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 x 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.

Keywords: cheesemaking, spore, free amino acid, arginine, electron microscopy

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., 2015). 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) (Cocolin, Innocente, Biasutti, & Comi, 2004; Rodriguez & Alatossava, 2010; Bassi, Puglisi, & Cocconcelli, 2015; Morandi, Cremonesi, Silvetti, Castiglioni, & Brasca, 2015). 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 (Martínez-Cuesta et al., 2010; Gómez-Torres, Ávila, Gaya, & Garde, 2014). 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 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 (Bassi et al., 2013) is deposited at DDBJ/EMBL/GenBank under the accession no. ANOE00000000. 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 & 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 et al., 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 minutes. 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^{TM} technology (FEI, Oregon, USA) under both low (130 Pa) and high ($7x10^{-5}$ Pa) vacuum conditions. Samples for TEM were prepared fixing 1 mL of tube content as described by D'Incecco, Faoro, Silvetti, Schrader, & Pellegrino, (2015). Ultrathin (50 to 60 nm) sections of resin inclusions were stained with uranyl acetate and lead citrate and examined with a Philips E208 microscope (Aachen, Germany).

124 2.5 Free Amino Acid analysis by ion exchange chromatography

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

131 2.6 Arginine utilization by *C. tyrobutyricum* in milk

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

137 2.7 Statistical analysis

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

141

142 **3. Results and Discussion**

143 With the aim of studying *C. tyrobutyricum* behaviour during the whole cheese manufacturing
144 process, we have set up an innovative experimental approach. Vegetative cells and spores of *C.*
145 *tyrobutyricum* were separately confined into dialysis tubes that were kept immersed in the milk
146 during the vat processing and then included into the cheese curd to undergo the subsequent steps of

147 acidification, brining and ripening. By this way, it was possible to recover both cells and spores that
148 had been directly in contact with the changing environment (milk, curd, cheese) outside the tube
149 and to analyse their behaviour in a real cheesemaking process.

150 3.1 *C. tyrobutyricum* counts in tubes sampled during cheese manufacture

151 The evolution in vegetative cell and spore numbers during the GP cheesemaking is represented in
152 Fig. 1 in relation to temperature and pH conditions. As expected, counts of *C. tyrobutyricum* at RE
153 stage were the same as at t=0, i.e. 10^7 CFU/mL vegetative cells in C-tubes and 10^8 CFU/mL spores
154 in S-tubes. During curd cooking at 54 °C, vegetative cells in C-tubes decreased to 8 x 10^5 CFU/mL
155 while 2 x 10^2 CFU/mL spores formed. No cultivable cells (<10/mL) were found at the following
156 sampling steps, suggesting the high temperature reached during vat processing to be lethal for *C.*
157 *tyrobutyricum* vegetative cells. A one-log increase in spore numbers was observed in 3C; due to the
158 lack of sampling in the time interval between ES and 3C, we supposed that, during this period,
159 spores could germinate, reproduce and sporulate. Limited information is available on the factors
160 triggering sporulation in clostridial species and the role of heat shock and exposure to oxygen is not
161 known. Kirk, Palonen, Korkeala, & Lindström (2014) showed that in *C. botulinum* sporulation
162 begins when the cells are in the exponential growth phase and we can speculate that a similar
163 behaviour could happen in *C. tyrobutyricum*.
164 Spore numbers in the S-tubes remained almost unchanged until the end of salting with only small
165 fluctuations, whereas few spores germinated during curd acidification (EA) (Fig. 1), when the drop
166 of pH created favourable conditions (Bassi, Cappa, & Cocconcelli, 2009). A two-log decrease in
167 spore concentration, with respect to the initial level, was observed in S-tubes taken from 3C and 6C.
168 However, very few vegetative cells (9.5 x 10^1 CFU/mL) were cultivable in those tubes. We
169 supposed that only a small portion of germinated spores was able to complete the cell cycle leading
170 to viable vegetative cells.

171 3.2 Scanning and Transmission Electron Microscopy

Both SEM and TEM were used to monitor morphological changes of *C. tyrobutyricum* cells and spores throughout the whole cheesemaking process. SEM analysis of the C-tube pellets gave overlapping results with data obtained with plate count method. At renneting, only vegetative cells were detected (Fig. 2a) whereas some spores began to be visible at the end of curd cooking (Fig. 2b) reflecting the two-log spore counts found at this step. At the curd extraction step, cells were almost lysed and a few spores were detectable (Fig. 2c). At the end of acidification (Fig. 2d) and after brining (not shown), we observed only damaged cells and some dormant spores. The S-tube taken at milk renneting contained only spores (Fig. 3a); spores appeared slightly to change their basal morphology as it was also observed at the end of curd cooking (Fig. 3b). Some vegetative cells were observed at the curd extraction (Fig. 3c) and at the end of acidification (Fig. 3d). The brining phase did not induce major modifications, keeping a mixed situation of faint cells in autolysis together with spores (data not shown).

Using TEM, only intact vegetative cells were detected in C-tube at renneting (Supplementary file 1), whereas some damaged vegetative cells were observed at the end of curd cooking (Supplementary file 2), consistently with SEM and plate count evidences. Interesting morphological changes of cells appeared at the CX step, after a severe heating (54 °C) and initial acidification (pH 6.3). In particular, a highly-dense black spot (Fig. 4a-c, arrows) appeared inside most of the cells and turned into a more complex oval structure (Fig. 4d-f) in the molded curd, which had slowly cooled down and acidified (pH 5.3). Starting from the outside, this structure consists of a 10-nm thick layer that encloses hundreds of circular substructures, and the black spot in the middle surrounded by a thin layer (Fig. 4f). To our knowledge, this intracellular structure, that we found in the majority of vegetative cells, was not previously observed in bacterial sporeformers. This structure differs from inclusion bodies usually found in bacteria and from the structures observed during the initial steps of sporulation (Garcia-Alvarado, Labbe, & Rodriguez, 1992; Al-Hinai, Jones, & Papoutsakis, 2015). The majority of vegetative cells appeared evidently damaged at the end of salting (Supplementary file 3) and afterwards, and very few spores were present.

