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Cell viability and resistance of probiotic bacteria in flavored rice byproducts beverage

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Abstract

In recent years, the development of fermented functional foods has raised, especially with the used of lactic bacteria which have probiotic features and can ferment many substrates, including cereals. The aim of this study was to assess the viability of the cultures *Lactobacillus acidophilus*, *Bifidobacterium* spp., and *Streptococcus thermophilus* in fermented rice extract with waxy maize starch (WMS) added during refrigerated storage (28 days at 5 °C), and to evaluate the resistance to acid and to bile salts of probiotic bacteria. The product's pH oscillated during the refrigerated storage. The *L. acidophilus* count was below 10^4 CFU/g after 14 days. The count of *Bifidobacterium* spp. at the beginning was 10^4 CFU/g and 0 after 14 days, while *S. thermophilus* 10^9 CFU/g and at the end of storage it was close to 10^5 CFU/g. *L. acidophilus* started close to 10 survived in small counts (1.87 10^3 and 1.16 10^3 CFU/g) at acidic conditions and in the presence of bile, respectively, conditions that were similar to the hostile conditions found in the digestive system, unlike *Bifidobacterium* spp., which did not survive at those conditions. The flavored fermented rice extract with WMS can only be considered probiotic after processing (time zero), indicating that the WMS did not help to increase the survival of these cultures. *L. acidophilus* proved to be more resistant to product conditions, acidity, and bile.

Keywords: *Oryza sativa* L., byproduct, vegetable extract, *Lactobacillus acidophilus*, bacteria survival

Introduction

Currently, there is a growing commercial interest in adding probiotic bacteria to fermented lactic products, due to recent discoveries in various areas of bioscience, which support the hypothesis that, along with nutrition, diet can modulate many functions in an organism.[1] Traditionally, yogurt is produced using *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. These lactic acid bacteria offer some health benefits, but they are not natural inhabitants of the intestine, nor do they survive in the gastrointestinal tract, performing few benefits to consumers' health. With the goal of making yogurts functional, probiotic bacteria are added, such as those belonging to the genera *Bifidobacterium* spp. and *Lactobacillus acidophilus*.[2] The addition of probiotic bacteria is a technique that has been widely adopted by the dairy industry. However, factors such as yogurt acidity, dissolved oxygen, interactions among species, inoculation practices, and storage conditions may interfere with the survival of probiotic microorganisms in fermented lactic products,[3] since there are not enough studies on probiotics' survival during refrigerated storage. In addition, the factors involved in the biomass production of one strain (medium pH, available sugars and their concentrations, and growth phase), the technological transformation, and the substrates into which the microorganisms are added, can significantly affect both its resistance to biological barriers (gastric acid and bile salts) and its capacity to interact with immune cells; therefore, conditioning its functionality.[4] Vegetable based extracts (soy, rice, corn, chestnuts, etc.) are used by those who are allergic to cow's milk protein and in cases of lactose intolerance.[5] Cereal based beverages possess enormous potential to meet consumers demand for non-dairy food, or as vehicles for the consumption of functional compounds, such as antioxidants, fiber, minerals, probiotics, and vitamins.[6] This is the main reason for which a large quantity of cereals is transformed into foods and fermented beverages before consumption. Therefore, more products need to be produced based on vegetable extracts with nutritional, technological, and sensorial properties similar to yogurts, and rice could be an alternative to be used as vegetable extract in beverages. Rice (*Oryza sativa* L.) is a staple food for more than 1.6 billion people around the world, mainly in Asian, South American, and African countries.[7] Rice bran is the outer layer of rice grain and it is obtained from the milling process, it is usually considered as residue by the industry and discarded. This bran has a great amount of nutrients, such as carbohydrates, protein, fat, dietary fiber, mineral, fatty acids, and antioxidants, such as oryzanol, tocoferol, tocotrienol, and ferulic acid.[8] Starch has been used to control the texture, consistency, and structure of many types of foods. Various studies have been conducted with waxy maize starch (WMS), to evaluate its functionality, exemplifying that WMS possesses a high content of slowly-digestible starch, which takes about 20 to 120 min to be digested in relation to other native starches. This brings benefits such as the slow and prolonged glucose release into the bloodstream,[9] which induces a low glycemic response. This is considered a benefit against metabolic disturbances, such as for example diabetes, pre-diabetes, cardiovascular problems and obesity.[10] In this context, the objective of this study was to evaluate if the strains of *Streptococcus thermophilus* (starter), *Lactobacillus acidophilus* (probiotic), and *Bifidobacterium* sp. (probiotic) can maintain their cell viability in strawberry flavored fermented rice

