

## Behavior of different *Bacillus* strains with claimed probiotic properties throughout processed cheese (“requeijão cremoso”) manufacturing and storage

Mariana B. Soares<sup>a</sup>, Carine N. Almada<sup>a</sup>, Caroline N. Almada<sup>a</sup>, Rafael C.R. Martinez<sup>a</sup>, Eliene P.R. Pereira<sup>a,b</sup>, Celso F. Balthazar<sup>b</sup>, Adriano G. Cruz<sup>b,c</sup>, C. Senaka Ranadheera<sup>d</sup>, Anderson S. Sant'Ana<sup>a,\*</sup>

<sup>a</sup> Department of Food Science, Faculty of Food Engineering, University of Campinas, Campinas, SP, Brazil

<sup>b</sup> Department of Food Technology, Faculty of Veterinary, Fluminense Federal University, Niterói, RJ, Brazil

<sup>c</sup> Department of Food, Federal Institute of Science and Technology of Rio de Janeiro, Rio de Janeiro, RJ, Brazil

<sup>d</sup> School of Agriculture & Food, Faculty of Veterinary & Agricultural Sciences, The University of Melbourne, Parkville, VIC 3010, Australia

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### ABSTRACT

Even though spore-forming bacteria have been mainly linked to spoilage or foodborne pathogens vehiculated through foods, some strains of *Bacillus* can potentially present probiotic properties. The advantage of incorporating probiotic *Bacillus* strains in foods relies mainly on the fact that these microorganisms present high resistance to harsh processing conditions. “Requeijão cremoso” is a type of processed cheese highly appreciated in Brazil. During processing, this product is submitted to several harsh conditions (heating at 90 °C, for instance), leading to the inactivation of probiotic bacteria belonging to *Lactobacillus* and *Bifidobacterium* genera. That fact has precluded the development of probiotic “requeijão cremoso” products; however, probiotic *Bacillus* strains may comprise a promising alternative to overcome the low resistance of traditional probiotics to food processing. The objective of this study was to evaluate the behavior of different *Bacillus* strains with claimed probiotic properties throughout processed cheese (“requeijão cremoso”) manufacturing. A total of five different *Bacillus* strains with claimed probiotic properties (*B. coagulans* MTCC 5856, *B. coagulans* GBI-30 6086, *B. subtilis* PXN 21, *B. subtilis* PB6, and *B. flexus* HK1) were individually inoculated at different stages of manufacture – curd pasteurization, coagulation, and fusion – of “requeijão cremoso” and their survival in each of these stages was determined. The survival of *B. coagulans* GBI-30 6086 was further assessed throughout “requeijão cremoso” production and shelf life (45 days at 6 °C). Besides, the chemical composition, level of proteolysis, and fatty acid profile of the treatments during shelf life were evaluated. The fusion stage was found as the most appropriate for the addition of *B. coagulans* GBI-30 6086, which allows the production of probiotic “requeijão cremoso” and facilitates the technological process while preventing the occurrence of final product recontamination.

### 1. Introduction

Cheeses are considered a proper food matrix for delivery of probiotic microorganisms due to their intrinsic characteristics [relatively high pH (4.8–5.6), high-fat content and low oxygen concentration], which protect them against the hostile conditions faced through GIT transit (GIT) (Boylston et al., 2004; Gomes da Cruz et al., 2009; Hayes et al., 2006). Among the several types of cheeses produced and commercialized in various countries, processed cheese is a featured product developed, aiming to expand the use and shelf-life of natural cheese

(Kapoor and Metzger, 2008).

Processed cheese is obtained by mixing different types of cheeses with emulsifying salts and other ingredients of dairy and non-dairy basis. The manufacturing is characterized by the application of heating and gently mixture of all ingredients to obtain a consistent food with a shelf-life of at least 3–4 months (Kapoor and Metzger, 2008). “Requeijão cremoso” is one sort of processed cheese typically manufactured and highly appreciated in Brazil. “Requeijão cremoso” is obtained by fusing a mass of drained and washed fresh curd added with butter or cream or anhydrous milk fat or butter oil, being reserved for

\* Corresponding author at: Rua Monteiro Lobato, 80, CEP: 13083-862, Campinas, SP, Brazil.

E-mail address: [and@unicamp.br](mailto:and@unicamp.br) (A.S. Sant'Ana).

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products whose milk base contains no fat or protein of non-milk origin (Oliveira et al., 2016).

Even though several dairy foods such as yogurt (Lourens-Hattingh and Viljoen, 2001), ice cream (Cruz et al., 2009), and fermented milk (Shiby and Mishra, 2013) have been considered adequate probiotic carriers, the processing steps of “requeijão cremoso” result in inactivation of common probiotic bacteria, limiting their application in this product. It is known that *Lactobacillus* and *Bifidobacterium* probiotic strains have low heat resistance and, consequently, would not survive the stage of curd fusion, currently conducted at 90 °C/5 min (Oliveira et al., 2018a). On the other hand, adding these microorganisms after curd fusion may lead to recontamination of the product (Menezes et al., 2013; Sunny-Roberts and Knorr, 2009), which may result in product's spoilage during shelf-life or in the growth of foodborne pathogens leading to economic losses and public health burden, respectively (Oliveira et al., 2018b). In this context, incorporating spore-forming bacteria strains with claimed probiotic properties seems to be the best alternative to overcome the drawbacks of traditional probiotics (*Lactobacillus* and *Bifidobacterium*) for production of probiotic “requeijão cremoso.” Additionally, bacterial spores can also withstand the adverse conditions found in the GIT (Cutting, 2011; Hong et al., 2005; Nithya and Halami, 2013), allowing probiotic cells to reach their site of action.

Among the spore-forming bacteria, strains of *Bacillus* have been the most frequently employed as probiotics (Cutting, 2011). Probiotic strains of spore-forming bacteria have been used recently as a supplement for animal feed, human food, and in medical treatments for intestinal disorders and urinary problems (Nithya and Halami, 2013). In this context, strains of the following species should be highlighted: *Bacillus clausii*, *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus coagulans* (Cutting, 2011). Health benefits of probiotic *Bacillus* strains have been described in the literature, such as improvements in protein carbohydrates digestion (Jäger et al., 2018; Keller et al., 2017), modulation of microbiota of elderly and improvement of dysbiosis (Nyangale et al., 2015; Nyangale et al., 2014), reduction of muscle damage and improvement of exercise recovery (Jäger et al., 2016), reduction of bloating and abdominal pain (Hun, 2009; Kalman et al., 2009), antimicrobial activity against pathogens in the gastrointestinal tract (Honda et al., 2011), improved parameters of colitis induced by *Clostridium difficile* in rats (Fitzpatrick et al., 2011), prevention of obesity parameters (Choi et al., 2016), among others. The safety of specific probiotic *Bacillus* strains has been reported (Salveti et al., 2016; Endres et al., 2009; Endres et al., 2011) and some have received the status of generally recognized as safe (GRAS) under intended conditions of use, such as *B. coagulans* GBI-30, 6086 (FDA, 2017), *B. coagulans* SNZ1969 (FDA, 2016a) and *B. coagulans* SBC37-01 (FDA, 2016b).