198 Spores within S-tubes looked unchanged until curd extraction (Supplementary files 1 and 2). The
199 typical dormant spore structure was observed, with well-organized layer-structured exosporium,
200 coat, cortex, inner membrane and core, as observed by SEM (Bassi, Cappa, & Cocconcelli, 2009).
201 Some spores changed their internal structure in the acidified curd (Fig. 5c), due to the beginning of
202 germination, confirming the plate count and SEM analysis. These spores had a larger core and a
203 smaller exosporium with respect to dormant spores that however were still present (Fig. 5a,b). After
204 core hydration, spore germination was reported to involve the core expansion (Setlow, 2003; Brunt,
205 Cross, & Peck, 2015). At the end of brine salting, cells appeared deeply damaged like those
206 observed in 6C, when spores always lacked of exosporium and the dense core was evident
207 (Supplementary file 4).
208 Overall, two main events were observed through our samplings: (i) vegetative cell sporulation
209 during the vat processing and (ii) spore germination at the end of curd acidification. The vegetative
210 cell sporulation was likely triggered by the exponential growth phase (Kirk, Palonen, Korkeala, &
211 Lindström, 2014). Differently, besides requiring favourable environmental conditions, the
212 germination process is known to be triggered by numerous molecules, the so-called germinants
213 (Setlow, 2003; Moir, 2006; Ramirez, Liggins, & Abel-Santos, 2010). Bassi, Cappa, & Cocconcelli
214 (2009) and Brunt, Cross, & Peck (2015) demonstrated a solution of L-alanine/L-lactate to have a
215 germinant effect toward spores of both *C. tyrobutyricum* and *C. sporogenes*. The high temperature
216 (52-56 °C for 6-8 h) and low pH (5.0-5.3) occurring within the moulded GP cheese (Pellegrino et
217 al., 1997) also represent favourable conditions inducing spore germination (Foster & Johnstone,
218 1990; Bassi, Cappa, & Cocconcelli, 2009), although the biochemical mechanisms behind these
219 phenomena have not yet been clarified.

220 3.3 Free amino acid metabolism of *C. tyrobutyricum* UC7086

221 The pattern of the FAA was determined within the C- and S-tubes as well as in the various matrices
222 (milk, whey, cheese) around them to have information on FAA utilization by *C. tyrobutyricum*

223 during the cheesemaking. The amounts of selected FAA are shown in Table 1. In this study, we
224 used dialysis tubes with a 50 kDa cut-off to contain either vegetative cells or spores and to allow
225 FAA to move freely in both directions and this was confirmed by preliminary assays (data not
226 shown). In order to compare the FAA levels within the tubes with those in the different matrices
227 outside them, it was necessary to consider the relative values for individual FAA.
228 No changes occurred in FAA pattern of milk until rennet coagulation (data not shown), confirming
229 previous observations (Pellegrino, Rosi, D'Incecco, Stroppa, & Hogenboom, 2015). At the curd
230 extraction from the vat, the contents of aspartate, glutamate, citrulline, valine, leucine, tyrosine, γ-
231 aminobutyric acid (GABA), arginine within the tubes were statistically ($P < 0.001$) different with
232 respect to those of the whey just drained off (Table 1). Among these, only GABA showed a higher
233 level within the tubes indicating that the formation of this non-protein amino acid was due to *C.*
234 *tyrobutyricum* cells metabolism and was so quick at the sampling moment that the equilibrium with
235 the outside (whey) could not be reached. Production of GABA, mediated by the glutamate
236 decarboxylase, represents a common way to contrast acidic conditions for several bacterial species
237 (Dhakal, Bajpai, & Baek, 2012; Brasca et al., 2016). However, the gene coding for the glutamate
238 decarboxylase is not present in the genome sequence of *C. tyrobutyricum* UC7086 (Bassi et al.,
239 2013) and in other fully sequenced strains (KCTC5387 and W428). Differently, these *C.*
240 *tyrobutyricum* strains harbour the gene coding for the 4-aminobutyrate transaminase (EC 2.6.1.19)
241 which can lead to the GABA biosynthesis by transamination. Low amounts of GABA were found
242 in the subsequent steps in spite of the large availability of glutamate.
243 At each of the subsequent sampling steps, one out of the four produced cheeses was cut to remove
244 the tubes. Furthermore, a sample representative of the whole cheese was taken, as well as two
245 portions (1-cm thick) around each tube: z1, in contact with the tube, and z2, surrounding z1. The
246 FAA level in these two portions allowed us to highlight the possible presence of a concentration
247 gradient indicating a movement of the individual FAA either inwards or outwards the tube. Data
248 were not different between z1 and z2 cheese portions taken around either C- or S-tubes, therefore

249 their mean values are presented (Table 1). No relevant differences were observed in the FAA levels
250 between C- and S-tubes at any sampling step, in accordance with the lack of an extensive cell
251 growth. Furthermore, none of the FAA was depleted, indicating that availability of FAA was not a
252 limiting factor for cell growth itself. This observation is supported by findings of Storari et al.,
253 (2016), who have recently reconstructed the presence of genes putatively involved in the
254 biosynthesis of 19 amino acids in the genomes of four *C. tyrobutyricum* strains isolated from hard
255 and semi-hard cheeses, including UC7086 used in this study. Alanine had an interesting behaviour
256 because its content was always lower in the S-tubes. As already mentioned, this amino acid in
257 combination with lactate can be a germinant of *C. tyrobutyricum* spores (Bassi, Cappa, &
258 Cocconcelli, 2009). However, probably more FAA can have this role depending on strain and
259 environmental conditions (Fisher & Hanna, 2005; Hornstra, de Vries, Wells-Bennik, de Vos, &
260 Abee, 2006), supporting the need for further investigations.
261 The most relevant impact of the presence of *C. tyrobutyricum* in cheese involved arginine (Table 1),
262 whose content progressively decreased ($P < 0.05$) within both the tubes and was depleted in 6-
263 month ripened cheese. The utilization of arginine within the tubes was confirmed by the decreasing
264 concentration observed from z2 to z1 cheese portions, indicating the slow permeation of the amino
265 acid into the tube itself to re-establish the equilibrium. An opposite behaviour was observed for
266 citrulline and ornithine (Table 1). In fact, these two non-protein amino acids generally derive from
267 the catabolism of arginine through the arginine deiminase (ADI) pathway (Zúñiga, Pérez, &
268 González-Candelas, 2002). Partial conversion of arginine to citrulline and, to a lower extent, to
269 ornithine also occurred in the samples representing the whole cheese at the various sampling steps
270 (Table 1). This is in fact the pattern we previously observed for those three FAA in GP cheese as
271 the result of the ADI pathway adopted by selected non starter LABs (D'Incecco et al., 2016). The
272 ADI pathway is used by a variety of microorganisms to contrast acid stress and, in the step leading
273 to ornithine, also to produce ATP (Price, Zeyniyev, Kuipers, and Kok, 2012; Fröhlich-Wyder et al.,
274 2015). However, the genomes of *C. tyrobutyricum* UC7086 (Bassi et al., 2013) and other *C.*

tyrobutyricum strains lack the arginine deiminase (EC 3.5.3.6), the enzyme that catalyses the deamination of arginine to citrulline. Differently, our strain harbours genes coding for argininosuccinate lyase (EC 4.3.2.1) and argininosuccinate synthase (EC 6.3.4.5) that regulate conversion of arginine into citrulline trough a different pathway. In common with the ADI pathway, *C. tyrobutyricum* UC7086 harbours the gene coding for ornithine carbamoyltransferase (EC 2.1.3.3) which promotes the convertion of citrulline to ornithine and carbamoyl-phosphate. Cheeses developing the LBD were reported to have higher pH than the control cheeses because of the metabolic activity of *Clostridium* (Le Bourhis et al., 2007; Gómez-Torres, Garde, Peirotén, & Ávila, 2015). However, to our knowledge no literature data concerning the adoption of a direct de-acidification mechanism is available for *C. tyrobutyricum.*