extract with the addition of WMS during refrigerated storage, to evaluate their *in vitro* resistance to gastrointestinal conditions, and to follow up their changes along the storage period.

Material and methods

Materials

Rice byproducts (bran and broken grains) were donated by the company "Arroz Cristal Ltda." located in Aparecida de Goiânia (Goiás, Brazil), WCS by the company "Febela – Fecularia Bela Vista" (Bela Vista de Goiás, Goiás, Brazil). Lactic ferment Rich®, constituted by strains of *Streptococcus thermophilus* (concentration not specified by the manufacturer), *Bifidobacterium* ssp. ($1x10^6$ CFU/g), and *Lactobacillus acidophilus* ($1x10^6$ CFU/g), granulated sugar (Cristal®), and fresh strawberry fruits were acquired at a local market (Goiânia, Goiás, Brazil).

Preparation of flavored fermented rice byproducts extract

Rice bran was heat treated in a microwave (Panasonic, NN-ST652W, Manaus, Brazil) (3min/ 900 W) for enough time to inactivate enzymes and prevent acidification. Next, it was roasted (direct heating) in batches of 500g in a stainless-steel container (40x20cm) at approximately 110ºC for 10 min. The roasted rice bran was sieved (30mesh), vacuum packed in laminated bags (polyethylene/nylon/ polyethylene), and stored at -18ºC until its processing. In order to process the rice byproducts extract, broken rice grains (920g) and roasted rice bran (80g) were mixed and cooked (25min) with water (1:3 w/v; by-products to water) in a stainless-steel container (10L) in order to obtain a final yield of cooked product of 300%. The cooked product was drained and disintegrated in batches of 750mL for 3 min using an industrial blender (Siemsen, LSB 25, Brusque, Brazil), then, water was added to facilitate the process (1:1 v/v; cooked product to water). The homogenized product was immediately cloth-filtered and then sifted (2mm aperture sieve). The permeated, an opaque and whitish liquid, was called water-soluble extract, to which granulated sugar was added (100g/L). This mixture was pasteurized (85ºC/ 30 min) in a water bath (Tecnal, TE-054-MAG, Piracicaba, Brazil) and cooled down to 45ºC. After reaching 45ºC, WCS was slowly added until it was fully dissolved in the sweetened extract. The mixture was once again heated (85ºC/5 min) and cooled down to 45ºC followed by the incorporation of lactic culture (400mg/L) as recommended by the manufacturer. The extract or fermented rice byproducts beverage (FRBB) was packed in screw-capped plastic flasks (50mL) and incubated in B.O.D. chambers (Tecnal, TE-4013, Piracicaba, Brazil) at controlled temperature (42ºC) until it reached pH 4.5. After this process, the FRBB was refrigerated for 12 h at 5 ± 1°C, and then added with artificial strawberry flavor (0.08% w/w), and natural strawberry syrup (30% w/v), resulting in FFRBB (Flavored Fermented Rice Byproducts Beverage). For the natural syrup processing, granulated sugar and water were mixed (100g/L) in a stainless-steel recipient (5 L) for approximately 10 min in order to obtain a 60 ºBrix syrup. Strawberries, previously sanitized, were manually chopped and boiled (30 min) into the previously prepared syrup. Metal capped glass flasks were filled in with the natural strawberry syrup, pasteurized in boiling water for 30 min and then cooled down do 37°C. Each syrup was added to the FFRBB in the proper concentration, manually homogenized, bottled in screw-capped glass jars (25mL), then stored at 5 ± 1°C until analysis.

pH determination

The pH was obtained by using a pH meter (Tecnal, TEC-51, Piracicaba, Brazil), according to the method proposed by the AOAC International.[11] The analysis was performed in triplicate.

