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Breads with Ora-pro-nobis flour and Bacillus clausii: Characterization and resistance to the gastrointestinal tract *in vitro*

Panes con harina de Ora-pro-nobis y Bacillus clausii: Caracterización y resistencia al tracto gastrointestinal *in vitro*

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Abstract

In this study breads containing ora-pro-nobis flour and spores of probiotic *Bacillus clausii* were developed. Physicochemical and microbiological characteristics of the breads and *in vitro* resistance of the probiotic to the gastrointestinal tract (GIT) were evaluated over 4 days of storage of the breads at 27 °C were evaluated. Acidity, total dry extract, moisture and ash of the breads from different treatments did not differ and the same happened for proteins, fats, carbohydrates and color. There was an increase in the viability of *B. clausii* over storage time, suggesting that the spores resisted baking, germinated and multiplied in the product. At the end of shelf life, viability in enteric phase II was 5.84 Log CFU/g of *B. clausii*. Considering daily consumption of 40 g of bread, approximately 7.44 Log CFU of *B. clausii* would be viable at the end of the GIT. Thus, the developed bread can be a potential carrier of this probiotic and a healthier option for the baking industry.

Keywords: Unconventional food plant; Bakery product; Probiotic; *In vitro* gastrointestinal resistance simulation; Healthy bread.

Resumen

En este estudio se desarrollaron panes que contienen harina de ora-pro-nobis y esporas del probiótico *Bacillus clausii*. Se evaluaron las características fisicoquímicas, microbiológicas de los panes y la resistencia *in vitro* del probiótico al tracto gastrointestinal (TGI) durante 4 días de almacenamiento de los panes a 27 °C. La acidez, extracto seco total, humedad y cenizas de los panes de los diferentes tratamientos no difirieron y lo mismo ocurrió para proteínas, grasas, carbohidratos y color. Hubo un aumento en la viabilidad de *B. clausii* durante el tiempo de almacenamiento, lo que sugiere que las esporas resistieron la cocción, germinaron y se multiplicaron en el producto. Al final de la vida útil, la viabilidad en fase entérica II fue de 5,84 Log UFC/g de *B. clausii*. Considerando el consumo diario de 40 g de pan, aproximadamente 7,44 Log UFC de *B. clausii* serían viables al final del TGI. Así, el pan desarrollado puede ser un portador potencial de este probiótico y una opción más saludable para la industria panadera.

Palabras clave: Planta alimenticia no convencional; Producto de panadería; Probiótico; Simulación de resistencia gastrointestinal *in vitro*; Pan saludable.

Introduction

Since ancient civilization, bread has been part of eating habits, but recently, the population has been looking for healthier bakery products (1). Brazil has an extensive biodiversity of plants rich in nutrients and, among these, is *Pereskia aculeata* Miller, an unconventional food plant (UFP's), widely used in culinary and popularly known in the country as ora-pro-nobis, which in Latin means ‘pray for us’. It’s nicknamed as “meat for the poor” due to its high protein content, superior to other vegetables commonly used as food, such as beans, corn or cabbage. This plant is also rich in total dietary fiber and has important mineral contents, vitamins A, C and folic acid, in addition to presenting a composition rich in phenolic compounds with antioxidant activity and essential amino acids such as lysine, leucine and valine, that are important for human health and nutrition (2).

The flour obtained from ora-pro-nobis leaves is also rich in nutrients and has been added to food preparations. Thus, its use in breads can be an alternative to improve the nutritional profile of these foods, promoting benefits to the health of the consumer. In addition to the benefits of this flour in breads, it has been shown in the literature that probiotic bacteria can resist and maintain their viability and benefits at the end of bread processing (3, 4, 5), making their addition to bakery products attractive (6).

