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Ethyl butanoate is synthesised both by alcoholysis and esterification by dairy lactobacilli and propionibacteria

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ABSTRACT

Esters are important flavour-active compounds in cheese and result from the activity of cheese bacteria. The bacteria and reactions involved, however are still largely unknown, with two main mechanisms, alcoholysis or esterification, potentially involved in ester synthesis from different precursors. The aim of this study was to compare the potential for ester synthesis *via* different reactions in ripening bacteria. Four strains, one *Lactobacillus fermentum,* one *L. casei,* and two *Propionibacterium freudenreichii*, were tested as growing cultures and cell-free extracts (CFE) for their capacity to synthesise ethyl butanoate (EtC4) in the presence of different precursors. In cultures, EtC4 was mainly produced *via* alcoholysis. The highest EtC4 levels were produced by *L. fermentum.* Propionibacteria were also able to synthesise esters *via* alcoholysis, as demonstrated here for the first time. CFE from *L. fermentum* synthesised EtC4 *via* alcoholysis, whereas the other three strains did it *via* esterification. Our results show that the EtC4 synthesis mechanisms and the amounts produced were markedly strain-dependent, and suggest that both esterification and alcoholysis could be involved in ester formation during cheese ripening. By providing a better understanding of EtC4 biosynthesis, this work paves the way for controlling its production in cheese, accelerating the formation of desirable flavour.

1. Introduction

Esters are common volatile compounds responsible for fruity flavour notes in many fermented foods and beverages (Gao, Wang, Jiang, Xu, & Xia, 2016; He et al., 2014; Liu, Crow, & Holland, 2009; Sumby, Grbin, & Jiranek, 2010). Regarding cheeses, they are part of the aroma array of many varieties, such as soft cheeses, Italian, Swiss-type, and goat and ewe milk cheeses (Curioni & Bosset, 2002; Liu, Holland, & Crow, 2004b). A range of esters is found in most cheese types, but the number and type of esters vary between cheese varieties. Swiss-type cheeses such as Emmental, Gruyére, Appenzell, and Comté contain not only the ethyl esters of short-chain (C2:0 to C8:0) fatty acids common to other cheese types, but also esters unique to these cheeses, such as several propionate esters (ethyl propionate, 1-methylpropyl propionate, butyl propionate) as well as branched-chain esters (ethyl 3-methylbutanoate, 3-methylbutyl acetate, 3-methylbutyl propanoate and 2-methylbutyl acetate) (Liu et al., 2004b; Thierry, Maillard, Hervé, Richoux, & Lortal, 2004; Thierry, Maillard, Richoux, & Lortal, 2006; Wolf, Peralta, Candioti, & Perotti, 2016). The contribution of esters to flavour depends on their concentration. Even though esters are normally present at low concentrations in cheeses (generally lower than 10 ng/g), they significantly contribute to the overall flavour balance due to their low perception thresholds (Liu et al., 2004b). Moreover, even at concentrations below their sensorial perception thresholds, esters can impact on cheese flavour through synergistic interactions between different esters and with other volatile compounds (Liu et al., 2004b). Esters may also attenuate or mask the impact of off-flavours (e.g., pungent, sharp) imparted by high levels of short-chain free fatty acids (FFA) (Liu et al., 2004b). However, excessive levels of ethyl esters of short-chain FFA (typically ethyl butanoate and ethyl hexanoate) cause a fruity flavour defect in Cheddar cheese (McGugan et al., 1975).

Microorganisms are the main agents of ester formation in fermented foods including cheeses. In Swiss-type cheeses, the microflora consists of two bacterial groups, lactic acid bacteria (LAB), mainly lactobacilli, used as starters or present as an indigenous microflora, and propionic acid bacteria (PAB), used as ripening cultures. Both LAB (Bouton, Buchin, Duboz, Pochet, & Beuvier, 2009; Liu et al., 2009) and PAB (Hong et al., 2017; Thierry et al., 2004, 2006; Wolf et al., 2016) have been associated with the formation of esters in cheese. However, their specific contribution is not known, and could result either indirectly

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from their ability to produce acids and alcohols, which are precursors of esters, or directly from their ester-synthesising enzymatic activity, or both.

