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Coconut Water Fermented by *Lactobacillus plantarum* with Inulin Addition: Development of a Potentially Synbiotic Beverage

Água de Coco Fermentada por *Lactobacillus plantarum* com Adição de Inulina: Desenvolvimento de uma Bebida Potencialmente Simbiótica

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Yolanda Graciela Kiesling
Graduada em Agroindustria Alimentaria pela Universidad Zamorano
Instituição: Universidad Zamorano
Endereço: Rodovia de Tegucigalpa a Danlí Km 30, Valle del Yeguare, Municipio de San Antonio de Oriente, Francisco Morazán, Honduras
E-mail: gracielakiesling95@gmail.com

Fernanda Silva Farinazzo
Doutoranda em Ciência de Alimentos pela Universidade Estadual de Londrina
Instituição: Universidade Estadual de Londrina
Endereço: Rodovia Celso Garcia Cid - Pr 445 Km 380, Londrina – PR, Brasil.
E-mail: fsfarinazzo@gmail.com

Maria Thereza Carlos Fernandes
Doutoranda em Ciência de Alimentos pela Universidade Estadual de Londrina
Instituição: Universidade Estadual de Londrina
Endereço: Rodovia Celso Garcia Cid - Pr 445 Km 380, Londrina – PR, Brasil.
E-mail: thereza.fernandes@hotmail.com

Carolina Saori Ishii Mauro
Doutoranda em Ciência de Alimentos pela Universidade Estadual de Londrina
Instituição: Universidade Estadual de Londrina
Endereço: Rodovia Celso Garcia Cid - Pr 445 Km 380, Londrina – PR, Brasil.
E-mail: carol.saori@gmail.com

Adriana Beatriz Di Iorio
Mestre em Nutrição pela Universidad de la Fraternidad de Agrupaciones Santo Tomás de Aquino (FASTA)
Instituição: Universidad Zamorano
Endereço: Rodovia de Tegucigalpa a Danlí Km 30, Valle del Yeguare, Municipio de San Antonio de Oriente, Francisco Morazán, Honduras.
E-mail: adi@zamorano.edu

Sandra Garcia
Doutora em Ciência de Alimentos pela Universidade Estadual de Londrina
Instituição: Universidade Estadual de Londrina
Endereço: Rodovia Celso Garcia Cid - Pr 445 Km 380, Londrina – PR, Brasil.
E-mail: sgarcia@uel.br

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ABSTRACT
The objective of this study was to develop a synbiotic beverage using green coconut water (*GCW*) and agave inulin. The supplementation of 0.5% (w/v) agave inulin and incubation at 32 °C provided the optimal conditions for viable cell counts of *Lactobacillus plantarum* BG 112, reaching 8.85 log CFU/mL, according to the factorial experimental design with face-centered star points (a = ±1). The probiotic count in *GCW* was 8.81 ± 0.94 log CFU/mL, and did not present significant difference during shelf life of 30 days, which is a satisfactory result for fermented products regarding viability. The increase of lactic acid (3.88 ± 0.61 % to 13.00 ± 1.48 %) caused a decrease in the pH of the beverage, reaching 3.66 ± 0.01 at the end of storage. In the sensory evaluation, all the parameters obtained average scores higher than 8.0, indicating a good acceptance of the product. Therefore, the synbiotic beverage developed presents a non-dairy alternative to deliver *L. plantarum*, with high acceptability and health-promoting properties to consumers.

Keywords: Agave inulin; *Cocos nucifera* L.; Optimization; Sensory evaluation.

RESUMO
O objetivo deste estudo foi desenvolver uma bebida simbiótica utilizando água de coco verde (*GCW*) e inulina de agave. A suplementação de inulina de agave a 0,5% (p/v) e a incubação a 32 ° C proporcionaram as condições ideais para contagens viáveis de células de *Lactobacillus plantarum* BG 112, atingindo 8,85 log UFC/mL, de acordo com o delineamento experimental fatorial com estrela centrada na face pontos (a = ± 1). A contagem de probióticos no *GCW* foi de 8,81 ± 0,94 log UFC/mL, e não apresentou diferença significativa durante o prazo de validade de 30 dias, resultado satisfatório para os produtos fermentados quanto à viabilidade. O aumento do ácido lático (3,88 ± 0,61% para 13,00 ± 1,48%) causou uma diminuição no pH da bebida, atingindo 3,66 ± 0,01 no final do armazenamento. Na avaliação sensorial, todos os parâmetros obtiveram escores médios superiores a 8,0, indicando boa aceitação do produto. Portanto, a bebida simbiótica desenvolvida apresenta uma alternativa não láctea para entregar *L. plantarum*, com alta aceitabilidade e propriedades promotoras de saúde para os consumidores.

