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Content and spatial distribution of dairy-related *Clostridium* spores in Grana Padano cheese during the ripening period

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ABSTRACT

This study investigated the stress factors that affect the content and the spatial distribution of *Clostridium* spores in Grana Padano (GP) wheels and in GP samples purchased from the market. The cheeses analyzed were produced adding lysozyme to prevent late blowing defects (LBD). The highest NaCl amount was detected in the external part (5.2 ± 0.6%) while a lower level was observed in the core (2.8 ± 0.3%). The clostridial community differed in cheeses with different ripening times along with pH and salt changes that occur slowly over time. The spore level in the core (0.24 ± 0.19 and 0.16 ± 0.14 MPN/g after 11 and 20 months of ripening) was significantly lower than in the peripheral zones (0.48 ± 0.26 and 0.45 ± 0.31 MPN/g). Moreover, in the wheels aged 11 months, the presence of *C. tyrobutyricum* was higher in the areas with the lowest salt content, while after 20 months of ripening when a homogeneous concentration of salt and pH within the wheel occurred, *C. sporogenes* was the prevalent species. Our results showed that in the GP wheels changes in the presence of clostridia responsible for LBD still occur until the salt content in the center of cheeses is higher than threshold NaCl needed to inhibit spore germination.

1. Introduction

Grana Padano (GP) is an extra hard, cooked cheese produced from raw milk that awarded the Protected Designation of Origin (PDO) certificate by the European Community in 1996.5,235,000 GP wheels were produced during the 2021 and about one third of them were exported mainly to Germany, France, US, UK, Switzerland, Spain and Canada (https://www.granapadano.it). GP is characterized by a long ripening period (9 months minimum) that promotes the development of the cheese flavor and support the growth of microbial communities able to survive to the cheese-making process. This can result in beneficial effects in the definition of a well-balanced and complex aroma profile or, alternatively, in spoilage defects that devalue the final product (Bassi, Puglisi, & Cocconcelli, 2015). Late blowing defect (LDB) is one of the major concerns of the GP manufacture and consists in formation of holes, cracks and splits, generally accompanied by unpleasant aroma and rancid flavor (Brasca, Morandi, & Silvetti, 2022). Even if a recent study evidenced a higher content of obligate homofermentative lactobacilli in GP samples with blowing defects (da Silva Duarte, Lombardi, Corich, & Giacomini, 2022), spore-forming bacteria belonging to the *Clostridium* genus are considered the main agents of this defect, since they are capable to convert lactate into butyrate, acetate, H_2 and CO_2. *Clostridium tyrobutyricum* is the primary cause of the LBD, but other clostridial species, such as *C. sporogenes*, *C. beijerinckii* and *C. butyricum* contribute significantly to the occurrence of blowing in cheese (Brändle, Domig, & Kneifel, 2016; Le Bourhis et al., 2007). This statement is confirmed by the fact that even though lysozyme is used to effectively counteract spore germination and growth in cheese, to date, about 2% of the GP wheels are affected by LBD and the economic loss related to this defect is about 36 million Euro/year (Giraffa, 2021).

Although LBD is a long-standing issue, its eradication is difficult since *Clostridium* spores are ubiquitous (soil, silage and feeds) and their presence is difficult to contain in milk at farm level (Borreani et al., 2019). Moreover, a spore content of 100 per L may be sufficient to cause the onset of defect (Zucali et al., 2015). PDO cheeses are considered particularly prone to LDB since their production regulations forbit the application of physical treatments (bactofugation or microfiltration) to reduce spore content in milk (Burtscher, Hobl, Kneifel, & Domig, 2020). On the other hand, some PDO cheese regulation allows the use of lysozyme to prevent the clostridial spore germination (Lodi & Stadhouders, 1990). In Italy, the use of lysozyme is quite widespread, in fact this enzyme is employed in the cheese-making of several semi-hard and

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Table 1
Origin and ripening period of the Grana Padano samples considered in this study.

