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Physicochemical characterization, viability and *in vitro* gastrointestinal resistance of probiotic *Bacillus* spp. in cheese bread

Caracterización fisicoquímica, viabilidad y resistencia gastrointestinal *in vitro* de *Bacillus* spp. probióticos en pan de queso

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Abstract
The objective was to develop cheese bread doughs containing spores of *Bacillus coagulans* BC30 or *Bacillus clausii* and to evaluate the physicochemical characteristics (moisture, ash, proteins and lipids), viability and *in vitro* gastrointestinal resistance of the probiotics. The control and probiotic bread doughs were kept at -20 °C for 90 days, with the product being baked after 0, 30, 60 and 90 days to carry out the analyses. There was no difference (p>0.05) in moisture, ash, lipids and carbohydrates between the masses of the three treatments. The viability of *B. coagulans* in dough, after processing and 90 days was > 7.71 log CFU/g, while in baked bread it was 5.24 log CFU/g and 6.03 log CFU/g at 0 and 90 days, respectively. The viability of *B. clausii* in dough at times 0 and after 90 days was > 7.32 log CFU/g and in bread baked at the same times it was, respectively, 5.39 log CFU/g and 5.95 log CFU/g. In enteric phase II, at 90 days, the viability of *B. clausii* was 4.77 log CFU/g, while that of *B. coagulans* was 4.11 log CFU/g. The addition of these probiotics to cheese bread is a promising alternative for the market.

Keywords: Bacillus coagulans; *Bacillus clausii*; Dough; Thermal resistance.

Resumen
El objetivo fue desarrollar masas de pan de queso que contengan esporas de *Bacillus coagulans* BC30 o *Bacillus clausii* y evaluar las características fisicoquímicas (humedad, cenizas, proteínas y lípidos), viabilidad y resistencia gastrointestinal *in vitro* de los probióticos. Las masas de pan control y probiótico se mantuvieron a -20 °C durante 90 días, horneándose el producto a los 0, 30, 60 y 90 días para realizar los análisis. No hubo diferencia (p>0.05) en humedad, cenizas, lípidos y carbohidratos entre las masas de los tres tratamientos. La viabilidad de *B. coagulans* en masa, después del procesamiento y después de 90 días fue > 7,71 log UFC/g, mientras que en pan horneado fue de 5,24 log UFC/g y 6,03 log UFC/g a los 0 y 90 días, respectivamente. La viabilidad de *B. clausii* en masa en los tiempos 0 y después de 90 días fue > 7,32 log UFC/g y en pan horneado en los mismos tiempos fue, respectivamente, 5,39 log UFC/g y 5,95 log UFC/g. En la fase entérica II, a los 90 días, la viabilidad de *B. clausii* fue de 4,77 log UFC/g, mientras que la de *B. coagulans* fue de 4,11 log UFC/g. La adición de estos probióticos al pan de queso es una alternativa prometedora para el mercado.

Palabras clave: Bacillus coagulans; *Bacillus clausii*; Masa; Resistencia térmica.

INTRODUCTION

The eating habits of the population, especially those individuals who care about health and well-being, have been undergoing changes related to better nutrition and body maintenance. In this sense, functional foods increasingly attract consumers' attention because they provide health benefits and help reduce health risks [1].

Probiotics are functional ingredients that, when administered in adequate amounts, provide health benefits [2] such as improving the immune system through the synthesis of vitamins, and enhancing the health of the gastrointestinal tract, preventing allergic and cancer-related diseases, among other benefits. These microorganisms are represented mainly by bacteria of the genera *Lactobacillus*, *Streptococcus*, *Bifidobacterium*, *Enterococcus* and *Bacillus* [3; 4].