Keywords: Inulina de agave*; Cocos nucifera L.;* Otimização; Avaliação sensorial

1 INTRODUCTION

Probiotics are live microbial food supplements that benefit consumer health by maintaining or improving their intestinal microbial balance, when administered in adequate amounts (*1*). Among functional foods, probiotics stand out for being well accepted and widely consumed in several countries. Probiotic microorganisms can act in the promotion of health due to various beneficial effects they exert in the host. These effects are usually due to the following mechanisms of action: resistance to pathogen colonization, regulation of intestinal transit, acid production, among others (*2*). Most of the microorganisms identified as probiotics are Gram-positive bacteria and belong to the genera *Bifidobacterium* and *Lactobacillus* spp. These bacteria genera have been used for food fermentation since the ancient times, and can serve a dual function by acting as a food fermenting agent and potentially health benefits provider (*3*).

Despite the growing demand for new products, the inclusion of probiotics in food matrices is

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still a challenging area of research in food technology (*4*). Dairy products, mainly fermented milks and yoghurt, are still the majority of probiotic products available in the market. However, a great part of the world population is affected by lactose-intolerance or milk allergies. In addition, there are people who choose not to consume milk products because of health conditions, like people with hypercholesterolemia, or personal preferences, such as strict vegetarians and vegans (*5*). In this context, it is necessary to develop and study nondairy fermented products to be introduced in the market.

In the last decade, several types of non-dairy food matrices have been successfully used to produce probiotic beverages (*6-11*). Many fruits and vegetables juices are considered suitable for the addition of probiotics as they are consumed regularly by people of all ages, and have carbohydrates, minerals, vitamins, dietary fibers, and antioxidants that could make them ideal substrates for probiotic growth (*12*).

Green coconut water (*GCW*) obtained from the endosperm of coconuts (*Cocos nucifera* L.) is colorless, sweet, naturally flavored and slightly acidic and is largely consumed in tropical countries (*13*). *GCW* is an isotonic drink rich in sugars, vitamins B and C, sodium, potassium, magnesium and calcium, minerals that can replenish the electrolytes in body dehydration (*14*). For this reason, *GCW* is highly consumed around the world and, consequently, represents one of the fastest growing beverage categories due to its natural hydrating qualities, enjoyable taste, functional health properties and nutritional benefits (*15*).

A trend in food technology is to combine probiotics and prebiotics. Products containing these both components are generically termed synbiotics (*16*). Prebiotic is defined as "a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (*17*). Prebiotics may exert a protective effect in probiotic food products, improving the survival and activity of probiotic bacteria during refrigerated storage, as well as during the passage through the gastrointestinal tract (*18*). The prebiotic inulin is a polysaccharide that can be extracted from plants of the families *Liliaceae*, *Amaryllidaceae*, *Gramineae*, *Compositae*, *Nolinaceae* and *Agavaceae*, although its main source is chicory (*Cichorium intybus*) (*19*). The addition of inulin to the *GCW* is an approach to improve the probiotic survival in the beverage, mainly because viability is an important parameter for developing probiotic foods to ensure their benefits for human health (*4*).

Therefore, due to the importance of providing products aimed at individuals with restriction on the consumption of lactose or who do not appreciate foods of animal origin, the objective of this study was to develop a synbiotic beverage, using coconut water and agave inulin.

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2 MATERIAL AND METHODS

2.1 EXTRACTION AND PREPARATION OF COCONUT WATER

Green coconuts (*Cocos nucifera* L.) were purchased in the local market of Londrina, Paraná – Brazil. The *GCW* used in this work was extracted by perforation with a manual, stainless-steel punch after the primary epicarp had been brushed and washed with water containing 100 ppm of active chlorine. The extracted *GCW* was maintained in plastic bags at - 20 °C. To prevent any microbial or enzymatic activity prior to its use in the fermentation, *GCW* was pasteurized at 90 °C for 5 min (*20*).

