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Impact of *Clostridium* spp. on cheese characteristics: Microbiology, color, formation of volatile compounds and off-flavors

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ABSTRACT

The impact of autochthonous and type-strains of *Clostridium tyrobutyricum*, *Clostridium butyricum*, *Clostridium beijerinckii* and *Clostridium sporogenes* on spoilage (late blowing defect, LBD), physico-chemical characteristics and volatile profile of cheese has been investigated. Five semi-hard cheeses were produced from ewe milk inoculated with 10^4 spores/mL of five *Clostridium* strains and ripened for 60 d. One cheese without clostridial spores served as control. *C. tyrobutyricum* CECT 4011 and INIA 68 resulted potent cheese spoilers, and caused the appearance of the earliest and greatest symptoms of LBD, affecting cheese pH and color, and leading to accumulation of volatile compounds like butyric, propionic and pentanoic acids and some aldehydes, alcohols and esters associated with cheese rancid and pungent off-odors. Cheeses contaminated with *C. beijerinckii* INIA 63 and *C. sporogenes* INIA 71 showed milder and late LBD symptoms, and a volatile profile characterized by higher levels of 2-butanone, 2,3-butanedione and 2-butanol than the rest of cheeses. Despite cheese inoculated with *C. butyricum* CECT 361 presented a slight blown-pack at the end of ripening, it showed physico-chemical characteristics and a volatile profile similar to control cheese. The first two axes of a principal component analysis (PCA) performed for the 21 significant volatile compounds out of 38, accounting for 91% of the variability between cheeses, separated cheeses made with *C. tyrobutyricum* CECT 4011 and INIA 68, with severe LBD symptoms, from the rest of cheeses, and also differentiated control cheese and cheese made with *C. butyricum* CECT 361, from cheeses with milder LBD symptoms made with *C. beijerinckii* INIA 63 and *C. sporogenes* INIA 71.

1. Introduction

Ripening of semi-hard and hard cheeses is a slow process which involves the metabolism of residual lactose, lactate and citrate, proteolysis and lipolysis of milk components throughout microbial, enzymatic and chemical transformations of fresh curd to give rise to a delicate balance of volatile and non-volatile compounds precursors or directly implicated in cheese flavor. Lactate may be metabolized by a number of pathways to various compounds which contribute to cheese flavor or off-flavors depending on variety, microbiota and ripening conditions (McSweeney & Sousa, 2000). Some undesirable microorganisms, like spores of certain species of the genus *Clostridium*, are able to survive the milk pasteurization and cheese-making processes, remaining in the cheese paste during ripening, where they can germinate into vegetative cells which subsequently metabolize lactate. In general, *Clostridium* metabolism of lactate includes the production of organic acids, mainly butyric acid, and some gases such as CO_2 and H_2, resulting in abnormal aroma and flavor and in cracks and slits in the cheese paste, also known as late blowing defect (LBD), a major cause of spoilage in semi-hard and hard cheeses (Garde, Ávila, Gómez, & Nuñez, 2013). The insolubility of H_2 in water is responsible for a very easy and quick cheese blowing (Fröhlich-Wyder & Bachmann, 2007). Clostridia able to metabolize not only lactate and residual sugars in cheese, but also citric acid (Garde, Ávila, Gaya, Arias, & Nuñez, 2012). LBD persists as a major cause of spoilage due to the ubiquitous presence and resistant nature of *Clostridium* spores and since low spore counts in milk can cause LBD if cheese conditions are suitable for the germination and growth of *Clostridium*. To minimize *Clostridium* contamination and development of LBD, it is possible to act at a preventive level at the dairy farm, by minimizing milk contamination (i.e. controlling silage quality, changing the feed, improving hygiene at milking and storage) or at a palliative

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level at the cheese industry by means of technological procedures (bactofugation or microfiltration of milk, addition of nitrate or lysozyme). However, the reduction in spore numbers achieved by bactofugation may be insufficient to impede LBD, microfiltration can only be applied to skim milk because milk fat globules are too large to pass through the membrane pores, and the use of chemicals is precluded by the current increasing demand for additive-free foods or by sanitary reasons (Garde et al., 2013).