Sample	n.	Ripening	Province
Whole wheel	1	11 months	Brescia
	2	11 months	Cremona
	3	11 months	Lodi
	4	11 months	Mantova
	5	12 months	Mantova
	6	11 months	Cremona
	7	11 months	Brescia
	8	11 months	Brescia
	9	19 months	Mantova
	10	20 months	Cremona
	11	20 months	Trento
	12	20 months	Piacenza
Sample from the market	13	>16 months	Mantova
	14	>9 months	Trento
	15	>9 months	Vicenza
	16	14 months	Vicenza
	17	>9 months	Cremona
	18	>9 months	–
	19	>9 months	Cremona
	20	>9 months	Cremona
	21	>9 months	Verona
	22	>9 months	Mantova

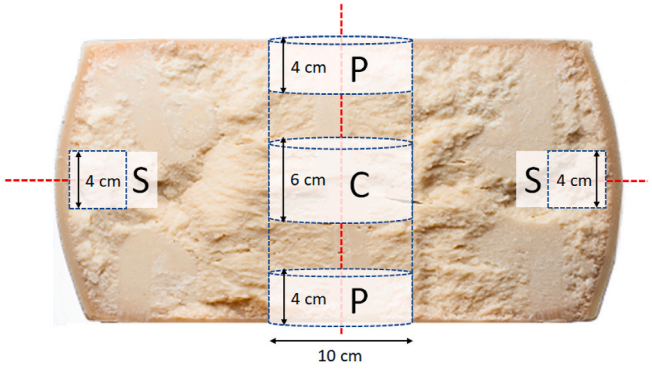


Fig. 1. Cheese sampling location.

hard PDO cheeses, such as Grana Padano, Montasio, Piave and Provolone (Brasca et al., 2013). The European Community (EU, 2011) authorized the use of this enzyme without suggesting any dosage limit (*quantum satis*), but usually it is usually added to milk in concentrations of about 500 Units/mL (Brändle et al., 2016; Silvetti, Morandi, Hintersteiner, & Brasca, 2017).

Currently, few data are available on the natural evolution of spores throughout the ripening period, although several investigations were performed on the spore content in raw milk (Brändle, Heinzle, et al., 2018; Burtscher et al., 2020), in farm environment (Borreani et al., 2019) and in blowing cheeses (Bassi et al., 2015; Brändle, Fraberger, et al., 2018) or experimental cheese-makings (Garde, Arias, Gaya, & Nuñez, 2011; Le Bourhis et al., 2007).

In order to provide a useful contribution to this issue, the aims of this study were: 1) to assess *Clostridium* spore content in GP wheels without LBD symptoms, obtained using the lysozyme in the cheese-making process; 2) to evaluate the spore distribution and species prevalence within GP wheels after 11 and 20 months of ripening; 3) to enumerate the spores present in GP cheeses purchased from market and 4) to identify the stress factors arising during GP aging that allow to avoid the blowing defect.

2. Materials and methods

2.1. Cheese samples collection

Twelve wheels of GP cheese without evident blowing defects were sampled after 11 (8 wheels) and 20 (4 wheels) months of ripening (Table 1). The wheels (diameter 35–45 cm, height 18–25 cm and weight 24–40 Kg) were collected in 5 different provinces. All cheeses were marked and therefore produced and ripened according to the production regulation (https://www.granapadano.it). GP wheels, From each wheel three different sampling areas were considered (under-rind on the lateral surface S; under-rind on the flat surface P and cheese core C) (Fig. 1). To obtain the cheese core samples, each wheel was cut lengthwise along the vertical axis and a cylindrical cheese section was obtained (10 cm of diameter). From this cheese section, an internal (C) and external (P) portion was successively sampled (Fig. 1). After the core sampling, the cheese wheels were cut along the horizontal axis to obtain two symmetrical halves and the under-rind samples (S) were taken (Fig. 1). One cm of the cheese rind was removed, and the samples thus obtained were transferred to the laboratory under refrigerated conditions and microbiological and chemical analyses were performed within 24 h of sample arrival.

Moreover, 10 pieces of GP cheeses, produced by 8 different dairies, were purchased from the market and subjected to microbiological analysis (Table 1).

2.2. Clostridial spore enumeration

Spore enumeration was carried out by the Most Probable Number (MPN) method according to the protocol previously set up by Morandi et al. (2021) allowing to measure also spore levels lower than 1/g.

In each area two sampling were subjected to dairy-related *Clostridium* spore determination. Spore

content of each sample was determined in duplicate.