2.2 MICROBIAL CULTURE STOCK PREPARATION

The strain *Lactobacillus plantarum* BG 112 was donated by Sacco (Sacco®, Brazil). The culture was activated (0.1% w/v) in pasteurized *GCW* containing 20 % (v/v) glycerol (Synth, Brazil) and divided into portions of 10 mL, which were kept frozen at - 20 °C. At the moment of use, the pre-inoculum were obtained through two activations in *GCW* at 37 ºC for 24 hours under aerobiosis to promote culture adaptation.

2.3 OPTIMIZATION OF FERMENTATION

The optimum fermentation conditions were studied through a face-centered star points experimental design. The probiotic culture fermentation was performed in 100 mL of *GCW* in sterile 250 mL flasks, adding 0 - 5 g/100 mL of agave inulin (Preventy-Mexico) (x_1) at an incubation temperature (x_2) of 25 - 45 °C (Table 1), according to the proposed experimental design, at an initial inoculum of 1% v/v. The time of 16 h for the end of the fermentation was established according to preliminary tests, in which *L. plantarum* had already reached the stationary phase of bacterial growth. The experimental design used was a 2^2 factorial design with face-centered star points ($a = \pm 1$) and two repetitions of central points (total of 12 experiments). This design was chosen because the axial points are at the center of each face of the factorial space, requiring only three levels for each factor (*21*). The levels of factors used are shown in Table 1, where (-1), (0), and (+1) indicate the low level of each factor, mid-level, and the high level, respectively. The experimental data were designed using Statistica® software version 10.0. A complete quadratic polynomial regression model was used to correlate the experimental data using Eq. 1:

$$Y_{viability} = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_1 x_1^2 + \beta_2 x_2^2 + \beta_{12} x_1 x_2 \quad (1)$$

Where, $Y_{viabiliy}$ = response variable to cell viability (log CFU/mL),

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x_1 and x_2 = coded independent variables (temperature and concentration of inulin), β = estimated coefficients of each term of the response surface model. The impact and meaning of each term (linear, quadratic, and interactions) in the regression equation was evaluated by analysis of variance (ANOVA).

Validation of Optimized conditions and predictive models were tested using the ideal conditions provided. Results of experiments performed under optimal conditions of each substrate and the average experimental values were compared with the expected values to determine the validity of the models.

2.4 PRODUCTION AND STORAGE OF THE FERMENTED *GCW* BEVERAGE

The fermentation was performed in 250 mL sterile glass flasks containing 100 mL of *GCW* that were inoculated with 1% (v/v) pre-inoculum with approximately 9 log CFU/mL, according to Optimized conditions, until pH 4.5. After that period, it was added 3% (m/v) of sucrose and 0.05% (v/v) of artificial coconut essence. Fermented *GCW* containing inulin and probiotics was dispensed into sterile glass flasks and stored at 4 °C. The aliquots were removed for analysis at the time intervals 0, 7, 15 and 30 days of shelf-life, for the viability of *L. plantarum* BG 112, pH, lactic acid, total soluble solids and color.

2.5 PHYSICOCHEMICAL ANALYSIS AND PROBIOTIC VIABILITY

The pH was determined using a digital potentiometer (Hanna® instrument, Romania). The level of total soluble solids (TSS), as °Brix, was assessed using a digital refractometer (Atago Co., Kyoto, Japan). A colorimeter (Minolta®, model CR400, Osaka, Japan) was used for the assessment of color parameters values, which were used to calculate ΔE (color loss), according to Eq. 2, where Δa, Δb e and ΔL represent the difference between color during the shelf-life of the product (*22*).

$$\Delta E = \sqrt{[(\Delta L*)^2 + (\Delta a*)^2 + (\Delta b*)^2]} \quad (2)$$

Lactic acid determinations were performed at Shimadzu liquid chromatographic system, Prominence LC-20A series HPLC (Shimadzu, Kyoto, Japan). The system consisted of a pump (LC-20AT) with solvent organizer and degas module (DGU-20A5), auto sampler (SIL-20AC), column oven (CTO-20A), refractive index (RID-10A) and a photodiode array detector (SPD-M20A), all managed by LC Solution software. A chromatographic column Shiseido Capcell Pak C18 MG (4.6 × 250 mm, 5 µ) was employed. The mobile phase consisted of 25.0 mmol/L of sodium phosphate buffer solution, with pH adjusted to 2.4 at a flow rate of 1.0 mL/ min. The column temperature was

maintained at 30 °C and a 20.0 µL volume was injected. Detection was performed simultaneously in a Refractive Index detector (RID-10A) and a Photodiode array detector (SPD-M20A), programmed at a fixed wavelength at 215 nm and in scan mode from 200 to 400 nm (*23*).

