Late blowing defect in *Grottone* cheese: detection of clostridia and control strategies

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Abstract

"Grottone" is a pasta filata hard cheese produced in Campania region from cow's milk and characterized by holes formation due to CO_2 development by Propionic Acid Bacteria. The contamination of raw milk with butyric acid-producing spore-forming clostridia represent a major concern for cheese producers since clostridia outgrowth may lead to the cheese late blowing defect during ripening. Detection of clostridial endospores in milk before processing and the use of antimicrobial compounds may represent an important control strategy. The present study is aimed to point out the most suitable procedure for the determination of clostridial spores in dairy samples, and to assess the inhibitory activity of several antimicrobial compounds against *Cl. sporogenes*. Based on results, MPN counts on Bryant and Burkey medium and CFU on RCM proved to be the most suitable protocols for routine testing. By using these procedures clostridial spores were detected in 10 out 13 milk samples and in all cheeses with late blowing defect. Within antimicrobial compounds, sodium nitrate is still the best choice for preventing late blowing, nevertheless a protective culture of *Lacticaseibacillus casei* proved to be a promising alternative. Nevertheless, the use of this protective culture in six Grottone cheese productions carried out at farm level, led to unsatisfactory results. Holes' development was hampered likely for an inhibition of the PAB starter and the expected 'Grouviera-type' taste was not perceived by panellists. Based on results, the use of protective cultures needs to be contextualized and interactions with starters needs to be evaluated case by case.

Introduction

"Grottone" is a pasta filata hard cheese ripened for at least two months, traditionally produced in the Matese Regional Park (Campania region, Southern Italy) from cow's milk and with the addition of salt, rennet, lactic, and propionic ferments. The cheese is characterized by holes formation due to CO_2 development by lactate metabolism of Propionic Acid Bacteria (PAB). Actually, "Grottone cheese" might be defined as a medley between a traditional Caciocavallo from Campania and a cheese with holes such as Emmenthal. After pasteurization at 68-72°C for 20 min, milk is added of the two starter cultures for direct vat use. Lactic Acid Bacteria (LAB), namely *Streptococcus* (*St.*) *thermophilus* and *Lactobacillus* (*Lb.*) *helveticus*, are responsible for the curd acidification, while *Propionibacterium freudenreichii* ssp. *shermanii* for the holes' formation during ripening. After 60-80 min, liquid rennet (Maxi Ren 180) is added (17 mL/100 L) and curd formation takes place in around 20-22 min. After further 30 minutes, curd is subject to a first cutting, which is followed, 20 minutes later, by a second one up to hazelnut size. Maturation is supposed to be completed when pH drops to 5.20 and curd may be stretched in water at 80-90°C. After salting in brine (30 min/Kg), cheeses (around 5 Kg each) are left ripening at about 10°C, 80-85% relative humidity, for at least two months.

Due to the specific production technology, the contamination of raw milk with spore-forming clostridia represents a major concern for cheese producers since conditions supporting PAB development during ripening may favour clostridia as well. As widely known, clostridial spores germination and outgrowth during the ripening may lead to an undesirable butyric acid fermentation (BAF) responsible for late blowing defect (LBD) in cheeses (Garde *et al*., 2020; Podrzaj *et al*., 2020). LBD is characterized by holes, cracks as well as an unpleasant flavour due to butyric acid development as well as protein catabolism. LBD is mainly caused by *Clostridium* (*Cl.*) *tyrobutyricum* capable of fermenting lactic acid with production of butyric acid, acetic acid, carbon dioxide, and hydrogen, but *Cl. butiricum*, *Cl. sporogenes*, and *Cl. beijerinckii* are often implicated too (Cocolin *et al*., 2004). Clostridial spores in raw milk, which commonly originate from silage, can survive to the pasteurization becoming, after germination, the dominant viable microflora in cheese (Borreani *et al*., 2019; Julien *et al*., 2008). Good farming practices are essential to prevent microbial contamination of milk (Reindl *et al*., 2014), but may be often not enough to manage the problem. Control strategies to prevent BAF involve the use of physical treatments, such as bactofugation or microfiltration before processing, but such methods, although reliable, require specific equipment and costly investments (Cosentino *et al*., 2013).