3.4 Arginine metabolism of *C. tyrobutyricum* UC7086 in milk

The capability of *C. tyrobutyricum* to deaminate free arginine was tested in a milk-based model system. Vegetative cells of *C. tyrobutyricum* UC7086 were inoculated in sterilized milk with and without (control) addition of lactate and arginine. In the control, counts only increased by one log after 10 days of incubation and the contents of free arginine significantly ($P < 0.05$) decreased (Table 2). When lactate and arginine were added to milk, counts increased by 4 log and approximately 30% of arginine was deaminated to citrulline and ornithine ($P < 0.05$) (Table 2). These observations confirmed that *C. tyrobutyricum* is able to metabolise free arginine producing citrulline and then ornithine.

4. Conclusions

The innovative experimental approach adopted in this work allowed us to give an important contribution to the knowledge on *C. tyrobutyricum* behaviour in real manufacturing of hard cheese because vegetative cells and spores were kept separated within the dialysis tubes and thus

299 specifically studied. The observed capability of vegetative cells to sporulate during the vat
300 processing indicated that also vegetative cells represent a potential risk for the insurgence of LBD
301 in cheese, thus discontinuing the association between this defect and the number of spores in the
302 cheese milk. This evidence highlights the need of having low numbers of both vegetative cells and
303 spores of *C. tyrobutyricum* in milk destined to hard cheese manufacturing but also of having
304 methods able to count both of them. Our results showed that *C. tyrobutyricum* UC7086 is capable to
305 adopt metabolic pathways leading to production of non-protein FAA, such as citrulline, ornithine
306 and GABA, depending on growth conditions. This capability was consistent with the genome of the
307 studied strain but could also be shared by other strains. Further investigation of these aspects could
308 support the understanding of *C. tyrobutyricum* adaptation to unfavourable environmental conditions
309 such as those encountered during hard cheese manufacturing.

310

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315

316 **References**

317 Al-Hinai, M.A., Jones, S.W., & Papoutsakis, E.T. (2015). The Clostridium sporulation programs:
318 diversity and preservation of endospore differentiation. *Microbiology and Molecular Biology*
319 *Reviews*, *79*, 19-37.

320

321 Ávila, M., Gómez-Torres, N., Hernández, M., & Garde, S. (2014). Inhibitory activity of reuterin,
322 nisin, lysozyme and nitrite against vegetative cells and spores of dairy-related *Clostridium*
323 species. *International Journal of Food Microbiology, 172*, 70-75.
324
325 Bassi, D., Cappa, F., & Cocconcelli, P.S. (2009). A combination of a SEM technique and X-ray
326 microanalysis for studying the spore germination process of *Clostridium tyrobutyricum*. *Research*
327 *in Microbiology, 160*, 322-329.
328
329 Bassi, D., Fontana, C., Gazzola, S., Pietta, E., Puglisi, E., Cappa, F., & Cocconcelli, P.S. (2013).
330 Draft genome sequence of *Clostridium tyrobutyricum* strain UC7086, isolated from Grana Padano
331 cheese with late-blowing defect. *Genome Announcements 1*, e00614-13.
332
333 Bassi, D., Puglisi, E., & Cocconcelli, P.S. (2015). Understanding the bacterial communities of hard
334 cheese with blowing defect. *Food Microbiology, 52*, 106-118.
335
336 Bermúdez, J., González, M.J., Olivera, J.A., Burgueño, J.A., Juliano, P., Fox, E.M., & Reginensi,
337 S.M. (2016). Seasonal occurrence and molecular diversity of clostridia species spores along
338 cheesemaking streams of 5 commercial dairy plants. *Journal Dairy Science, 99,* 1-9.
339
340 Brasca, M., Hogenboom, J.A., Morandi, S., Rosi, V., D'Incecco, P., Silvetti, T., & Pellegrino, L.
341 (2016). Proteolytic Activity and Production of γ-Aminobutyric Acid by *Streptococcus thermophilus*
342 Cultivated in Microfiltered Pasteurized Milk. *Journal of Agriculture and Food Chemestry, 64*,
343 8604-8614.
344
345 Brunt, J., Cross, K.L., & Peck, M.W. (2015). Apertures in the *Clostridium sporogenes* spore coat
346 and exosporium align to facilitate emergence of the vegetative cell. *Food Microbiology, 51*, 45-50.

347

348 Caplan, Z., Melilli, C., & Barbano, D.M. (2013). Gravity separation of fat, somatic cells, and
349 bacteria in raw and pasteurized milks. *Journal of Dairy Science, 96,* 2011-2019.

350

351 Cocolin, L., Innocente, N., Biasutti, M., & Comi, G. (2004). The late blowing in cheese: a new
352 molecular approach based on PCR and DGGE to study the microbial ecology of the alteration
353 process. *International Journal of Food Microbiology, 90,* 83-91.

354

355 D'Incecco, P., Gatti, M., Hogenboom, J.A., Bottari, B., Rosi, V., Neviani, E., & Pellegrino, L.
356 (2016). Lysozyme affects the microbial catabolism of free arginine in raw-milk hard cheeses. *Food*
357 *Microbiology, 57,* 16-22.

358

359 D'Incecco, P., Faoro, F., Silvetti, T., Schrader, K., & Pellegrino, L. (2015). Mechanisms of
360 Clostridium tyrobutyricum removal through natural creaming of milk: A microscopy study. *Journal*
361 *of Dairy Science, 98,* 5164-5172.

362

363 Dhakal, R., Bajpai, V.K., & Baek, K.H. (2012). Production of GABA (γ-aminobutyric acid) by
364 microorganisms: a review. *Brazilian Journal of Microbiology, 43,* 1230-1241.