Bacillus coagulans is a species that presents strains with probiotic characteristics, such as *B. coagulans BC30*, which presents spores highly resistant to acids and able to adapt to

environments in the intestinal tract with a low presence of oxygen, remaining stable during processes involving heat treatment and when stored at low temperatures [5]. In addition to *B. coagulans*, *Bacillus clausii* is also a sporulated probiotic that is tolerant to heat, acid and salt, as well as resistant to the human gastrointestinal tract [6].
The addition of *Bacillus* probiotics has been studied in several food products, as the spores of these microorganisms are resistant to heat treatment and survive after industrial baking processes, which is an additional advantage compared to usual probiotics, such as lactobacilli. Thus, *Bacillus* have already been used in pasta [7; 8], breads [9; 10], cake mix [11], among other foods subjected to heat treatment.
Cheese bread is a widely consumed product in Brazil. Its formulation includes ingredients such as egg, cheese, cassava starch, milk or water, butter or vegetable oil and salt [12]. The addition of functional ingredients, such as probiotics resistant to heat treatment in bakeable products is an innovative idea and arouses interest among consumers and the food sector due to the scarcity of bakery and confectionery products containing probiotics. Therefore, this study aimed to develop cheese bread doughs added with *Bacillus coagulans* BC30 spores and *Bacillus clausii* spores, and to evaluate their physicochemical characteristics, as well as the viability of probiotics and their resistance to the simulated gastrointestinal tract (GIT) *in vitro* in baked cheese breads.

MATERIAL AND METHODS

Development of cheese breads

For the development of cheese breads, sunflower oil and salt were used, purchased from local stores in Rio Pomba – MG, Brazil; pasteurized liquid egg (Cecoti, Juiz de Fora, MG, Brazil); half-cured cheese; salted butter; and whole milk powder donated by Porto Alegre (Ponte Nova, MG, Brazil) and starch mix (Poduim Alimentos, Tamboara, PR, Brazil), according to Table 1. *B. clausii* (Enterogermina Plus®, Sanofi, São Paulo, SP, Brazil) was added to the formulation in suspension, and *B. coagulans* BC30 (Kerry/Ganeden Biotech BC30®, USA) was added in freeze-dried powder form.

Table 1. Formulation of cheese breads.

Treatments	Portions
Control cheese bread - CCB	500g of dough
Cheese bread with *B. coagulans* – CBBCO	500g of dough + 18 g of freeze-dried powder of *B. coagulans* BC30 with 1,0 x 10^9 CFU
Cheese bread with *B. clausii* – CBBCLA	500 g of dough + 5 mL with 4,0 x 10^9 spores of *B. clausii*

Initially, all powdered ingredients were transferred to a dough mixer (Arno, SX33, Brazil) and mixed until complete homogenization. Then, sunflower oil, butter, and liquid egg were added, while maintaining homogenization. Subsequently, water was added until the dough became consistent and homogeneous, and the cheese was added last. The mass obtained was divided into three portions as shown in Table 2.

Table 2. Percentages of ingredients used in the preparation of cheese bread.

Ingredients	CCB	CBBCO	CBBCLA
Mix of starches	33.98	33.98	33.98
Half cured Minas cheese	14.77	14.77	14.77
Butter with salt	1.85	1.85	1.85
Whole liquid egg	10.62	10.62	10.62
Whole milk powder	2.31	2.31	2.31
Salt	0.92	0.92	0.92
Sunflower oil	5.54	5.54	5.54
Water	30.01	30.01	30.01
B. clausii	-	-	5 mL
B. coagulans BC30	-	18 g	-

Source: adapted from Castro [13]. *The formulation was prepared for 100g of dough. However, CBBCLA dough contains 105g and CBBCO contains 118g.

The three treatments were weighed and formed into 25g portions. The dough from the different treatments was analyzed for the physicochemical characteristics and the viability of the probiotics at time zero (T0) of manufacturing.
At the same time, the bread samples from the different treatments were transferred to a stainless-steel pan and baked at 180 °C for 30 minutes in an oven that had been preheated (Arno, SX33, Brazil) for 10 minutes. The remaining cheese bread doughs prepared were stored in styrofoam trays covered with plastic film and frozen at -20 °C.

