LWT - Food Science and Technology 92 (2018) 32–39

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Growth factors affecting gas production and reduction potential of vegetative cell and spore inocula of dairy-related *Clostridium* species

Tiziana Silvetti, Stefano Morandi*, Milena Brasca

Institute of Sciences of Food Production (ISPA), National Research Council (CNR), 20133, Milan, Italy

ARTICLE INFO

Keywords:
Clostridium
Spore
Vegetative form
Redox potential
Cheese blowing
Cheese ripening

ABSTRACT

Cheese late blowing defect caused by the *Clostridium* growth results in flavour and texture flaws and huge economical losses. The aim of this study was to evaluate simultaneously the influence of pH (6.50, 5.75, 5.00), NaCl (0, 1, 2, 4%) and temperature (10, 12, 15, 20, 37 °C) on gas production and reduction potential (E_h) of vegetative cell and spore inocula of dairy related clostridia (*Clostridium beijerinckii, C. butyricum, C. sporogenes* and *C. tyrobutyricum*). Clostridia showed a high reduction capacity in milk ($E_{h7min} < -280$ mV in 24 h). Spores exposed to harsh environment generated cells less sensitive to adverse conditions. A temperature ≤15 °C was effective in preventing the gas formation by vegetative cells for over 70 days, but a combination of temperature ≤15 °C, pH ≤ 5.00 and osmotic stress (NaCl 2%) or a temperature ≤ 10 °C was necessary to avoid spores outgrowth and gas production.

Gas production resulting from *C. tyrobutyricum* spore germination and growth was not affected by acidity and salt concentration at 20 °C. Some strains of *C. sporogenes* were capable of producing gas even at 12 °C, pH 5.00 and 1% of NaCl. A ripening temperature below 10 °C represents a potential strategy to prevent LBD occurrence.

1. Introduction

The genus *Clostridium* consists of a diverse group of obligately anaerobic, Gram-positive, endospore-forming microorganisms, which are distributed in different environments such as soil, water, manure, silage, forage and raw milk (Doyle et al., 2015).

The clostridial spores may survive milk pasteurization and cheese-making process and can germinate into vegetative cells during the ripening period, giving late blowing defect (LBD). Late blowing defect appears when butyric acid fermentation takes place during cheese ripening as a consequence of metabolism of butyric acid bacteria such as *Clostridium butyricum*, *C. tyrobutyricum* and *C. beijerinckii*. This metabolism includes the fermentation of lactic acid, produced by lactic acid bacteria during the cheese-making process, and the production of butyric acid, acetate, CO_2 and H_2. This defect may also be caused by *C. sporogenes*, which can produce gas due to proteolysis and degradation of amino acids via the Stickland reaction (Doyle et al., 2015). The pressure of involved gases causes cracks and splits, generally accompanied by unpleasant aroma and rancid flavour, especially in hard and semi-hard cheeses (Cremonesi, Vanoni, Silvetti, Morandi, & Brasca, 2012; Garde, Arias, Gaya, & Nuñez, 2011; Le Bourhis et al., 2007).

These defects are due to the ubiquitous presence and resistant nature of *Clostridium* spores that can cause LBD also at low concentrations if cheese conditions are suitable for their germination and growth. *Clostridium tyrobutyricum* is considered the primary cause of LBD, but *C. beijerinckii*, *C. butyricum* and *C. sporogenes* contribute to this defect in cheese, too (Cremonesi et al., 2012; Garde, Arias et al., 2011; Klijn, Nieuwenhof, Hoolwerf, van derWaals, & Weerkamp, 1995; Le Bourhis et al., 2007). Although LBD is an old problem, very well known by cheese manufacturers, to eradicate it is still a difficult issue. The spores in milk can be reduced at dairy farm level (Zucali et al., 2015) or by physical treatment (bactofugation or microfiltration), while the spore germination can be prevented using some additives (Brändle, Domig, & Kneifel, 2016). Centrifugation or microfiltration of the cheese milk can reduce the spore load by up to 99%, but at the present time, physical treatments are not permitted for the production of many Protected Designation of Origin (PDO) cheeses, while the addition of nitrate, lysozyme, bacteriocin or bacteriocin producer bacteria in milk can inhibit the spore germination of *C. tyrobutyricum* (Ávila, Gómez-Torres, Delgado, Gaya, & Garde, 2017; Brändle et al., 2016; Ávila, Gómez-Torres, Hernández, & Garde, 2014; Lodi, 1990). Germination and growth of *Clostridium* spores is also affected by intrinsic and extrinsic factors such as pH, salt concentration and temperature. However, the potential of these parameters to prevent LBD in cheese has not been extensively investigated (Spolaor, Andrighetto, Lombardi, Brasca, & Morandi, 2014). Moreover, the influences of stress factors on the