The viability of the probiotic in the fermented *GCW* was determined by the plate count method. *L. plantarum* BG112 counts were performed in Man Rogosa and Sharpe (MRS) agar (Merck®) and incubated under aerobic conditions at 37 °C for 48 h. The results were expressed as log of colony forming units per mL (log CFU/mL).

2.6 SENSORY EVALUATION

The sensory panel was composed of 100 untrained individuals (52 women and 48 men). The acceptance test of attributes (color, aroma, flavor, texture and overall acceptance) were made using a 9-point hedonic scale (1-disliked very much and 9-liked extremely) and was performed on day 1 of product storage. The judges evaluated the formulation of potentially synbiotic *GCW* in an assessment session. The formulation (25 mL) was coded with 3-digit numbers and served at a temperature of 4 °C in plastic cups. The tests were performed in individual cabins with fluorescent lamps. The microbial evaluation of the coliforms and *Salmonella* sp. were carried out before sensory evaluation, in accordance with Brazilian law to ensure safe consumption of the beverage (*24*).

3 RESULTS AND DISCUSSION

3.1 OPTIMIZATION OF FERMENTATION

The responses obtained from the experimental data for *L. plantarum* BG 112 cell viability ($Y_{viability}$) after *GCW* fermentation are shown in Table 1. The response variable showed values ranging from 6.00 to 8.70 log CFU/mL of *L. plantarum* in fermented *GCW* with and without agave inulin addition. The experimental data were used to develop a quadratic polynomial regression with linear and quadratic terms.

Table 1 - Coded/real values and the observed/predicted responses obtained by the application of a central composite design in *GCW* fermentation by *Lactobacillus plantarum* BG 112.

Assay	Agave inulin/ x_1 (g/100mL)	Temperature/ x_2 (°C)	Cell viability/$Y_{viability}$ (log CFU/mL)	
			Observed	Predicted
1	0 (-1)	25 (-1)	8.31	8.19
2	0 (-1)	45 (+1)	7.32	7.16
3	5 (+1)	25 (-1)	8.40	8.53
4	5 (+1)	45 (+1)	6.00	6.09
5	0 (-1)	35 (0)	8.55	8.84
6	5 (+1)	35 (0)	8.69	8.47
7	2.5 (0)	25 (-1)	8.30	8.30

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8	2.5 (0)	45 (+1)	6.48	6.56
9	2.5 (0)	35 (0)	8.61	8.60
10	2.5 (0)	35 (0)	8.70	8.60
11	2.5 (0)	35 (0)	8.48	8.60
12	2.5 (0)	35 (0)	8.66	8.60

The ANOVA of the quadratic models and coefficient estimates for *L. plantarum* was performed (Table 2). It was possible to observe that linear inulin concentration value (x_1) and both linear and quadratic temperature values (x_2) were statistically significant ($p \leq 0.05$).

Table 2 - Analysis of Variance (ANOVA) of the Quadratic Models and Coefficient Estimates for the Response (cell viability of *L. plantarum*) of fermented *GCW*.

Source model	Sum of squares	df	Mean square	F value	P
Inulin (L)	0.201667	1	0.201667	23.3927	0.016856*
Inulin (Q)	0.010045	1	0.010045	1.1652	0.359440
Temperature (L)	4.525753	1	4.525753	524.9736	0.000182*
Temperature (Q)	3.632482	1	3.632482	421.3568	0.000253*
Inulin × Temp.	0.495616	1	0.495616	57.4899	0.004759*
Lack of fit	0.203754	3	0.067918	7.8783	0.061959
Pure error	0.025863	3	0.008621		
Total SS	9.407232	11			

L: linear, Q: quadratic. R^2= 0.976, R_{adj}= 0.955, * Significant terms.