Ten grams of each cheese sample were homogenized with 90 mL of reconstituted skim milk (10% w/v) (Sacco srl, Cadorago, Italy) supplemented with A-solution (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) for 2 min in a Stomacher BagMixer (Interscience, St. Nom, France) at 45 °C. The entire homogenate was then equally dispensed into 10 sterile tubes. All the tubes were sealed with 2 mL of sterile vaseline/paraffin (1:1 w/w), heated at 80 °C for 10 min to kill the vegetative bacterial cells and to promote spore germination, and then incubated at 37 °C for 7 days. MPN counts related to gas positive tubes were expressed as MPN/g according to ISO 7218:2007/Amd 1:2013 (ISO 7218, 2013). The limit of detection of this MPN method is 0.11 MPN/g (Morandi et al., 2021).

2.3. Detection of Clostridium species by multiplex PCR analysis

Multiplex-PCR was applied to detect the *Clostridium* species (*C. beijerinckii*, *C. butyricum*, *C. sporogenes* and *C. tyrobutyricum*) present in the different areas of GP wheels (Cremonesi, Vanoni, Silvetti, Morandi, & Brasca, 2012). Moreover, DNA was extracted from of each positive MPN tube, as described by Cremonesi et al. (2006). Multiplex-PCR reaction was performed using the AccuPrime Taq DNA polymerase (Thermo Fisher Scientific, Walthman, MA, USA). All determinations were performed in duplicate.

2.4. Physico-chemical determination

Physico-chemical analyses of the different GP samples (moisture and NaCl concentration) were conducted by Neotron SPA according to ISO 5534 (ISO 5534, 2004) and ISO 8070 (ISO 8070, 2007) procedures. The pH of the cheese samples was measured using a multi-channel pH-meter

Table 2
Humidity, NaCl, pH and spores content detected during the Grana Padano cheese ripening (11–12 and 19–20 months).

	Sampling	Ripening			
		11–12 months		19–20 months	
		mean	SD	mean	SD
Moisture (g/100 g)	S	32.68^{Ba}	0.85	30.60^{Aa}	0.50
	P	33.66^{Ba}	0.70	31.30^{Aa}	0.55
	C	36.31^{Bb}	0.85	34.35^{Ab}	0.55
NaCl in moisture (%)	S	5.2^{c}	0.6	5.5^{b}	1.0
	P	3.4^{b}	0.4	4.1^{a}	0.5
	C	2.8^{Aa}	0.3	4.1^{Ba}	0.5
pH	S	5.5	0.1	5.5	0.0
	P	5.5	0.1	5.5	0.1
	C	5.5	0.1	5.5	0.1
Lysozyme content (mg/kg)	S + P + C	123.1	17.0	110.5	24.2
Spores (MPN/g)	S	0.35^{Ab}	0.16	0.57^{Bc}	0.46
	P	0.48^{b}	0.26	0.45^{b}	0.31
	C	0.24^{a}	0.19	0.16^{a}	0.14
	market	0.46	0.27		

SD: standard deviation.
S: under-rind on the lateral surface; P under-rind on the flat surface; C cheese core.
Means with different capital letters in the same line are significantly different ($p < 0.05$).
Means with different lowercase letters in the same column are significantly different ($p < 0.05$).

(Acidification Monitoring System and Analyser AMSA, Star Ecotronics, Milan, Italy) equipped with an electrode InLab Solids Pro-ISM (Mettler Toledo, Greifensee, Switzerland).

2.5. Determination of the lysozyme in cheese samples

Lysozyme content in cheese samples was determined by HPLC-FLD method following the procedure described in the ISO method (ISO 27105, 2016). A homogeneous mixture of each wheel was obtained by grating 30 g of every sampling area (S, P and C) by a knife mill.

2.6. Statistical analysis

Statistical analysis was performed with the software package MINITAB ver. 14.13 (Minitab Inc., State College, PA, USA). Data were analyzed by ANOVA using the Tukey multiple comparisons method. A p value of 0.05 or less was considered significant.

3. Results and discussion

The physico-chemical properties of the inner and outer areas of the GP cheeses are reported in Table 2. After 11 months of ripening, the moisture in the inner region (C) was significantly higher than the P and C areas ($p < 0.001$). Similar data were observed also after 20 months ripening (Table 2). These results agree with those reported in previous studies (Malacarne et al., 2009; Tosi, Sandri, Tedeschi, Malacarne, & Fossa, 2008), that highlighted differences in moisture values (≥2 units) between the core of Parmigiano Reggiano cheese and its peripheral portions. This difference begins during the brining process and lasts until about 24 months of maturation (Tosi et al., 2008).

