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The late blowing defect of hard cheeses: Behaviour of cells and spores of *Clostridium tyrobutyricum* throughout the cheese manufacturing and ripening

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ABSTRACT

The late blowing defect still represents a problem for hard cheeses. Thus, the behaviour of the cheese spoiling bacterium *C. tyrobutyricum* was studied throughout the cheesemaking and ripening of Grana Padano using an innovative approach. Cells and spores, independently sealed within dialysis tubes, were kept in the vat during the entire cheesemaking and then into cheese until 6-month ripening. At each sampling step, morphological changes of cells and spores were monitored by electron microscopy and supported with plate counts. Vegetative cells died during curd cooking and then were no longer cultivable. However, 2×10^2 spores appeared at the end of this stage, likely triggered by the exponential growth phase, and were present until 6-month ripening. In cheese, *C. tyrobutyricum* UC7086 proved to convert free arginine to citrulline and then to ornithine, and to produce γ-aminobutyric acid by glutamate transamination rather than by decarboxylation. Compartmentalization of vegetative cells and spores into dialysis tubes was effective in studying their respective behaviour in a real cheesemaking. This approach allowed to demonstrate that the number of vegetative cells in milk in addition to that of spores should be considered for the eradication of the late blowing defect.

1. Introduction

Hard cheeses are susceptible to defects that may develop during the prolonged ripening. Gas-producing clostridia, gram-positive endospore-forming, anaerobic bacteria, are responsible for the late blowing defect (LBD) (Le Bourhis et al., 2007; Gómez-Torres, Garde, Peirotén, & Ávila, 2015; Bermúdez et al., 2016). When favourable environmental conditions occur in cheese, spores can germinate into vegetative cells that produce acetic acid, butyric acid, carbon dioxide, and hydrogen by the fermentation of lactate (Garde, Ávila, Gaya, Arias, & Nuñez, 2012). The abundant gas causes cracks and holes to form within the cheese, generally in combination with an unpleasant flavour. *C. tyrobutyricum* is considered the principal responsible for LBD in hard cheeses such as Grana Padano (GP) (Bassi, Puglisi, & Cocconcelli, 2015; Cocolin, Innocente, Biasutti, & Comi, 2004; Morandi, Cremonesi, Silvetti, Castiglioni, & Brasca, 2015; Rodriguez & Alatossava, 2010). Many approaches were proposed to prevent LBD in cheese: bactofugation or microfiltration of milk (Elwell & Barbano, 2006), addition of nitrate or lysozyme (Ávila, Gómez-Torres, Hernández, & Garde, 2014), and addition of lactic acid bacteria (LAB) strains biologically active against gram-positive bacteria (Gómez-Torres, Ávila, Gaya, & Garde, 2014; Martínez-Cuesta et al., 2010). However, these methods have technical or legal limitations. The consolidated GP cheesemaking is described in the product specification (European Union, 2011) since this cheese is registered as a Protected Designation of Origin (PDO) cheese (European Union, 2012). The raw milk is partly skimmed by natural creaming to a fat content to 2.1–2.2 g/100 mL. During the 8–10 h of natural creaming, fat globules stably interact with both spores and vegetative cells (D'Incecco, Faoro, Silvetti, Schrader, & Pellegrino, 2015) which are thus removed with the cream (Caplan, Melilli, & Barbano, 2013). After the addition of the natural

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whey starter, milk is rennet coagulated at 33–34 °C. The curd is cut into small granules under gentle stirring and heating up to 53–54 °C. When stirring is stopped, the curd granules deposit at the bottom of the vat to compact under the hot whey for about 1 h. The curd taken from the vat is cut into two portions that are kept in molds for about 48 h. During this period, the fast growth of thermophilic LAB lowers pH to 5.2–5.3. Subsequent steps are brine salting for 20–25 days and ripening for a minimum of 9 months.

Although spores of *C. tyrobutyricum* remaining in milk after creaming likely survive the cheesemaking (Farkye, 2000), no information is available on the behaviour of vegetative cells. The aim of this work was to investigate how cheesemaking conditions of GP impact *C. tyrobutyricum* cell cycle. Using an innovative experimental approach, both vegetative cells and spores of *C. tyrobutyricum* were separately submitted to the whole process and sampled at the most crucial steps, including cheese ripening. Their behaviour was thus directly highlighted using electron microscopy techniques and interpreted with the support of microbiological data. The adopted approach also allowed obtaining information on the capability of *C. tyrobutyricum* to metabolise selected amino acids.

2. Materials and methods

2.1. Bacterial strain and spore production

C. tyrobutyricum strain UC7086, previously isolated from a cheese with LBD and part of the Università Cattolica del Sacro Cuore culture collection, was used. The genome sequence of this strain is deposited at DDBJ/EMBL/GenBank (Bassi et al., 2013). The strain was cultured in Reinforced Clostridial Medium (RCM) (Oxoid, UK) with 1.4 g/100 mL sodium lactate (Merck, Germany) and incubated at 37 °C for 48 h in anaerobic chamber (Don Whitley Scientific, Shipley, UK). Spore suspensions of *C. tyrobutyricum* UC7086 were prepared according to Bassi, Cappa, and Cocconcelli (2009). Purified spore crops were plate counted and stored at 4 °C until use.