Gut health is an area of growing interest in the food and nutrition industries because of the relationship between the gut microbiome and a range of beneficial health outcomes. The role of the intestinal microbiota is fundamental in maintaining human health, as the aging process changes the profile of the microbiota in the intestine, increasing the risk of disease (7). Therefore, ingredients that confer benefits to the gut microbiome are increasingly present in a number of foods and supplements. Probiotics and prebiotics are the most widely incorporated in functional foods, with significant levels of investment in the development of new technologies and foods that are carriers of probiotics (8, 9, 10). However, due to the high temperatures used in the manufacture of bread, the usual probiotics are not able to resist and, thus, spore-forming bacteria are shown as an alternative for the development of probiotic breads, as they resist baking (11).

Among sporulated probiotics, *Bacillus clausii* has good viability and stability compared to other non-sporulated probiotic species (12). There is a growing number of researches in the food area regarding the use of sporulated probiotics, since in addition to being more resistant to heat treatments, they have a longer shelf life compared to those traditionally used (13) and can thus reduce cost of production and distribution (14). In this context, the aim of this study was to prepare bread containing ora-pro-nobis flour and *B. clausii* spores and to evaluate the physical-chemical and microbiological characteristics besides to the *in vitro* survival of the probiotic after simulating gastrointestinal conditions, verifying the probiotic potential of the prepared breads.

Material and Methods

Obtaining ora-pro-nobis flour

To obtain the flour, leaves of ora-pro-nobis were used, previously washed and sanitized in a 200 mg/L chlorinated solution (Sumaveg, Johnson Diversey, SP, Brazil), rinsed in potable water, and dried in an air circulation oven (Nova Ethics/400/4ND, Brazil) at 60 °C for 24 hours. Then, the material was crushed in a domestic blender (Arno, São Paulo, SP, Brazil), sieved at 18 mesh (mesh opening 1mm), and the flour obtained was packed in glass jars with hermetic lids, which were stored away from light, for 10 days, until use.

Elaboration of breads containing ora-pro-nobis flour and inoculation of *B. clausii*

Wheat flour was used for making the breads - extraction rate 75% (1000 g); ora-pro-nobis flour (25 g); refined sugar (120 g); salt (20 g); powdered milk (30 g); butter (125 g); dry biological yeast (12 g) and water (380 mL). The ingredients were mixed in a planetary mixer (Philips Walita RI7912/41, Brazil) for approximately 6 minutes until the optimal gluten development. The dough was left to rest for 35 minutes and then weighed and divided in half, being half used for breads containing ora-pro-nobis flour (control treatment) and the other half for breads containing ora-pro-nobis flour and the probiotic bacteria. 4.0×10^9 *B. clausii* spores (9.6 Log spores of Enterogermina Plus®, Sanofi) were added for each 40 g of mass.

After adding the probiotic, the dough was again homogenized, rounded, and left to rest for another 40 minutes, then divided into 40 g portions, and fermented for 1 hour and 45 minutes at 27 °C. Breads from both treatments were baked in a gas oven (Electrolux Celebrate 56DT, São Paulo, Brazil) for 30 minutes at 250 ºC and, after cooling, were packed in high-density polyethylene (HDPE) plastic bags and stored at room temperature for up to 4 days, to carry out the analyzes at times 0 and on the fourth day of storage. The work was carried out in 3 repetitions.

Determination of physical-chemical characteristics of breads

The chemical composition of the breads was determined after baking (at time 0) and on the fourth day of storage at room temperature according to the Association of Official Analytical Chemists, AOAC (15). All physicochemical analyzes were performed in triplicate.

The pH of the breads was determined using a digital pH meter (Tecnopon NT PHM, Piracicaba, São Paulo, Brazil) and the total acidity was analyzed by potentiometric volumetry.

Moisture, total dry extract (EST) and ash analyzes were carried out using the gravimetric method, with moisture and EST being determined after drying in an oven at 105 ºC (Nova Ética/400/4ND, Brazil) until a constant weight was obtained, and ash after incineration of organic matter in a muffle furnace (Marconi MA 385/3, Piracicaba, São Paulo, Brazil) at 550 ºC for 6 h (Method 923.03, AOAC 2000) (15).