The growth of *Clostridium* species in cheese is influenced by the lactic acid and salt content, as well as by pH, fat content, ripening conditions, temperature, and presence of antimicrobial compounds (Goudkov & Sharpe, 1966). Regarding antimicrobial compounds, the European regulation 1333/08 and any subsequent amendment authorizes the addition of nitrate (E251 and E252) at a maximum amount of 150 mg/kg (expressed as sodium nitrite) and lysozyme (E1105) at "*quantum satis*". Without the addition of these additives to cheese, the level of spore-forming clostridia may become critical; however, the possible health risks associated with nitrates and nitrites, *i.e.* formation of carcinogenic nitrosamines and also with lysozyme, *i.e.* adverse allergic reactions, create concern

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among the consumers and, therefore food business operators are searching for a reliable alternative (Reindl *et al*., 2014; Senyk *et al*., 1989).

BAF control can be also performed by enumerating clostridial spores in raw milk to be processed, but reliable methods for spores quantification are still needed (Reindl *et al*., 2014). Therefore, the aims of the present work were: i) to compare the commonly used approaches for spore-forming clostridia detection of in dairy samples and to point out the most suitable procedure; ii) to evaluate the occurrence of *Clostridium* spp. spores in milk and to trace a correlation with LBD occurrence in Grottone cheese samples, and iii) to assess the inhibitory activity of several antimicrobial compounds, *i.e.* nisin, lysozyme, nitrite, and of a protective culture of *Lacticaseibacillus* (*Lb.*) *casei* against *Cl. sporogenes*.

Materials and Methods

Evaluation of methods for clostridial spores' enumeration in milk and dairy samples

For the initial screening, 13 samples – 11 milk, one curd, and one brine samples - were collected in a dairy plant - Iaquilat Trade srl - located in the province of Benevento (Southern Italy). Samples were transported at 4°C to the laboratory and processed within few hours. Samples were analysed according to the following routine procedures for spore-forming clostridia: MPN method in Bryant and Burkey medium (BBB - Bryant & Burkey, 1956) according to the procedure detailed by Garde *et al*. (2011); MPN in Skin Milk (Oxoid, Basingstoke, UK), modified according to Cremonesi *et al*. (2012); MPN in modified Reinforced Clostridial Medium (mRCM - Hirsch e Grinstead, 1954) and in Tryptone Sulphite Neomycin Agar (TSN - Mossel, 1959). The principle of TSN relies on the ability of sulphite-reducing anaerobic bacteria to reduce sulphites to sulphide which, in the presence of ferric citrate, forms a black precipitate of iron sulphide (black colonies). Neomycin, polymyxin and sulphide render the medium inhibitor with regard to secondary flora. Similarly, RCM for MPN was made differential for sulphite-reducing clostridia by the addition of sodium sulphite and ferric citrate. Additionally, the 13 samples were analysed by a CFU method: plating on Reinforced Clostridial Medium (RCM) coupled to incubation in anaerobic jars with an H_2 plus CO_2 generating kit (AnaeroGen, Oxoid). As further test, *Cl. sporogenes* ATCC 1143 after 16 h of incubation in RCM (Oxoid) was analysed with all protocols, except the one using skim milk proposed by Cremonesi and coworkers (2012). The concentrations of vegetative cells were determined by direct counting at microscope in Petroff-Hausser chamber as well.

Subsequently, 24 samples – 13 milk samples and 11 Grottone cheeses with LBD - were collected in the same farm and analysed according to two selected protocols: MPN in BBB medium and CFU on RCM plates. Pure cultures of *Cl. sporogenes* ATCC1143 were used as control. Cheese samples (10 g) were homogenized with 90 mL of sterile 2% (w/v) sodium citrate solution at 45°C in a Colworth Stomacher 400 (A.J. Seward Ltd, London, UK) and subject to decimal serial dilutions in sterile physiological solution (NaCl 0,9%).