The mathematical statistical model represents the response function equivalent to Eq. 3. The significant terms at 5% are shown with an asterisk (*). The linear value of x_2 presented a p = 0.000182 and the quadratic value of x_2, p = 0.000253. This means that there has been Optimization of cell counts, which can be achieved when optimum temperature levels are employed. The interactions of inulin (x_1) and temperature (x_2) were significant (p = 0.004759), indicating that the main effects (x_1 and x_2) cannot be evaluated separately.

$$Y_{viability} = 8.595 - 0.3667*x_1 + 1.227x_1^2 - 1.737*x_2 - 2.334*x_2^2 - 0.704*x_1x_2 \quad (3)$$

All significant terms had negative coefficients. The negative coefficient showed that with a lower concentration of inulin and a lower incubation temperature, *L. plantarum* cell production increased. The signal and the value of the quantitative effect represent the trend and magnitude of the influence on the response, respectively (*25*).

To verify the prediction and fit of the developed regression equations, ANOVA was performed. The non-significant terms were maintained in the model, because when they were removed, there was a reduction in the value of R^2. The regression model were able to explain 97.6% the values observed for the production of *L. plantarum* cells. Lack of fit was not significant (p =

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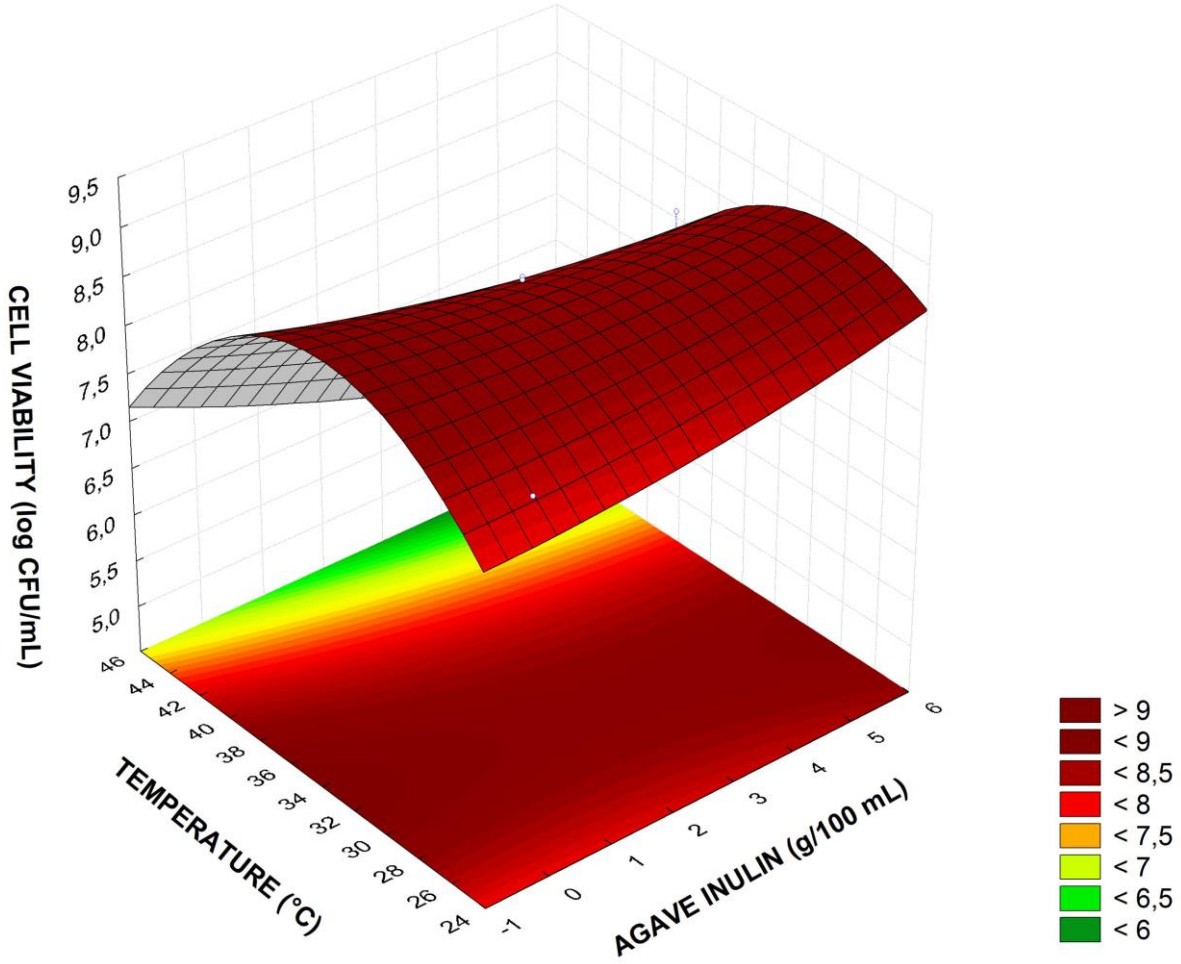
0.061959), suggesting that the model does accurately fit to the data, confirming the validity of the predictive analytic model.