No significant difference in the pH values of the three cheese positions (C, P and S) were observed (Table 2). Similar results were reported by Tosi et al. (2008) and Malacarne et al. (2009), who detected only slight pH changes in Parmigiano Reggiano during the ripening period.

Considering the salt content in the wheels with 11 months of aging, a different NaCl in moisture distribution was still observed (Table 2). The highest salt amount was detected in the external part near the lateral surface S (5.2 ± 0.6%), while a lower NaCl concentration in moisture was observed in the under-rind of the flat surface P (3.4 ± 0.4%) and even less in the cheese core C (2.8 ± 0.3%). These differences resulted to be statistically significant ($p \leq 0.001$). Also, after 20 months of ripening the highest salt amount was detected in the area S (5.5 ± 1.0%), while no significant differences were detected in the P and C portions (Table 2). As described by Fox, Guinee, Cogan, and McSweeney (2017) the mutual migrations of water and salt in opposite directions result in a decreasing NaCl gradient from the surface to the center of the cheese, and a decreasing moisture gradient from the core to the rind of the wheel. These gradients disappear if the ripening time is long enough, and the time needed to reach the equilibrium depends on cheese size and shape, but also on cheese composition and storage conditions.

Table 2 shows the spore count of the cheese samples. MPN-based clostridial spore numbers in GP wheels ranged from 0.16 ± 0.14 to 0.57 ± 0.43 MPN/g sample.

It is interesting to notice that the spore level in the core portion (0.24 ± 0.19 in cheese ripened 11 months and 0.16 ± 0.14 MPN/g in cheese

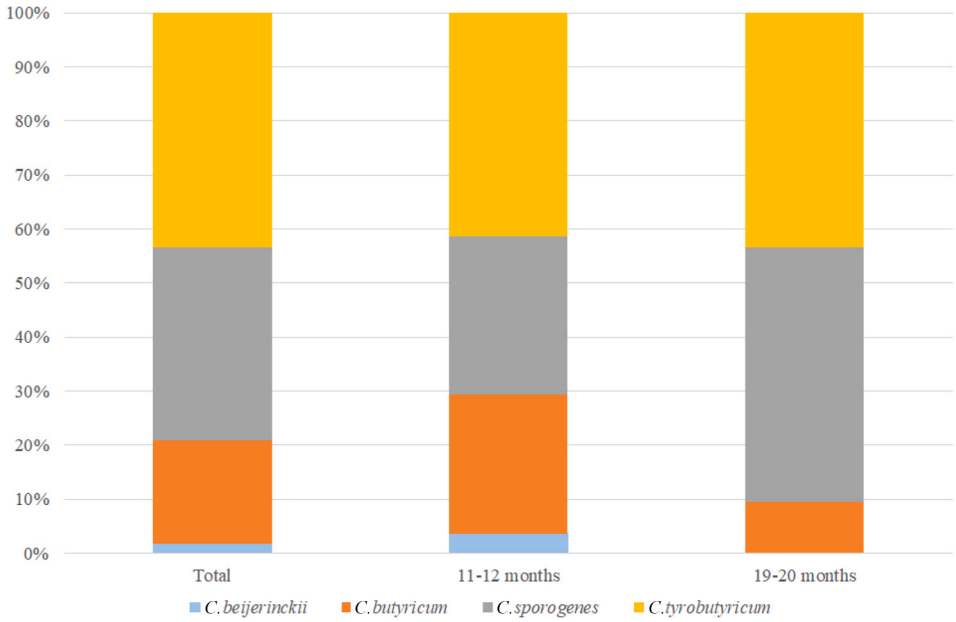
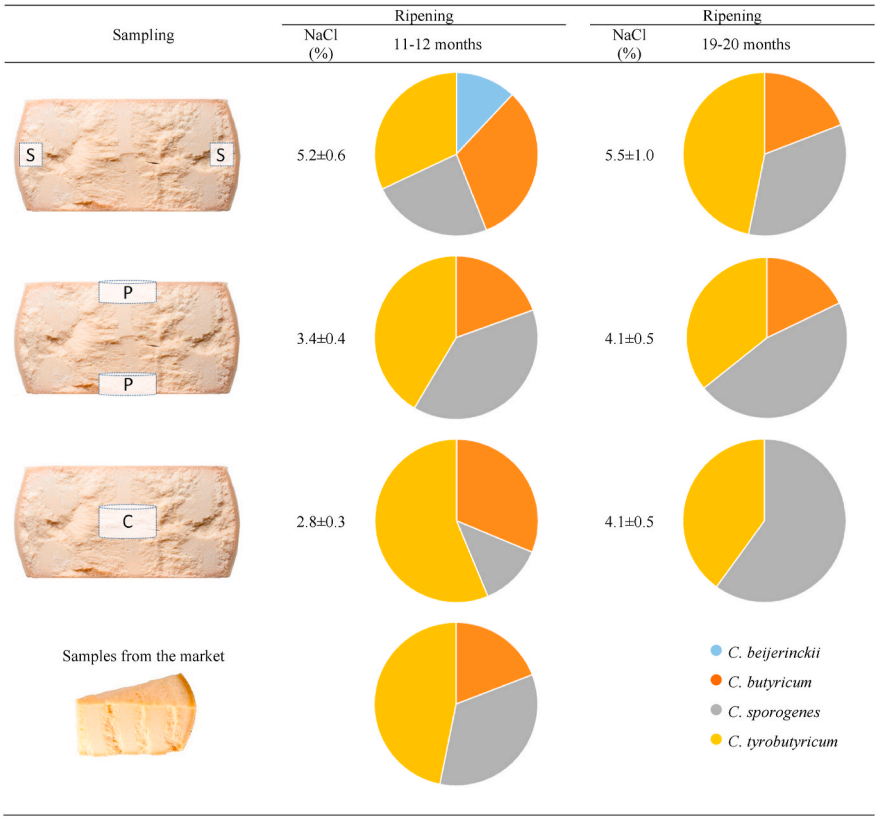