Ester synthesis can occur *via* four reactions: esterification (reaction of an acid and an alcohol), alcoholysis (reaction of an ester and an alcohol), acidolysis (reaction of an ester and an acid) and transesterification (reaction of two esters) (Holland et al., 2005). Esterification and alcoholysis are thought to be the two mechanisms involved in ester synthesis in cheese (Holland et al., 2005; Liu et al., 2004b). Ester biosynthesis reactions are catalysed by carboxylic ester hydrolases (esterases, EC 3.1.1). Esterases catalyse both the synthesis and the hydrolysis of esters (Bornscheuer, 2002). The equilibrium existing between these reactions depends on the environmental conditions prevailing in cheese [water activity, pH, temperature and availability of substrates] (Bornscheuer, 2002; Liu et al., 2004b). Concerning ester precursors, ethanol is the most abundant alcohol in cheese (McGugan et al., 1975), thus explaining the prevalence of ethyl esters in cheeses. Ethanol results from the activity of obligatory heterofermentative lactobacilli and/or from yeasts (Chamba & Irlinger, 2004), but can also be produced at low amounts by thermophilic starter lactobacilli (Richoux, Maillard, Kerjean, Lortal, & Thierry, 2008). 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).

LAB possess intracellular and cell-surface associated esterases (Fenster, Parkin, & Steele, 2003; Gobbetti, Smacchi, & Corsetti, 1997). Cell-free extracts (CFE) of strains of different LAB species showed esterolytic activities (Oliszewski, Medina, Gonzalez, & Perez-Chaia, 2007), and these strains also contributed to the release of short-chain FA during cheese ripening (Abeijón Mukdsi, Medina, Katz, Pivotto, Gatti, & Gonzalez, 2009b). CFE of lactobacilli strains displayed ester-synthesising ability in an aqueous medium *via* both esterification and alcoholysis mechanisms (Abeijón Mukdsi, Medina, Alvarez, & Gonzalez, 2009a; Costello, Siebert, Solomon, & Bartowsky, 2013; Hong et al., 2018; Liu, Holland, & Crow, 2003).

Concerning PAB, 12 esterases have been identified in *Propionibacterium freudenreichii* genome, including one extracellular lipolytic esterase active on milk fat (Dherbécourt et al., 2010; Abeijón Mukdsi et al., 2014); nevertheless, little information is available about their ester-synthesising activity. Few studies reported the ability of PAB strains (growing and non-growing cells) to produce ethyl esters *via* esterification (Liu, Holland, & Crow, 1998), whereas the capacity of PAB to synthesise esters *via* alcoholysis has never been evaluated. Therefore, the available knowledge about the ester-synthesising activity of LAB and PAB appears as scarce and difficult to compare, since the experiments have been performed under different conditions.

The aim of the present study was to compare the ester-synthesising ability of mesophilic lactobacilli and PAB strains, and ascertain in particular whether EtC4 biosynthesis results from esterification and/or alcoholysis reactions. Our strategy combined assays performed using two strains of each group, tested as growing cells and as CFE in the presence of different combinations of substrates.

2. Materials and methods

2.1. Microorganisms and growth conditions

Four strains were selected because they are known lipolytic/esterolytic strains. *Lactobacillus fermentum* CRL1446 and *Lactobacillus casei* CRL1430, provided by the Centro de Referencia para Lactobacilos (CERELA-CONICET, Tucumán, Argentina), have been isolated from Argentinean goat milk cheese. *Propionibacterium freudenreichii* CIRM-BIA1^T and CIRM-BIA508 were obtained from the Centre International de Resources Microbiennes - Bactéries d'Intérêt Alimentaire (CIRM-BIA, INRA, Rennes, France). CIRM-BIA508 was isolated from Gruyère cheese. Lactobacilli strains were grown at 37 °C in MRS broth (Becton Dickinson, France), and PAB at 30 °C in yeast extract-lactate (YEL) broth (Malik, Reinbold, & Vedamuthu, 1968). All strains were grown in glass tubes without agitation, and activated two times before each experimental use.