Although probiotic spore-forming bacteria seems to be very attractive to expand the incorporation and development of probiotic foods, there has been a lack of data regarding their survival through food processing and product's shelf life (Sorokulova, 2008). To date, no studies have been published in the scientific literature that assesses the survival of *Bacillus* strains with claimed probiotic properties during the production of processed cheeses. Given the above, in this work the behavior of five *Bacillus* strains with claimed probiotic properties added at different stages of the manufacture of “requeijão cremoso” and during the product's storage period (45 days at 6 °C) is reported for the first time.

## 2. Material and methods

### 2.1. Strains

Lyophilized preparations (in the form of spores) of *B. coagulans* GBI-30 6086 (Ganeden Biotech, Mayfield Heights, United States), *B. coagulans* MTCC 5856 (Sabinsa Corporation, New Jersey, United States), *B. subtilis* PXN 21 (Protexin, Lopen Head, United Kingdom), *B. subtilis* PB6 (Kemin, Indaiatuba, Brazil), and *B. flexus* HK1 (Mysore, India) with

claimed probiotic properties were used for direct vat set (DVS) at the different stages of “requeijão cremoso” processing.

### 2.2. Manufacture of “requeijão cremoso”

#### 2.2.1. Raw materials and ingredients

“Requeijão cremoso” was manufactured with type A semi-skimmed pasteurized milk (Xandô, São Paulo, Brazil), pasteurized cream – 35% fat (Atilate, Itatiba, Brazil), 1:10 (v/v) lactic acid solution (Dinâmica, Diadema, Brazil), sodium chloride (Dinâmica, Diadema, Brazil), emulsifying salt JOHA S9 (ICL Food Specialties, São Bernardo do Campo, Brazil), and potassium sorbate (Dinâmica, Diadema, Brazil).

#### 2.2.2. Preparation of “requeijão cremoso”

The milk was heated at 70 °C, following the addition of 0.28% (v/v) of a lactic acid solution. This combination was agitated manually until complete homogenization. The mix was set aside at rest for 30 min for coagulation of the curd (thermal acid coagulation). Subsequently, the gel formed was separated into equal portions, and the curd was drained with the aid of cloth sieves and washed with cold, sterile distilled water until the curd reached pH 5.2. Finally, the curd was pressed and kept refrigerated overnight. After complete whey draining, the other ingredients were added: cream, water, sodium chloride, and emulsifying salt in the proportions of 37%, 20%, 1.5%, and 1.5%, respectively, concerning the basic mass (w/w) obtained. Then, the fusion stage (90 °C/5 min) was performed under manual agitation in Bunsen burner until complete fusion. The product was then cooled to 50 °C, a temperature at which potassium sorbate was added in the concentration of 1 g/kg of “requeijão cremoso.” Finally, the “requeijão cremoso” was hot-filled into polystyrene plastic containers with 100 mL capacity. The containers were previously sanitized with a 200 ppm sodium hypochlorite solution. The final product was stored at 6 °C for 45 days.

### 2.3. Inoculation of *Bacillus* strains with claimed probiotic properties at different stages of “requeijão cremoso” processing

The *Bacillus* strains with claimed probiotic properties were inoculated individually at the concentration of  $10^6$ – $10^7$  spores/g or mL at different stages of “requeijão cremoso” production, i.e., before milk pasteurization, before curd coagulation, and before curd fusion. Such stages were selected in order to determine the most appropriate time for addition of *Bacillus* strains. Thus, the *Bacillus* strains were added directly to the milk, following pasteurization at 65 °C for 30 min. In the curd coagulation stage, the *Bacillus* strains were inoculated when the milk temperature reached 70 °C, and the lactic acid solution was added to obtain the fresh curd. In the fusion stage, the *Bacillus* strains were added once the other ingredients were mixed with the fresh curd, and the temperature reached 90 °C.

#### 2.3.1. Sampling collection throughout “requeijão cremoso” processing

During the different stages of “requeijão cremoso” processing, aliquots (1 mL) were collected at predetermined time intervals aiming to determine the counts of *Bacillus* strains with claimed probiotic properties. During milk pasteurization, samples were collected at  $t_{0\text{min}}$ ,  $t_{10\text{min}}$ ,  $t_{20\text{min}}$ , and  $t_{30\text{min}}$ . During coagulation, the samples were collected at  $t_{0\text{min}}$  (coagulation 1),  $t_{30\text{min}}$  (coagulation 2), in addition to  $t_m$  (curd),  $t_d$  (after draining) and  $t_{ov}$  (pressed curd and left to rest overnight – 12 h). Finally, during the curd fusion, samples of “requeijão cremoso” were obtained at  $t_{0\text{min}}$ ,  $t_{2.5\text{min}}$ ,  $t_{5\text{min}}$ , and  $t_{20\text{min}}$ . These time intervals were selected based on preliminary experiments (data not shown).

### 2.4. Enumeration of *Bacillus* strains with claimed probiotic properties

The enumeration of *Bacillus* strains with claimed probiotic properties in the collected samples was performed after the application of heat shock (80 °C/10 min), using a water bath (Quimis model Q334M-28,

**Table 1**  
*Bacillus* strains with claimed probiotic properties and culturing conditions.

Strain	Culture media	Incubation conditions	Composition/manufacturer
<i>B. flexus</i> HK1 (Mysore, India)	Tryptone agar, yeast extract and NaCl (LB)	37 °C/24 h	Formulated <sup>a</sup>
<i>B. subtilis</i> PB6 (Kemin, Indaiatuba, Brazil)	Tryptone soy yeast extract agar (TSAYE)	30 °C/16 h	Formulated <sup>a</sup>
<i>B. subtilis</i> PXN 21 (Protexin, Lopen Head, UK)	Mannitol egg yolk polymyxin agar (MYP)	37 °C/24 h and 20 °C/24 h	Acumedia (Lansing, USA)
<i>B. coagulans</i> GBI-30 6086 (Ganeden Biotech, Mayfield Heights, EUA)	Glucose yeast extract agar (BC)	40 °C/48 h	Formulated <sup>a</sup>
<i>B. coagulans</i> MTCC 5856 (Lactospore, New Jersey, EUA)	Glucose yeast extract agar (GYEA)	37 °C/72 h	Formulated <sup>a</sup>

<sup>a</sup> The composition of the culture media are provided in the supplementary material.