Cell viability of starter and probiotic cultures

Samples of FRBB (25g) were weighed in sterile bags, where 225mL of peptone water (0.1g/100g) were added and then, homogenized in a stomacher (Logen Scientific, 1251, Diadema, Brazil). Decimal dilutions were performed in tubes containing 9 mL of peptone water (0.1%) until the dilution of 10^{-9}. Then, in-depth inoculation of 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} dilutions were carried out using appropriate selective media and incubation parameters for each microorganism. For *Lactobacillus acidophilus*, MRS medium - De Man Rogosa Sharpe (Himedia®) added with 0.5 mg/100 g of Clindamycin (Teuto®) sterilized by filtration[12] was used. For *Bifidobacterium* spp., MRS medium supplemented with L-cysteine HCl (Gemini®) (0.05%), lithium chloride (Neon®) (0.3%), and sodium propionate (Neon®) (0.9%) was used and all of them were sterilized by membrane filtration.11 The microbial culture media were incubated at 37°C/ 72 h with added overlay under anaerobic conditions, using jars containing anaerobic environment generating sachets (Anaerobac®, Probac, Brazil). For Streptococcus thermophilus, M17 medium with glycerol phosphate (Himedia®) was used and incubated for 48 h/ 37°C, at aerobic conditions.[13] The microbial count was carried out according to the following characteristics of Lactobacillus acidophilus: typical wrinkled, depressed, and brownish colonies, with irregular borders, and diameters between 0.1 and 1.0mm.[14] For *Bifidobacterium* spp. the following characteristics were considered: white, smooth, bright, round, small colonies with diameters between 0.7 and 1.2mm,[15] while for Streptococcus thermophilus, small colonies with yellowish borders and with diameter between 0.1 and 0.5mm were the characteristics considered.[15] To obtain the final result, the number of colonies was multiplied by the inverse of the dilution. The colonies were confirmed according to the criteria of positive reaction to Gram staining.

Resistance of probiotics to hydrochloric acid and bile salts

Conditions similar to the human gastrointestinal tract were adopted to evaluate the resistance of probiotic microorganisms (*Bifidobacterium* spp. and *Lactobacillus acidophilus*) to acids and bile salts.[16] For the hydrochloric acid tolerance test, a sample of 1 g of product was placed in tubes with 9mL of 0.08M HCl (Synth®) sterile solution containing 0.2% of NaCl, pH adjusted to 1.55, and then incubated at 37°C for 30, 60, 90, and 120 min in a bacteriological incubator. At each time, plate counts using MRS-clindamycin and MRS-LP agars for Lactobacillus acidophilus and *Bifidobacterium* spp., respectively, were performed. These intervals simulated the average time of food permanence in the stomach, the organ in which microorganisms are exposed to acidity. The test of resistance to bile salts consisted of transferring 1 g of sample to tubes containing 9mL of sterile simulated intestinal juice: KH_2PO_4 (Synth®) (0.05M) containing 0.6% of bile salts (Himedia®) at pH 7.4. Tubes were incubated at 37°C/ 150 min in bacteriological incubator (Fanem, 32683/210441, São Paulo, Brazil), and then, plate count was performed using the media as described for cell count. All the analyses were performed in two independent repetitions.

