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Prevention of late blowing defect by reuterin produced in cheese by a *Lactobacillus reuteri* adjunct

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ABSTRACT

In this study, reuterin-producing *Lactobacillus reuteri* INIA P572 was added to cheese as an adjunct culture together with 50 or 100 mM glycerol (required for reuterin production), with the aim of controlling *Clostridium tyrobutyricum* CECT 4011 growth and preventing the late blowing defect (LBD) of cheese caused by this strain. *L. reuteri* survived cheese manufacture and produced reuterin *in situ*, detected at 6 and 24 h. However, the produced reuterin was enough to inhibit the growth of *Clostridium*, showing undetectable spore counts from day 30 onward and, therefore, to prevent cheese LBD during ripening (60 d, 14 °C). The acidification of these cheeses was not affected, although from day 14 they showed significantly lower lactococci counts than cheese made only with the starter (control cheese). Cheeses with LBD showed lower levels of lactic acid than control cheese and the formation of propionic and butyric acids, but cheeses with reuterin showed the same organic acids profile than control cheese. The cheese made with *L. reuteri* and 100 mM glycerol showed a light pink colour, not observed in the cheese made with *L. reuteri* and 50 mM glycerol. These results demonstrated a potent anti-clostridial activity of reuterin produced in an actual food product like cheese, and proved to be a novel approach to prevent LBD of cheese.

1. Introduction

Cheeses are susceptible to spoilage of the final product, resulting in severe economic losses for the cheese industry. The development of defects and alterations depends on the control of those microbiological and technological factors that influence cheese making and ripening such as variations in milk quality, milk pre-treatment (pasteurization), hygiene practices, differences in starter culture activity and acidity profiles, manufacture technology, compositional parameters, and ripening temperature/environments (O'Sullivan et al., 2013). Among them, microbiological factors are the most difficult to control. Undesirable cheese spoilage microorganisms include, among others, coliforms, yeasts, heterofermentative lactic acid bacteria and spore-forming bacteria, which cause early or late blowing defects in cheeses, depending on the microorganism (Ledenbach and Marshall, 2009). Late blowing defect (LBD) appears during the ripening of semi-hard and hard cheeses as *Clostridium* strains, mainly *Clostridium tyrobutyricum*, carry out the butyric acid fermentation. When lactic acid is metabolized, other organic acids, mainly butyric acid, and gases such as carbon dioxide and hydrogen are produced. The pressure of evolved gases causes cracks and splits, which are generally accompanied by unpleasant aroma and rancid flavour caused by the acids. Although LBD of cheese is an old problem, very well known by cheese manufacturers, eradicate it is a difficult issue since *Clostridium* spores are ubiquitous, much more resistant to heat, chemicals, irradiation and desiccation than vegetative cells, and as few as 50–1000 spores per litre of milk is enough to induce the defect if cheese conditions are suitable for the germination and growth of *Clostridium* (Garde et al., 2013).

The control of *Clostridium* development in cheese has been traditionally done by addition of nitrate or lysozyme, bactofugation and microfiltration (Lodi, 1990; Stadhouders, 1990; van den Berg et al., 2004), but the application of new anti-clostridial cultures is very promising, especially for the cheese industry. The inoculation of milk with bacteriocinogenic lactic acid bacteria in cheese manufacture has been successfully investigated to prevent LBD (Bogovic Matijasic et al., 2007; Garde et al., 2011b; Martínez-Cuesta et al., 2010; Rilla et al., 2003). Furthermore, in a previous work, the antimicrobial compound reuterin present in a cell-free supernatant

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from *Lactobacillus reuteri* INIA P572 exhibited a broader anti-clostridial spectrum than lysozyme and nitrite, inhibiting the growth of spores and vegetative cells of all *Clostridium* strains tested, including *C. tyrobutyricum* strains (Ávila et al., 2014). *L. reuteri* is a heterofermentative lactic acid bacteria that belongs to the microbiota of humans and animals gut (Vollenweider and Lacroix, 2004). In addition, it has been isolated from a variety of food products including milk products (Casas and Dobrogosz, 2000), and it is used as a probiotic in the health care of humans and animals (Vollenweider and Lacroix, 2004). Some *L. reuteri* strains produce reuterin (β-hydroxypropionaldehyde) as an intermediate step in the conversion of glycerol to 1,3-propanediol (Lüthi-Peng et al., 2002). Reuterin is water-soluble, active at a wide range of pH values, resistant to proteolytic and lipolytic enzymes and with a broad inhibitory activity. It has been reported direct addition of reuterin as an additive to prevent the growth of pathogenic microorganisms in milk and dairy products (Arqués et al., 2004, 2008a; b; 2011; El-Ziney and Debevere, 1998), and *in situ* reuterin production by *L. reuteri* plus glycerol in dairy products has been recently proved (Langa et al., 2013). Hence, here we evaluate the addition of reuterin-producing *L. reuteri* INIA P572 to cheese milk, as an adjunct culture, together with 50 or 100 mM glycerol, in order to control *C. tyrobutyricum* CECT 4011 growth and to prevent cheese LBD caused by this strain.

