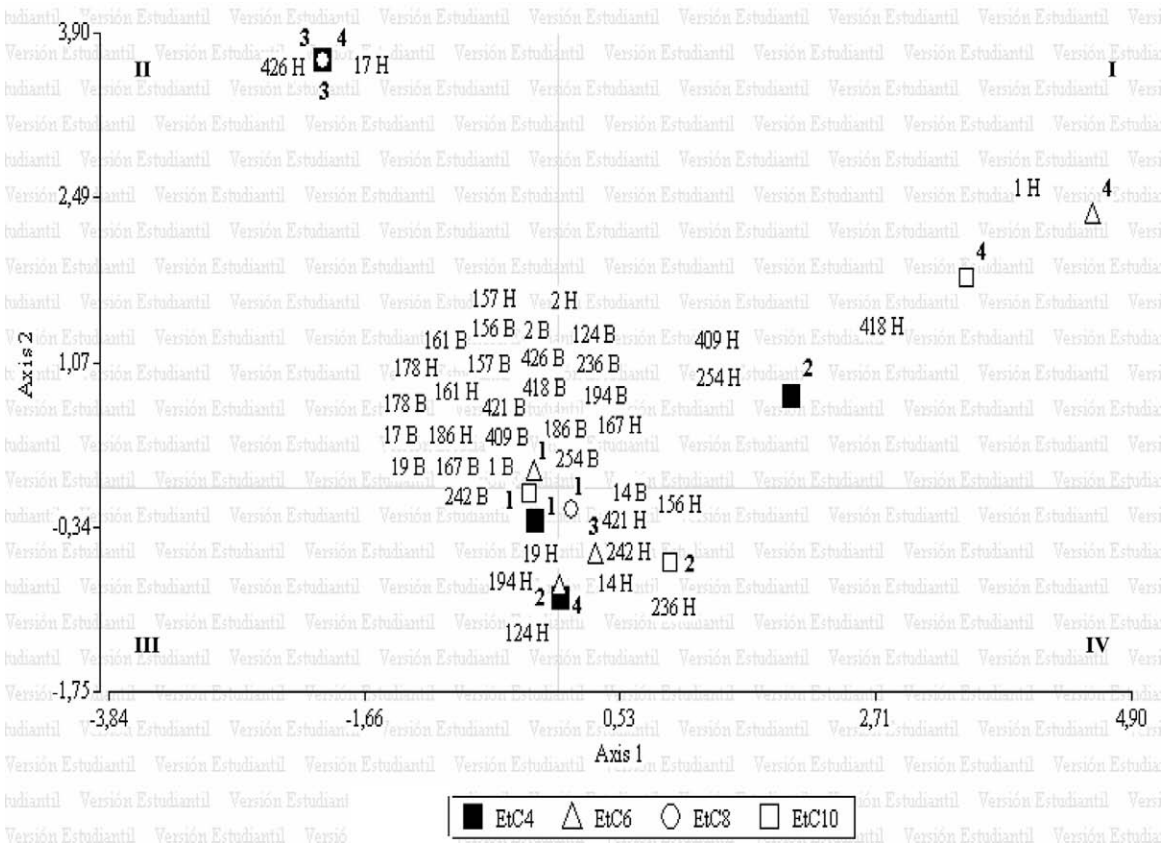


**Fig. 2.** Biplot obtained by Multiple Correspondence Analysis of short-chain fatty acids produced by the mechanism of esterification, expressed in ppm. Points are coded by strain (1, *L. fermentum* ETC1; 2, *L. delbrueckii* subsp. *bulgaricus* ETC2; 14, *L. rhamnosus* ETC14; 17, *L. plantarum* ETC17; 19, *L. casei* ETC19; 124, *E. faecium* ETC124; 418, *E. faecium* ETC418; 156, *L. plantarum* Ov156; 161, *L. plantarum* Ov161; 186, *L. plantarum* Ov186; 236, *L. plantarum* Ov236; 157, *E. faecium* Ov157; 167, Ov167; 178, Ov178; 194, Ov194; 242, Ov242; 254, Ov254; 426, Ov426; 409, Ov0409; 421; *E. durans* Ov421) and substrate (B, butanoic acid; H, hexanoic acid). EtC2, ethyl acetate; EtC4, ethyl butanoate; EtC6, ethyl hexanoate ; EtC8, ethyl octanoate; EtC10, ethyl decanoate. The position of some points was slightly modified to avoid overlapping of the labels.