365

366 Elwell, M.W., & Barbano, D.M. (2006). Use of microfiltration to improve fluid milk quality.
367 Journal of Dairy Science 89, E20-E30.

368

369 European Union. 2011. Regulation no 584/2011. Approving non-minor amendments to the
370 specification for a name entered in the register of protected designations of origin and protected
371 geographical indications (Grana Padano PDO). OJ L 160, 18.06.2011, 65-70.

372

373 European Union. 2012. Regulation no 1151/2012 of the European Parliament and of the Council on
374 Quality Schemes for Agricultural Products and Foodstuffs. OJ 343, 14.12.2012, 1-29.
375
376 Farkye, N.Y. (2000). Microbiology of cheese making and maturation. *Encyclopaedia of Food*
377 *Microbiology, 1,* 381-387.
378
379 Fisher, N., & Hanna, P. (2005). Characterization of Bacillus anthracis germinant receptors in vitro.
380 *Journal of Bacteriology, 187,* 8055-8062.
381
382 Foster, S.J., & Johnstone, K. (1990). Pulling the trigger: the mechanism of bacterial spore
383 germination. *Molecular Microbiology, 4,* 137-141.
384
385 Fröhlich-Wyder, M.T., Bisig, W., Guggisberg, D., Irmler, S., Jakob, E., & Wechsler, D. (2015).
386 Influence of low pH on the metabolic activity of *Lactobacillus buchneri* and *Lactobacillus*
387 *parabuchneri* strains in Tilsit-type model cheese. *Dairy Science & Technology, 95,* 569-585.
388
389 Garcia-Alvarado, J.S., Labbe, R.G., & Rodriguez, M. A. (1992). Sporulation and enterotoxin
390 production by *Clostridium perfringens* type A at 37 and 43 degrees C. *Applied Environmental*
391 *Microbiology*, 58, 1411-1414.
392
393 Garde, S., Ávila, M., Gaya, P., Arias, R., & Nuñez, M. (2012). Sugars and organic acids in raw and
394 pasteurized milk Manchego cheeses with different degrees of late blowing defect. *International*
395 *Dairy Journal, 25,* 87-91.
396
397 Gómez-Torres, N., Ávila, M., Gaya, P., & Garde, S. (2014). Prevention of late blowing defect by
398 reuterin produced in cheese by a Lactobacillus reuteri adjunct. *Food Microbiology, 42,* 82-88.

399

400 Gómez-Torres, N., Garde, S., Peirotén, Á., & Ávila, M. (2015). Impact of Clostridium spp. on
401 cheese characteristics: Microbiology, colour, formation of volatile compounds and off-flavors.
402 *Food Control, 56,* 186-194.

403

404 Hogenboom, J.A., D'Incecco, P., Fuselli, F., & Pellegrino, L. (2017). Ion-exchange
405 chromatographic method for the determination of the free amino acid composition of cheese and
406 other dairy products: an inter-laboratory validation study. *Food Analytical Methods, 10,* 3137-3148.
407 Hornstra, L.M., de Vries, Y.P., Wells-Bennik, M.H., de Vos, W.M., & Abee, T. (2006).
408 Characterization of germination receptors of *Bacillus cereus* ATCC 14579. *Applied Environmental*
409 *Microbiology, 72,* 44-53.

410

411 Jonsson, A. (1990). Enumeration and confirmation of *Clostridium tyrobutyricum* in silages using
412 neutral red, D-cycloserine, and lactate dehydrogenase activity. *Journal of Dairy Science, 73,* 719-
413 725.

414

415 Kirk, D.G., Palonen, E., Korkeala, H., & Lindström, M. (2014). Evaluation of normalization
416 reference genes for RT-qPCR analysis of spo0A and four sporulation sigma factor genes in
417 *Clostridium botulinum* group I strain ATCC 3502. *Anaerobe, 26,* 14-19.

418

419 Le Bourhis, A.G., Doré, J., Carlier, J.P., Chamba, J.F., Popoff, M.R., & Tholozan, J.L. (2007).
420 Contribution of *C. beijerinckii* and *C. sporogenes* in association with *C. tyrobutyricum* to the
421 butyric fermentation in Emmental type cheese. *International Food Microbiology, 113,* 154-163.

422

Martínez-Cuesta, M.C., Bengoechea, J., Bustos, I., Rodríguez, B., Requena, T., & Peláez, C. (2010). Control of late blowing in cheese by adding lacticin 3147-producing *Lactococcus lactis* IFPL 3593 to the starter. *International Dairy Journal, 20,* 18-24.

Moir, A. (2006). How do spores germinate? *Journal of Applied Microbiology, 101*, 526-530.

Morandi, S., Cremonesi, P., Silvetti, T., Castiglioni, B., & Brasca, M. (2015). Development of a triplex real-time PCR assay for the simultaneous detection of *Clostridium beijerinckii*, *Clostridium sporogenes* and *Clostridium tyrobutyricum* in milk. *Anaerobe, 34*, 44-49.

Pellegrino, L., Battelli, G., Resmini, P., Ferranti, P., Barone, F., & Addeo, F. (1997). Effects of heat load gradient occurring in moulding on characterization and ripening of Grana Padano. *Lait, 77*, 217-228.

Pellegrino, L., Rosi, V., D'Incecco, P., Stroppa, A., & Hogenboom, J.A. (2015). Changes in the soluble nitrogen fraction of milk throughout PDO Grana Padano cheese-making. *International Dairy Journal, 47,* 128-135.

Price, C.E., Zeyniyev, A., Kuipers, O.P., & Kok, J. (2012). From meadows to milk to mucosa–adaptation of Streptococcus and Lactococcus species to their nutritional environments. *FEMS Microbiology Reviews, 36,* 949-971.

Ramirez, N., Liggins, M., & Abel-Santos, E. (2010). Kinetic evidence for the presence of putative germination receptors in *Clostridium difficile* spores. *Journal of Bacteriology, 192,* 4215-4222.

448 Rodriguez, L.M., & Alatossava, T. (2010). Effects of copper on germination, growth and
449 sporulation of *Clostridium tyrobutyricum*. *Food Microbiology, 27,* 434-437.
450
451 Setlow, P. (2003). Spore germination. *Current Opinion in Microbiology, 6.6,* 550-556.
452
453 Storari, M., Kulli, S., Wüthrich, D., Bruggmann, R., Berthoud, H., & Arias-Roth, E. (2016).
454 Genomic approach to studying nutritional requirements of *Clostridium tyrobutyricum* and other
455 Clostridia causing late blowing defects. *Food Microbiology, 59,* 213-223.
456
457 Zúñiga, M., Pérez, G., & González-Candelas, F. 2002. Evolution of arginine deiminase (ADI)
458 pathway genes. *Molecular Phylogenetic and Evolution, 25,* 429-444.