* Corresponding author.
E-mail address: stefano.morandi@ispa.cnr.it (S. Morandi).

https://doi.org/10.1016/j.lwt.2018.02.014
Received 9 October 2017; Received in revised form 1 February 2018; Accepted 7 February 2018
Available online 10 February 2018
0023-6438/

metabolic activity of *Clostridium* species implicated in LBD have not been examined in-depth, and the few reports available are focused on the single effect of pH, salt concentration or temperature exclusively on the *C. tyrobutyricum* growth (Ruusunen, Surakka, Korkeala, & Lindström, 2012). Recent studies suggest that not only clostridial spores, but also vegetative cells originally present in the raw milk can contribute to the origin of LBD in cheeses (D'Incecco, Pellegrino, Hogenboom, Cocconcelli, & Bassi, 2018), and Ávila et al. (2014) highlighted a different sensibility of clostridial spores and vegetative cells to antimicrobial compounds; thus it would be newsworthy to delve into their behaviour of both clostridial forms under conditions resembling the cheese ripening.

Clostridia growth requires an anaerobic and reduced atmosphere. Oxidation-reduction potential (E_h) is an important physicochemical parameter that, together with pH and temperature, determines the microenvironment and the types of microorganisms that can grow during cheese ripening. The change of E_h, from positive to negative values during the cheese-making, reflects the formation of an anaerobic system in which only facultatively or obligately anaerobic microorganisms can grow (Abraham, Cachon, Colas, Feron, & De Coninck, 2007). Different studies have shown that lactic acid bacteria have the capacity to reduce their environmental E_h (Brasca, Morandi, Lodi, & Tamburini, 2007; Morandi, Silvetti, Tamburini, & Brasca, 2016), but few information is available on the reducing activity of dairy-related *Clostridium* strains (Langeveld & Galesloot, 1971).

The aims of this work were to investigate the combined effects of temperature, pH and NaCl concentration on the reduction potential and gas production of dairy-related *Clostridium* vegetative cells and spore inocula, under conditions resembling the ripening of cheeses involved in late blowing defects.

2. Materials and methods

2.1. Experimental design

A first step was performed to evaluate the influence of the combined effect of temperature, salt concentration and pH on the activities (redox potential and gas production) of vegetative cell and spore inocula of four *Clostridium* type strains. The effectiveness of these stress factors were monitored in two different media: enriched milk (EM) and Reinforced Clostridial Medium (RCM) broth (Oxoid, Milan, Italy). The values of temperature (10, 12, 15, 20 and 37 °C), salt concentration (0, 1, 2 and 4% of NaCl) and pH (5.00, 5.75 and 6.50) were chosen considering the ripening condition of semi-hard and hard cheeses susceptible to LBD (Table 1). RCM broth was tested only at 37 °C, since this medium and this temperature are generally applied for the most probable number analysis (Garde, Arias et al., 2011).

In a second step, the environmental inhibitory conditions preliminary established on the four type strains (pH 5.75–5.00; NaCl 0, 1, 2 and 4%; 10, 12, 15 and 20 °C) were further studied up to 70 days of incubation on vegetative cell and spore inocula of 25 *Clostridium* strains isolated from cheeses affected by LBD (2 *C. beijerinckii,* 14 *C. sporogenes* and 9 *C. tyrobutyricum*).