The lipid content was measured using the Soxhlet method. Extraction from a 5 g sample was carried out using petroleum ether as a solvent in a Soxhlet extractor (Solab SL/2002, Piracicaba, São Paulo, Brazil) according to AOAC (method number 920.39) (16). The samples were extracted continuously for about 6 h at 65 °C. The excess of solvent was removed by evaporation at room temperature, and the lipid content (%) was determined by weighing and using the equation: 100 x N/P, where N = g lipid and P = g sample.

Crude protein was determined using digestion equipment (Solab Co. SL 42/25). The digestion product was distilled using a nitrogen distiller (Lucadema LUCA-74, Ribeirão Preto, São Paulo, Brazil) and titrated with 0.1 mol/L HCl (Impex) (Kjeldahl method 979.09, AOAC 2000) (15). The nitrogen:protein convertion factor used was 6.25.

The total carbohydrate content was obtained by the difference: (100 - (% humidity + % ash + % lipids + % crude proteins)), according to James *et al*. (17).

The analyzes of the color of the crust and crumb of the breads were expressed in L*, a* and b* values (coordinates of the CIELAB system), using a Colorimeter (CR 10, Konica Minolta, Osaka, Japan).

Microbiological evaluation of breads

The breads were analyzed following the microbiological quality standards recommended by Brazilian legislation (18), which establishes criteria for *Escherichia coli*; *Salmonella* sp.; *Bacillus cereus* presumptive and molds and yeasts. Furthermore, coliforms were determined at 35 °C. Coliforms and *E. coli* were determined using Petrifilm™ (3M™ Plate 6404, Sumaré, São Paulo, Brazil). The absence/presence of *Salmonella* sp. was determined by mixing of 25 mL of the product in 225 mL of 1% buffered peptone water (Kasvi, São José dos Pinhais, PR, Brazil) following the methodology described by Andrews *et al*. (19). *B. cereus* analysis was done according to Bennett and Belay (20) in mannitol yolk polymixin agar (MYP, MicroMed/Isofar, Duque de Caxias, RJ, Brazil). Petrifilm (3M Petrifilm 6407/6417/6445) was used for filament fungi and yeasts, incubating the plates in a B.O.D. oven (Nova Técnica, NT704, Piracicaba, SP, Brazil) at 25 °C ± 1 °C for 5 days.

These analyzes were carried out in duplicate, at times 0 and 4 days of storage of the bread, according to the methodologies described by the American Public Health Association, APHA (21).

Viability of *B. clausii* in the breads

Probiotic viability analysis was performed in duplicate, using Tryptone Soybean Agar, TSA (Kasvi, São José dos Pinhais, PR, Brazil) by the in-depth plating method, after the preparation of the bread (time 0) and after 2 and 4 days of storage. Petri dishes (Cial, Paulina, São Paulo, Brazil) were kept at 37 ºC for 72 hours and after incubation, the colony forming units (CFU) of *B. clausii* in the product were enumerated and counted manually.

Resistance of *B. clausii* to simulated in vitro gastrointestinal conditions

The resistance of *B. clausii* to the *in vitro* simulated gastrointestinal tract (GIT) was evaluated by simulating gastric and enteric phases I and II, at times 0 and 4 days of bread storage. The simulation of gastric and enteric conditions with gastrointestinal tract enzymes was performed according to the methodology of Bedani *et al*. (22).

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 carried out and the count of *B. clausii* surviving the gastrointestinal conditions was performed.

The percentage of bacterial survival in relation to the initial counts, before the assay, was also calculated, according to Guo *et al*. (23), using the following equation:

(1) $$\text{Survival rate (\%)} = (\text{Log CFU } N_1/\text{Log CFU } N_0) \times 100$$

Where N_1= probiotic cell count at the end of the *in vitro* assay and N_0 = probiotic cell count before the *in vitro* assay.