2.2. Cell-free extract preparation and protein determination

Cells cultured in 400 mL of MRS or YEL broth were harvested at late exponential phase by centrifugation (10,000x*g* for 10 min at 4 °C), washed twice with 100 mM sodium phosphate buffer (pH 7), and resuspended in the same buffer to reach an optical density at 650 nm (OD_{650}) of ca. 30. Cell suspensions were disrupted using zirconium beads (diameter, 0.1 mm; BioSpec Products, Bartlesville, OK) in a bead beater (MM301; Retsch, Haan, Germany; 30 Hz, 20 min). Cellular debris were removed by centrifugation (20,000x*g* for 30 min at 4 °C) and the supernatant (containing intracellular and cell wall-membrane fractions) was used as CFE. The efficiency of breakage, estimated based on spectrophotometric measurements, ranged from 74 to 93%, depending on the strain considered. Protein concentrations were determined by the method of Bradford (Bradford, 1976), using a kit from Bio-Rad (CA, USA) and bovine serum albumin (Sigma, Saint Quentin Fallavier, France) as standard.

2.3. Ester synthesis in cultures

LAB and PAB cells were grown in MRS and YEL medium, respectively, supplemented with different EtC4 precursors. Media without any supplementation were used as controls. Media were supplemented with: E medium: ethanol, 2 mM final concentration; EB medium: ethanol and butanoic acid, 2 mM each; ETB medium: ethanol and tributyrin, 2 mM each; Eem medium: ethanol, 2 mM, and milk fat emulsified with a sodium caseinate solution, final fat concentration of 30% w/w in the broth medium, as previously described (Dherbécourt et al., 2010). Cells were cultured (1% v/v inoculum) at 37 °C and 30 °C, for LAB and PAB, respectively. Non-inoculated media (blanks) were also incubated to evaluate possible non-enzymatic formation of esters. Cells were harvested (10,000x*g* for 10 min at 4 °C) during the exponential and stationary phase of growth, and culture supernatants stored at −80 °C until analysis.

Population levels were monitored by measuring the OD_{650}. Duplicate independent cultures were performed for each strain.

2.4. Ester synthesis by cell-free extracts

Ester synthesis was evaluated in an assay mixture containing CFE (2 mg protein/mL), 100 mM sodium phosphate buffer pH 7, 100 mM ethanol, and either 100 mM butanoic acid or 33 mM tributyrin to assess esterification or alcoholysis, respectively. Assay mixtures were incubated at 37 °C (lactobacilli) or 30 °C (PAB) for 24 h, and stored at −80 °C until analysis. Controls lacking substrates were included to check for endogenous ester production. Blanks lacking CFE were also included to check for the absence of non-enzymatic formation of esters. Net productions were calculated by subtracting the ester levels detected in controls and blanks. All incubations were performed in duplicate.

2.5. Quantification of esters

Esters were analysed by dynamic head space – gas chromatography – mass spectrometry (DHS-GC-MS) using a Clarus 680 gas chromatograph coupled to Clarus 600T quadrupole mass spectrometer (Perkin Elmer, Courtabœuf, France). Briefly, a Turbo Matrix HS-40 HS Trap (Perkin Elmer) was used as a head space sampler. A 2.5 g sample (supernatant or assay mixture) was placed in 20 mL-Perkin Elmer vial. The HS-trap sampling parameters were previously described in detail (Pogačić et al., 2015). Volatile compounds were separated on an Elite 5MS capillary column (60 m length × 0.25 mm i.d. × 1 μm film

thickness; Perkin-Elmer), with He as the mobile phase. The oven was temperature-programmed at 35 °C for 5 min, followed by increasing the temperature at a rate of 7 °C/min up to 140 °C, and then at 13 °C/min up to 280 °C. The mass spectrometer was operated in the scan mode within a mass range of *m/z* 29 to 206, scan time: 0.2 s and inter scan delay: 0.1 s. Ionisation was done by electronic impact at 70 eV.