Experimental design and analysis of the results

Completely randomized design (CRD), with five treatments (0, 7, 14,

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21, and 28 days of refrigerated storage) and two original repetitions were used, totaling ten experimental units. The pH and cell viability data were submitted to the analysis of variance and regression at 5% probability using the software Statistica 10.0.[17] Descriptive analysis was used to present the results for microbiological analysis and resistance to hydrochloric acid and to bile salts using Excel (Microsoft, version 2016, Redmond, EUA).

Results and discussion

From the results of the analysis, analysis of variance, and regression, mathematical models for pH, *Lactobacillus acidophilus*, *Bifidobacterium* spp., and *Streptococcus thermophilus* were obtained (Table 1). All the models were significant at 1% probability and explained 75 to 92% of the responses. The product's pH varied slightly during refrigerated storage, with a slight decrease at the beginning and an increase after 14 days, retroacting to the level verified at time zero (Figure 1A). It is important to emphasize that at the end of the fermentation, before the addition of the strawberry syrup and packaging, the average pH of the fermented extract based on bran and broken rice grains (8:92) was 4.5. As expected, the decrease of 0.3 units of pH was due to continued fermentation during cooling until the product temperature reached 5 ºC. The same behavior was observed by,[18] who worked with probiotic yogurt and found a decrease of 0.17 unit in pH during the product cooling after finishing incubation with pH 4.5. The reduction of pH during storage is due to the continuing conversion of the sugars into lactic acid and acetic acid by the inoculated microorganisms,[19] which explains the decrease found at the beginning of the FFB storage. However, at some point, the microorganisms stop producing the acids responsible for the reduction in pH, and this period also culminates in the death of some of their cells. In addition, this medium is slightly alkalized by the presence of other components, produced through hydrolysis during the fermentation of complex B vitamins, proteins, and fats,[20] which stops the decrease of pH. The slight buffer capacity of the medium explained the pH small increase that was found 14 days after storage. The counts of probiotic and starter cultures decreased over the 14 days. *Lactobacillus acidophilus* count was close to 10^6 CFU/g at time zero, decreasing to 10^5 CFU/g at 7 days, and then it went below 10^4 CFU/g (Figure 1B), while *Bifidobacterium* spp. count was close to 10^4 CFU/g at the beginning of storage and zero after 14 days (Figure 1C). *Streptococcus thermophilus* count began close to 10^9 CFU/g and at the end of storage was close to 10^5 CFU/g, which was the highest count among the three evaluated microorganisms (Figure 1D). Typical counts (10^7 CFU/g) of probiotics were obtained in the fresh product as required by[21] for yogurt, but at the seventh day this amount could only be considered as a therapeutic dose. According to,[22] in order to receive the nomenclature "probiotic food", milk fermented with yogurt should contain 10^6 to 10^7 viable cells per gram or mL of product, while the minimal therapeutic dose is 10^5 CFU/g. Demirci et al.[8] reported that *S. thermophilus* counts ranged from 8.22 to 9.83 log CFU/g during the whole storage time in rice bran yogurt, and at the first day of cold storage, S. thermophilus counts varied from 8.39 to 9.83 log CFU/g in all yogurts co-fermented with *L. casei*. Probiotic microorganisms cell viability can be affected by many factors, including the composition of the medium that is to be fermented during the biological processes of the cultures. In a study conducted by Fuchs et al.,[23] the viability of the culture used and the similarity of the count levels in yogurts supplemented with prebiotics and those without supplementation were due to the fact that the medium used for probiotic fermentation (reconstituted milk powder) was rich in lactose, lactic acid bacteria's preferred substrate, which must have