The response surfaces were generated in order to Optimize the process. Figure 1 shows the interactive effect of inulin and temperature on the cell viability ($Y_{viability}$) of *L. plantarum* after *GCW* fermentation. The regions with high cell concentrations were near 0 or 5 g of agave inulin/100mL and 30 °C. The highest (optimum) viability of *L. plantarum* BG 112 in the *GCW* was obtained at a 0.5% (w/v) of inulin concentration and fermentation temperature of 32 °C, reaching 8.85 log CFU/mL (critical point of Equation 3, according to Statistic program).

Figure 1 - Response surface 3D contour plot for *L. plantarum* BG 112 cell viability (log CFU/mL) in coconut water.



Other authors found probiotic cell viability in *GCW* similar to that found in this study. Kantachote et al. (*26*), while studying mature *GCW* for fermentation, obtained a beverage with *L. plantarum* DW12 viability of 8.4 log CFU/mL after 48 h of fermentation at 35 °C.

In rice extract, the concentration of 2% (w/w) of chicory root inulin (Orafti®) as a prebiotic was the optimum observed to support the growth of probiotic *L. plantarum* TISTR 2075. After

fermentation at 37 °C for 24 h, the viable cell number was above 8.94 log CFU/mL. The results indicated that the extract supplemented with 2 % (w/w) inulin exhibited a higher specific growth rate (0.157/h) compared to the samples without inulin addition. However, the addition of 3% inulin was not significantly different compared with 1% inulin (*27*). In the present study, the agave inulin concentration necessary to promote optimal growth of *L. plantarum* BG 112 was lower compared to the work cited.

On the other hand, in blended carrot and orange juices, the growth of *L. plantarum* CECT 220 was not affected by the presence of inulin in the substrate. The viability of *L. plantarum* was investigated in the juices after the addition of artichoke inulin in concentrations of 0, 1% and 2% (w/w), and after 24 h of fermentation at 37 °C, the growth of *L. plantarum* was about 9.13 log CFU/mL in all juices, regardless of the addition of inulin. The results showed that inulin was not the main carbon energy source for *L. plantarum* fermentation, and the prebiotic is fermented more slowly compared to glucose and fructose (*28*).

3.2 PHYSICOCHEMICAL ANALYSIS AND PROBIOTIC VIABILITY

The physicochemical analysis and cell viability of *L. plantarum* BG 112 in the fermented *GCW* during storage are presented in Table 3.

The *L. plantarum* viability in *GCW* after 16 h fermentation at 32 °C was 8.81 ± 0.94 log CFU/mL and did not present significant difference during 30 days of storage. The result obtained in our study was superior to that found by Dharmasena et al. (*29*), who reported a *L. plantarum* Lp 115-400B viability of 6.99 ± 0.27 log CFU/mL after fermentation of oatmeal and *GCW* with addition of inulin. Our viability results were also superior to that found in coconut water without prebiotic supplementation in the beverage developed by Kantachote et al. (*26*), which presented *L. plantarum* DW12 viability of 8.50 log CFU/mL in fermented mature *GCW*.

Table 3 - Cell viability and physicochemical analysis of *GCW* during shelf life of 30 days.

Storage day	*L. plantarum* BG112 (log CFU/mL)	% lactic acid	pH	ΔE	Brix
0	$8.81^{a} \pm 0.94$	$3.88^{c} \pm 0.61$	$4.64^{a} \pm 0,00$	-	$11.25^{a} \pm 0.04$
7	$8.79^{a} \pm 0.20$	$8.02^{b} \pm 1.70$	$3.99^{b} \pm 0,03$	$10.60^{a} \pm 0.98$	$11.10^{a} \pm 0.10$
15	$8.77^{a} \pm 0.06$	$9.16^{b} \pm 1.37$	$3.89^{b} \pm 0,01$	$10.04^{a} \pm 1.50$	$10.60^{b} \pm 0.09$
30	$8.79^{a} \pm 0.03$	$13.00^{a} \pm 1.48$	$3.66^{c} \pm 0,01$	$10.30^{a} \pm 1.20$	$10.55^{b} \pm 0.04$

Different letters in the same column indicate significant differences between treatments ($P<0.05$).