EtC4 was identified by comparison of mass spectra and retention times with those of an authentic standard (99% purity; Aldrich, Saint Quentin Fallavier, France). It was quantified by the areas of fragment 116 from the regression curve of the standard, using external standard calibration, under the GC conditions described above. Peaks were quantified (*m/z*).

2.6. Statistical analysis

All experiments were repeated twice. The results were submitted to one-way analysis of variance using R software (http://www.R-project.org). Fisher's least significant difference (LSD) test was performed for multiple mean comparisons ($P < 0.05$).

3. Results

3.1. Synthesis of ethyl butanoate in cultures

The two LAB strains reached the end of exponential phase of growth after approx. 6 h (1st sampling time), and went on growing at a slower rate until approx. 18 h. They were further incubated for 18 h in the stationary phase (2nd sampling time at 26 h), during which *L. casei* cultures kept the same OD_{650} values, while *L. fermentum* cultures showed a ca. 20% OD_{650} decrease. The two PAB strains stopped growing after 45 h incubation (1st sampling time), and then showed a 20% OD_{650} decrease during the stationary phase (2nd sampling time at 90 h). The addition of EtC4 precursors did not alter the growth of any of the strains evaluated (data not shown).

No significant EtC4 synthesis was observed in neither control lacking substrates nor E media (ethanol only) by any of the four strains. Only very low EtC4 levels were detected in the blanks (non-inoculated media) (Fig. 1).

The concentration of EtC4 in the media containing different ester precursors is shown in Fig. 1. Significant differences in the amount of EtC4 produced were observed depending on the strain, the medium, and the growth phase.

Concerning the growth phase, higher EtC4 levels were generally produced during the stationary phase than during the exponential phase. This difference was in particular very marked for cultures of *L. fermentum* CRL1446, with factors of 9, 104 and 2 in the stationary phase compared to the exponential phase in EB, ETB and Eem media, respectively.

Concerning the strains, *L. fermentum* CRL1446 was the best EtC4 producer in all the media assayed. It synthesised significantly higher EtC4 levels than *P. freudenreichii* CIRM-BIA1, the second best producer (e.g. 10-, 32- and 5-fold higher in EB, ETB and Eem during the stationary phase cultures, respectively). The highest EtC4 concentration (ca. 430 ng/g) was observed in ETB medium. This concentration was 11- and 20-fold higher compared to that observed in EB and Eem cultures at the same growth phase. *L. casei* CRL1430 synthesised significant levels of EtC4 (ca. 7 ng/g) only in Eem medium. *P. freudenreichii* CIRM-BIA1 synthesised similar concentrations of EtC4 (4–5 ng/g) in EB and Eem media, at both stages of growth evaluated. The highest EtC4 concentrations were detected in ETB cultures (3-fold higher than in EB at the stationary phase). *P. freudenreichii* CIRM-BIA508 produced significant amounts of EtC4 only in ETB medium, at both exponential and stationary phase of growth (10 and 15 ng/g, respectively).

Concerning the acyl-donors present in the medium, the four strains were capable of synthesising EtC4 in ETB and/or Eem, i.e. *via* alcoholysis, whereas only two strains, *L. fermentum* CRL1446 and *P. freudenreichii* CIRM-BIA1, also did it in EB, i.e. *via* esterification.

3.2. Synthesis of ethyl butanoate by cell-free extracts

The results of EtC4 production by CFE are shown in Fig. 2. CFE of *L. fermentum* CRL1446 produced EtC4 in the assay mixture containing tributyrin and ethanol, i.e. *via* alcoholysis, whereas for CFE of *L. casei* CRL1430 and both PAB strains, EtC4 synthesis occurred only in the presence of butanoic acid and ethanol, i.e. *via* esterification (Fig. 2). CFE from PAB strains synthesised 2–4-fold higher EtC4 levels than LAB (20, 10, and 5 μg/g in *P. freudenreichii* CIRM-BIA508, CIRM-BIA1, and LAB, respectively).