Statistical analysis

The product characterization experiment was set up in a Completely Randomized Design (CRD) in a 2 X 2 factorial scheme with 2 treatments (addition or not of probiotics) and 2 times (time 0 and 4 days). In this experiment, the physical-chemical characteristics of pH, acidity, TDE (total dry extract), humidity, ash, protein, lipids and carbohydrates and the colorimetric characteristics, L*, a* and b* of the crust and crumb of the breads were evaluated. Statistical analyzes for the physical-chemical and colorimetric characteristics of the developed breads were performed using analysis of variance (ANOVA) at 5% probability and means test.

The probiotic viability experiment in breads added with ora-pro-nobis was set up in a CRD Design with the evaluations carried out at times 0, 2, and 4 days. Counts were converted to log CFU/g of product and analysis of variance (ANOVA) at 5% probability was performed. Means were grouped using the Scott-Knott test at 5% probability.

The *in vitro* gastrointestinal resistance experiment was carried out in a CRD Design, with comparisons between the phases at each time and between the times for the different phases. Counts were converted into log CFU/g and analysis of variance (ANOVA) at 5% probability was performed. Means were compared by Tukey's test at 5% probability.

The analyzes were carried out with the R Software (24) and the ExpDes.pt Package (25).

Table 1: pH, total acidity (mg organic acid %) and centesimal composition (%) of breads containing ora-pro-nobis flour, from the control treatment and added with probiotic during storage.

Treatment	Days	pH	Total acidity	Protein	Lipid
Control bread	0	5,95 ± 0,01 Aa	2,80 ± 0,01 a	9,15 ± 0,20 a	4,44 ± 0,82 a
	4	6,08 ± 0,02 Ab	2,60 ± 0,21 a	9,17 ± 0,19 a	4,47 ± 0,52 a
Bread with *B. clausii*	0	5,88 ± 0,04 Ba	3,10 ± 0,75 a	9,11 ± 0,05 a	4,54 ± 0,16 a
	4	5,89 ± 0,02 Ba	3,40 ± 0,73 a	9,10 ± 0,06 a	4,51 ± 0,67 a

Treatment	Days	Carbohydrate	TDE	Moisture	Ashes
Control bread	0	15,50 ± 5,80 a	31,20 ± 1,38 a	68,80 ± 1,38 a	1,90 ± 0,07 a
	4	15,83 ± 0,98 a	31,90 ± 9,14 a	68,10 ± 9,14 a	2,10 ± 1,12 a
Bread with *B. clausii*	0	14,50 ± 2,70 a	32,40 ± 0,80 a	67,60 ± 0,81 a	1,40 ± 0,02 a
	4	14,17 ± 6,27 a	28,70 ± 2,03 a	71,30 ± 2,00 a	2,20 ± 0,04 a

* Capital letters compare the different treatments for a single time and means followed by the same lowercase letter in the column do not differ from each other by Tukey's Test at 5% probability.

Results and Discussion

Determination of physical-chemical characteristics of breads

There was a significant pH difference ($p<0.05$) between control bread and bread containing *B. clausii* spores within the same time for both treatments. However, there was no pH difference for the breads containing the probiotic over the storage period ($p>0.05$), indicating the stability of the product over the four days of storage (Table 1).

During storage, the levels of total acidity, total dry extract (TDE), moisture, and ash of the control bread and bread containing *B. clausii* spores did not differ from each other ($p>0.05$). This result shows that the addition of the probiotic did not interfere with these characteristics, which is desirable when preparing a new product, as it maintains the original characteristics of the product (Table 1).

The macronutrient contents and centesimal composition of the breads of both treatments, after processing (time 0) and after 4 days of storage also did not differ from each other ($p>0.05$), indicating that the addition of the probiotic maintained the bread macronutrient profile (Table 1).