Diadema, Brazil), followed by immediate cooling in an ice bath. Then, serial decimal dilutions were performed using the following diluents recommended by the manufacturers of the *Bacillus* strains with claimed probiotic properties: saline solution 0.9%, for *B. coagulans* MTCC 5856; peptone water 1% (w/v) for *B. coagulans* GBI-30 6086 6086 and HF1; peptone water 2% (w/v) for *B. subtilis* PB6; peptone water 1% (w/v) added with saline solution of NaCl (0.8%, w/v) for *B. subtilis* PXN 21. Aliquots of the dilutions were inoculated in the appropriate selective culture media, as per the manufacturer's instructions as detailed in Table 1. The analysis was done in duplicate, and the results were expressed as the logarithm of the number of spores/g of product.

#### 2.5. The survival of *B. coagulans* GBI-30 6086 inoculated at different steps during “requeijão cremoso” shelf life

The survival of *B. coagulans* GBI-30 6086 during “requeijão cremoso” shelf-life was studied as this strain showed a better survival rate (smaller number of decimal reductions) compared with the other strains described in Section 2.4. Thus, the strain was inoculated at the concentration of 10<sup>8</sup> spores/g at different stages of the process: RP – milk pasteurization at 65 °C/30 min; RF – curd fusion at 90 °C/5 min; RPF – after curd fusion stage (cooling at the temperature of 50 °C). A control treatment (RC) with no addition of *B. coagulans* GBI-30 6086 was also included.

The “requeijão cremoso” were produced as described in 2.1.2 and stored for 45 days under refrigeration (6 °C). Samples from each formulation were analyzed weekly to assess the survival of *B. coagulans* GBI-30 6086 during “requeijão cremoso” shelf life. The results were expressed as the logarithm of the number of spores/g of product.

#### 2.6. The centesimal composition of “requeijão cremoso”

The centesimal composition (g/100 g) of the different treatments was determined in triplicate at the initial time of shelf life. The moisture content was determined through direct drying of 5 g of food, using a heating chamber heated to a temperature of 100–105 °C for 24 h (AOAC, 2007). Total nitrogen or crude protein quantification employed the classical Kjeldahl method using 200 mg of “requeijão cremoso” and applying the correction factor corresponding to 6.38 (AOAC, 2007). Lipid quantification was conducted according to the Gerber method, which employs acid hydrolysis with sulfuric acid to extract the fat from the food (AOAC, 2007).

#### 2.7. Proteolysis and profile of fatty acids

The level of proteolysis was determined at the start and final of “requeijão cremoso” shelf life using a spectrophotometer method as described by Gomes et al. (2011), Felicio et al. (2016) and Pereira et al. (2016).

The profile of fatty acids (FA) was determined as detailed in Matera et al. (2018). Lipids extraction from “requeijão cremoso” was done as

proposed by Silva et al. (2011). The quantification of the fatty acids through gas chromatography as well as analytical conditions, data collection and classification of FA into short-chain FA (SCFA from C2 to C4), medium-chain FA (MCFA from C6 to C12), and long-chain FA (LCFA from C14 to C24) are detailed in as detailed in (Felicio et al., 2016; Silva et al., 2017; Silva et al., 2018). The analysis was performed in triplicate.

#### 2.8. Statistical analysis

The probiotic “requeijão cremoso” formulations and all the experiments were replicated twice. The data obtained were processed using the Assisat 7.7 Beta program (Campina Grande, Brazil) and using analysis of variance (ANOVA), followed by *t* and Tukey tests, when appropriate. The significance level adopted in the tests was *p* < 0.05.

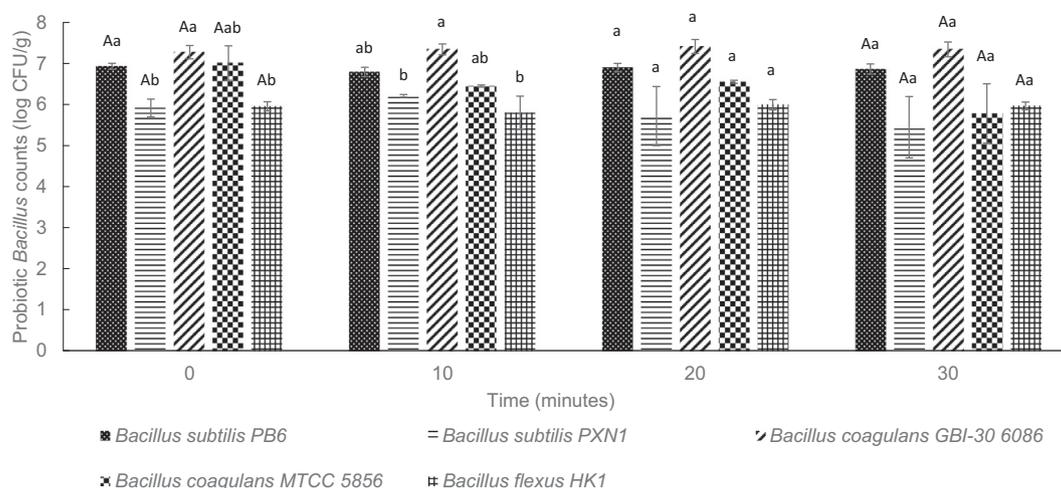
### 3. Results and discussion

#### 3.1. The behavior of different *Bacillus* strains with claimed probiotic properties added at different stages of “requeijão cremoso” processing

##### 3.1.1. Pasteurization

Fig. 1 presents the counts of spores of the *Bacillus* strains with claimed probiotic properties during milk pasteurization (65 °C/30 min) for the production of “requeijão cremoso.” The populations of spores of probiotic bacilli studied remained high (above 6 log cycles/g) at the end of the milk pasteurization, being similar to those determined at the beginning of the process (*p* > 0.05), except for *B. coagulans* MTCC 5856 (Fig. 1). The counts of spores of *B. coagulans* GBI-30 6086 (7.3 log CFU/g) and *B. subtilis* PB6 (6.2 log CFU/g) were higher (*p* < 0.05) than those observed for *B. subtilis* PXN 21 (5.9 log CFU/g) and *B. flexus* HK1 (5.9 log CFU/g) at *t*<sub>0min</sub> (Fig. 1).