Physicochemical evaluation of cheese bread doughs during the storage period

The analyses of moisture, ash, proteins, and lipids were carried out at time 0 according to the methodology of the Association of Official Analytical Chemists [14] for the preparation of the cheese bread doughs (CCB, CBBCO e CBBCLA).
Humidity determination was conducted using the gravimetric method, based on the weight loss of the samples in an oven heated to 105 °C until constant weight was reached. The ash was obtained from fixed mineral residue after incineration of the sample in a muffle furnace at a temperature of 550 °C for 12 hours.

For protein determination, the *Kjeldahl* method was used with a nitrogen-to-protein conversion factor of 6.38, and the quantification of lipids was carried out using the *Soxhlet* method, with residues being weighed and quantified after removing the solvent.

After obtaining the results of the analyses described above, the carbohydrate content was determined by difference through the following calculation: % Carbohydrates = 100 – (% moisture + % ash + % lipid + % protein).

Viability of *B. coagulans* BC30 and *B. clausii* (Enterogermina Plus®) in cheese bread during the storage period

To determine the viability of probiotics, 10 g of dough and baked cheese bread from both treatments were diluted in 90 mL of peptone saline solution [0.85 % de NaCl (Synth, Diadema, São Paulo, Brazil) and 0.1 % of peptone (Acumedia, Michigan, EUA)], and the mixture was homogenized to carry out serial dilutions. The pour plate method was adopted by adding 1 mL of dilutions 10^2 to 10^6 into each Petri dish (Cial, Paulina, São Paulo, Brazil), followed by approximately 15-20 mL of TSA agar (Tryptone Soy Agar). They were incubated at 37 °C for 48 hours in the case of *B. clausii* and 72 hours in the case of *B. coagulans* BC30 [15]. At the end of the incubation period, the count of *B. coagulans* BC30 and *B. clausii* was expressed in Colony Forming Units (CFU) to determine the amount of probiotic bacteria in the product.

Viability was evaluated in the dough after formulation and after baking the breads (time 0), and after 30, 60, and 90 days of freezing the products at -20 ºC (CBBCO e CBBCLA).

Evaluation of the resistance of *B. coagulans* BC30 and *B. clausii* to simulated *in vitro* gastrointestinal conditions

To evaluate resistance to gastrointestinal conditions, baked cheese breads were subjected to *in vitro* testing by simulating gastric and enteric phases I and II, at times 0, 30, 60, and 90 days of storage at -20 °C, according to the methodology of Bedani, Rossi and Saad. [16].

At the end of each phase (gastric, enteric I and II), 1 mL aliquots were removed, and serial dilutions were made in saline solution (0.85% NaCl). Then, in-depth plating on TSA Agar was performed, and the counts of *B. coagulans* BC30 and *B. clausii* surviving the gastrointestinal conditions were determined.

The percentage of bacterial survival in relation to the initial counts prior to the assay, was also calculated according to Guo et al. (2009) [17], using the following equation: Survival rate (%) = (Log CFU N_1/Log CFU N_0) x 100; where N_1= probiotic cell counts at the end of the *in vitro* assay and N_0 = probiotic cell counts before the *in vitro* assay.

Statistical analysis

The experiment for the physicochemical characterization of moisture, ash, proteins, lipids, and carbohydrates of the products was performed under a in a Completely Randomized Design (CRD).

The viability of probiotics was also analyzed by CRD in a 3 × 4 factorial scheme (3 treatments: control and 2 probiotics × 4 times: 0, 30, 60, and 90 days). Analysis of variance (ANOVA) was performed at 5% probability. The means were generated and evaluated using the Tukey Test at a 5% probability level.