Fig. 2. Clostridial species distribution (%) detected in positive tubes of cheese samples.

Table 3
Distribution of *C. beijerinckii*, *C. butyricum*, *C. sporogenes* and *C. tyrobutyricum* in Grana Padano cheese samples at different ripening period.



ripened 20 months) was significantly lower than that of the peripheral zones (Table 2; $p \leq 0.001$).

This result is explicable considering the different stress factors (pH, salt and moisture content, time and temperature of aging) that affect the metabolic activity of *Clostridium* strains. After brining and during ripening, a more hostile environment is generated in the peripheral areas of cheeses that hinders clostridial spore germination. On the contrary, in the core of the cheese, the low NaCl concentration and the high moisture support the germination of spores into vegetative cells, thus reducing spore content (Gómez-Torres, Ávila, Gaya, & Garde, 2014; Morandi et al., 2021). It is interesting to note that different authors evidenced the presence of cracks, splits and irregular eyes in Valtellina Casera and Gouda cheeses containing low levels of spores (respectively 0.18 and 0.40 MPN/g) (Herman, De Block, & Van Renterghem, 1997; Morandi et al., 2021). In GP samples, we detected a spore concentration comparable with those found in Valtellina Casera and Gouda, but no LBD symptoms were observed. This fact could be explained considering the salt concentration in moisture that was higher than 2.0% (threshold suggested by Silvetti, Morandi, and Brasca (2018) for inhibiting gas production by clostridia at the GP ripening temperature (15–22 °C)) and by the presence of lysozyme in the cheese matrix. The concentration of this antimicrobial enzyme in GP samples was 123.1 ± 17.0 and 110.5 ± 24.2 mg lysozyme/kg in 11–12 and 19–20 months respectively (Table 2). Our results agreed with Schneider, Werkmeister, Becker, and Pischetsrieder (2011), who determined the presence of lysozyme in commercial GP samples and showed that this enzyme remains relatively stable also after 54 weeks of ripening.

It is important to consider that the clostridial germination as well as the gas production are strain-dependent and highly variable, even among strains of the same species (Podrzaj, Burtscher, Küller, & Domig, 2020; Silvetti et al., 2018) hence the simultaneous application of the technological parameters IS necessary to inhibit the most resistant and to be certain of preventing the occurrence of the defect. Moreover, the association of two or more strains is often a crucial factor for the appearance of LBD (Garde, Gaya, Arias, & Nuñez, 2012; Le Bourhis et al., 2007).