Ten minutes after the beginning of the milk pasteurization (*t*<sub>10min</sub>), the concentration of spores of strains *B. subtilis* PXN 21 and *B. flexus* HK1 in the food were lower (*p* < 0.05) than those observed for *B. coagulans* GBI-30 6086 (7.3 log CFU/g), indicating higher sensitivity of these strains to heating (Fig. 1). Twenty minutes after the beginning of the heat treatment stage, the populations of spores of all *Bacillus* strains with claimed probiotic properties remained stable, with no significant reductions observed in the counts determined (*p* > 0.05) (Fig. 1). Even though all strains showed excellent thermal stability, *B. coagulans* GBI-30 6086 was the most resistant strain, as the spores counts remained virtually unchanged throughout milk pasteurization. Bacterial spores are highly heat resistant and can survive milk pasteurization (Gopal et al., 2015; Silva and Gibbs, 2004; Wilbey, 2014). Therefore, the addition of *Bacillus* strains with claimed probiotic properties in the raw milk to be further pasteurized for use in the production of “requeijão cremoso” seems feasible. These results comprise a significant achievement to overcome technological obstacles posed by probiotic strains of *Lactobacillus* and *Bifidobacterium* which are known to be very sensitive to pasteurization conditions normally employed in dairy processing



**Fig. 1.** Counts of different *Bacillus* strains with claimed probiotic properties (log CFU/g) at 0, 10, 20 and 30 min during milk pasteurization (65 °C per 30 min) for “requeijão cremoso” production. Different capital letters indicate significant difference ( $p < 0.05$ ) between the counts of the same strain determined at the beginning (0) and at the end (30 min) of pasteurization ( $t$ -test). Different lowercase letters indicate significant difference ( $p < 0.05$ ) between the counts obtained for different strains at the same time interval (Tukey’s Test).

(Tripathi and Giri, 2014). Various adaptations have been tried to overcome the technological obstacles associated with the incorporation of probiotic strains of *Lactobacillus* and *Bifidobacterium* in foods. These adaptations include the addition of probiotic microorganisms at different stages of food processing, the use of different techniques such as fermentation in two stages, micro-encapsulation, milder temperatures, special packaging, and strict control of storage temperature (Boylston et al., 2004; Cruz et al., 2009). For instance, during cheese production, probiotic strains of the genera abovementioned are usually added, along with starter cultures, in the cheese processing tank to previously pasteurized milk (Gomes da Cruz et al., 2009). However, this procedure is not feasible for “requeijão cremoso,” as the product will be further subjected to other stressful unit operations, such as curd fusion.

### 3.1.2. Coagulation

The stage of thermal acid coagulation of milk proteins was conducted to obtain the basic curd of “requeijão cremoso.” In this process, the temperature ranges from 70 to 80 °C and pH is slightly acid (5.4 to 5.8), representing stressful conditions for probiotic bacteria belonging to *Lactobacillus* and *Bifidobacterium* genera. Probiotic bacteria traditionally used in the manufacture of cheeses are inoculated after curd draining (Cárdenas et al., 2014). Also, coagulated curd washing can lead to significant losses of these microorganisms, and their viability may be reduced in the final product (Gomes da Cruz et al., 2009). Thus, the use of more resistant strains, such as *Bacillus* strains with claimed probiotic properties is justified as these microorganisms are expected to tolerate the sublethal conditions of the coagulation step.

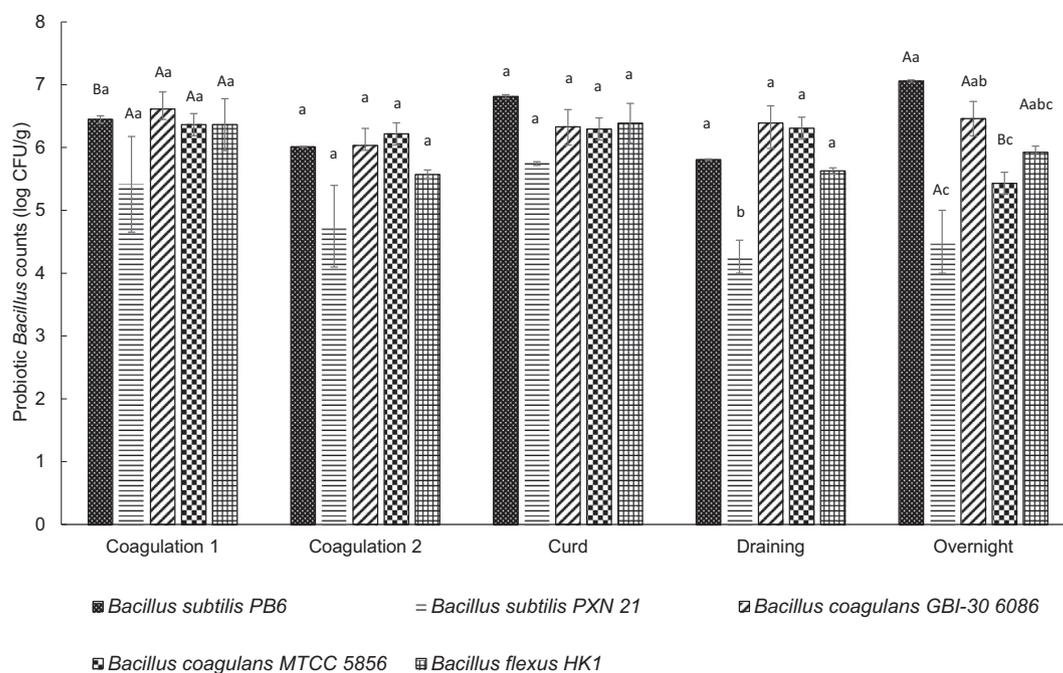
All *Bacillus* strains with claimed probiotic properties were inoculated at the concentration of 6 log CFU/g of “requeijão cremoso,” at the beginning of the coagulation stage (Fig. 2). However, at the initial time of the process ( $t_{0min}$ ), *B. subtilis* PXN 21 showed smaller counts concerning the initial inoculum (5.4 log CFU/g), although no significant difference was observed between both ( $p > 0.05$ ). At times  $t_{30min}$  and on the first day after obtaining the coagulated curd (Fig. 2), there was no significant difference between the counts of probiotic spores ( $p > 0.05$ ). After curd draining, only the population of *B. subtilis* PXN 21 showed a significant reduction (1.2 log CFU/g) (Fig. 2), indicating its higher sensitivity to curd washing compared to the other probiotic strains ( $p < 0.05$ ).