When analyzing the macronutrient content of French bread, traditionally consumed in Brazil, as described in the Brazilian Food Composition Table – TACO (26), it was observed contents of 8.0% of proteins, 3.1% of lipids and 58.6% of carbohydrates. The values of proteins and lipids found in the breads of the present study are close to those of the traditional French bread, which makes the product attractive to the consumer, as it has similar characteristics to those already traditionally consumed. However, the carbohydrate content of the breads containing probiotic and ora-pro-nobis flour, are lower when compared to the Brazilian TACO table, which is also attractive to consumers looking for diets with reduced carbohydrates and calories.

Probiotic breads with ora-pro-nobis flour have higher moisture contents when compared to breads to the Brazilian TACO table, which is 28.5%. Breads with vegetables have a greater tendency to retain water and, consequently, increase moisture due to the greater amount of water-soluble fiber present in vegetables. This result is directly related to the reduction of carbohydrate contents, which tend to be less concentrated at higher moisture values, also contributing to greater bread softness, thus improving its texture. It is important to emphasize that the prepared breads were not added additives and preservatives and, therefore, are healthier compared to commercial breads.

Ranawana *et al*. (27) added vegetables to breads and found 43.7% moisture and 68.1% carbohydrates in broccoli breads and 43.6% moisture and 69.9% carbohydrates in beetroot breads. In the same study, for the control breads, 41.2% and 75.9% of moisture and carbohydrates were found, respectively. These results indicate that moisture was higher in breads with vegetables than in control breads, and this increase reduces carbohydrate levels, as was also observed in the present study.

In a study with the addition of chia flour to bread, Sayed-Ahmad *et al*. (28) added flour in proportions of 2, 4, and 6% and found that the moisture content increased significantly with the increase in flour added, as well as in the present study, in which the developed breads had a higher moisture content when compared to the Brazilian TACO table values. Thus, the results of the present study are in line with the literature and with some products already known on the market, confirming their potential.

Analysis of the color of the crust and crumb of the control bread and bread containing *B. clausii* spores were also carried out. The probiotic did not alter the luminosity (L*) of the bread crust and crumb, since there was no difference ($p>0.05$) between treatments and over the storage period (Table 2). The same was verified for the variables a* of the crumb and b* of the crust and crumb, with a predominance of greenish-yellow color in the crust and in the crumb, compatible with the color of ora-pro-nobis flour used.

Table 2: Color (L*, a*, b*) of crust and crumb of bread containing ora-pro-nobis flour, from the control treatment and added of probiotic, at times 0 and 4 days.

Treatment	Day	L* crust	L* crumb	a* crumb	b* crust	b* crumb
Control bread	0	41,0 ± 2,4 a	39,7 ± 2,4 a	0,0 ± 0,3 a	16,6 ± 1,6 a	11,8 ± 0,8 a
	4	41,6 ± 2,8 a	40,2 ± 1,6 a	0,2 ± 0,3 a	16,9 ± 1,9 a	11,7 ± 0,6 a
Bread with *B.clausii*	0	42,1 ± 1,1 a	39,1 ± 2,4 a	-0,2 ± 0,33 a	18,0 ± 0,7 a	10,8 ± 1,0 a
	4	41,6 ± 2,8 a	44,9 ± 3,7 a	0,6 ± 0,7 a	17,9 ± 1,8 a	12,5 ± 1,3 a

* Means followed by the same letter do not differ from each other by Tukey's Test at 5% probability.

Chlorophyll is a pigment widely distributed in green plants. However, these pigments can undergo degradation during processing, exposure to high temperatures, and storage, resulting in color variation from bright green to dark yellow (29). This degradation was evidenced in the present study when observing the color of the bread after baking, with a predominance of the yellow hue (Figures 1A and 1B).