Captions to figures

Fig. 1. Evolution of cell (C-tube) (black bar) and spore (S-tube) (grey bar) concentrations counted within the respective tubes and temperature (—) and pH (- - -) variations recorded at selected steps throughout the Grana Padano cheesemaking: (RE) milk renneting, (EC) end of curd cooking, (CX) curd extraction, (EA) end of acidification, (ES) end of salting, (3C) three month ripened cheese, and (6C) six month ripened cheese.

Fig. 2. SEM analysis of C-tube content at selected steps of Grana Padano cheesemaking: a) renneting; b) end of curd cooking (54°C) (EC); c) curd extraction (CX); d) end of curd acidification (EA). Images of cells (C) and spores (S) of *Clostridium tyrobutyricum* captured at the different steps support the sporulation of the cell after the vat processing. Scale bars are 500 nm in length in panels "a", "b", "c" and 400 nm in length in panel "e".

Fig. 3. SEM analysis of S-tube content at selected steps of Grana Padano cheesemaking:a) renneting; b) end of curd cooking (54°C) (EC); c) curd extraction (CX); d) end of curd acidification (EA). Cells (C) and spores (S) of *Clostridium tyrobutyricum* captured at different steps. Vegetative cells appeared after the curd extraction.Scale bars are 500 nm in length.

Fig. 4. TEM micrographs of cells in C-tube content at selected steps of Grana Padano cheesemaking: a-c) curd extraction; arrows: black spot in the cytoplasm; d-f) acidified curd; arrows: oval structures surrounding the black spot. Cell morphology evolves during the cheesemaking.

Fig. 5. TEM micrographs of spores in S-tube content at acidified curd step of Grana Padano cheesemaking. Images support the germination phase of spores. Black frames in panel "a" are shown with higher magnification in panels "b" and "c", where ultra-structural details of the spore

485 are tagged: (Ex) exosporium; (Ct) coat; (Cx) cortex and (Cr) core. Panel “c” shows a spore during
486 the germination phase, when the core increases in size.
487

Table 1. Concentration of FAAs (g/100 g FAA) throughout Grana Padano cheesemaking and ripening. Depending on the sampling step, analysed samples are: cell and spore tube contents; the whey taken at curd extraction; the cheese portion (z1) in contact with the tube; the cheese portion (z2) surrounding z1; a portion representing the whole cheese. Data are means of triplicate analyses.

Sampling	Sample	Asp	Asn	Glu	Gln	Ala	Cit	Val	Ile	Leu	Tyr	Gaba	Orn	Lys	His	Arg
Curd at the extraction	cell tube	3.04	0.73	43.38	2.57	6.49	0.42	1.43	1.46	1.91	1.16	6.73	0.50	5.33	0.80	3.44
	spore tube	3.03	0.63	42.67	3.23	5.74	0.47	1.40	1.67	1.75	1.04	7.26	0.42	5.34	0.99	3.39
	whey	3.20*	0.55	40.31*	3.40	5.60	0.89*	4.29*	1.72	2.44*	1.66*	2.23*	0.56	5.68	0.63	4.63*
Cheese at the end of acidification	cell tube	2.15	1.47	9.95	7.75	11.44	0.00	2.05	3.34	7.36	2.33	2.28	0.06	13.71	4.17	7.38[a]
	spore tube	2.16	1.55	10.33	7.79	10.61	0.00	2.10	3.47	7.53	2.63	2.42	0.30	13.25	4.58	7.61[a]
	z1	2.25	2.95	9.99	6.59	10.75	0.00	5.10	3.50	8.31	3.93	0.58	0.00	13.13	3.46	7.40
	z2	2.20	2.83	9.71	6.94	10.84	0.00	5.56	3.10	8.24	3.53	0.00	0.00	14.38	3.42	7.27
	whole cheese	2.55	2.30	10.80	6.38	9.73	0.00	5.70	3.20	7.80	3.24	0.00	0.00	13.91	3.03	7.26
Cheese at the end of salting	cell tube	2.69	3.53	13.23	11.13	5.63	0.05	6.65	4.24	8.40	2.43	0.61	0.22	9.85	2.81	4.57[b]
	spore tube	2.44	3.61	13.72	11.49	4.45	0.08	6.75	3.74	9.66	2.07	0.86	0.29	10.24	2.09	4.43[b]
	z1	2.21	4.09	13.49	7.90	4.70	0.00	6.20	4.19	9.16	2.61	0.00	0.32	12.47	3.60	5.39
	z2	2.30	3.90	13.42	7.24	4.84	0.00	6.02	4.24	9.32	2.88	0.00	0.21	12.71	3.51	5.67
	whole cheese	2.34	4.32	12.43	5.78	4.43	0.00	6.34	4.42	9.90	3.13	0.00	0.00	11.75	3.24	7.74
Cheese after 3 months ripening	cell tube	3.65	3.88	17.47	7.39	5.34	1.05	5.15	5.24	8.94	1.43	0.44	1.56	11.64	2.15	1.22[c]
	spore tube	3.54	4.24	18.52	6.54	4.19	1.04	4.92	5.16	9.01	1.01	0.64	1.56	11.54	2.28	1.29[c]
	z1	2.06	4.68	16.58	5.14	3.91	0.93	7.65	4.57	10.32	1.32	0.00	2.46	12.25	4.25	1.75
	z2	1.58	4.91	15.55	5.72	3.21	0.00	7.16	4.09	10.81	2.61	0.00	0.22	11.51	3.93	6.36
	whole cheese	1.87	4.39	15.89	5.81	2.92	0.00	7.10	4.85	9.32	2.59	0.00	0.14	11.64	3.79	5.44
Cheese after 6 months ripening	cell tube	5.86	2.83	22.54	2.77	4.45	2.29	2.92	6.72	9.82	0.42	0.29	1.60	12.63	1.37	0.00 [d]
	spore tube	5.28	3.34	22.62	2.80	3.57	2.35	2.83	6.65	9.39	0.67	0.55	1.64	12.35	1.23	0.00 [d]
	z1	2.78	3.18	19.71	3.45	3.46	1.28	8.75	5.22	10.53	2.58	0.08	2.37	13.16	2.20	0.27
	z2	2.40	4.22	18.43	2.08	3.21	0.60	8.16	4.69	10.84	2.77	0.00	0.51	11.53	3.53	4.22
	whole cheese	2.49	4.22	17.84	2.52	2.64	1.28	7.63	5.15	9.68	2.83	0.00	0.19	11.47	3.14	3.44
Pooled standard deviation		0.85	0.26	1.83	1.73	0.46	0.20	3.36	0.27	0.37	0.59	1.24	0.40	0.57	0.63	2.77