Clostridium tyrobutyricum is considered the primary cause of LBD, but *Clostridium butyricum, Clostridium sporogenes* and *Clostridium beijerinckii* also contribute to the appearance of this defect in cheese (Cocolin, Innocente, Biasutti, & Comi, 2004; Garde, Ávila, Arias, Gaya, & Nuñez., 2011; Klijn, Nieuwenhof, Hollwerf, Vanderwaals, & Weerkamp, 1995; Le Bourhis et al., 2005; 2007). High levels of butyric acid have been found in different cheese varieties with LBD such as Saint-Nectaire (Mayenobe, Didienne, & Pradel, 1983), Gouda (Klijn et al., 1995; Mayenobe et al., 1983), Grana Padano (Cocolin et al., 2004), Emmental, Gruyère, Comté, Beaufort and Ossau-Iraty (Le Bourhis et al., 2005), and Manchego (Garde, Ávila, et al., 2012) cheeses, the latter also showing high levels of acetic acid and low levels of citric acid. Although LBD has generally been associated with high butyric acid concentrations, exceptions have been reported (Cocolin et al. 2004; Le Bourhis et al., 2005). Propionic acid can also be found in late blowing cheeses, and its production by *Clostridium* in cheese and milk has been described as strain-dependent (Garde, Arias, Gaya, & Nuñez, 2011; Le Bourhis et al., 2007). In addition, different sugar profiles have been described in Manchego cheeses with different LBD degrees, recording lower levels of lactose and galactose than faultless cheeses (Garde, Ávila, et al., 2012). However, the volatile fraction of cheeses with LBD, other than the mentioned organic acids, remains quite uncharacterized, and so it does the specific contribution of the different *Clostridium* species to the production of cheese off-flavors closely related to LBD. To date, only the volatile fraction of an LBD ewe milk cheese artificially contaminated with spores of *C. beijerinckii* has been investigated (Garde, Ávila, et al., 2011). Hence, here we evaluate the effect of the addition of *C. tyrobutyricum, C. beijerinckii, C. sporogenes* and *C. butyricum* spores, isolated from LBD Manchego cheeses, on LBD, physico-chemical characteristics and volatile profile of ewe milk cheese.

2. Material and methods

2.1. Bacterial strains and propagation

Commercial freeze-dried mesophilic lactic culture Choozit™ MA 16 LYO 25 DCU from Danisco (kindly provided by Larbus S.A., Madrid, Spain), with no gas production, and consisting of limited *Lactococcus lactis* subsp. *lactis* and *cremoris* strains, was used in cheese-making. The packet of MA 16 was resuspended in reconstituted skim milk to achieve a lactococci concentration of 4×10^9 cfu/mL, aliquoted and frozen at −40 °C until use. The day before cheese-making, one aliquot was thawed and used to inoculate at 0.1% reconstituted skim milk and incubated at 30 °C for 18 h.

C. tyrobutyricum INIA 68, *C. beijerinckii* INIA 63 and *C. sporogenes* INIA 71 were isolated from Manchego cheeses with pronounced late blowing defect (Garde, Arias, et al., 2011, Garde, Ávila, et al., 2012; Garde, Gaya, Arias, & Nuñez, 2012). These strains were selected on the basis that they were some of the best candidates to reproduce LBD in trial cheeses because they presented different pulsotypes represented by several isolates which produced high amounts of gas and butyric acid in milk and in Bryant and Burkey broth (containing sodium lactate). *C. tyrobutyricum* CECT 4011 and *C. butyricum* CECT 361 were obtained from the Spanish Type Culture Collection (Colección Española de Cultivos Tipo, CECT, Valencia, Spain). Given the ability of *C. tyrobutyricum* CECT 4011 to cause LBD in a similar type of cheese (Gómez-Torres, Ávila, Gaya, & Garde, 2014) we included this strain as a positive control of development of LBD. All *Clostridium* strains were maintained at −80 °C in Reinforced Clostridial Medium (RCM, Difco, Detroit, USA) with 5% glycerol and subcultured in RCM and incubated at 37 °C for 48 h in anaerobic jars with an H_2 plus CO_2 generating kit (AnaeroGen, Oxoid, Basingstoke, UK) before spore preparation.