had the same protective effect as the oligofructose and inulin used in the beverages. Regarding the FFBB, the fermented medium did not contain lactose, and possibly because of this, the probiotic microorganisms metabolized other types of sugars, such as the sucrose present in the syrup and the oligosaccharides naturally present in the extract, which could hinder the microbial survival during product storage. Probiotic microorganisms are also affected by starter cultures, which normally predominate on the probiotic microorganisms themselves during storage, acidifying the medium and interfering in the probiotic's survival. Any pH below 4.3 significantly affects the viability of bifidobacterial,[24] which was observed throughout the storage of FFBB. Similarly,[25] reported that the most important factor for the bifidobacteria mortality in yogurt was the low pH. *Lactobacillus acidophilus* has been reported to be more acid tolerant than bifidobacteria.[26] The same tendency was found in this study, since the Bifidobacterium culture disappeared after 14 days, while L. acidophilus culture remained during the entire storage period, although in relatively low quantities. This can also be explained by the fact that the Bifidobacterium genus does not ferment foods and it is taxonomically different from other lactic acid bacteria.[27] FFB 4.2 pH negatively affected the cell viability. The fermentation interruption at pH above 4.5 could help to preserve the probiotic cultures, since they probably would not decrease during refrigeration. In a study conducted by Shah et al.,[28] with five different types of commercial yogurts with probiotic potential containing *Lactobacillus* and *Bifidobacterium*, the same phenomena was observed, the *Bifidobacterium* count decreased in all products, in three of them the fresh sample presented a count ≤ 10^3 CFU/g, and at the end of five weeks, the viable cells of this microorganism disappeared. The starter culture of the fermented extract (*S. thermophilus*) produces hydrogen peroxide through its metabolism, which could cause a partial lesion of the probiotic cells (*Bifidobacterium* and *L. acidophilus*), leading to a quick decline in viable cells, and also the antagonism among the present microorganisms.[18] Another important factor for probiotic microorganisms' viable cell count is the range type of culture mediums and the plating techniques used by various authors, and mainly because of the difficulties related to a mixed culture, as it is the case of this work. As Talwalkar and Kailasapathy[29] confirmed, there is a need to standardize a single culture medium that provides viable cell counts of *L. acidophilus*, *Bifidobacterium* spp., and *L. casei* in different products in the presence of starter cultures. However, the plating method also influences the recovery rate of these microorganisms cells. According to Roy,[30] the technique of depth plating is preferred in relation to the surface technique for Bifidobacterium, although Bifidobacterium species can present different recovery rates for propagation in plates due to their sensitivity to oxygen, which is highly variable among diverse species of this microorganism. Overall, the physiological growth needs may vary widely among bifidobacterial that are industrially available, so the selection of a single medium and a single form of plating is very difficult to accomplish. In general, the total cell count for the starter and the probiotic cultures remained high until the end of storage (5 x 10^5 CFU/g). The majority of the count was due to the presence of S. thermophilus, which was responsible for product acidification. This is not considered a probiotic in Brazil once it is unable to withstand the adverse conditions within the human organism. However, some authors have reported the survival of this microorganism under acidic conditions and in the presence of bile acids.[31] Freitas et al.[3] reported a viable cell count ranging from 10^7 to 10^8 CFU/g of *S. thermophilus* in fermented milk (added to bifidobacteria cultures) stored at 4 and 10 °C for 28 days, obtained by M17 culture media by conventional plating. They affirmed that viable