In the cheese samples collected from market, obtained from the peripheral portion P of GP wheels, the mean spore content was 0.46 ± 0.27 MPN/g (Table 2). Interestingly, this value does not differ from the value detected in the peripheral portions P of the 11 GP wheels analyzed for the spatial distribution of the clostridial spores, confirming that the mean values determined in this area of the wheels correspond to the current average spore content of GP cheese.

Multiplex-PCR performed on the MPN positive tubes allowed us to identify the clostridial species present in the different areas of the GP wheels (Fig. 2). Considering all the samples, *C. tyrobutyricum* resulted to be the most prevalent species (41.5%) followed by *C. sporogenes* (29.3%), *C. butyricum* (25.6%), and *C. beijerinckii* (3.7%). These results were in agreement with Cocolin, Innocente, Biasutti, and Comi (2004) and Bassi et al. (2015), who detected the same clostridial species in GP samples with LBD symptoms.

Multiplex PCR serves as an estimation of relative abundance, not providing unambiguous insights, nevertheless it is a useful tool to point out relative changes.

Comparing the multiplex PCR results, *C. sporogenes* (29.3% at 11 months and 47.2% at 20 months) and *C. tyrobutyricum* (about 42.0%) were the most abundant clostridial species detected at the two ripening periods. Differently, in the wheels ripened up to 19 months, *C. beijerinckii* disappeared while *C. butyricum* was present in low percentage (9.4%) and the relative abundance of *C. tyrobutyricum* did not

change (Fig. 2).

These data confirm the adaptability of *C. sporogenes* and *C. tyrobutyricum* to different stress factors (pH, salt and moisture content, time and temperature of aging) involved in cheese ripening (Silvetti et al., 2018).

The salt gradient from the surface to the core of the wheels revealed to be a key factor affecting the various clostridia species in a different way (Table 3). In the wheels aged 11 months, the *C. tyrobutyricum* percentage increases (from 32.0% in S to 56.3% in C) with the decrease of the NaCl concentration, while with the advancement of the ripening process and with a homogeneous concentration of salt within the wheel, *C. sporogenes* was the prevalent species in C and P areas (Table 3).

These data support the evidence that changes occurring during the aging period (changes in moisture and NaCl concentration and the complex biochemical events) influence the microbial communities and select the bacterial species that are better adapted to growth under specific conditions. The greater occurrence of *C. tyrobutyricum* and *C. sporogenes* in the cheese matrix with the lowest NaCl content confirm, as previously pointed out by *in vitro* experiments by Silvetti et al., 2018, that a slow diffusion determining a low salt content can favor the gas production therefore the onset of the blowing defect.

Considering the 10 samples recovered from the market, *C. tyrobutyricum* was the dominant species (48.8%) followed by *C. sporogenes* (34.0%) and *C. butyricum* (19.2%) (Table 3). The distribution of the clostridial species in these samples was quite similar to those obtained in the peripheral portions confirming the MPN results (Table 2).

4. Conclusions

Our findings highlight that clostridial population (species and spore number) evolves throughout ripening as a result of pH and salt changes that occur slowly over time. In GP cheeses without LBD symptoms are present clostridial spores, indeed in wheels after 11 and 20 months of ripening, we found spores belonging to *C. butyricum*, *C. sporogenes* and *C. tyrobutyricum* species. Their content varied inside the cheese matrix and was related to the NaCl concentration. In large-size wheels, the decreasing salt gradient (from the rind to the center of the cheese) can support the germination of spores, where low NaCl and high moisture levels are present. Concerning that point, the GP wheels aged 11 months could be still susceptible to LBD, since the salt content in the center of cheeses (mean value 2.8%) is slightly higher than threshold NaCl concentration needed to inhibit the spore germination. Thus, the use of milk with a low content of clostridial spores (less than 100 per L) and a strict control of NaCl migration inwards during the entire ripening process of Grana Padano (15–22 °C) are needed to avoid the use of lysozyme in the cheese-making process and the germination of clostridial spores.

CRediT authorship contribution statement

S. Morandi: Investigation, Validation, Methodology, Formal analysis, Writing – original draft. **T. Silvetti:** Investigation, Writing – review & editing. **M. Brasca:** Supervision, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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