513 * FAA statistically different ($P < 0.001$) between whey and tube contents

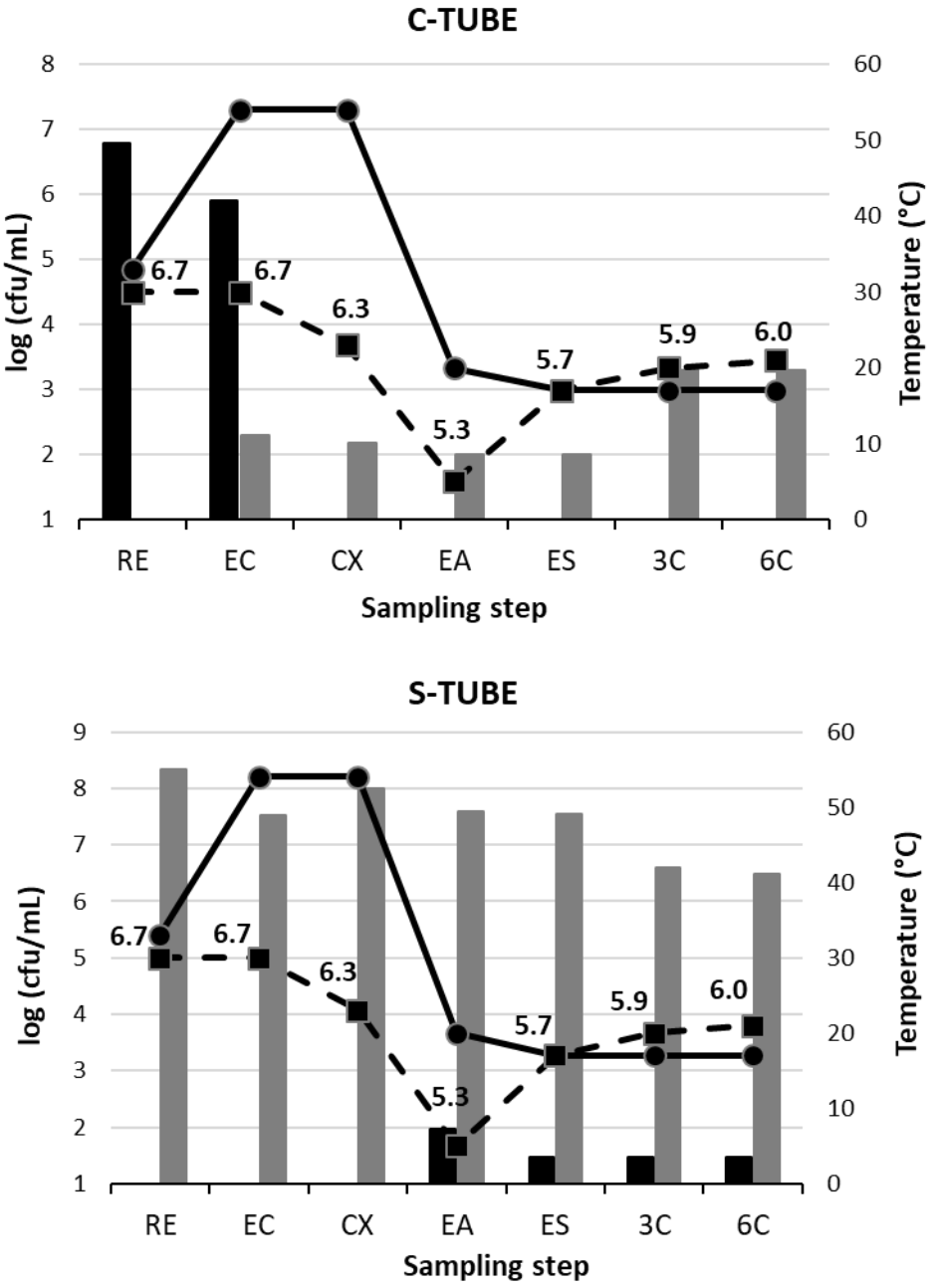
514 [a,b,c,d] Different letters in the arginine column represent statistically different ($P < 0.05$) values among sampling steps

Table 2. Trials carried out to confirm the capability of *Clostridium tyrobutyricum* to use free arginine. Data of arginine, citrulline and ornithine (g/100g FAA) and plate counts of *C. tyrobutyricum* (CFU/mL) in milk with and without addition of arginine (0.5 g/L) and lactate (14 g/L). Data are means of two distinct incubations analysed in duplicate.

	Milk + *C. tyrobutyricum*		**Milk + *C. tyrobutyricum* + Arginine + Lactate**	
	0 days	**10 days**	**0 days**	**10 days**
Arg	3.56 ± 0.02^{a}	2.74 ± 0.41^{b}	78.84 ± 3.31^{c}	56.86 ± 8.63^{d}
Cit	0.85 ± 0.08^{a}	0.84 ± 0.03^{a}	0.95 ± 0.03^{a}	6.27 ± 1.26^{b}
Orn	0.66 ± 0.03^{a}	0.94 ± 0.19^{a}	0.65 ± 0.02^{a}	13.59 ± 5.67^{b}
Counts	$2.1 \times 10^{2} \pm 21^{a}$	$5.5 \times 10^{3} \pm 7.1 \times 10^{2}{}^{b}$	$2.1 \times 10^{2} \pm 21^{a}$	$1.9 \times 10^{6} \pm 2.1 \times 10^{5}{}^{c}$

^{a,b,c,d} Different letters along the same line represent statistically different ($P < 0.05$) values.