At the end of the coagulation stage (after the curd’s overnight rest), the strain *B. subtilis* PB6 presented higher spore counts (7.1 log CFU/g) compared to the other strains ( $p < 0.05$ ), although it did not differ

from the populations of *B. coagulans* GBI-30 6086 (6.5 log CFU/g) ( $p > 0.05$ ). These results indicated that *B. subtilis* PB6 and *B. coagulans* GBI-30 6086 were the most resistant strains to acid coagulation employed for “requeijão cremoso” production. On the other hand, *B. subtilis* PXN 21 showed lower counts at the end of the coagulation process (4.5 log CFU/g), featuring as the most sensitive strain to acid coagulation at  $> 70$  °C.

### 3.1.3. Fusion

The results indicated that the counts of the different *Bacillus* strains with claimed probiotic properties presented no significant reduction ( $p > 0.05$ ) between the beginning and end of fusion. The counts of these strains remained around 6 log CFU/g, except for *B. subtilis* PB6, for which a decrease of 1.2 log cycles was observed at the end of the fusion stage. During the curd fusion stage ( $t_{2.5min}$  and  $t_{5min}$ ), no significant difference ( $p < 0.05$ ) was observed between the counts of all *Bacillus* strains with claimed probiotic properties studied (Fig. 3). After the cooling of the “requeijão cremoso” ( $t_{20min}$ ), the counts determined for *B. subtilis* PXN 21 were lower than those observed for *B. coagulans* GBI-30 6086 ( $p < 0.05$ ). In the “requeijão cremoso” manufacturing process, the curd fusion is the most aggressive stage against the probiotic microorganisms traditionally added to foods. The high temperatures (90–95 °C) employed inactivate probiotic vegetative cells, which makes their addition at this stage impracticable. The very few tries to produce probiotic “requeijão cremoso” were done adding probiotic bacteria after curd fusion (De Oliveira Gaino et al., 2012; Souza, 2017). Different temperatures for the addition of probiotic strains of *Lactobacillus* and *Bifidobacterium* in “requeijão cremoso” are mentioned in the literature, such as 45 °C (Fernanda and Cardoso, 2011); 50 °C (De Oliveira Gaino et al., 2012) after the curd fusion stage. Thus, the common practice of adding these microorganisms after curd fusion demonstrates their sensitivity to high temperatures, which impacts their viability in the final product and throughout the product’s shelf life. Thus, the possibility to add *Bacillus* strains with claimed probiotic properties to “requeijão cremoso” at curd fusion step enables the dairy industry to overcome significant technological barriers inherent to the manufacture of probiotic dairy products by enabling proper probiotic homogenization and avoiding the recontamination of the product.

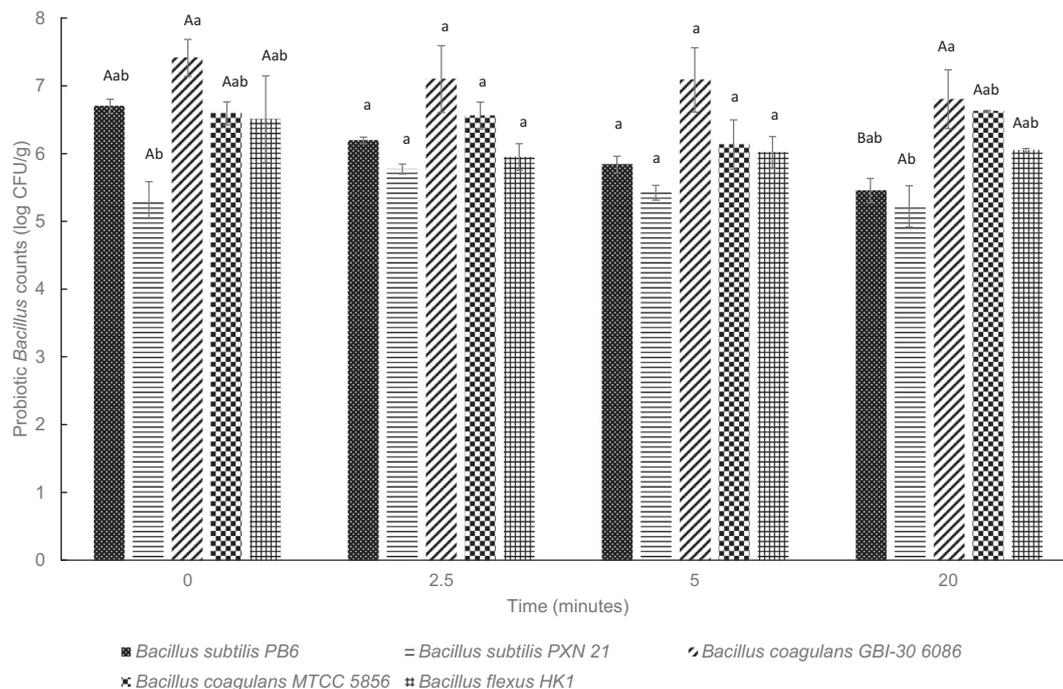


**Fig. 2.** Counts of different *Bacillus* strains with claimed probiotic properties (log CFU/g) during the coagulation step of “requeijão cremoso” production. The samples were collected at  $t_{0min}$  (coagulation 1),  $t_{30min}$  (coagulation 2), in addition to  $t_m$  (curd),  $t_d$  (after draining) and  $t_{ov}$  (pressed curd and left to rest overnight – 12 h). Different upper capital letters indicate significant difference ( $p < 0.05$ ) between the counts of the same strain determined at the beginning (0) and at the end (overnight) of coagulation step ( $t$ -test). Different lowercase letters indicate significant difference ( $p < 0.05$ ) between the counts obtained for different strains, at the same time interval (Tukey's Test).

**3.1.4. The overall number of log reductions of different *Bacillus* strains with claimed probiotic properties subjected to milk pasteurization, coagulation and curd fusion**

As depicted in Table 2, the number of logarithmic reductions observed for the different *Bacillus* strains with claimed probiotic

properties subjected to each unitary operation of “requeijão cremoso” manufacture was not significantly different ( $p > 0.05$ ). In general, increase, or reduction of up to 0.5 logarithmic cycles in microbial populations is related mainly to microbiological methods uncertainty and are disregarded when assessing the growth potential of pathogens in



**Fig. 3.** Counts of *Bacillus* strains with claimed probiotic properties (log CFU/g) during the fusion step (0, 2.5, 5 and 20 min) of “requeijão cremoso” production. Different upper capital letters indicate significant difference ( $p < 0.05$ ) between the counts of the same strain determined at the beginning (0) and at the end (20 min) of fusion step ( $t$ -test). Different lowercase letters indicate significant difference ( $p < 0.05$ ) between the counts obtained for different strains, at the same time interval (Tukey's Test).

**Table 2**

Difference ( $\gamma$ ) (mean and standard deviation) of *Bacillus* strains with claimed probiotic properties counts (log CFU/g) between the beginning ( $N_0$ ) and the end ( $N_T$ ) of each unit operation during “Requeijão cremoso” processing<sup>1</sup>.

