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Effect of reuterin-producing *Lactobacillus reuteri* coupled with glycerol on the volatile fraction, odour and aroma of semi-hard ewe milk cheese

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ABSTRACT

The effect of the biopreservation system formed by *Lactobacillus reuteri* INIA P572, a reuterin-producing strain, and glycerol (required for reuterin production), on the volatile fraction, aroma and odour of industrial sized semi-hard ewe milk cheese (Castellano type) was investigated over a 3-month ripening period. The volatile compounds were extracted and analyzed by SPME-GC-MS and cheese odour and aroma profiles were studied by descriptive sensory analysis. Control cheese was made only with a mesophilic starter and experimental cheeses with *L. reuteri* were made with and without glycerol. The addition of *L. reuteri* INIA P572 to milk enhanced the formation of six volatile compounds. Despite the changes in the volatile compounds profile, the use of *L. reuteri* INIA P572 did not noticeably affect the sensory characteristics of cheese. On the other hand, the addition of *L. reuteri* INIA P572 coupled with 30 mM glycerol enhanced the formation of twelve volatile compounds, but decreased the formation of five ones. The use of the biopreservation system did not affect overall odour and aroma quality of cheese although it resulted in a significant decrease of the odour intensity scores. In addition, this cheese received significant higher scores for "cheesy" aroma and significant lower scores for the aroma attributes "milky", "caramel" and "yogurt-like". The first two axes of a principal component analysis (PCA) performed for selected volatile compounds and sensory characteristics, accounting for 75% of the variability between cheeses, separated cheeses made with *L. reuteri* INIA P572 and glycerol from the rest of cheeses, and also differentiated control cheese from cheeses made with *L. reuteri* INIA P572 from day 60 onward. Our results showed that the reuterin-producing *L. reuteri* INIA P572 strain, when coupled with glycerol, may be a suitable biopreservation system to use in cheese without affecting odour and aroma quality.

1. Introduction

Reuterin is an antimicrobial compound that consists of monomeric, hydrated monomeric, and cyclic dimeric forms of 3-hydroxypropionaldehyde (3-HPA), with a broad spectrum of antimicrobial activity towards a wide range of pathogens and food spoilage organisms including gram-positive and gram-negative bacteria, yeasts and moulds (Vollenweider and Lacroix, 2004). It induces oxidative stress in cells by modifying thiol groups in proteins and small molecules, ultimately resulting in cell death (Schaefer et al., 2010; Vollenweider et al., 2010). Reuterin is water-soluble, active at a wide range of pH values, and resistant to proteolytic and lipolytic enzymes. All these properties make of reuterin a potential food biopreservative. *Lactobacillus reuteri* is the only species that produces high levels of reuterin in the presence of glycerol, while being highly resistant to the antimicrobial activity of reuterin (Schaefer et al., 2010). *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 dairy products (Casas and Dobrogosz, 2000), and it is used as a probiotic in the health care of humans and animals (Vollenweider and Lacroix, 2004).

Application of reuterin as an alternative strategy for decontamination and preservation of dairy products has been described. Direct addition of reuterin as an additive to prevent the growth of pathogenic microorganisms in milk and dairy products has been proposed (Arqués et al., 2004, 2008a, 2008b, 2011; El-Ziney and Debevere, 1998), but to date reuterin is not legislated as a food preservative. To overcome this limitation, biopreservation systems consisting of a reuterin-producing *L. reuteri* strain and glycerol (registered in the European Union as food additive E 422) have been recently proposed for dairy products (Angiolillo et al., 2014; Gómez-Torres et al., 2014; Langa et al., 2013). Langa et al. (2013) successfully proved the *in situ* production of reuterin by *L. reuteri* plus glycerol in yogurt and cheese model systems. The application of an active coating enriched with *L. reuteri* and glycerol to Fior di Latte cheese prolonged its microbial quality and, thus, its shelf life (Angiolillo et al., 2014). Late blowing defect of cheese caused by

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Clostridium tyrobutyricum strains was prevented by the use of reuterin-producing *L. reuteri* INIA P572 coupled with glycerol as a biopreservation system in model and industrial sized cheese manufacture (Gómez-Torres et al., 2014). Nonetheless, in the search for innovative and alternative preservation strategies to prevent microbial spoilage and poisoning of cheese, it is also essential to provide knowledge concerning their effects on the ripening process and sensory characteristics, a gap in some studies, in view of potential industrial application. Therefore, in this work we investigate the effect of *L. reuteri* INIA P572 coupled with glycerol on the formation of volatile compounds and on the aroma and odour profiles of industrial sized semi-hard ewe milk Castellano cheese during 3 months of ripening.

2. Materials and methods

2.1. Lactic cultures and cheese manufacture

Commercial freeze-dried mesophilic lactic culture Choozit™ MA 11 from Danisco (Laboratorios Arroyo, Santander, Spain), and consisting of limited *Lactococcus lactis* subsp. *lactis* and *cremoris* strains, was used in cheese making following the manufacturer's instructions.

L. reuteri INIA P572, with a good performance in dairy products (Gómez-Torres et al., 2014; Langa et al., 2013), was used for reuterin production in cheese. This strain was maintained at −80 °C in MRS broth (Biolife, Milano, Italy) with 5% glycerol and sub-cultured twice in MRS broth at 37 °C for 24 h in anaerobic conditions. Just before use in cheese manufacture, the grown culture was centrifuged (5000 ×*g*, 15 min, 20 °C) and the pellet was washed twice and resuspended in milk.