2. Materials and methods

2.1. Bacterial strains and propagation

The reuterin-producing *L. reuteri* INIA P572 was selected from the INIA culture collection due to their high reuterin yield (Langa et al., 2013; Rodríguez et al., 2003). This strain was maintained at −80 °C in MRS broth (Biolife, Milano, Italy) with 5% glycerol and subcultured twice in MRS broth at 37 °C for 24 h in anaerobic jars with an H_2 plus CO_2 generating kit (AnaeroGen, Oxoid, Basingstoke, UK). Just before use in cheese manufacture, the grown culture was centrifuged (5000 × *g*, 15 min, 20 °C) and the pellet was washed twice with sterile distilled water and resuspended in reconstituted skimmed milk. Commercial mesophilic lactic culture MA 016, kindly provided by Larbus S.A. (Madrid, Spain) and consisting of limited *Lactococcus lactis* subsp. *lactis* and *cremoris* strains, was cultured in reconstituted skim milk at 30 °C for 18 h before use in cheese manufacture. *C. tyrobutyricum* CECT 4011 from the Spanish Type Culture Collection (Colección Española de Cultivos Tipo, CECT, Valencia, Spain) was used as cheese blowing agent. It was maintained at −80 °C in Reinforced Clostridial Medium (RCM, Difco, Detroit, USA) with 5% glycerol and subcultured in RCM and incubated at 37 °C under anaerobic conditions for 48 h.

2.2. Spore preparation

Spores of *C. tyrobutyricum* CECT 4011 were obtained following inoculation in RCM (without agar and sodium acetate) and incubation at 37 °C under anaerobic conditions for 3 days. After centrifugation (5000 × *g*, 15 min, 20 °C), the pellet was washed twice with sterile distilled water, resuspended in reconstituted skimmed milk and heat-shocked at 80 °C for 20 min to kill off vegetative cells before use in cheese manufacture. Spore counts were determined on RCM agar (1.5%, w/v) after anaerobic incubation at 37 °C for 3 days.

2.3. Cheese manufacture

Model cheeses were manufactured from pasteurized cow's milk in duplicate experiments, carried out on different days. Each experiment consisted of eight vats, each containing 2 L of milk, which was heated at 32 °C. Calcium chloride and a culture of the commercial starter MA 016 were added at 0.01% and 1% (approximately 7 log cfu/mL milk), respectively, to all vats. *L. reuteri* INIA P572 was added at 0.1% to vats 5–8 (approximately 6 log cfu/mL milk), and vats 2–7 were deliberately contaminated with approximately 3 log spores/mL milk of *C. tyrobutyricum* CECT 4011. Rennet (0.025 g/L, Laboratorios Arroyo, Santander, Spain) was added to milk 20 min later to all vats. At this stage of cheese making, glycerol was also added at a final concentration of 50 mM to vats 3 and 6, and of 100 mM to vats 4 and 7. After 40 min, the curds were cut into 6–8 mm cubes and scalded at 38 °C for 15 min. Whey was drained off and curds were washed with 800 mL of distilled and sterilized water at 37 °C, and distributed into cylindrical moulds. One cheese, of approximately 200 g in weight, was obtained from each vat. The cheeses were pressed overnight at 20 °C, vacuum packaged in Cryovac plastic bags and ripened at 14 °C for 60 d.

2.4. Microbiological determinations

Representative cheese samples (5 g) were homogenized with 45 mL of a sterile 2% (w/v) sodium citrate solution at 45 °C in a Stomacher 400 (A. J. Seward Ltd, London, UK). Decimal dilutions of cheese homogenates were prepared in sterile 0.1% (w/v) peptone solution. *L. reuteri* counts were determined on duplicate plates of Rogosa agar (Difco) and incubated at 37 °C for 48 h under anaerobic conditions, and lactococci counts from commercial starter in duplicate on Tryptic Glucose Yeast agar (Biolife) with 0.1% added skim milk (Biolife) for 24 h at 30 °C. Spore counts were determined, after heat-shock (80 °C, 20 min) of the cheese homogenate dilutions, on RCM agar, as described in Section 2.2.

2.5. Production and quantification of reuterin

Reuterin production in cheese by *L. reuteri* INIA P572 was measured on an extract obtained after homogenizing 2 g of cheese with 4 mL of MilliQ water using an Ultra-Turrax T8 homogenizer (IKA, Labortechnik, Staufen, Germany), followed by centrifugation (12000 × *g*, 5 min, 20 °C). Whey drained from cheese after 6 and 24 h was also centrifuged for reuterin quantification. The concentration of reuterin in the supernatants from cheese and whey samples was determined on duplicate following the colorimetric method described by Lüthi-Peng et al. (2002). For obtaining the standard curves of reuterin (0–10 mM) in cheese and whey, cheese and whey samples from cheese only made with commercial starter MA 016 were homogenized with different amounts of cell-free supernatant of *L. reuteri* INIA P572 containing 65 mM reuterin (obtained as described by Ávila et al., 2014).

2.6. Detection of late blowing defect

During the ripening, cheeses were subjected to regular visual and odour inspections to monitor formation of gas and butyric acid. Blowing cheeses were identified by the gas formation in the package bag, the appearance of irregular eyes, cracks and splits within the cheese matrix and the rancid odour caused by butyric acid.

2.7. Physico-chemical determinations

Cheese pH was measured in duplicate by means of a Crison pH meter (model GPL 22, Crison Instruments, Barcelona, Spain) using a Crison penetration electrode (model 52-3.2). Dry matter content was determined in duplicate after drying to constant weight in a vacuum oven at 100 °C (IDF, 1982). Water activity (a_w) was