*E. durans* Ov421, *E. faecium* Ov242 and *L. plantarum* Ov156, since they produce similar amounts of EtC6.

The strain *L. fermentum* ETC1 differs significantly from the rest, being associated to the highest levels of EtC6 and EtC10 production in the presence of hexanoic acid and ethanol (Quadrant I). *E. faecium* ETC418 is positioned not far but distinguishable from this first strain, in the same quadrant, since their EtC10 production is similar (both level 4).

*Lactobacillus plantarum* ETC17 and *E. faecium* Ov426 are very close situated in quadrant II, since they produce very similar amounts of EtC4 (level 3). They are also related to high levels of EtC8 production (level 4 and 3, respectively). *L. rhamnosus* ETC14 and *E. faecium* ETC124 are very closely located in the limit between quadrant III and IV, presenting the highest production of EtC4. This latter strain is also situated near to *E. faecium* Ov194 and *L. casei* ETC19, since they are all related to level 2 production of EtC6.

*Enterococcus faecium* ETC124 is associated to the highest EtC4 production by both mechanisms evaluated: alcoholysis (Fig. 1) and esterification (Fig. 2). These results could be explained by the presence of more than one esterase. Multiple esterases were found in several LAB (Abeijón et al., 2006; Katz et al., 2002; Oliszewski et al., 2007; Sarantinopoulos et al., 2001).

When comparing the ester-synthesising activity of the assayed strains by the two mechanisms evaluated (Tables 3 and 4), in general, higher ester-forming activities by esterification were observed. Enterococci strains were able to synthesise esters by alcoholysis in the presence of tributyrin, which would be related to their higher esterolytic activities. Other authors reported that in an aqueous environment, the yield of ester biosynthesis *via* alcoholysis was much higher than that of esterification (Liu, Baker, et al., 2004). In an environment where the water activity is low, such as ripened cheese, these strains may contribute to ester formation by both mechanisms (esterification and alcoholysis).

## 4. Conclusions

The results show that strains evaluated in this work were able to synthesise esters of short-chain fatty acids, mainly ethyl butanoate and ethyl hexanoate, which are desirable key odours in goat's and ewe's milk cheeses.

The highest ester-synthesising activities were observed when CFE were incubated in the presence of free fatty acids and ethanol, indicating that esterification is the prevalent mechanism in these strains. In enterococci strains the alcoholysis mechanism is also involved. These strains could be useful as adjunct cultures for small ruminants' dairy products and would contribute to the development of fruity flavour notes. Great variability in ester-forming activities between strains highlights the need for selecting appropriate cultures.

These results contribute to the knowledge of ester synthesis mechanisms by lactic acid bacteria and provide information about this topic in *Enterococcus* genus, which has not been reported at present. A deeper understanding of these mechanisms would allow to control ester production in fermented milk and cheese, assisting the manufacturers in their endeavour to obtain products with appropriate organoleptic characteristics.

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## References

Abdi, H., & Valentin, D. (2007). Multiple correspondence analysis. In N. J. Salkind (Ed.), *Encyclopedia of measurement and statistics* (pp. 651–657). Thousand Oaks, CA: Sage.

Abeijón, M. C., Medina, R. B., Katz, M. B., & González, S. N. (2006). Technological properties of *Enterococcus faecium* isolated from ewe's milk and cheese with importance for flavour development. *Canadian Journal of Microbiology, 52*, 237–245.

Abeijón Mukdsi, M. C., Medina, R. B., Katz, M. B., Pivotto, R., Gatti, P, & González, S. N. (2009). Contribution of lactic acid bacteria esterases to the release of fatty acids in miniature ewe's milk cheese models. *Journal of Agricultural and Food Chemistry, 57*, 1036–1044.

Bornscheuer, U. T. (2002). Microbial carboxyl esterases: Classification, properties and application in biocatalysis. *FEMS Microbiology Review, 26*, 73–81.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–255.

Cabral, M. E. (2005). *Flavour compound formation from citrate by enterococci in small ruminant's milk*. Bachelor Thesis, Nacional University of Tucumán: Argentine.

Cabral, M. E., Abeijón Mukdsi, M. C., Medina, R. B., & González, S. N. (2007). Citrate metabolism by *Enterococcus faecium* and *Enterococcus durans* isolated from goat's and ewe's milk: Influence of glucose and lactose. *Canadian Journal of Microbiology, 53*, 607–615.