The simulated *in vitro* gastrointestinal resistance experiment was carried out in CRD, with comparisons made between phases at each time point and across time points within each phase. Counts were converted into log CFU/g and analysis of variance (ANOVA) was performed. The means were compared using the Tukey Test at a 5% probability level.

The analyses were carried out with the R Software (R CORE TEAM, 2021) and the ExpDes.pt Package [18].

RESULTS AND DISCUSSION

Physicochemical characteristics of control and cheese bread doughs added with *Bacillus* probiotics

There was no difference in moisture, ash, lipids and carbohydrates among the different treatments (p>0.05), indicating that the addition of probiotic *Bacillus* did not interfere with the physicochemical quality of the cheese bread dough. However, only the protein content of the control bread dough was lower (p<0.05) in relation to doughs containing probiotics (Table 3).

Table 3. Average percentages (%) of the physicochemical characteristics of control cheese bread doughs (CCB) and those added with *B. clausii* (CBBCLA) and *B. coagulans* BC30 (CBBCO).

Treatments	Moisture	Ashes	Protein	Lipids	Carbohydrates
CCB	41.56 ± 1.02^a	2.56 ± 0.06^a	5.30 ± 0.06^b	13.12 ± 0.47^a	38.38 ± 0.77^a
CBBCLA	40.75 ± 3.33^a	2.13 ± 0.05^a	6.48 ± 0.36^a	13.52 ± 0.04^a	38.59 ± 2.29^a
CBBCO	35.70 ± 0.06^a	2.75 ± 0.86^a	6.22 ± 0.16^a	13.24 ± 0.46^a	42.76 ± 2.62^a

*Different letters in the same column indicate a significant difference (p<0.05) between treatments.

In the study by Almada-Érix et al. [10], the incorporation of *B. coagulans* BC30 also did not affect humidity or other evaluated characteristics, such as specific volume, texture and color parameters, water activity, and pH of the breads.

Bakery products and some baked foods are mostly composed of carbohydrates, having low nutritional value but high energy value [19]. In the present study, the addition of *Bacillus* probiotics did not affect the carbohydrate content of the samples.

Viability of *B. coagulans* and *B. clausii* in cheese bread during the storage period

A reduction in the viability ($p<0.05$) of *B. coagulans* and B. *clausii* was found after baking the cheese breads (Figure 1A and 1B). The viability of *B. coagulans* in cheese bread dough was 7.77 log CFU/g at T0 (after processing) and 7.71 log CFU/g after 90 days, whereas for the baked bread it was 5.24 log CFU/g at T0 and 6.03 log CFU/g after 90 days (Figure 1A). The viability of *B. clausii* in bread dough was 7.73 log CFU/g at T0 and 7.32 log CFU/g after 90 days, whereas in baked bread at the same times it was 5.39 log CFU/g and 5.95 log CFU/g, respectively (Figure 1B). It was also observed that the time factor did not interfere with the viability ($p>0.05$) of *B. coagulans* and *B. clausii* in either dough or baked bread (Figure 1A and 1B).

Time (days)	0	30	60	90
Rate of survival (%)	67.46	77.33	79.84	78.23

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Rate of survival (%)	67.46	77.33	79.84	78.23