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cells were within the limits recommended for this product, even knowing that this count could be related to the previously inoculated mixed culture, or to any other microorganisms present in that culture. Hauly et al.[31] evaluated the commercial validity of "soy extract yogurt" containing *S. thermophilus* and *L. bulgaricus*, and obtained a total count of 5.36 log CFU/g at day 21, and concluded that the product can no longer be considered probiotic as a result of the mixed culture. The total microorganism count obtained in this study remained at a dose regarded as therapeutic until the end of storage and remained a probiotic food until 14 days of storage. According to Corrêa et al.,[32] *S. thermophilus* culture associated with *Bifidobacterium* in doses ranging from 10^6 to 10^7 CFU/g of the formulated product is indicated for diarrhea prevention in children caused by ingestion of antibiotics or rotavirus infection. The native WMS did not show a probiotic effect, as shown in this study, and therefore, did not perform the role of protecting the probiotics throughout their storage. This is most likely due to the composition of the medium, since the FFB is low in the nutrients required for the multiplication and maintenance of these microorganisms. Thus, it is necessary to improve the composition of the extract in further studies, by the addition of other ingredients, such as simple sugars (glucose and fructose) in order to replace lactose - the main sugar for metabolization of these microorganisms. According to Ross et al.,[33] the development of non-lactic probiotic products is a challenge for the food industry due to the difficulty of probiotic microorganisms to grow and survive in hostile environments. After the isolation and staining of some colonies, it was observed that the media used were selective and suitable for counting the different microorganisms present in the FFB, as the morphology and colony colors observed are compatible with those found in the literature for microorganisms of the genera *Lactobacillus*,[34] *Bifidobacterium*,[35] and *Streptococcus*.[36] The analysis of cell viability showed that the composition of the product still needs to be improved in order to prolong the survival of these microorganisms throughout the entire shelf-life period. Using freeze-dried probiotic cultures separate from the fermented culture could be an alternative, since it is easier to standardize pH and incubation temperature for one type of microorganism, also, by adding the probiotic cultures after the fermentation of the product so that they do not suffer from pH fluctuation during fermentation. The survival rates of the species (*Lactobacillus acidophilus* and *Bifidobacterium* spp.) were not satisfactory (Table 2), however, *L. acidophilus* survived (in small counts) the hostile conditions of the simulated human digestive system, in contrast to what was observed for *Bifidobacterium* spp. These results can probably be explained by the fact that the substrate was not suitable for the adaptation and multiplication of both colonies, which was also verified by the cell viability analysis. Nevertheless, the tested conditions were extreme. For the survival in the stomach, it must be taken into consideration that there is usually simultaneous ingestion of foods along with the bacteria, resulting in an increase of pH in the stomach. Rönkä et al.[37] studied the resistance of *Lactobacillus brevis* in MRS broth at pH 4.0 during 3 h, and observed that the studied bacterial strains maintained their viability, however, at pH 2.0 there was a decrease of 8 log of the number of viable bacteria (CFU/mL) after 3 h of incubation. In this study, at lower pH (1.55) a reduction close to 3 log was found after 2 h of incubation for both probiotic microorganisms, as well as in the test with 0.6 g/100 g of bile after 2.5 h of incubation. According to Charalampopoulos et al.,[38] probiotic survival during gastrointestinal transit is influenced by the food physicochemical properties used as cell carriers, with the most significant factors being buffering capacity and the medium pH.