Inhibitory activity of nisin, lysozyme, nitrite and *Lb. casei* against *Cl. sporogenes*

All tests were carried out by using the strain *Cl. sporogenes* ATCC 1143 as indicator. Strain was routinely cultured in RCM (Oxoid). *Lb. casei* Lyofast LC 4P1 was obtained by Sacco System (Como, Italy). The strain is traded as bioprotective culture against LBD in cheese. For the trial, LC 4P1 was cultured in MRS broth (Oxoid) at 30°C for 48 h. After centrifugation at 14.000 rpm for 15 min, the filter-sterilized (0,22 µm) supernatant was stored at −40°C until use.

Tests were carried out according to the protocol proposed by Ávila *et al*. (2014). Basic media for MIC analysis were Litmus milk (LM - Oxoid) and RCM without cysteine hydrochloride (RCM1) that could enhance the inhibitory effect of nitrite (Johnston & Loynes, 1971). Stock solutions of nisin (Sigma, St. Louis, Mo., USA; 2,5% pure nisin, potency of 10^6 IU/g) were prepared at concentrations of 0,25 g/L, in 0,02 N HCl (for MIC assays in RCM) or in distilled water (for MIC assays in LM). Lysozyme (Fluka Biochemica, Buchs, Switzerland ~70,000 U/mg) was dissolved in distilled water to yield a stock solution of 8 g/L (5.6×10^5 U/mL). Sodium nitrite (Sigma) stock solution of 40 g/L was also prepared. All solutions were filter-sterilized (0,22 µm) and stored at −40°C until use. The MIC of *Lb. casei* supernatant, nisin, lysozyme, and sodium nitrite were determined as the lowest concentration that showed a complete inhibition of the growth of the assayed *Clostridium* strain. Serial two-fold dilutions of the antimicrobial compounds were prepared in distilled water to obtain a final concentration range of 250 to 0,489 µg/mL for pure nisin, 8.000 to 15,63 µg/mL for lysozyme, and 40 to 0,078 mg/mL for sodium nitrite. Then, 150 µL of each dilution were added into wells of a sterile 96-well microplates with lid. The clostridial strain was grown overnight and diluted to approximately 10^6 vegetative cells/mL in double strength RCM broth or LM, and then 150 µL were pipetted into the wells containing the antimicrobial compounds, in duplicate. Positive clostridia growth controls were prepared without any antimicrobial compound. Negative controls were prepared without *Cl. sporogenes* inoculation. Microplates were incubated at 37°C under anaerobic conditions for 7 days and bacterial growth was confirmed visually. Wells showing absence of growth in RCM and LM by comparing to negative control wells were interpreted as negative. Experiments were performed in duplicate.

Inhibitory activity of commercial lysozyme preparations

To evaluate if different commercially available lysozymes may differ in antimicrobial activity, two different formulations (A and T) were compared to a lysozyme of analytical grade standard purchased from Sigma-Aldrich (S). Three different concentrations - 0,5, 0,33, and 0,25 mg/mL – were tested against *Cl. sporogenes* ATCC1143 and *Staphylococcus xylosus* (*St*.) DSM 20267. Broth cultures of *Cl. sporogenes* and *St. xylosus* in RCM or BHI (Oxoid) respectively, were centrifuged and pellets were resuspended in sterile saline to obtain a final concentration of about 10^3-10^4 CFU. Cell suspensions were added of lysozyme and incubated at 37°C up to 120 h in anaerobic (*Cl. sporogenes*) or aerobic (*St. xylosus*) conditions. Bacterial loads at 24, 72 and 120 h were assessed by drop method (Collins, 1989) onto RCM and Mannitol Salt Agar (Oxoid), for clostridia and staphylococci, respectively. In a second set of trials, cells collected by centrifugation of *Cl. sporogenes* or *St. xylosus* were resuspended in RCM and BHI (Oxoid), respectively. After lysozyme adding (10, 5, 2,5 and 1,25 mg/mL), cultures were incubated for 72 h and counted as above described.