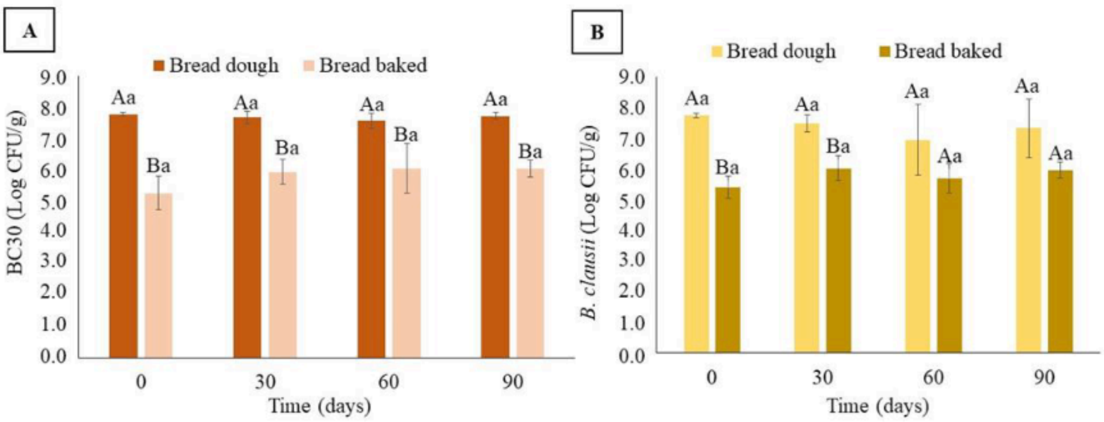

Figure 1. Viability in log CFU/g of *B. coagulans* BC30 (A) and *B. clausii* (B) in cheese bread dough and baked cheese bread over 90 days.

*Different capital letters indicate a significant difference ($p<0.05$) before and after baking at the same time. Different lowercase letters indicate significant differences ($p<0.05$) over time for each treatment.

Almada-Érix et al. [10] also found a significant reduction in the viability of *B. coagulans* BC30 in bread after baking at T0. During the baking stage, the researchers observed a significant difference in the probiotic counts between the crusts of white and wholemeal breads after baking, which decreased by 1.9 log CFU/g and 1.6 log CFU/g, respectively. In the crumb, reductions of 1.6 log CFU/g and 1.5 log CFU/g in probiotic counts were observed, respectively. In the entire slice of both breads, there was a reduction in the count of 1.5 log CFU/g.

Regarding the survival rate of probiotics, this was represented by the viability in the baked cheese bread (final count) in relation to the viability in the dough (initial count) at each time point. Thus, at T0 the survival rate of *B. coagulans* was above 67% and by the end of the storage period it was above 78%. For *B. clausii*, the rate was approximately 70% at T0 and above 82% after 90 days of storage (Figure 1A and 1B). Both probiotics showed an excellent survival rate throughout storage, this being higher for *B. clausii* (Figure 1B).

According to Cinbas, Tontul and Akin [20], the high content of oils and fats present in the formulation of some foods are capable of exerting a thermal insulating effect on the spores when subjected to high cooking temperatures, ensuring the stability of the probiotics.

Thus, the use of sporulated probiotics is considered to be of great industrial interest due to their ability to withstand processing steps, such as mechanical homogenization and heat treatment. Although the time-temperature binomial affected spore survival compared with the dough, counts above 10^5 log CFU/g of both probiotics were able to survive the high baking temperatures to which the cheese breads were subjected (Figure 1). A portion of cheese bread is defined as 50 g, equivalent to two medium-sized units. Therefore, by consuming this portion daily, the consumer would be ingesting the internationally suggested amount of > 10^6 required to exert a beneficial effect on health [21; 22], suggesting that cheese bread can be considered a potential carrier matrix for these microorganisms. In the present study, frozen cheese breads were also found to be an excellent carrier matrix for probiotics, as storage at low temperatures maintained the viability of both probiotics over time.

Survival of *B. coagulans* and *B. clausii* in cheese bread after simulated *in vitro* gastrointestinal (GIT) conditions

When comparing the viability of *B. coagulans* after the *in vitro* test, a significant difference was observed between the phases at 0, 60, and 90 days (p<0.05) (Table 4).

During the first 30 days of storage, more than 5.6 log CFU/g remained viable in enteric phase II (FEII), which simulates the large intestine, whereas after 60 and 90 days, a reduction of more than 1.0 log CFU/g was observed. However, regardless of the shelf life, to guarantee consumer benefits, with counts >10^6 CFU/g, it is necessary to consume 100g of cheese bread, which is equivalent to 4 units, throughout the storage period.