Chamba, J. F., & Irlinger, F. (2004). Secondary and adjunct cultures. In P. F. Fox, P. L. H. McSweeney, T. M. Cogan, & T. P. Guinee (Eds.), *Cheese. chemistry, physics and microbiology* (3rd ed., pp. 191–206). London: Elsevier.

Collins, Y. F., McSweeney, P. L. H., & Wilkinson, M. G. (2003). Lipolysis and free fatty acid catabolism in cheese: A review of current knowledge. *International Dairy Journal, 13*, 841–866.

Crow, V., Curry, B., & Hayes, M. (2001). The ecology of non-starter lactic acid bacteria (NSLAB) and their use as adjuncts in New Zealand Cheddar. *International Dairy Journal, 11*, 275–283.

Crow, V. L., Holland, R., Pritchard, G. G., & Coolbear, T. (1994). The diversity of potential cheese ripening characteristics of lactic acid starter bacteria: 2. The levels and subcellular distribution of peptidase and esterase activities. *International Dairy Journal, 4*, 723–742.

Dahl, S., Tavaria, F. K., & Malcata, F. X. (2000). Relationships between flavour and microbiological profiles in Serra da Estrela cheese throughout ripening. *International Dairy Journal, 10*, 255–262.

Freitas, A. C., & Malcata, F. X. (1998). Lipolysis in Picante cheese: Influence of milk type and ripening time on free fatty acid profile. *Le Lait, 78*, 251–258.

Gandolfi, R., Gaspari, F., Franzetti, L., & Molinari, F. (2000). Hydrolytic and synthetic activities of esterases and lipases of non-starter bacteria isolated from cheese surface. *Annals of Microbiology, 50*, 183–189.

Georgala, A., Moschopoulou, E., Aktypis, A., Massouras, T., Zoidou, E., Kandarakis, I., et al. (2005). Evolution of lipolysis during the ripening of traditional Feta cheese. *Food Chemistry, 93*, 73–80.

Ha, J. K., & Lindsay, R. C. (1992). Influence of $a_w$ on volatile free fatty acids during storage of cheese bases lipolyzed by kid goat pregastric lipase. *International Dairy Journal, 2*, 179–195.

Holland, R., Liu, S.-Q., Crow, V. L., Delabre, M.-L., Lubbers, M., Bennett, M, et al. (2005). Esterases of lactic acid bacteria and cheese flavour: Milk fat hydrolysis, alcoholysis and esterification. *International Dairy Journal, 15*, 711–718.

Horwood, J. F., Lloyd, G. T., & Stark, W. (1981). Some flavour components of Feta cheese. *Australian Journal of Dairy Technology, 36*, 34–37.

Horwood, J. F., Stark, W., & Hull, R. R. (1987). A "fermented, yeasty" flavour defect in Cheddar cheese. *Australian Journal of Dairy Technology, 42*, 25–26.

Katz, M. B. (2005). *Microbial flora of ewe's milk and cheese: Contribution to development of flavour*. Ph.D. Thesis, National University of Tucumán: Argentina.

Katz, M., Medina, R., González, S., & Oliver, G. (2002). Esterolytic and lipolytic activities of lactic acid bacteria isolated from ewe's milk and cheese. *Journal of Food Protection, 65*, 1997–2001.

Law, B. A. (1998). Research in support of cheese technology – An European perspective. *Australian Journal of Dairy Technology, 52*, 36–40.

Le Quere, J.-L., Pierre, A., Riaublanc, A., & Demaizieres, D. (1998). Characterization of aroma compounds in the volatile fraction of soft goat cheese during ripening. *Le Lait, 78*, 279–290.

Liu, S.-Q., Baker, K., Bennett, M., Holland, R., Norris, G., & Crow, V. L. (2004). Characterisation of esterases of *Streptococcus thermophilus* ST1 and *Lactococcus lactis* subsp. *cremoris* B1079 as alcohol acyltransferases. *International Dairy Journal, 14*, 865–870.

Liu, S.-Q., Holland, R., & Crow, V. L. (1998). Ethyl butanoate formation by dairy lactic acid bacteria. *International Dairy Journal, 8*, 651–657.

Liu, S.-Q., Holland, R., & Crow, V. L. (2003a). Ester synthesis in aqueous environment by *Streptococcus thermophilus* ST1 and other dairy lactic acid bacteria. *Applied Microbiology and Biotechnology, 63*, 81–88.

Liu, S.-Q., Holland, R., & Crow, V. L. (2003b). Synthesis of ethyl butanoate by a commercial lipase in aqueous media under conditions relevant to cheese ripening. *Journal of Dairy Research, 70*, 359–363.