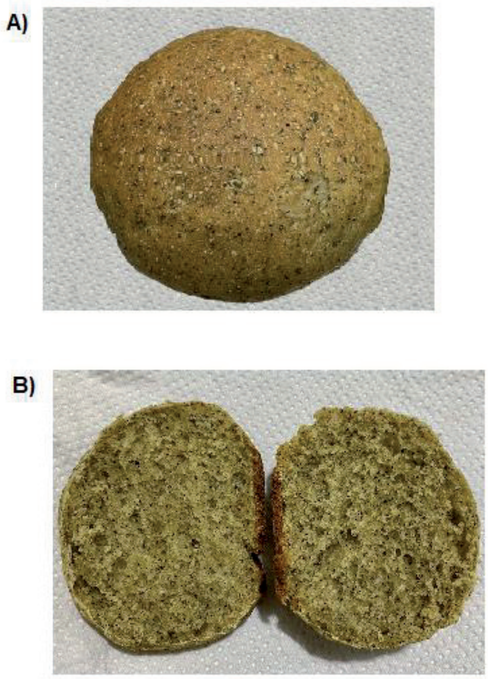

Figure 1: Bread color. A) crust color; B) crumb color.

Microbiological evaluation of breads

It was verified in the breads of both treatments stored at 27 ºC, counts <1.0 x 10^1 for coliforms at 35 °C and *Escherichia coli*, absence of *Salmonella* sp. in 25 g and <1.0 x 10^1 for *Bacillus cereus* and molds and yeasts, being the product suitable for human consumption until the 4th day of storage, as it meets the standards established by Brazilian legislation (18). These results reflect good manufacturing practices during bread processing, in addition to temperature control during preparation and proper storage, which are essential for the quality of the final product.

Viability of *B. clausii* in the breads

There was a significant increase in the viability of *B. clausii* ($p<0.05$) over the bread storage time (Figure 2).

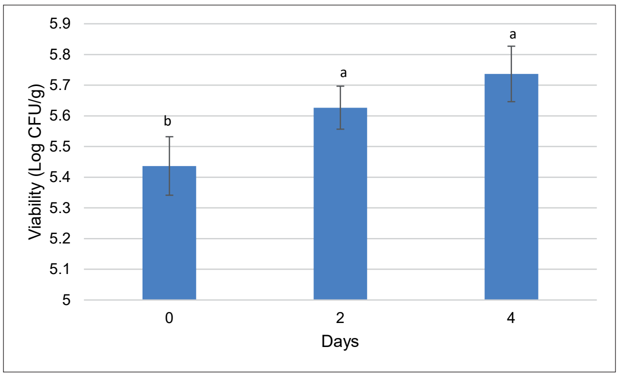

Figure 2: Viability of B. clausii in bread containing ora-pro-nobis flour at times 0, 2 and 4 days. *Means followed by the same letter do not differ according to the Scott-Knott Test at 5% probability.

The initial count of *B. clausii*, before the baking stage, was 9.6 Log of spores and after this stage, there was a reduction in bacterial viability at time zero, with the count being 5.43 Log CFU/g, showing that temperature affected the viability of probiotics in breads (Figure 2). The pH of the medium also directly interferes with bacterial growth. Claus and Berkeley (30) reported that the ideal pH for the growth of *B. clausii* varies between 7.0 and 10.5 and as in the present study, the breads had lower pH values, this may also have contributed to a lower viability of the product.

However, even with the initial reduction, an increase in probiotic viability was observed over time (Figure 2) and on the 4th day of storage, it was observed a count of 5.73 Log CFU/g. This increase in probiotic viability also occurred in the study of Zhang *et al*. (4), who supplemented bread with *L. plantarum* and evaluated the effects of baking temperature and storage time on bacterial viability. The researchers observed that after 5 days of storage there was an increase in viability, which went from 4.0 Log CFU/g to 6.0 Log CFU/g, suggesting that cells that survive after roasting need time to recover the cell damage suffered by the heat, requiring adaptation to the new environment, to reestablish its growth, which certainly also occurred in our study.

Resistance of *B. clausii* to simulated *in vitro* gastrointestinal conditions

When incorporating probiotic microorganisms into food matrices, it is important to carry out studies on their survival both during food processing conditions and during gastrointestinal transit stress conditions (31). The *in vitro* assay was analyzed between the different phases, gastric, enteric I and enteric II of the gastrointestinal tract, at the same analysis time (T0, T4) and within the same phase, throughout the shelf life (Figure 3).