When analyzing the breads containing *B. clausii*, a difference in viability between the gastric phase and the enteric phases I and II was observed only at day 30 (p<0.05). In enteric phase II, the viability was > 5.33 log CFU/g for *B. clausii* at 0, 30, and 60 days, and equal to 4.77 log CFU/g at 90 days, suggesting that only a 50 g portion of bread is sufficient to achieve satisfactory counts throughout the shelf life (Table 4).

Table 4. Viability of *B. coagulans* and *B. clausii* (log CFU/g) in cheese bread after *in vitro* gastrointestinal resistance test, at times 0, 30, 60 and 90 days of frozen storage for the gastric phase (GP), and enteric phase I (EI) and II (EII).

Probiotic	Phases	Times			
		0	30	60	90
B. coagulans	GP	6.76 ± 0.02^a	5.15 ±0.06^a	4.44 ± 0.05^b	4.20 ± 0.19^b
	EI	5.95± 0.1 6^b	5.33 ± 0.22^a	4.56 ± 0.01^{ab}	4.90 ± 0.07^a
	EII	5.62 ± 0.06^c	5.65 ± 0.03^a	4.61 ± 0.09^a	4.11 ± 0.06^b
B. clausii	GP	5.77 ± 1.78^a	5.12 ±1.16^b	5.80 ± 0.65^a	4.39 ± 5.27^a
	EI	5.73 ± 0.92^a	5.43 ± 0.57^a	5.71 ± 4.40^a	4.77 ± 1.46^a
	EII	5.60 ± 1.20^a	5.33 ± 2.01^a	5.37 ± 12.54^a	4.77 ± 1.47^a

*Different letters for each probiotic evaluated separately, in the same column, indicate a significant difference (p<0.05) between the phases at the same time.

In the EII phase (Figure 2A), *B. coagulans* presented approximately 108.09% viable cells at the initial time (T0). After 90 days of storage (p<0.05), around 69.37% of the probiotics remained viable at the end of the simulated GIT. On the other hand, the survival rate of *B. clausii* went from 96.53% at T0 to 77.63% at the end of 90 days of storage (p>0.05) (Figure 2B). Therefore, it is observed that *B. clausii* showed a higher survival rate in the final intestine phase (EII) after the bread storage period. However, both probiotics showed sufficient survival (> 69% of viable cells) to provide health benefits to the consumer after passing through the simulated GIT, indicating that their use is promising in bakery and bakeable products. Alves [9] developed breads with ora-pro-nobis flour and observed that the survival rate of *B. clausii* at the end of enteric phase II was 98.8% at time 0 and 101.9% after 4 storage days. These values further reinforce that *B. clausii* spores are capable of resisting the time and temperature stress involved in bread manufacturing and storage, as well as the simulated *in vitro* digestion process.