4. Discussion

This study is the first report comparing under the same experimental conditions the ester-synthesising ability of the main ripening flora of Swiss-type cheeses, namely mesophilic lactobacilli and PAB. While their presence has been associated with the formation of esters in Emmental, Comté, and Pategrás cheeses (Bouton et al., 2009; Thierry et al., 2004; Wolf et al., 2016), the ability of these bacteria to generate esters remains unclear. We focused on EtC4 production since it is one of the major esters found in cheese, and has been identified as a flavour impact compound in several cheeses including Swiss-type cheese (Curioni & Bosset, 2002; Dunkel et al., 2014). A better understanding of the origin of EtC4 is needed to control and accelerate the formation of desirable cheese flavour.

Our strategy consisted in investigating the effect of two factors on EtC4 synthesis by four strains: i) the use of both growing cells and CFE, because both alive bacteria and their intracellular enzymes released after cell lysis can contribute to ripening reactions (Thierry et al., 2015), and ii) the presence of different acyl-donors and ethanol, to investigate whether ester synthesis occurred *via* esterification or *via* alcoholysis. Our results show that the EtC4-synthesising ability is highly variable among the strains evaluated, not only regarding the amount of ester produced, but also the mechanism involved (Figs. 1 and 2). These findings are in agreement with those reported by other authors (Costello et al., 2013; Hong et al., 2018).

Two mechanisms have been described for the synthesis of esters by LAB: esterification and alcoholysis. The ability of LAB to synthesise EtC4 *via* esterification was first reported using CFE (Hosono, Elliot, & McGugan, 1974) and later using non-growing cells (Liu et al., 1998). It occurs in a both species- and strain-dependent manner. Esterase activity in LAB is exclusively cell-associated, i.e. due to cytoplasmic and cell wall-anchored enzymes (Fenster et al., 2003; Gobbetti et al., 1997). Most often the presence of more than one esterase has been shown in LAB including lactobacilli (Oliszewski et al., 2007). The first evidence of ester synthesis *via* alcoholysis was reported by Liu et al. (2003), who observed that growing cells and CFE from dairy LAB can synthesise substantial amounts of EtC4 from tributyrin and ethanol in an aqueous system**s** *via* a transferase reaction (alcoholysis). This reaction is catalysed by an acyltransferase, which directly transfers the butyryl groups from tributyrin to ethanol (Liu et al., 2004a).

The two LAB strains used in this study exhibited different ester-synthesising abilities, which markedly depended on the conditions (cell vs. CFE, and nature of the acyl-donor). *L. fermentum* CRL1446 produced the highest amounts of EtC4 *via* both esterification and alcoholysis. However, alcoholysis appeared as the main mechanism involved in EtC4 synthesis in this strain (Figs. 1 and 2), suggesting that at least one esterase displays acyltransferase activity in this strain, in agreement with previous findings on another *L. fermentum* strain, B4017 (Liu et al., 2003). The high EtC4-synthesising activity observed during the stationary phase in *L. fermentum* CRL1446 cultures could at least partly result from the activity of intracellular esterase(s) released after bacterial cell lysis, estimated from the OD_{650} decrease observed during