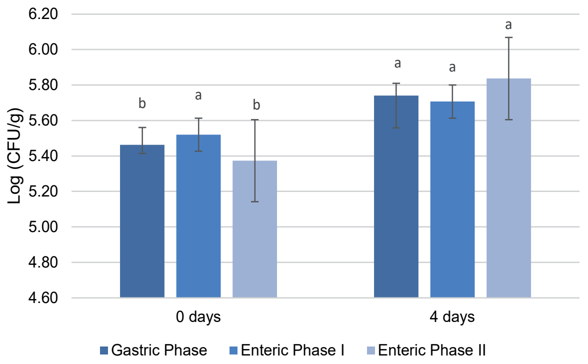

Figure 3: *In vitro* gastrointestinal resistance of *B. clausii* in the breads after processing (0 day) and after 4 days of storage. *Letters indicate the comparison of viability between the different times of the same phase. Means followed by the same letter do not differ from each other by Tukey's Test at 5% probability.

There was a difference in viability values ($p<0.05$) between the gastric and enteric phases II at different times, with the average viability in the gastric phase at time zero being 5.46 Log CFU/g, increasing to 5.74 Log CFU/g after 4 days, and 5.37 Log CFU/g at time zero in enteric phase II, increasing to 5.84 Log CFU/g after 4 days. This increase in viability after digestion simulation is in agreement with what was previously observed in the plate count during the shelf life, suggesting that the storage time of the breads contributes to recovery from damage suffered by cells after exposure to heat. However, analyzing the behavior of *B. clausii* over the bread storage time, it was found that there was no difference in viability ($p>0.05$) between the phases of the *in vitro* test at the end of shelf life (Figure 3).

At the end of shelf life, the viability in enteric phase II, the final portion of the gastrointestinal tract that simulates the large intestine, was 5.84 Log CFU/g of *B. clausii* Figure 3). The breads are sold in portions of approximately 40 to 50 grams. Therefore, the daily consumption of a portion of 40 grams offers the consumer 7.44 Log CFU/portion, a sufficient amount to guarantee the effective action of the probiotic in the health of the gastrointestinal tract, which makes the bread potentially probiotic.

The survival rate of *B. clausii* at the end of enteric phase II was 98.8% at time 0 and 101.9% at time 4 days. These values corroborate the data presented, reinforcing once again that the spores of *B. clausii* can survive the stress of time and temperature involved in bread making, and also the digestion process, reaching the large intestine viable, making the developed product a great carrier of probiotic.

Foods derived from the cereal group, especially bread, have significant potential for delivering probiotic microorganisms to the consumer. The use of flour obtained from grains can protect probiotics during their passage through the gastrointestinal tract, increasing their colonization and, consequently, their viability (32). Associated with this, the fact that bread is widely consumed worldwide makes it a promising matrix to reach a significant portion of the population, giving them improvements in health, obtained by daily consumption of probiotic breads.

Conclusions

The addition of *B. clausii* spores did not modify the composition of the breads, which proved to be similar to breads already consumed by the population, indicating that the developed product may be well accepted by the consumer. The viability of the probiotic increased during the bread storage period, indicating that the matrix is promising to carry this microorganism and the product was considered safe for human consumption. At the end of the *in vitro* gastrointestinal simulation, 5.84 Log CFU/g of viable *B. clausii* reached the large intestine, suggesting that the consumption of a 40 g portion carries 7.44 Log CFU/ portion of *B. clausii*, that will promote functional effects to consumers.

This study demonstrates that breads made with ora-pro-nobis flour can effectively carry *B. clausii* and that it survived the baking of the product. This finding highlights an innovative alternative for the bakery industry aiming to incorporate functional vegetables and sporulated probiotics into their products, catering to the health-conscious public that seeks a healthy diet.

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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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