Use of Lb. casei Lyofast LC 4P1 in 'Grottone' cheese manufacturing

Grottone cheese manufactures were carried out at Iaquilat Trade srl on 200 L milk batches. Experiments were performed in parallel by using the same milk. A total of nine combinations were tested. Since only three vats could be used, experiments were carried across three consecutive weeks during spring 2018. In the three set of trials, one vat was used to produce Grottone cheese according to the routine process adopted by the producer and served as con-

trol (C1, C2 and C3). In detail, both LAB and PAB starters – CSL and HD 094 - were supplied by Mofin Alce Group, (Novara, Italy). In the six trials supplemented with the *Lb. casei* LC 4P1 culture, LAB and PAB were provided by Sacco System as well. In detail, Lyofast YHL 094F containing *Streptococcus thermophilus*, *Lactobacillus helveticus, Lactobacillus delbrueckii* subsp. *bulgaricus*, and subsp. *lactis* was used for lactic fermentation, while Lyofast PB 1 was used to inoculate *Propionibacterium freudenreichii* ssp. *shermanii*. In one batch, the protective culture was added at the same time of the starters (A), while in the remaining five manufacturing, it was added in a batch of milk for a pre-maturation. The percentage of pre-fermented milk was 10 (B), 36 (C), 50 (D), 70 (E) and 100% (F). The pre-maturation was followed by pH measurement up to get an initial pH in the vat of about 6,5, so its length was shorter as higher was the inoculum. In the case of 10% it lasted around 3 h, while when the entire batch was pre-fermented, it was 20 min. After brining, five Grottone cheeses for each manufacture were randomly marked and cut in half after two months of ripening. The appearance of the cheese in terms of holes presence, size, and distribution, was analysed. Samples were sensory evaluated by ten well-trained panellists selected within company staff. Judges were asked to express a value judgment 'yes or no' on only two points: LBD symptoms and presence of the expected sensory features.

Results and Discussion

Comparison of protocols for Clostridia detection

The contamination of raw milk with spores of butyric acid-producing clostridia represents a major concern for cheese producers since spores' germination and clostridia outgrowth may lead to the LBD in cheese. When LBD occurs, economic losses are already inevitable therefore the determination of clostridial endospores in milk before processing is of great importance (Brändle *et al*., 2016). Indeed, in case of not satisfying results, cheese producers may use contaminated milk for other purposes. Detection of clostridial endospores through molecular methods, although reliable, is still too expensive for cheese producers (Brändle *et al*., 2018), so conventional counting protocols remain the only way forward.

In the present work, 13 different dairy samples were analysed by five different counting methods. Among them, the Bryant and Burkey broth proposed in 1956 resulted to be the most successful medium enabling the detection of the highest level of clostridial spores (Table 1), whilst all other methods proved to be bias by several issues. MPN in BBB medium is quite time consuming but the resazurine toning depending on redox potential, coupled to the lifting of the paraffin septum, make results' interpretation extremely easy (Brändle *et al*., 2016). According to data, only two milk samples out of 13 – C and E – proved to be contaminated by clostridial spores (Table 1).

Even in the MPN method proposed by Cremonesi *et al*. (2012), tubes are covered by paraffin seals, but in this case, the base medium is skim milk. The turbidity of the milk combined to its coagulation due to thermal treatments and to microbial growth greatly jeopardizes the data collection. As matter of fact, no certain result could be retrieved (Data not shown).

mRCM proved to be poorly selective allowing the growth of other anaerobe and facultative anaerobe microorganisms, even if it was made more selective by the supplementation of iron citrate and sodium sulphite (Gibbs & Freame, 1965). As shown in Table 1, growth occurred in almost all samples, but in no case, it was possible to observe blackening around colonies. TSN use is a MPN method in tube with agarized medium as well. It is usually adopted for *Cl. perfringens* detection and may highlight the presence of clostridia through the appearance of a black halo surrounding colonies due to iron-ammonium citrate precipitation. Also, in this case, growth occurred, despite the neomicin selectivity, but no blackening could be noticed (Table 1).

Due to ease of use, CFU counts on RCM is the most popular method. As expected, colonies could be counted in almost all samples. Indeed, according to suppliers, *Clostridium* spp. presence should be verified by subculturing on Columbia Agar plates, eventually followed by other confirmatory biochemical testing. In such light, this method would become even more time-consuming than MPN in BBB medium.