523 Fig. 1



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526 Fig. 2

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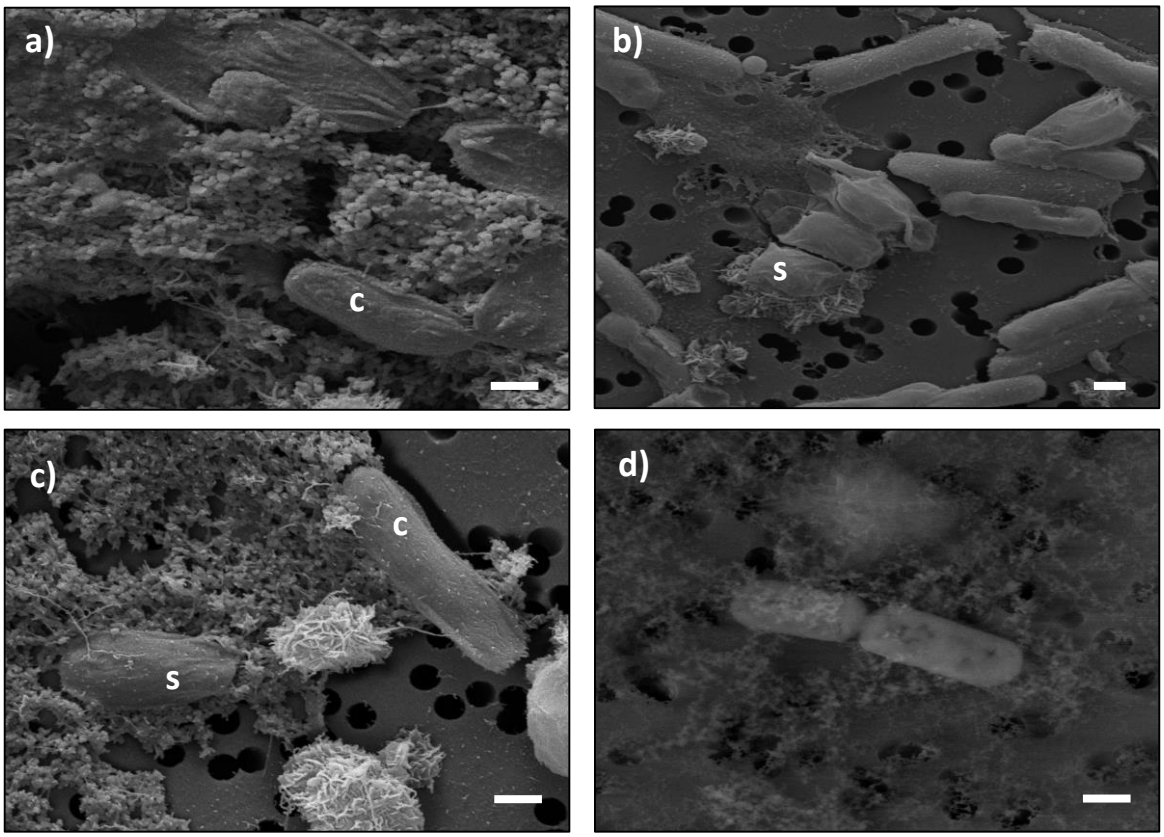

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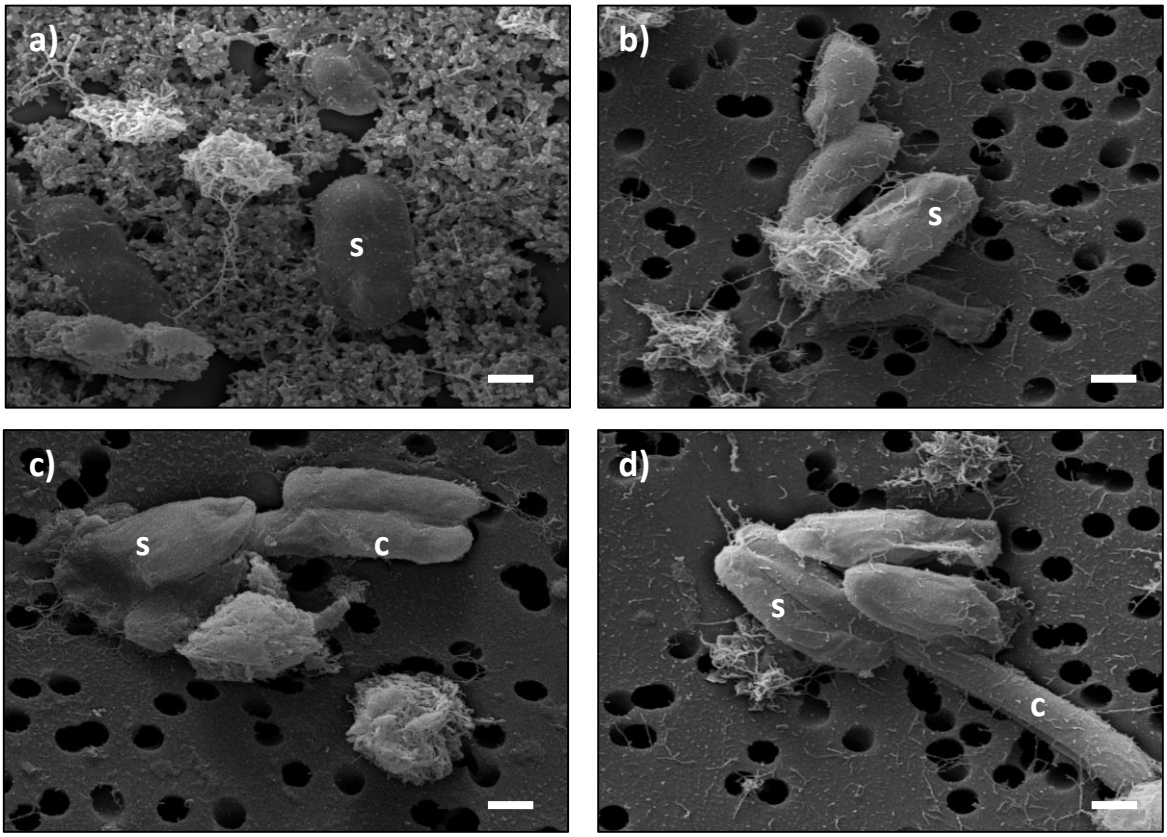
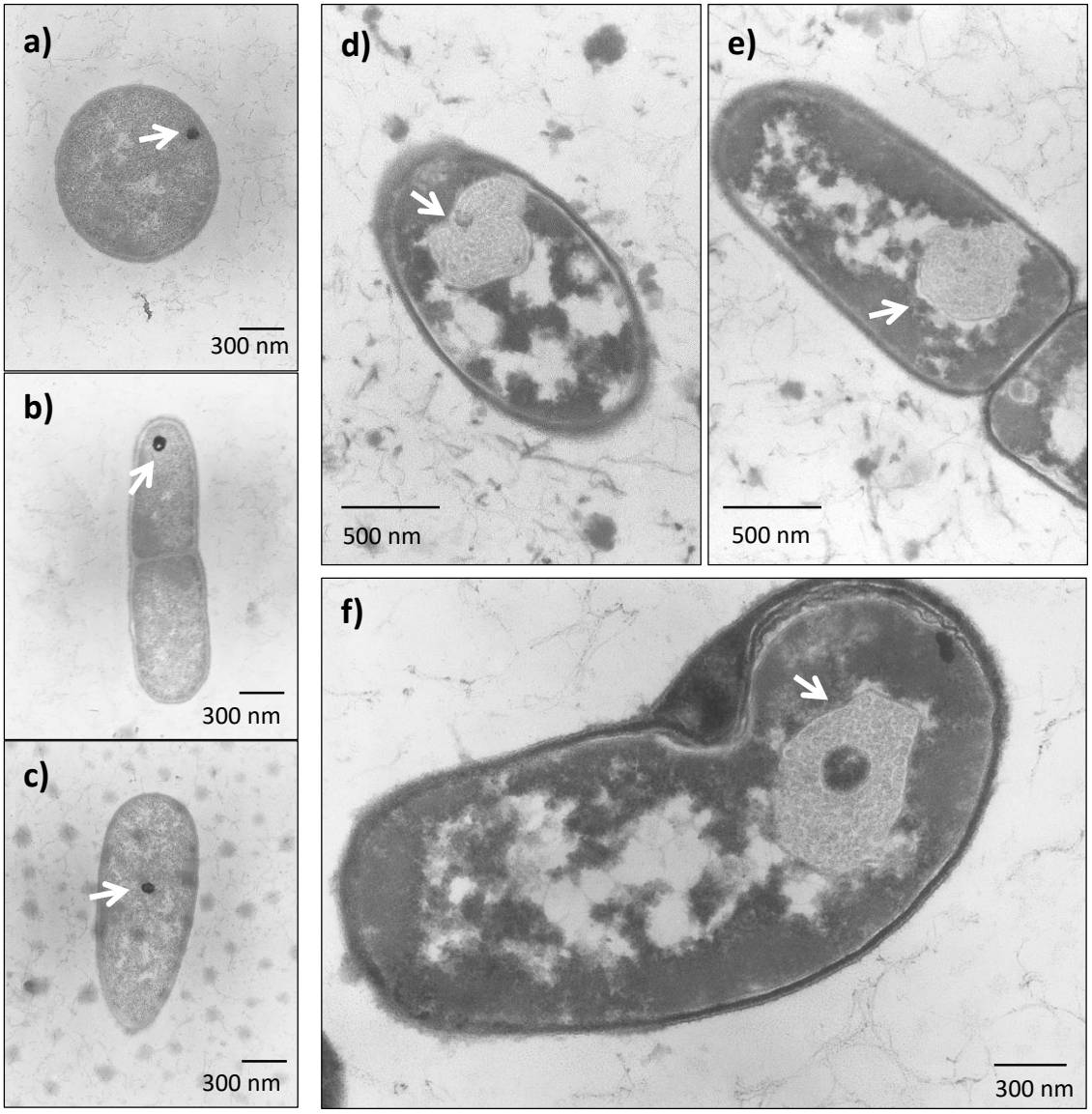
Fig. 3