The probiotic viability is an important factor to be considered in functional foods development

because these microorganisms must survive in the food matrix during shelf life, and then resist to the stressing gastrointestinal conditions after ingestion (*4*). It has been suggested that probiotics should be present in food to a minimum concentration of 6 log CFU/mL at the expiry date (*30*). In this context, the viability of *L. plantarum* BG 112 throughout the shelf life of *GCW* was adequate for a drink with probiotic properties.

The amount of lactic acid and pH values underwent statistically significant changes during the storage period, as shown in Table 3. The quantification of lactic acid in the sample increased from 3.88 ± 0.61% after fermentation to 13.00 ± 1.48% at the end of 30 days. During the shelf life of a fermented product, the organic acids are important indicators of the biochemical metabolic processes that may occur (*10, 31*).

The increase of lactic acid caused a decrease in the pH of *GCW*, reaching 3.66 ± 0.01 at the end of storage. Shahabbaspour et al. (*32*) and Santos Filho et al. (*33*) also observed a decrease in the pH of probiotic beverages during the storage period and attributed this decline to post-fermentation of the lactic acid bacteria. The addition of glucose and presence of residual glucose and fructose in *GCW* can support continuous metabolic activity of the strain and generate, as observed, a post-acidification in the beverage (*10*)

The level of TSS (Table 3) showed a significant decrease from day 15 until day 30, possibly due to the consumption of the sugars by the strain used in this work. The color results (ΔE) did not show significant difference in 30 days of storage, which guarantees a stability in the product appearance during the period tested. This is a good result because the aesthetic and sensorial parameters are examples of the main requirements taken into consideration by consumers when evaluating the safety of food (*34*).

3.3 SENSORY EVALUATION

The average sensory evaluation scores for the synbiotic beverage are shown in Table 4. All the parameters obtained an average score higher than 8.0, which corresponds to “liked very much”. The parameters aroma and flavor obtained the higher acceptance scores, with 8.53 ± 0.29 and 8.34 ± 0.36, respectively. The lowest score reached was for the color parameter, with an average of 8.27 ± 0.26.

Table 4 - Sensory evaluation scores

Parameter	Color	Aroma	Flavor	Texture	Overall Acceptance
Score	8.27 ± 0.26	8.53 ± 0.29	8.34 ± 0.36	8.33 ± 0.33	8.3 ± 0.37

Prado et al (*10*) developed a functional product of *GCW* fermented by *L. plantarum* in the presence of yeast extract, soy protein hydrolysate and sucrose. The sensory acceptance scores ranged from 4.0 to 5.5 (hedonic rating scale from 1 to 7) for the parameters color, aroma, texture, flavor and overall acceptance, showing a lower acceptance score compared to that obtained in this work. Kantachote et al. (*26*) reported that their *GCW* beverage formulations fermented by *L. plantarum* were only moderately acceptable, according to the sensory evaluation of their panelists. Comparing the acceptance of the products developed by the other authors with the beverage elaborated in this work, the synbiotic *GCW* generally presented higher acceptance values, which demonstrates its potential use as a functional food.

The beverage developed in the present work had a high percentage of acidity, as mentioned previously. In this way, sucrose and artificial coconut flavor were added in order to enhance the beverage liking and promote a higher acceptance of the product. According to Hoffman et al. (*35*), in a review study of the flavour preferences among young and adults, papers described sweet taste preference among adults and rated sour as significantly less pleasant than sweet. Thus, the sensory evaluation scores obtained for our drink demonstrates the commercial potential of fermented *GCW* beverages with inulin addition. We conclude that the sensory evaluation scores obtained for our drink demonstrates the commercial potential of fermented *GCW* beverages with inulin addition.

4 CONCLUSIONS

The temperature of incubation and agave inulin addition to the *GCW* beverage fermented by *Lactobacillus plantarum* BG 112 had a positive effect in the growth of the probiotic bacteria. After 30 days of shelf life, the beverage had *L. plantarum* BG 112 cell viability according to the recommended daily ingestion of probiotic foods. Futhermore, the fermented *GCW* showed potentially synbiotic properties and a high acceptability by consumers in the sensory evaluation test.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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