As general consideration, more than one discrepancy was recorded by comparing the MPN counts on BBB medium and CFU on RCM: the populations level on the two media was never comparable (Table 1). To obtain further indications about the five methods, a culture of *Cl. sporogenes* ATCC 1143 after 16 h of incubation was analysed with all protocols, except the one using skim milk (Cremonesi *et al*., 2012). Cells counted at microscope were $7.3 \pm 0.8 \times 10^8$. Microbial levels detected by MPN were 4.5×10^7 cell/mL for both mRCM and BBB (Data not shown), but without blackened colonies in mRCM, while in all BBB positive tubes the lifting of the paraffin seal was recorded. BBB thus allowed counting the bacterium, but the level of detection was one Log lower than that recorded by microscope count. On TSN growth occurred up to 10^7 cell/mL, but even in this case, no blackening occurred. Finally, on RCM, presumptive *Clostridium* loads were around 9×10^7 CFU/mL (data not shown). In other words, MPN in BBB tubes and CFU on RCM agar plates provided around the same values, but, in both cases, one Log lower than that retrieved by spores counting at the microscope, an approach that is well known to lead to a viable cells' over-estimation (D'incecco, 2017).

Table 1. Dairy samples analysed for clostridia detection according to four protocols: MPN in Bryant and Burkey medium (BBB), in modified Reinforced Clostridial Medium (mRCM), and in Tryptone Sulphite Neomycin Agar (TSN), plus CFU on Reinforced Clostridial Medium (RCM).

Method		Curd	Brine	Milk										
				M1	A	B	C	D	E	F	G	H	I	N
BBB (cell/mL or g)	Growth	<10	0	25	250	250	45	250	25	9	9	25	45	250
	Gas	<10	0	0	0	0	45	0	3	0	0	0	0	0
mRCM (cell/mL or g)	Growth	<10	0	25	25	25	950	25	25	25	25	25	45	45
	Blackening	<10	0	0	0	0	0	0	0	0	0	0	0	0
TSN (cell/mL or g)	Growth	<10	0	25	25	25	25	25	250	25	250	25	25	21000
	Blackening	<10	0	0	0	0	0	0	0	0	0	0	0	0
RCM (CFU/mL or g)	Growth	<10	<10	$>10^3$	<10	3.00×10	1.10×10^2	$>10^3$	$>10^3$	$>10^3$	$>10^3$	1.10×10^5	1.28×10^5	2.05×10^5

Recovery of spore-forming clostridia in milk and cheese samples

Based on results, two procedures – namely MPN counts in BBB medium and CFU on RCM - were adopted to analyse a total of 13 milk samples and 11 Grottone cheese with LBD. Clostridial spores were detected in 10 out of 13 milk samples (Table 2). MPN spores' counts in contaminated milk samples ranged from 0,3 to 95 cell/mL (mean MPN counts = 10,78 cell/mL) (Table 2). Even though the spores' count in milk samples was low, it has been previously reported that even very low amounts of clostridial spores may however lead to pronounced LBD in cheeses (Burtscher *et al*., 2020). Moreover, the onset of LBD does not only depend on the spores count itself, but also on the environmental conditions in cheese allowing spores' germination (Burtscher *et al*., 2020). In the present study, clostridial spores were detected in all cheeses with LBD (Table 3). However, the observed levels of spores were lower than those reported by Garde *et al*. (2011) and Dasgupta and Hull (1989) in Manchego and Swiss-type cheeses with LBD. With reference to milk samples, results obtained with the two approaches were quite coherent, except for two samples of raw bulk milk and one milk collected from one producer (Data not shown). In the latter samples, according to MPN no clostridia were detectable, while on RCM few colonies could be counted (Table 2). In cheese, microbial loads recorded with the two approaches were rather different (Table 3). Discrepancies between the two procedures, however, are not surprising since the BBB medium undergoes initial laboratory pasteurization which destroys the vegetative cells (Bryant & Burkey, 1956). Moreover, in sample C7, no growth was recorded in tube, although the cheese was, like every other, affected by LBD (Table 3).