538 Fig. 4



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541 Fig. 5

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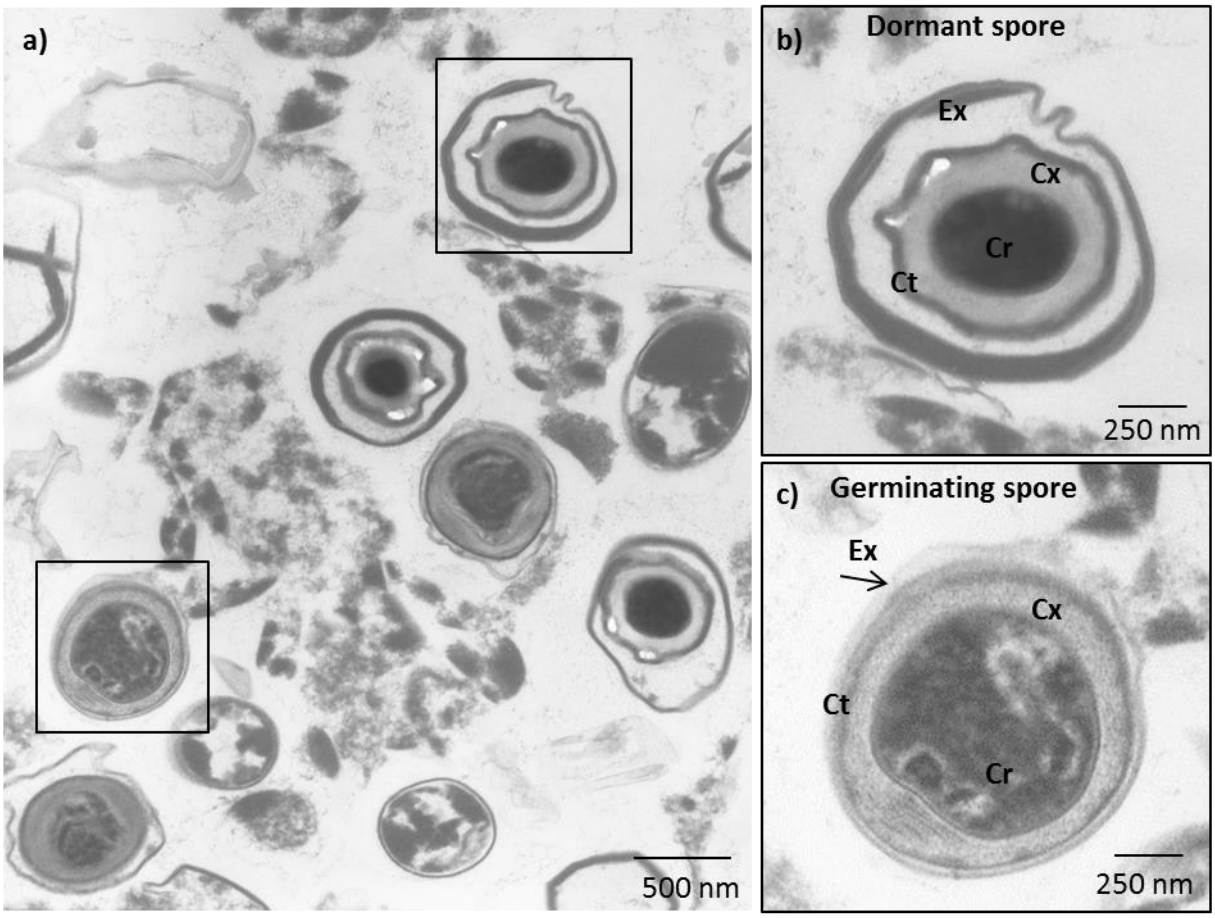

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