2.2. Spore preparation

Spores of all *Clostridium* strains were obtained after incubation at 37 °C for 3 days under anaerobic conditions. Spores of *C. tyrobutyricum* CECT 4011 and INIA 68, and *C. sporogenes* INIA 71 were prepared by inoculation in modified RCM (without agar and sodium acetate), and spores of *C. beijerinckii* INIA 63 and *C. butyricum* CECT 361 were obtained by inoculation in Bryant and Burkey broth (Merck, Darmstadt, Germany) and in BHI (Biolife, Milano, Italy), respectively. After centrifugation (5000× *g*, 15 min, 20 °C), the pellets were washed twice with sterile distilled water and resuspended in reconstituted skimmed milk. Spore suspensions were maintained at −40 °C until their use in cheese manufacture. The spore concentrations in the suspensions were determined, after heat treatment (80 °C, 20 min), on RCM agar (1.5%, w/v) after anaerobic incubation at 37 °C for 3 days. Concentration of spore suspensions ranged from 10^7 to 10^9 spores/mL.

2.3. Cheese manufacture

Cheeses were manufactured from pasteurized (72 °C for 15 s) ewe milk from the Manchega breed in duplicate experiments, carried out on different days. The Manchega herd was fed with home-made feed and vetch hay, and no silage was included in the diet. Milk samples showed mean values of 8.34% total fat, 6.34% total protein, 20.21% dry matter (DM), 5.40×10^4 total bacteria cfu/mL and 1.85×10^2 coliforms cfu/mL. Each experiment consisted of six vats, each containing 1.2 L of milk, which was heated at 32 °C. Calcium chloride and commercial starter MA 16 cultured in skimmed milk were added at 0.01% and 1% (approximately 10^7 cfu/mL), respectively, to all vats. Vats 2-6 were deliberately contaminated with approximately 10^4 spores/mL of *C. tyrobutyricum* CECT 4011 and INIA 68, *C. butyricum* CECT 361, *C. beijerinckii* INIA 63 and *C. sporogenes* INIA 71, respectively. Faultless control cheese from vat 1 was not contaminated with any clostridia. Powdered calf rennet (0.025 g/L milk, 1:150,000 strength, chymosin ≥94%, bovine pepsin ≤6%; Laboratorios Arroyo, Santander, Spain) was added to all vats 20 min later. After 40 min, the curds were cut into 6–8 mm cubes and scalded at 38 °C for 15 min. Whey was drained off through a colander with a cheesecloth, followed by a light hand pressure, and each curd was distributed into one cylindrical mold with holes. The cheeses were pressed overnight at 20 °C and 0.015 kg/cm^2 pressure to drain excess of whey out the holes of the molds. One cheese, of approximately 200 g in weight, was obtained from each vat. After pressing, cheeses were vacuum packed in two multilayer plastic barrier bags of 42 μm (Cryovac HT3050; 200 × 300 mm; permeability to oxygen = 15 $cm^3 \times m^{-2} \times day^{-1} \times bar^{-1}$ at 23 °C and 0% RH, Cryovac Sealed Air Corporation, Milano, Italy) and ripened at 14 °C for 60 d.

2.4. Microbiological determinations

Representative cheese samples (5 g), including both cheese surface and core, were homogenized with 45 mL of a sterile 2% (w/v) sodium citrate solution at 45 °C in a Stomacher 400 (A. J. Seward