Inhibitory effect of nisin, lysozyme, sodium nitrite and Lb. casei Lyofast LC 4P1

The MICs of nisin, lysozyme, sodium nitrite and *Lb. casei* Lyofast LC 4P1 were assessed against *Cl. sporogenes* ATCC 1143. The effectiveness of the four antimicrobial compounds varied with the culture media used. Even at the maximum tested concentration (8000 µg/mL), lysozyme did not inhibit the growth of *Cl. sporogenes* in LM, whereas, in RCM1 a variable effect was observed at concentration equal or higher than 1.000 µg/mL (Data not shown). According to Ávila *et al*. (2014), lysozyme at the concentration usually employed in cheese factories (400 µg/mL), does not inhibit the growth of *Cl. sporogenes*.

The minimal concentration of sodium nitrite required for *Cl. sporogenes* ATCC 1143 inhibition in LM (20 mg/L) was higher than that required in RCM1 (10 mg/mL), while nisin did not inhibit the growth of *Cl. sporogenes* either in RCM1 or in LM (Data not shown). Results are in agreement with those reported by Ávila *et al*. (2014) for sodium nitrite, but not for nisin. According to authors, all tested clostridia strains were sensitive to this latter antimicrobial compound, even if MICs were quite variable. Actually, *Clostridium* sensitivity to nisin has already been proved to be strain-specific (Hofstetter *et al*., 2013).

The inhibitory effect of *Lb. casei* Lyofast LC 4P1 was evaluated since strains of this species may exhibit an antagonistic effect towards microorganisms such as *Cl. sporogenes* (Zavisic *et al*., 2012). In RCM1, the supernatant of *Lb. casei* Lyofast LC 4P1 prevented the growth of *Cl. sporogenes* even after a dilution 1/2, although a slight inhibition was observed up to a concentration reduced at 1/8. On the other hand, no activity was observed when LM medium was used (Data not shown).

Inhibitory activity of different commercial lysozymes

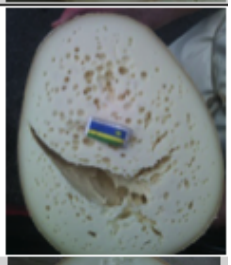
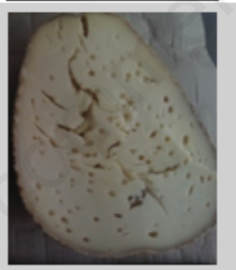
The antimicrobial activity against *Cl. sporogenes* and *St. xylosus* of two commercial lysozymes - A and T -was compared to that exerted by an analytical grade lysozyme (S). In the first set of trials, experiments were carried out by using cell suspensions in sterile saline of both strains. *St. xylosus* was sensitive to concentrations equal or higher than 0.25 mg/mL already after 24-h of incubation (Data not shown). *Cl. sporogenes* was inhibited (CFU/mL <1) by lysozyme concentrations of 0.5 and 0.33 mg/mL. When the concentration was lowered to 0.25 mg/mL some differences could be noted at 120 h: only the commercial lysozyme A was able to exert an antimicrobial activity (CFU/mL <1), while for lysozymes S and T, *Cl. sporogenes* loads were still 1.66×10^4 and 2.50×10^5, respectively (Data not shown). According to outcomes, a lysozyme concentration in the range 0.30–0.50 mg/mL should be adequate to control the target strain.

The experiment was repeated by using the same strain, but cells collected by centrifugation were resuspended in a proper growth medium in order to understand what happens in conditions closer to reality, since the dairy environment is an extremely rich ecosystem. In such trials, both species proved to express a higher resistance. In detail, *Cl. sporogenes* proved to be able to survive even at a lysozyme concentration of

Table 2. Milk samples analysed by MPN in BBB medium and CFU on RCM agar.

Sample	Code	MPN in BBB (cell/mL)		CFU on RCM (CFU/mL)
		Growth	Gas	
Vat - raw	P	25	0	30
Vat - raw	V	9	0	10
Single producer - raw	L1	24	0.9	20
Single producer - raw	L2	9.5	0	20
Single producer - raw	L3	25	0.3	70
Single producer - raw	L4	15	0.4	10
Single producer - raw	L5	95	95	120
Vat – raw	V1	25	0.9	4
Vat - pasteurized	V2	25	0.9	21
Single producer - raw	L6	25	4.5	110
Single producer - raw	L7	45	0.9	3
Vat – raw	V3	45	1.5	100
Vat - pasteurized	V4	25	2.5	40

Table 3. LBD Grottone cheese samples analysed by MPN in BBB medium and CFU on RCM agar.