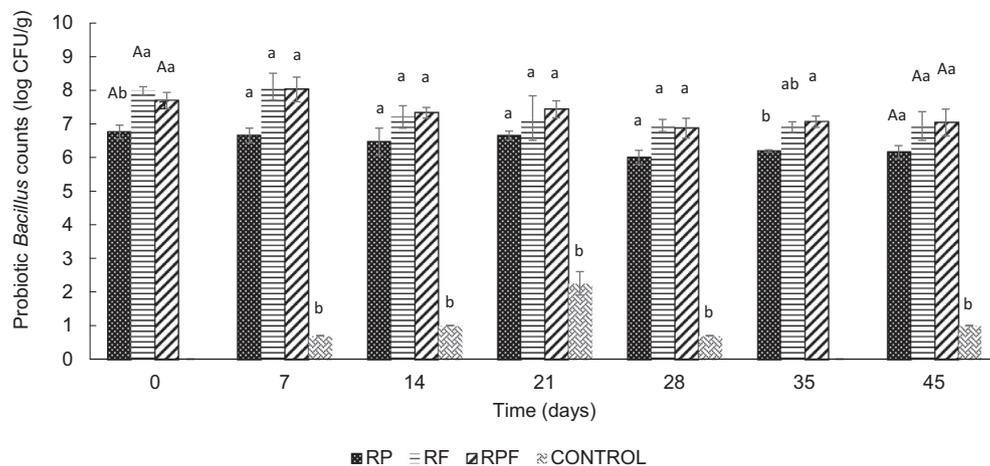
Strain	“Requeijão cremoso” processing steps		
	Pasteurization	Coagulation	Melting
<i>B. subtilis</i> PB6	-0.07 <sup>a</sup> ± 0.07	0.61 <sup>a</sup> ± 0.11	-1.24 <sup>a</sup> ± 0.11
<i>B. subtilis</i> PXN 21	-0.47 <sup>a</sup> ± 0.75	-0.91 <sup>a</sup> ± 1.79	-0.09 <sup>a</sup> ± 0.82
<i>B. coagulans</i> GBI-30 6086	0.07 <sup>a</sup> ± 0.02	-0.15 <sup>a</sup> ± 0.62	-0.61 <sup>a</sup> ± 0.23
<i>B. coagulans</i> MTCC 5856	-1.23 <sup>a</sup> ± 1.62	-0.93 <sup>a</sup> ± 0.16	0.03 <sup>a</sup> ± 0.23
<i>B. flexus</i> HK1	0.01 <sup>a</sup> ± 0.02	-0.44 <sup>a</sup> ± 0.72	-0.46 <sup>a</sup> ± 0.86

<sup>1</sup> Lowercase letters indicate significant difference ( $p < 0.05$ ) between the counts obtained for different probiotic strains, at the same processing step (Tukey's Test).

foods (NACMCF, 2010). Therefore, the lower variations in the counts of *Bacillus* strains with claimed probiotic properties in “requeijão cremoso” compared with those reported for probiotic microorganisms such as *Lactobacillus* and *Bifidobacterium* in dairy products (Granato et al., 2010; Shori, 2015), indicating the higher tolerance of the former to deleterious conditions when compared to the latter.

### 3.2. The survival of *Bacillus coagulans* GBI-30 6086 inoculated at different steps of processing during “requeijão cremoso” shelf life

The behavior of *B. coagulans* GBI-30 6086 added at different steps of processing during the shelf life of “requeijão cremoso” (45 days) is shown in Fig. 4. Evaluating the survival of probiotic microorganisms throughout food's shelf life is essential to ensure the delivery of probiotic cells (Martinez et al., 2015). From Fig. 4, it can be seen that the sooner *B. coagulans* GBI-30 6086 was added to the product, i.e., in the milk to be pasteurized, the lower its counts throughout the storage of “requeijão cremoso.” Despite this, the counts of spores of *B. coagulans* GBI-30 6086 inoculated in the milk pasteurization stage (RP) were always above 6 log spores/g. The counts of *B. coagulans* GBI-30 6086 in “requeijão cremoso” samples inoculated at the fusion stage (RF) and after the fusion stage (RPF) were above 7–8 log CFU/g until the end of their storage period (Fig. 4). Therefore, according to these data, adding *B. coagulans* GBI-30 6086 at the pasteurization stage for “requeijão cremoso” production would not be the best choice. Nonetheless, an alternative for the food industry to add such probiotic strain before the milk pasteurization would be to employ a higher concentration of spores to ensure a specific count (7–8 log CFU/g, for instance) is found in the final product till the finish of its shelf-life. However, the main limitation of this strategy would be a higher cost of the final product due to the addition of higher amounts of the probiotic strain.



**Fig. 4.** Counts of *Bacillus coagulans* GBI-30 6086 (log CFU/g) during shelf-life of “requeijão cremoso” when incorporated in different processing steps (RP: before milk pasteurization, RF: fusion, RPF: after fusion, and control - no probiotic added). Different upper capital letters indicate significant difference ( $p < 0.05$ ) between the shelf-life (0 and 45 days) for the same treatment ( $t$ -test). Different lowercase letters indicate significant difference ( $p < 0.05$ ) between the treatments for same period of storage (Tukey's Test).

In this study, the addition of spore-forming probiotics at the fusion stage (90 °C/5 min), i.e., the product's most aggressive unit operation, had no negative impact on the viability of *B. coagulans* GBI-30 6086. The counts of spores of this strain were high and similar to those determined in the RPF treatment, in which milder conditions are employed. These findings are of great value for the dairy industry, mainly for processed cheese such as “requeijão cremoso,” as the addition of probiotic *Bacillus* strains in the curd fusion stage enables better homogenization of the probiotics, prevents product's recontamination as well as does not affect probiotic's viability. Therefore, to ensure probiotic spore viability until ingestion by consumer, curd fusion seems to be the adequate stage for the addition of *B. coagulans* GBI-306086 during “requeijão cremoso” production. Such information is unprecedented from the technological point of view since in the scientific literature there is a predominance of studies in which probiotic strains of *Lactobacillus* and *Bifidobacterium* were added after one or more heat treatment. This procedure commonly demands the application of protection approaches of probiotic bacteria such as microencapsulation, especially in cheese manufacture (Alves, 2009; Buriti et al., 2007; Buriti et al., 2005a, 2005b; Santini et al., 2012; Soares e Barros and Delfino, 2014). Several studies report good viability of probiotic lactic acid bacteria strains added to different types of fresh cheese, after stages that would be deleterious to their survival, finding adequate concentrations (above 6 log CFU/g or mL) as recommended by the scientific community (Buriti et al., 2007; Buriti et al., 2005a, 2005b; Soares e Barros and Delfino, 2014). On the other hand, other studies showed reductions in populations of probiotic LAB in some cheeses (Alves, 2009; Santini et al., 2012; Vinderola et al., 2000). Considering all these studies with strains of probiotic vegetative cells, the variability of survival of these in the matrices studied depends not only on the step of adding to foods but also on the food storage time and type of strain used.