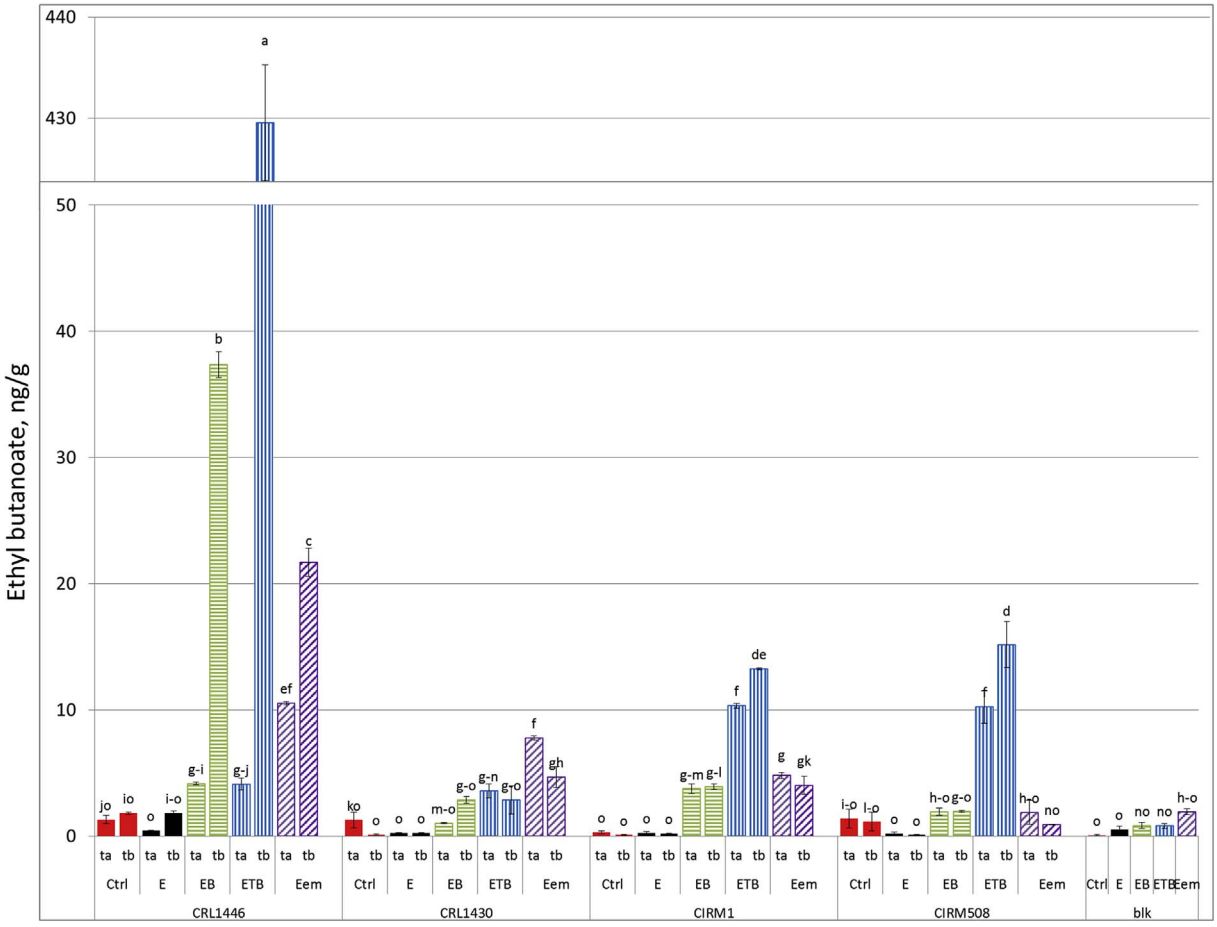

Fig. 1. Synthesis of ethyl butanoate in cultures of four strains (*L. fermentum* CRL1446, *L. casei* CRL1430, *P. freudenreichii* CIRM-BIA1 and CIRM-BIA508) grown in the presence of different precursors of esters and harvested at two stages of growth. ta: exponential phase, tb: stationary phase. Media: Ctrl = Control media (without any supplementation). Media supplemented with: E, 2 mM ethanol; EB, ethanol and butanoic acid, 2 mM each; ETB, ethanol and tributyrin, 2 mM each; Eem, 2 mM ethanol and milk fat emulsion, final fat concentration of 30% w/w. Blanks are non-inoculated media. Results are expressed in ng/g. Bars and error bars show the means and the standard errors of two independent experiments. Bars with different letters (a–o) significantly differed according to LSD test ($P < 0.05$).