Code	MPN in BBB (cell/g)	CFU in RCM (CFU/g)	Appearance	Code	MPN in BBB (cell/g)	CFU in RCM (CFU/g)	Appearance
C1	9	20		C7	<10	2.4×10^5	
C2	2.5×10^4	1.5×10^5		C8	$2.5x\times10^2$	3.6×10^4	
C3	9.5×10^3	3.8×10^4		C9	1.5×10^3	180	
C4	4.5×10^2	2.4×10^4		C10	20	120	
C5	9.5×10^2	1.8×10^5		C11	20	60	
C6	2.5×10^2	1.5×10^5					

10 mg/mL, with a reduction of the population level only after 72 h of incubation (Figure 1 – Panel A).

Situation appeared to be completely different for *St. xylosus*. An inhibition was evident after 24 h, but a strain's adaptation to the antimicrobial occurred since populations' levels were higher at 72 h. The most controlling lysozymes were the analytical grade one and the commercial type coded as A: population were completely inhibited by concentrations of 10 and even 5 mg/mL (Figure 1 – Panel B).

Indeed, commercially available preparations of lysozyme vary significantly in terms of purity, enzyme concentration and potency (Brasca *et al*., 2013).

Implications of protective culture Lyofast LC 4P1 on Grottone cheese quality

Nine Grottone cheese manufactures were carried out at farm level. In six trials the *Lb. casei* culture Lyofast LC 4P1 was used jointly with LAB and PAB starters provided by the same supplier. In batch A, the protective culture was added at the same time of the starters, while in manufacturing B, C, D, E and F the protective culture was used for a milk pre-fermentation that involved a percentage of the final volume of 10, 36, 50, 70 and 100%, respectively. After two months of ripening, five Grottone cheeses for each manufacture - for a total 45 wheels -were cut in two halves and analysed for their appearance. At the date of analyses, none of the produced Grottone wheels, other than the 45 initially marked, presented obvious signs of LBD. Actually, this defect is known to occur even later during the ripening, and it is not always possible to assess its oncoming before the wheel's opening (Cosentino *et al*., 2015).

With reference to the samples analysed, the five wheels collected within the same trial always shared the same appearance, except in case of trial F (one wheel out of five presented an adequate holes' amount), and C1 (3 out of five wheels showed an undesirable cavernous appearance) (Table 4). According to data, the use of Lyofast LC

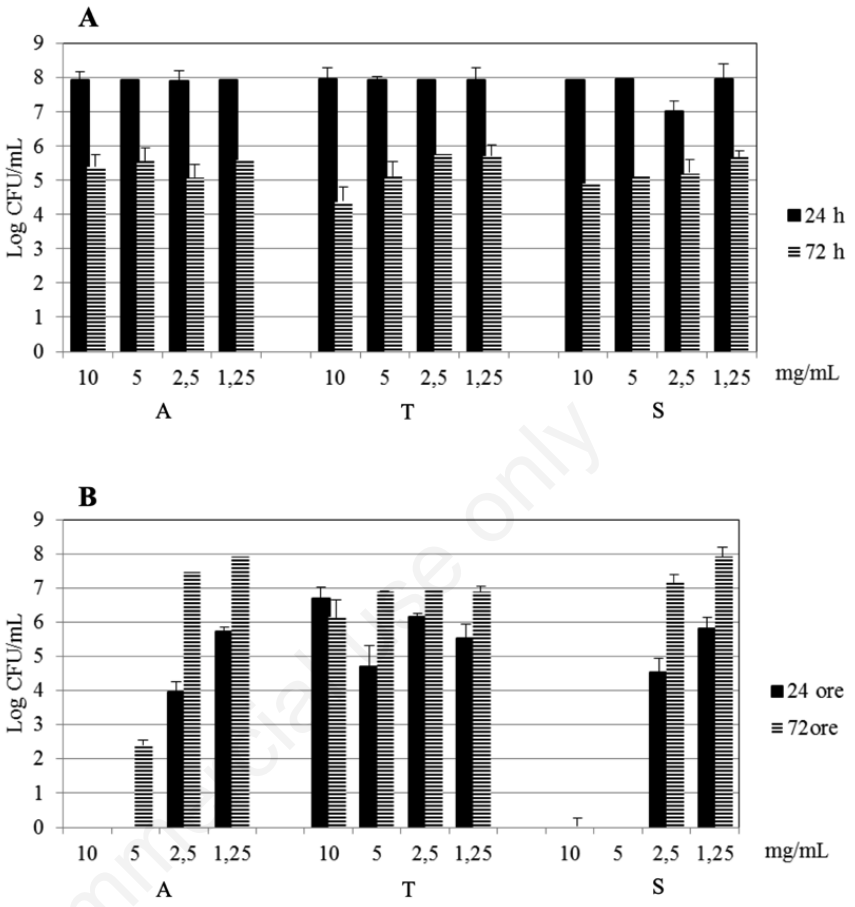
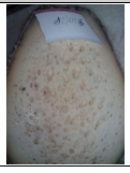