Liu, S.-Q., Holland, R., & Crow, V. L. (2004). Esters and their biosynthesis in fermented dairy products: A review. *International Dairy Journal, 14*, 923–945.

Macedo, A. C., Costa, M. L., & Malcata, F. X. (1996). Changes in the microflora of Serra cheese: Evolution throughout ripening time, lactation period and axial location. *International Dairy Journal, 6*, 79–94.

Malcata, F. X., Reyes, H. R., Garcia, H. S., Hill, C. G., Jr., & Amundson, C. H. (1992). Kinetics and mechanisms of reactions catalysed by immobilized lipases. *Enzyme and Microbial Technology, 14*, 426–446.

Martínez-Castro, I., Sanz, J., Amigo, L., Ramos, M., & Martín-Alvarez, P. (1991). Volatile components of Manchego cheese. *Journal of Dairy Research, 58*, 239–246.

McSweeney, P. L. H., & Sousa, M. J. (2000). Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review. *Le Lait, 80*, 293–324.

Medina, R., Katz, M., González, S., & Oliver, G. (2001). Characterization of the lactic acid bacteria in ewe milk and artisanal cheese from argentine northwest. *Journal of Food Protection, 64*, 559–563.

Moio, L., & Addeo, F. (1998). Grana Padano cheese aroma. *Journal of Dairy Research, 65*, 317–333.

Oliszewski, R., González, S., & Pérez Chaia, A. (2006). Identification and technological characterization of lactic acid bacteria isolated from goat's milk and artisanal cheese of the Argentine northwest. *Revista Argentina de Lactología, 24*, 47–58.

Oliszewski, R. (2006). *Goat's milk: Its quality and evaluation of regional caprine lactic starters in the manufacture of fermented products*. Ph.D. Thesis, National University of Tucumán: Argentina.

Oliszewski, R., Medina, R. B., González, S. N., & Pérez Chaia, A. B. (2007). Esterase activities of indigenous lactic acid bacteria from Argentinean goats' milk and cheeses. *Food Chemistry, 101*, 1446–1450.

Ortigosa, M., Torre, P., & Izco, J. M. (2001). Effect of pasteurization of ewe's milk and use of a native starter culture on the volatile components and sensory characteristics of Roncal cheese. *Journal of Dairy Science, 84*, 1320–1330.

Randazzo, C. L., Pitino, I., De Luca, S., Scifò, G. O., & Caggia, C. (2008). Effect of wild strains used as starter cultures and adjunct cultures on the volatile compounds of the Pecorino Siciliano cheese. *International Journal of Food Microbiology, 122*, 269–278.

Sarantinopoulos, P., Andrighetto, C., Georgalaki, M. D., Rea, M. C., Lombardi, A., Cogan, T. M., et al. (2001). Biochemical properties of enterococci relevant to their technological performance. *International Dairy Journal, 11*, 621–647.

Tavaria, F. K., & Malcata, F. X. (2003). Enzymatic activities of non-starter lactic acid bacteria isolated from a traditional Portuguese cheese. *Enzyme and Microbial Technology, 33*, 236–243.

Tavaria, F. K., Silva Ferreira, A. C., & Malcata, F. X. (2004). Volatile free fatty acids as ripening indicators for Serra da Estrela cheese. *Journal of Dairy Science, 87*, 4064–4072.

Tavaria, F. K., Tavares, T. G., Silva-Ferreira, A. C., & Malcata, F. X. (2006). Contribution of coagulant and native microflora to the volatile-free fatty acid profile of an artisanal cheese. *International Dairy Journal, 16*, 886–894.

Tsakalidou, E., Manolopoulou, E., Kabaraki, E., Zoidou, E., Pot, B., Kersters, K., et al. (1994). The combined use of whole cell protein extracts for the identification (SDS-PAGE) and enzyme activity screening of lactic acid bacteria isolated from traditional Greek dairy products. *Systematic and Applied Microbiology, 17*, 444–458.

Urbach, G. (1997). The flavour of milk and dairy products: II. Cheese: Contribution of volatile compounds. *International Journal of Dairy Technology, 50*, 79–89.

Whitfield, F. B., Jensen, N., & Shaw, K. J. (2000). Role of *Yersinia intermedia* and *Pseudomonas putida* in the development of a fruity off-flavour in pasteurized milk. *Journal of Dairy Research, 67*, 561–569.