In this work, the populations of *B. coagulans* GBI-30 6086 remained stable during “requeijão cremoso” shelf-life (Fig. 4), no matter the step of their incorporation (RP, RF, and RPF). This finding demonstrates that under the conditions studied no spore germination and outgrowth occurred.

### 3.3. Centesimal composition, proteolysis and fatty acid composition of “requeijão cremoso” containing *B. coagulans* GBI-30 6086

The results for the chemical composition, proteolysis, and fatty acid composition of the four treatments of “requeijão cremoso” (control and probiotic) studied are shown in Tables 3–5. The centesimal composition analysis of “requeijão cremoso” indicated no significant differences among the products studied and that the formulation is consistent with Brazilian legislation (Mercosul, 1996). The data also indicated no significant differences in the proteolysis and fatty acid constitution of the

**Table 3**

Mean and standard deviation of chemical composition values of control and “requeijão cremoso” containing *Bacillus* strains with claimed probiotic properties<sup>1</sup>.

Treatments <sup>2</sup>	Moisture (g/100 g)	Fat (g/100 g)	Protein (g/100 g)
Control	63.2 <sup>a</sup> ± 0.35	22.2 <sup>a</sup> ± 0.56	13.4 <sup>a</sup> ± 0.23
RP	63.4 <sup>a</sup> ± 0.25	22.7 <sup>a</sup> ± 0.66	13.2 <sup>a</sup> ± 0.68
RF	62.9 <sup>a</sup> ± 0.27	22.5 <sup>a</sup> ± 0.71	13.1 <sup>a</sup> ± 0.58
RPF	63.6 <sup>a</sup> ± 0.32	22.9 <sup>a</sup> ± 0.14	13.6 <sup>a</sup> ± 0.74

<sup>1</sup> Lowercase letters indicate significant difference ( $p < 0.05$ ) between the treatments for the same analyses (Tukey's test).

<sup>2</sup> RP: before milk pasteurization, RF: fusion, RPF: after fusion, and control - no probiotic added.

**Table 4**

Mean and standard deviation of proteolysis level of control and “requeijão cremoso” containing *Bacillus* strains with claimed probiotic properties (0 and 45 days of storage)<sup>1,2</sup>.

Treatments <sup>3</sup>	Proteolysis (absorbance at 340 nm)	
	0	45 days
Control	0.345 <sup>ab</sup> ± 0.03	0.478 <sup>ab</sup> ± 0.05
RP	0.378 <sup>ab</sup> ± 0.06	0.633 <sup>ba</sup> ± 0.08
RF	0.389 <sup>ab</sup> ± 0.05	0.432 <sup>ba</sup> ± 0.06
RPF	0.399 <sup>ab</sup> ± 0.02	0.701 <sup>ba</sup> ± 0.08

<sup>1</sup> Capital letters indicate significant difference ( $p < 0.05$ ) for the same treatment between the beginning and the end of storage (45 days).

<sup>2</sup> Lowercase letters indicate significant difference ( $p < 0.05$ ) between the treatments for the same storage time.

<sup>3</sup> RP: before milk pasteurization, RF: fusion, RPF: after fusion, and control - no probiotic added.

“requeijão cremoso” studied (Tables 4 and 5). Proteolysis is characterized as the biochemical process in which the product's proteins are broken through the action of enzymes of the product itself, yeasts or contaminant bacterial enzymes, hence originating further volatile compounds such as amines, acids (isobutyric, valeric, and isovaleric),

**Table 5**

Mean and standard deviation fat acids content control and “requeijão cremoso” containing *Bacillus* strains with claimed probiotic properties<sup>1</sup>.

Fat acids	Treatments <sup>2</sup>			
	Control	RP	RF	RPF
	g/100 g of fat			
Butanoic (C4:0)	1.10 <sup>a</sup> ± 0.04	1.20 <sup>a</sup> ± 0.05	1.40 <sup>a</sup> ± 0.03	1.30 <sup>a</sup> ± 0.04
Hexanoic (C6:0)	1.50 <sup>a</sup> ± 0.03	1.30 <sup>a</sup> ± 0.06	1.60 <sup>a</sup> ± 0.04	1.50 <sup>a</sup> ± 0.05
Octanoic (C8:0)	1.20 <sup>a</sup> ± 0.03	1.30 <sup>a</sup> ± 0.07	1.40 <sup>a</sup> ± 0.03	1.00 <sup>a</sup> ± 0.01
Docanic (C10:0)	2.20 <sup>a</sup> ± 0.03	2.10 <sup>a</sup> ± 0.01	2.40 <sup>a</sup> ± 0.02	2.10 <sup>a</sup> ± 0.07
Dodecanoic (C12:0)	2.50 <sup>a</sup> ± 0.05	2.00 <sup>b</sup> ± 0.05	2.00 <sup>b</sup> ± 0.09	2.00 <sup>b</sup> ± 0.06
Tetradecanoic (C14:0)	10.1 <sup>a</sup> ± 0.68	8.50 <sup>c</sup> ± 0.06	9.50 <sup>b</sup> ± 0.08	9.70 <sup>b</sup> ± 0.06
Pentadecanoic (C15:0)	1.60 <sup>a</sup> ± 0.02	1.10 <sup>b</sup> ± 0.05	1.70 <sup>a</sup> ± 0.08	1.80 <sup>a</sup> ± 0.05
Palmitic (C16:0)	33.0 <sup>a</sup> ± 0.78	34.1 <sup>a</sup> ± 0.97	31.5 <sup>b</sup> ± 0.77	31.2 <sup>b</sup> ± 0.85
Palmitoleic (C16:1)	1.70 <sup>a</sup> ± 0.05	1.74 <sup>a</sup> ± 0.05	1.80 <sup>a</sup> ± 0.07	1.90 <sup>a</sup> ± 0.59
Stearic (C18:0)	10.4 <sup>b</sup> ± 0.65	10.7 <sup>b</sup> ± 0.77	11.2 <sup>a</sup> ± 0.66	9.50 <sup>c</sup> ± 0.92
Trans-vaccenic (C18:1 t)	2.80 <sup>a</sup> ± 0.08	2.50 <sup>a</sup> ± 0.66	2.70 <sup>a</sup> ± 0.06	2.30 <sup>b</sup> ± 0.06
Oleic (C18:1c)	29.0 <sup>b</sup> ± 0.56	31.2 <sup>a</sup> ± 0.76	30.4 <sup>b</sup> ± 0.88	31.5 <sup>a</sup> ± 0.95
Linolenic (C18:2)	0.70 <sup>a</sup> ± 0.04	0.60 <sup>a</sup> ± 0.06	0.80 <sup>a</sup> ± 0.06	0.90 <sup>a</sup> ± 0.04
α-Linolenic acid, ALA (C18:3)	0.40 <sup>b</sup> ± 0.05	1.20 <sup>a</sup> ± 0.07	0.60 <sup>b</sup> ± 0.02	1.10 <sup>a</sup> ± 0.05
Conjugated acid linoleic (CLA)	1.80 <sup>b</sup> ± 0.04	2.20 <sup>a</sup> ± 0.03	1.50 <sup>b</sup> ± 0.05	2.60 <sup>a</sup> ± 0.03
Short-chain fatty acids (SCFA)	1.10 <sup>a</sup> ± 0.06	1.40 <sup>a</sup> ± 0.03	0.80 <sup>b</sup> ± 0.02	1.30 <sup>a</sup> ± 0.05
Medium-chain fatty acids (MFCA)	7.40 <sup>a</sup> ± 0.07	5.40 <sup>b</sup> ± 0.06	7.40 <sup>a</sup> ± 0.03	6.60 <sup>b</sup> ± 0.05
Long-Chain Fatty Acids (LCFA)	91.5 <sup>a</sup> ± 0.97	93.8 <sup>a</sup> ± 0.08	91.7 <sup>a</sup> ± 0.88	92.5 <sup>a</sup> ± 0.45
Medium-chain fatty acids (MUFA)	33.5 <sup>b</sup> ± 0.58	35.4 <sup>a</sup> ± 0.06	34.90 <sup>b</sup> ± 0.96	35.7 <sup>a</sup> ± 0.57
Polyunsaturated fatty acid (PUFA)	1.10 <sup>b</sup> ± 0.06	1.80 <sup>a</sup> ± 0.03	1.40 <sup>b</sup> ± 0.54	2.00 <sup>a</sup> ± 0.03
Total	100	100.64	99.9	100.4