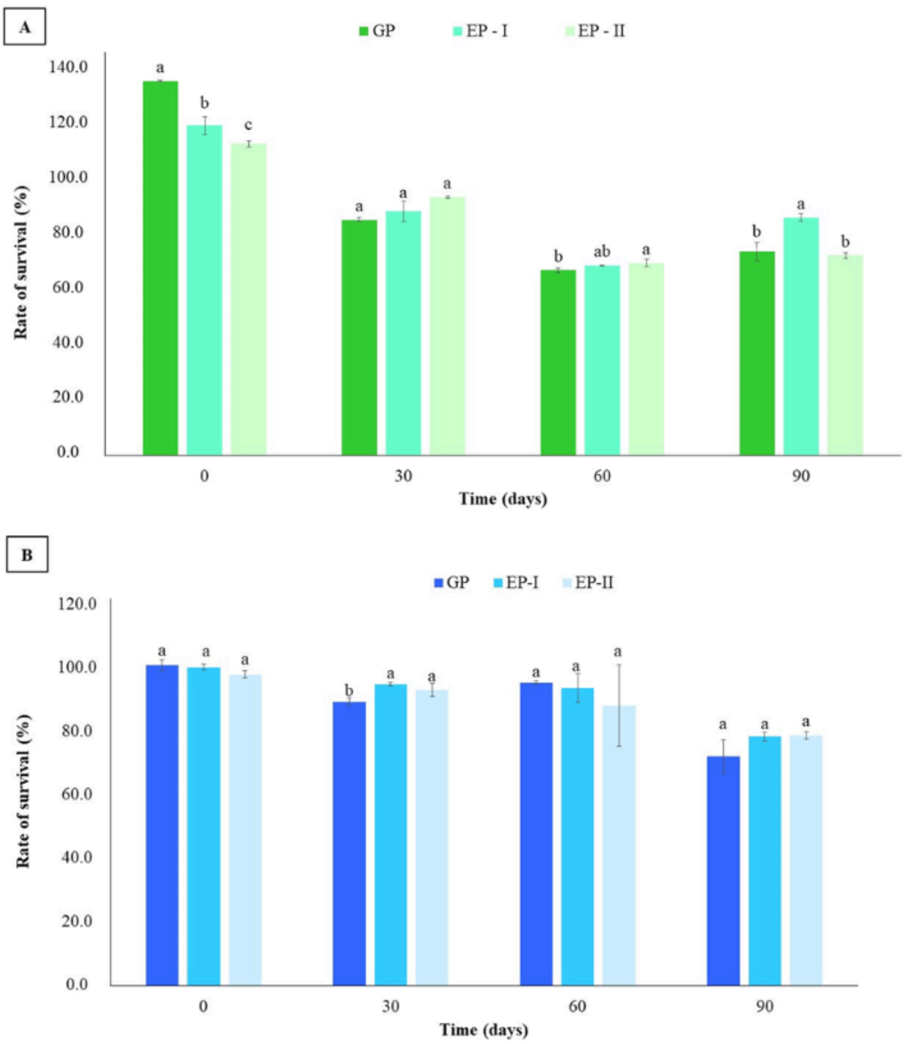

Figure 2. Survival rate of *B. coagulans* (A) and *B. clausii* (B) in cheese breads, after *in vitro* gastrointestinal resistance testing, at times 0, 30, 60, and 90 days of storage for the gastric phase and enteric phases I and II.

*Different letters in the same bars indicate a significant difference ($p<0.05$) between phases at the same time.

The ability of *Bacillus* species to form endospore is a crucial characteristic for their survival under environmental stress conditions, as it contributes to the tolerance of pH, bile fluid levels, high temperatures, dehydration, salt, among other environments [23].

Therefore, the addition of *B. coagulans* and *B. clausii* to cheese bread is advantageous, as they resist high baking temperatures as well as freezing conditions, thereby expanding the possibilities of their addition to baked products.

CONCLUSIONS

The addition of *B. coagulans* and *B. clausii* did not alter the moisture, ash, lipids and carbohydrates of the cheese breads.

The viability of probiotic *Bacillus* in raw cheese bread dough remained above 7.0 log CFU/g throughout the 90 days of storage. In baked breads, viability remained above 5.0 log CFU/g and storage time did not influence their viability either in raw dough or the baked breads.

The *in vitro* gastrointestinal simulation showed that, for more than 10^6 CFU/g of viable cells to reach the large intestine, it is necessary to consume 50 g of cheese bread containing *B. coagulans* at 0 and 30 days of storage, and 100 g at 60 and 90 days of storage. In contrast for cheese bread containing *B. clausii*, only a portion of 50 g, which is equivalent to two loaves of bread, is sufficient throughout the product's entire useful life for the consumer to obtain the benefits.

Both *Bacillus* probiotics resisted the cheese bread baking process, expanding the possibilities of their addition to bakery products subjected to heat treatment.

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CONFLICT OF INTEREST

There is no conflict of interest from the authors. The authors are solely responsible for the content of this article.

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