The combined effect of temperature, pH and salt concentration was evaluated performing the entire experimental design in duplicate.

2.2. Bacterial strains

Type strains used in this study were *C. beijerinckii* DSM 791^T, *C. butyricum* DSM 10702^T from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig (Germany), *C. sporogenes* ATCC 3584^T provided by the American Type Culture Collection (U.S.A.) and *C. tyrobutyricum* IN15b from the Institute of Sciences of Food Production collection (ISPA-CNR, Milan, Italy) (Morandi, Cremonesi, Silvetti, Castiglioni, & Brasca, 2015). A total of 25 *Clostridium* strains (2 *C. beijerinckii*, 14 *C. sporogenes* and 9 *C. tyrobutyricum*), isolated from different Italian cheeses with pronounced LBD, previously identified as reported by Cremonesi et al. (2012) were also used.

Before each experiment *Clostridium* strains were grown in RCM broth and incubated at 37 °C for 48 h in jars with an anaerobic incubation system (Anaerocult A, Merck Millipore, Darmstadt, Germany). Concentration of vegetative cells, determined on RCM agar incubated anaerobically at 37 °C for 72 h, ranged from 10^6 to 10^7 CFU/mL.

2.3. Preparation of spore suspensions

Clostridium spores were prepared according to Ávila et al. (2014). Spore suspensions were obtained by spread-plating 48 h clostridial cultures on RCM agar, followed by incubation until 80–90% sporulation was observed by phase contrast microscopy (7–15 days later). Prior to their use in the experiments, spores were heat-shocked at 80 °C for 10 min to kill the possible vegetative bacterial cells and to promote the spore germination. After heating, the spore concentration was determined on RCM agar incubated anaerobically at 37 °C for 72 h. Concentration of spore suspensions ranged from 10^5 to 10^7 spores/mL.

2.4. Growth media

The growth media used in this study were enriched milk (EM) and RCM broth. EM was obtained from reconstituted skim milk (10% w/v) (Sacco srl, Cadorago, Italy) supplemented with yeast extract (1%) (Formedium, Hunstanton, UK), sodium lactate (3.36%) (Merck KGaA, Darmstadt, Germany), sodium acetate (1%) (Carlo Erba, Cornaredo, Italy), cysteine (0.2%) (Sigma-Aldrich, St. Louis, MO). In this study we used EM because milk in itself is not an ideal substrate for supporting growth of all *Clostridium* strains, since some clostridial species are not able to ferment lactose (*C. butyricum* and *C. tyrobutyricum*) and for this reason they do not grow in milk unless their appropriate carbon sources

Table 1
Ripening conditions, pH and NaCl content in European hard and semi-hard cheeses involved in late blowing defect.

Cheese	Countries	Firmness	Ripening temperature (C°)	Ripening time (months)	pH	NaCl (%)
Beaufort	FR	hard	10–15	6–14	5.4	1.6–1.8
Cheddar	UK	hard	7–15	6–24	5.5	1.5
Comtè	FR	semi hard	14–19	4–18	5.8	1.0
Edam	NL	semi hard	12–14	2–12	5.7	2.0
Emmentaler	CH	hard	11–14	4–12	5.1–5.3	0.7
Grana Padano	IT	hard	15–22	9–20	5.7	1.6
Gouda	NL	semi hard	10–17	1–36	5.8	2.0
Kasseri	GR	semi hard	15–18	3–12	5.2–5.4	2.2
Noord-Hollandse Gouda	NL	semi hard	14	1–18	5.2	3.3–3.6
Parmigiano Reggiano	IT	hard	≥16	12–30	5.3	1.4
Queso Manchego	ES	hard	3–16	1–24	4.8–5.8	2.3

Data for ripening conditions were taken from: Commission Regulation (EC) No 129/2012 (2012); Anastasiou et al. (2009); Mucchetti and Neviani (2006); Guinee and Fox (2004); Mout (2004); Delacroix-Buchet and Marie (1994); Berdagué, Jeunet, Grappin, and Duboz (1987).