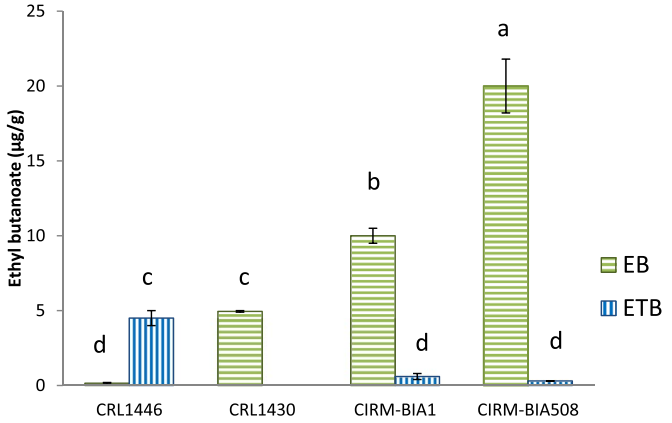

Fig. 2. Net production of ethyl butanoate by cell-free extracts of four strains (*L. fermentum* CRL1446, *L. casei* CRL1430, *P. freudenreichii* CIRM-BIA1 and CIRM-BIA508) in the presence of ethanol and either tributyrin (ETB) or butanoic acid (EB). Values were corrected by subtracting the levels of esters formed in controls (lacking substrates) and blanks (lacking cell-free extracts). Results are expressed in µg/g of protein. Bars and error bars show the means and the standard errors of two independent experiments. Bars with different letters (a–d) significantly differed according to LSD test ($P < 0.05$).

incubation (ca. 20%). Esterases are quite stable enzymes (Esteban-Torres, Santamaría, de las Rivas, & Muñoz, 2014; Esteban-Torres, Mancheño, de las Rivas, & Muñoz, 2015) and therefore could have remained active once released from cells to the medium. Previous results concerning the esterase activity of *L. fermentum* CRL1446 support the existence of at least two distinct enzymes: one cytoplasmic and one cell surface-associated esterase (Abeijón Mukdsi, personal communication), as also observed in another *L. fermentum* strain, DT41, used as an Italian cheese starter (Gobbetti et al., 1997). Interestingly, in *L. fermentum* CRL1446, growing cells but not CFE produced EtC4 *via* esterification, in contrast to the results observed in the other three strains tested. This could be explained by the fact that the cell surface-associated esterase would be the main enzyme catalysing EtC4 synthesis *via* esterification during growth, but would lose its stability and/or activity once the cells are disrupted. The cytoplasmic enzyme would thus be involved in the EtC4 synthesis *via* alcoholysis, observed in both CFE experiments and cultures. The high EtC4 levels produced in cultures of *L. fermentum* CRL1446 compared to the other three strains tested (Fig. 1) could also be related to the endogenous ethanol production by the strain during growth (data not shown), as expected for an obligatory heterofermentative LAB species. Ethanol is a limiting factor for ethyl ester biosynthesis in some cheese varieties, more ethyl esters and a significantly enhanced fruity flavour perception being detected in ethanol-added cheeses compared to controls (Hong et al., 2018, 2017; Liu et al., 2004b; Richoux et al., 2008; Thierry et al., 2006). *L. casei* CRL1430 only produced significant amounts of EtC4 in Eem, in which butyryl groups from milk fat, preferentially esterified at the *sn*-3 position of triglycerides, are transferred directly to ethanol, as described for several lipases (Lubary, ter Horst, Hofland, & Jansens, 2009). Great differences in the ability to synthesise esters were reported for 12 lactobacilli strains

evaluated as potential adjunct cultures for Camembert-type cheese (Hong et al., 2018). Out of the 12 strains, CFE from only 4 strains showed EtC4-synthesising activity *via* esterification and 11 *via* alcoholysis, indicating that alcoholysis is the prevalent mechanism, in agreement with our results.