Figure 1. Populations' levels of *Cl. sporogenes* ATCC1143 (A) and *St. xylosus* DSM 20267 (B) after 24 and 72 h in presence of 10, 5, 2,5 and 1,25 mg/mL of commercially available lysozymes (A and T) or analytical grade lysozyme (S). Cells suspended in RCM or BHI media.

Table 5. Grottone cheese manufactures at farm level. Trial A: *Lb. casei* culture Lyofast LC 4P1 added with starters; Trial B, C, D, E and F: LC 4P1 used for milk pre-fermentation at 10, 36, 50, 70 and 100% of the total volume. C1, C2 and C3: controls.

Manufacure conditions	Trial A	B	C	D	E	F	C1	C2	C3
Protective	LC 4P1	LC 4P1	LC 4P1	LC 4P1	LC 4P1	LC 4P1	-	-	-
PAB	PB1	PB1	PB1	PB1	PB1	PB1	CSL	CSL	CSL
LAB	YHL 094F	YHL 094F	YHL 094F	YHL 094F	YHL 094F	YHL 094F	HD 097	HD 097	HD 097
Pre-ferm. vol.	-	20 L (10%)	72 L (36%)	100L (50%)	140 L (70%)	200 L (100%)	-	-	-
Pre-ferm. lenght	-	~3 h	~3 h	~2 h	~40 min	~20 min	-	-	-
Appearence	Few holes	Few holes	Few holes	Few holes	Few holes	Few holes and crackings	Cavernous	Compliant	Compliant
									

4P1 in combination with PAB and LAB provided by the same supplier hampers holes development (Table 4). Moreover, in samples treated with the protective culture, panellists were not able to perceive the expected 'Grouviera-type' taste traditionally associated to Grottone cheese (Data not shown). From this perspective, the lack of holes could be due to an inhibition of the PAB starter development, rather than to an adverse interaction between PAB and protective culture.

Conclusions

LBD in hard and semi-hard cheeses could be avoided by determining the initial content of clostridial endospores in milk before processing. Based on results, MPN counts on BBB and CFU on RCM resulted to be the most suitable procedures for routine testing. By using these approaches clostridial spores were detected in 10 out 13 of the analysed milk samples and all cheeses with LBD, even if several discrepancies were recorded. The growth of clostridia in cheese can be controlled by using antimicrobial compounds. Lysozyme is the most common choice, but outcomes may be largely affected by the type of product, since commercial lysozymes greatly vary in terms of effectiveness. Biopreservation by means of protective LAB cultures may represent a promising opportunity, but the interaction with starters needs to be carefully investigated. As matter of fact, when the protective culture Lyofast LC 4P1 was used in combination with PAB and LAB in Grottone cheese manufacturing, the holes' development was hampered, likely due to an inhibition of the PAB starter development as even proved by the absence of the expected 'Grouviera-type' taste.

As general consideration, the manufacture of Grottone cheese stands on a faint balance between lactic and propionic bacteria that leads to a qualitative inconsistency of the product, besides the creation of environmental conditions favourable to clostridia development.

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