<sup>1</sup> Lowercase letters indicate significant difference ( $p < 0.05$ ) between the treatments for the same fat acid (Tukey's test).

<sup>2</sup> RP: before milk pasteurization, RF: fusion, RPF: after fusion, and control - no probiotic added.

thiols, and others (Bezerra et al., 2016). At the end of the 45 days, all treatments showed augmented levels of proteolysis. As no difference was observed between the treatments, this increase can be attributed to the action of enzymes of the product itself and not to the germination and outgrowth of *B. coagulans* GBI-30 6086. It is crucial to notice that this phenomenon is often observed during the cheese ripening stage. The presence of natural enzymes of milk, such as plasmin, may have caused some modification on food stability and influenced the level of proteolysis during the cheeses' shelf life. The proteolysis extension index is fundamentally associated with the indigenous proteinases of milk and of the coagulant agent, which break the protein down into high molecular weight peptides (Fox, 1989). Although one of the possible causes of proteolysis may also be the contamination of raw materials, for example, by psychrotrophic microorganisms (Wolfschoon-Pombo and Lima, 1989), in this study such hypothesis was disregarded, since the raw materials used to produce “requeijão cremoso” presented adequate microbiological quality and no formation of gases was observed in the final product.

All the “requeijão cremoso” presented similar fatty acids contents. Cow milk, used in the manufacture of “requeijão cremoso” is rich, mainly, in saturated fatty acids, among them the long-chain fatty acids (Alves, 2009). Studies using cheese as a probiotic matrix found higher amounts of saturated fatty acids in symbiotic cream cheese (minimum and maximum fat, respectively, 226.7 ± 0.3 mg/g and 411.1 ± 20.7 mg/g). However, a reduced concentration of unsaturated fatty acids was observed during the product's storage period (Alves, 2009). In contrast, no significant alteration was found among the contents of unsaturated fatty acids determined during the storage of cheeses made with buffalo milk added with probiotic cultures (Van Nieuwenhove et al., 2007). In this work, the values for unsaturated fatty acids determined in the different treatments of “requeijão cremoso” produced were low concerning the saturated fatty acids, with a mean of 1.58 g/100 g of fat of the cheeses. Some studies state that the addition of probiotic cultures leads to increase in the amount of conjugated linoleic acids (CLA) in dairy products during shelf-life (Akalin et al., 2007; Ekinci et al., 2008; Van Nieuwenhove et al., 2007). On the contrary, CLA contents were not changed in cream cheese stored at 8 °C

for 45 days containing probiotic strains (Alves, 2009). In this study, it has been found that the samples submitted to treatments RP and RPF presented higher CLA contents ( $p < 0.05$ ) as compared to those found in control “requeijão cremoso” (no addition of *B. coagulans* GBI-30 6086). As conflicting data on the effects of processing on CLA contents in dairy products are reported (Bainbridge et al., 2017), Avilez Ruiz et al., 2016), further studies are needed to investigate this finding.

#### 4. Conclusion

Herein, all *Bacillus* strains with claimed probiotic properties evaluated were able to withstand the unit operations employed during “requeijão cremoso” manufacturing. Among the strains evaluated, *B. coagulans* GBI-30 6086 exhibited the best performance retaining high populations during “requeijão cremoso” shelf life (45 days at 6 °C). Besides, *B. coagulans* GBI-30 6086 did not impact on “requeijão cremoso” proteolysis and fatty acid profile during storage, indicating that no germination and outgrowth took place. Curd fusion was found as the adequate stage for the addition of *B. coagulans* GBI-30 6086 to produce probiotic “requeijão cremoso” because it allowed a proper mixture of the culture to the curd and would be followed by heating at 90 °C/5 min, which avoids product's recontamination.

The findings of this study show that the tested *Bacillus* strains with claimed probiotic properties comprise a promising and innovative option for the production of probiotic foods subjected to deleterious unit operations to probiotic strains of *Lactobacillus* and *Bifidobacterium*. As such, an expansion in the range and diversification of products carrying probiotic bacteria becomes possible by employing *Bacillus* strains with claimed probiotic properties. The study also highlights that, additionally to a proper selection of the probiotic strain, the choice of the step to incorporate the *Bacillus* strains with claimed probiotic properties is of chief importance concerning microorganism's viability throughout the product's manufacture and shelf-life.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijfoodmicro.2019.108288>.

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