Industrial sized semi-hard Castellano cheeses were manufactured in duplicate experiments on different days from pasteurized ewe milk. Each experiment consisted of three 50-L vats. After pasteurization (60 °C for 30 min), the milk was cooled to 32 °C and calcium chloride (0.01%) and the commercial lactococci starter MA 11 (approximately 7 log cfu/mL milk) were added to all vats. *L. reuteri* INIA P572 was added at 0.1% to vats 2 and 3 (approximately 6 log cfu/mL milk). Rennet (18 mL/50 L milk, 1:15,000 strength; Laboratorios Arroyo) was added to all vats 25 min after lactic cultures inoculation, and food-grade glycerol was added at a final concentration of 30 mM to vat 3 to promote reuterin synthesis in cheese. The curds were cut 30 min after rennet addition into 6–8 mm cubes and scalded at 37–38 °C for 40–50 min. The whey was drained off and curds were distributed into cylindrical moulds. Three cheeses, of approximately 3 kg in weight, were obtained from each vat. They were pressed at 1.5–2 atm until curd pH was 5.4, salted for 17 h at 12 °C in brine (190 g of NaCl/L), and ripened at 12 °C and 85% relative humidity for 90 d. Cheese rinds were protected against mould growth by coating them on day 7 with 2 layers of a commercial antifungal formulation containing natamycin and potassium sorbate (Fungirol DP 10 TLS, Laboratorios Arroyo).

2.2. Microbiological determinations

Representative cheese samples (10 g) were homogenized with 90 mL of a sterile 2% (*w*/*v*) sodium citrate solution at 40 °C in a Stomacher 400 (A. J. Seward Ltd., London, UK). Decimal dilutions were prepared in sterile 0.1% (*w*/*v*) peptone solution. Starter lactococci and lactobacilli counts in cheese samples were determined on duplicate, on Tryptic Glucose Yeast agar (Biolife) with 0.1% added skim milk powder (Biolife) after 24 h at 30 °C, and on Rogosa agar (Difco) after 48 h at 37 °C in anaerobic conditions, respectively.

2.3. Analysis of volatile compounds

Cheese pieces wrapped in aluminium foil were vacuum packed and frozen at −40 °C at 30, 60, and 90 d of ripening, until analysis. Volatile compounds of ewe milk cheeses were extracted in duplicate by automated solid-phase microextraction (SPME) using a CTC CombiPal autosampler (Agilent, Palo Alto, CA, USA) and analyzed by gas chromatography–mass spectrometry (GC-MS) (HP 6890-MSD HP 5973, Agilent) as described by Gómez-Torres et al. (2015). Ten grams of cheese were homogenized in a mechanical grinder with 15 g of Na_2SO_4 and 30 μL of an aqueous solution of 543 mg/L cyclohexanone. Five grams of the mixture were weighed in a 20 mL headspace glass vial sealed with a PTFE faced silicone septum (Supelco, Bellefonte, PA, USA). Vials were placed on autosampler tray and subjected to SPME. Both equilibration and extraction phases were carried out at 37 °C for 20 and 30 min, respectively. A 2 cm 50/30 mm StableFlex Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) coated fiber (Supelco) was used for headspace extraction. Desorption into the GC injection port was at 260 °C for 10 min in splitless mode. Before use, the fiber was conditioned in the GC injection port at 270 °C for 1 h as recommended by the manufacturer. Chromatographic separation was carried out in a Zebron 100% polyethylene glycol capillary column (60 m long; 0.25 mm internal diameter; 0.50 mm film thickness; ZB-WAXplus, Phenomenex, Torrance, CA) with 1 mL/min helium flow, with the following temperature program: 16 min at 45 °C, first ramp 4 °C/min to 110 °C, 9 min at 110 °C, second ramp at 15 °C/min to 220 °C and 5 min at 220 °C, final ramp to 240 °C at 10 °C/min and 2 min at 240 °C. The detection, identification and semi-quantification of volatile compounds were carried out as described by Garde et al. (2002). Mass detection was performed in the scan mode, from 33 to 220 amu at 2.23 scan/s, and ionization by EI at 70 eV. Data were collected with the HP ChemStation program, and volatile compounds were identified by comparison of spectra with the Wiley 275 library and by comparison of their retention times with authentic standards (Sigma Chemical Co.). Relative abundances of compounds were expressed as percentages of their peak areas on the peak area of the internal standard cyclohexanone.

2.4. Determination of free amino acids

Free amino acids were extracted from duplicate samples of cheese as described by Krause et al. (1995). Analysis of individual amino acids was carried out by reverse-phase HPLC using a Beckman System Gold chromatograph, after derivatization with Waters AccQ Fluor Reagent, using a Waters AccQ Tag (Waters, Milford, MA, USA) column.

2.5. Sensory evaluation

A descriptive test was developed for cheeses based on the guidelines of hard and semi-hard cheeses given by Bérodier et al. (1997). Ten trained panellists evaluated the odour and aroma of cheeses after 30, 60, and 90 d of ripening for quality (overall acceptance) and intensity (overall intensity) of odour and aroma on a 0–10-point scale, using a horizontal line anchored in the middle and at both ends. Odour was defined as the olfactory sensation felt directly by the nose, and aroma as the olfactory sensation felt retronasally upon mastication. After removal of the rind, cheeses were cut into representative triangular slices (15–20 g). Slices of the three cheeses per session from each of the three vats manufactured on the same day, coded with random three-digit numbers, were presented to panellists in random order. The routine of sensory tasting was that the cheeses were first scored for odour by smelling and then introduced in the mouth and scored for the retronasal odorous sensation. Bread and water were provided to cleanse the mouth between cheeses.

For both odour and aroma a series of attributes were scored separately on a 1 to 7-point scale (nil or very slight to very intense perception), belonging to six families, namely, family "lactic", including the descriptors "milky", "buttery", "yogurt-like", and "cheesy"; the family "toasted", including the descriptor "caramel"; the family "animal", including the descriptors "sheepy"; the family "fruity-flowery"; the family