2.2. Cheese manufacturing and sampling

Two vats (1000 L milk each) were worked in parallel at a GP dairy, using the usual conditions previously described (D'Incecco et al., 2016), and a total of four cheeses (~40 kg each) were obtained. Aliquots (10 mL) of vegetative cell culture (10^7 CFU/mL whey) and of spore suspension (10^8 CFU/mL water) of *C. tyrobutyricum* UC7086 were separately put into Spectra/Por® 6 dialysis tubes (50,000 Da MWCO, 28 mm flat width, 2.5 mL/cm volume/length, Spectrum Laboratories Inc. CA, US) that were carefully sealed and differently labelled by colour bands to allow identification. Seven cell-containing tubes (C-tubes) and seven spore-containing tubes (S-tubes) were kept suspended into each vat during the cheesemaking. When stirring was interrupted, after curd cutting and cooking, the tubes were deposited at the bottom of the vat, where the curd grains were aggregating and compacting. Overall, one C-tube and one S-tube were taken at the following steps of processing: rennet addition (RE) (t = 12 min); end of curd cooking at 54 °C (EC) (t = +20 min); curd extraction from vat (CX) (t = +30 min); end of curd acidification in mould (EA) (t = +48 h); end of brine salting (ES) (t = +18 d); after 3-month (3C) and after 6-month ripening (6C). The sampling steps EA, ES, 3C and 6C implied the destruction of one cheese each to take the tubes out. Sampled tubes were all processed in the same way. Briefly, the tube content was recovered with distilled water to a volume of 10 mL and divided into four portions destined to: (i) plate counts, (ii) scanning electron microscopy (SEM), (iii) transmission electron microscopy (TEM), and (iv) free amino acid (FAA) analysis.

2.3. Plate counts

The tube contents were preliminary diluted with physiological solution and plated on RCM agar medium (Oxoid Ltd., Wade Road, Basingstake, Harnpshire, Engl.) with the addition of 0.005 g/100 mL of neutral red solution and 200 mg/L of D-cycloserine (Jonsson, 1990) for selectively enumerating yellow colonies of *C. tyrobutyricum* vegetative cells. Spores were counted on the same medium after a treatment at 80 °C for 10 min. All plates were incubated at 37 °C for 48 h in anaerobic conditions. Counts were carried out in duplicate.

2.4. Scanning and transmission electron microscopy

Samples for SEM were prepared as follows: 1 mL of tube content was centrifuged, the pellet was recovered with physiological solution and 5 μL fixed on a positively charged nylon membrane (Roche Diagnostics GmbH Germany). The membrane was then dehydrated in an ethanol series (75 mL/100 mL, 85 mL/100 mL, 95 mL/100 mL and 100 mL/100 mL) at room temperature. Critical point drying was performed in a Baltec CPD030 dryer. Specimens were coated with gold by sputtering (Balzer Union Med 010) and analysed with a Quanta SEM microscope ESEM™ technology (FEI, Oregon, USA) under both low (130 Pa) and high (7×10^{-5} Pa) vacuum conditions.

Samples for TEM were prepared fixing 1 mL of tube content as described by D'Incecco et al. (2015). Ultrathin (50–60 nm) sections of resin inclusions were stained with uranyl acetate and lead citrate and examined with a Philips E208 microscope (Aachen, Germany).

2.5. Free amino acid analysis by ion exchange chromatography

The pattern of free amino acids (FAA) was determined as described by Hogenboom, D'Incecco, Fuselli, and Pellegrino (2017). Briefly, solid samples (curd, cheese) were solubilized with sodium citrate buffer, homogenized, and deproteinized with sulfosalicylic acid. The obtained extracts as well as the liquid samples (milk, supernatant of tube material) were diluted using an equal volume of lithium citrate buffer at pH 2.2, filtered and analysed by IEC. A Biochrom 30plus (Biochrom Ltd, Cambridge, UK) amino acid analyser was used. Analyses were performed in triplicate.

2.6. Arginine utilization by C. tyrobutyricum in milk

Tubes containing 20 mL of (a) sterilized milk and (b) sterilized milk added with arginine (0.5 g/L) and lactate (14 g/L) were both inoculated with 1.2×10^2 CFU of logarithmic phase cells of *C. tyrobutyricum* UC7086 and incubated for 10 days at 37 °C in anaerobic conditions. Samples were then analysed for bacterial counts and FAA as above described. Duplicate incubations were performed and analysed in duplicate.

2.7. Statistical analysis

Statistical treatment of data was performed by means of SPSS Win 12.0 program (SPSS Inc., Chicago, IL, USA). A *t-test* was used to analyse the mean values among FAA. A $P < 0.05$ was assumed as significance limit, unless differently indicated.

3. Results and discussion

With the aim of studying *C. tyrobutyricum* behaviour during the whole cheese manufacturing process, we have set up an innovative experimental approach. Vegetative cells and spores of *C. tyrobutyricum* were separately confined into dialysis tubes that