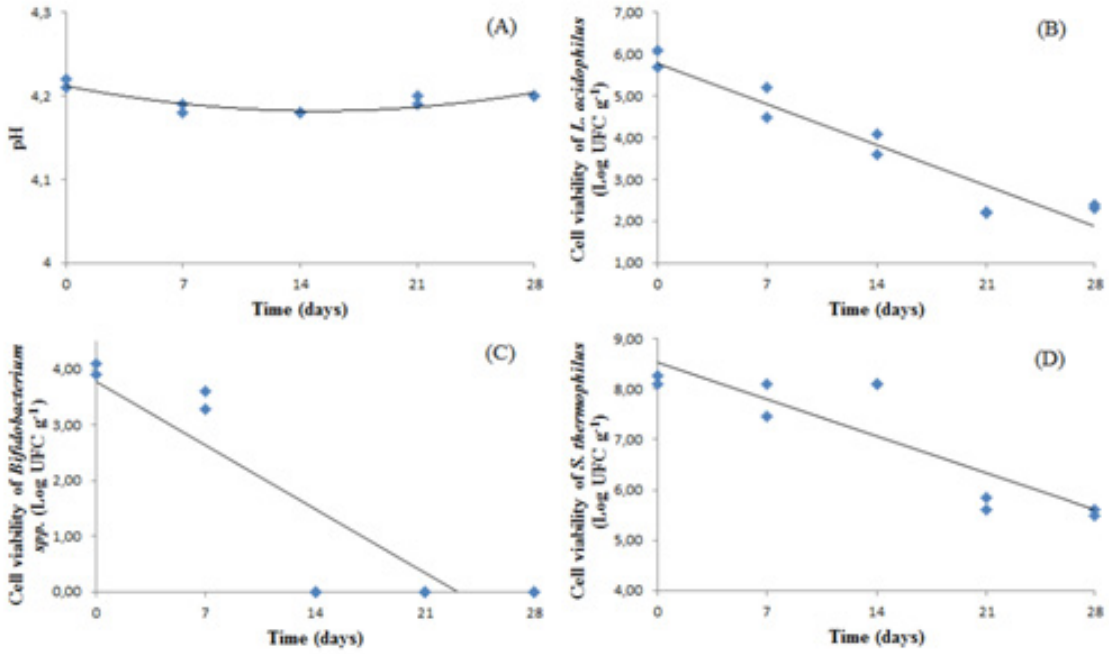

Figure 1 pH (A) and cell viability of *Lactobacillus acidophilus* (LA) (B), *Bifidobacterium* spp. (c), and *Streptococcus thermophilus* (ST) (D) of the bran and broken rice grains extract (8:92) fermented with flavored waxy maize starch during different storage times.

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Table 1 Adjusted models, significance level (p), and determination coefficient (R^2) for pH and cell viability of *Lactobacillus acidophilus*, *Bifidobacterium* spp., and *Streptococcus thermophilus* (y_1 to y_4, respectively) of bran and broken rice grains flavored extract (8:92) fermented with waxy maize starch during storage periods (days) (x).

Parameter	Model	p	R2
pH	Y_1=4.212-0.004x+0.0001x^2	0.007836	0.75
Lactobacillus acidophilus	Y_2=5.780-0.1391x	0.000014	0.92
Bifidobacterium spp.	Y_3=3.776-0.1634x	0.000718	0.78
Streptococcus thermophilus	Y_4=8.535-0.1047x	0.000988	0.76

Table 2 Average survival obtained for *L. acidophilus* and *Bifidobacterium* spp. after different time points in the simulation of gastrointestinal tract

	L. acidophilus survival (CFU/g)	Bifidobacterium spp. survival (CFU/g)
R.A.1 30 min in pH 1.55	1.0 x 10^2	0
R.A.1 60 min in pH 1.55	1.2 x 10^2	0
R.A.1 90 min in pH 1.55	1.72 x 10^3	0
R.A.1 120 min in pH 1.55	1.87 x 10^3	0
R.B.2 155 min in pH 7.4	1.16 x 10^3	0

Conclusion

Typical counts of probiotics were obtained in the fresh product as predicted in the literature (min. 10^7 CFU/g), but after 7 days of storage these counts could only be considered as a therapeutic dose. The drastic reduction in counts may be related to the type of fermented beverage, and depending on its chemical composition, it may behave quite differently from traditional dairy beverages with probiotics. The native WMS did not favor the cell viability of the probiotics during storage. Regarding resistance of the probiotic microorganisms to conditions similar to the gastrointestinal tract, the results were only positive for *Lactobacillus acidophilus*, which survived under the tested conditions. Further studies could use WMS modified for functional improvement of the product or interrupt fermentation when the pH is around 4.8, since due to post-acidification the pH of the product decreases to around 0.3 units, which is close to lethal to the probiotic bacterial survival.

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Authors' contributions

KKFDC, TCP, MSSJ, and MC conceived and designed the research. KKFDC, ÉRO, ARO, and AECR conducted experiments and analyzed data. KKFDC and ÉRO wrote the manuscript. All authors read and approved the manuscript.

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None.

Conflicts of interest

The authors declare no conflicts of interests.

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