This study demonstrates for the first time that PAB are also capable of synthesising esters *via* alcoholysis. Previous reports showed that non-growing cells of two strains of *P. freudenreichii* produced EtC4 *via* esterification, with dramatic differences in their EtC4-synthesising activity (variation factor of 187) (Liu et al., 1998). The highest EtC4 production was detected in ETB cultures for both *P. freudenreichii* strains, demonstrating that alcoholysis was the prevalent EtC4-synthesising mechanism in cultures. Twelve (putative) esterases were predicted from the genome sequence of *P. freudenreichii* CIRM-BIA1: PF#279 is the sole secreted esterase, PF#774 is predicted to be surface-exposed, and the ten remaining esterases are predicted to be intracellular (Dherbécourt et al., 2010; Abeijón Mukdsi et al., 2014). Both PF#279 and PF#774 esterases display lipolytic activity on milk fat, but PF#279 is the main responsible for lipolysis of a milk fat emulsion (Abeijón Mukdsi et al., 2014). In the presence of tributyrin as acyl-donor, both PAB strains assessed produced EtC4 in cultures (Fig. 1), but not the corresponding CFE (Fig. 2). This result suggests that the secreted esterase PF#279 is the main enzyme responsible for EtC4 synthesis *via* alcoholysis, whereas the cell wall-associated and/or the intracellular esterases would be involved in the synthesis of EtC4 *via* esterification.

In an aqueous environment, ester synthesis is expected to be strongly disadvantaged. In cultures containing tributyrin or milk triglycerides as acyl-donor, EtC4 resulted from the balance between its synthesis *via* alcoholysis and its hydrolysis, but detectable amounts of EtC4 were produced by the four strains tested. Liu et al. (2003) demonstrated that the rate and yield of ester synthesis *via* alcoholysis were greater than those of FFA release when non-growing cells of *Streptococcus thermophilus* ST1 were incubated in presence of ethanol and di- and monoglycerides. They suggest that ethanol is a preferred substrate, thus favouring ester synthesis *via* alcoholysis rather than its hydrolysis. In CFE of several dairy LAB (Hong et al., 2017) and wine LAB strains (Costello et al., 2013), ester-synthesising activities *via* alcoholysis were generally greater than *via* esterification. In contrast, Abeijón Mukdsi et al. (2009a) showed that the ester-forming activity in CFE of various lactobacilli strains isolated from goat and ewe milk cheeses occurred *via* both mechanisms, with in general a higher activity *via* esterification. In the present study, however, only one mechanism was used by CFE from the four strains evaluated. These observations demonstrate that several factors can be involved in the variability in ester-synthesising ability observed among strains and conditions, such as different substrate specificity of the enzymes, accessibility to substrates, enzyme stability, ethanol concentration, among others.

5. Conclusion

We clearly showed that the strains evaluated were capable of synthesising EtC4, which is essential for characteristic flavour development in Swiss cheeses. In general, more EtC4 was synthesised in cultures containing tributyrin or milk fat as acyl donors, indicating that alcoholysis is the prevalent mechanism. However, the pattern of ester synthesis differed for the four strains tested depending on the conditions, which stresses both the need for selecting appropriate cultures for cheese manufacture, and the importance of the experimental conditions and appropriate controls for screening purposes. Moreover, this study is the first providing information about the ester synthesis mechanisms in PAB. *L. fermentum* CRL1446 and *P. freudenreichii* CIRM-BIA1 appear as suitable strains to be tested as aroma-producing cultures in Swiss cheese manufacture. A deeper understanding of the ester-synthesising mechanisms would allow modulating ester production in cheese, providing a more controlled impact on cheese flavour. This would be achievable by a rational approach that would adapt the strategies to modulate the availability of the different precursors or to induce cell lysis depending on the mechanisms of ester synthesis activity of the cultures selected.

Conflict of interest

Authors declare that they have no conflict of interest.

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