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Article

Probiotic Functional Carbonated Whey Beverages: Development and Quality Evaluation

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Abstract: Whey proteins have excellent nutritional characteristics due to their levels of essential amino acids with high bioavailability. However, it has a high biochemical oxygen demand (BOD) and a considerable polluting potential, thus food manufacturers have opted to add whey to food formulations. The demand for beverages containing vitamins, probiotics, prebiotics, minerals, and bioactive compounds (antioxidants) with health benefits has increased and driven market growth. Therefore, this study aimed to develop a probiotic functional carbonated beverage from cheese whey and evaluate its microbiological, and physicochemical characteristics soon after the production and during storage. The viability and stability of probiotic, the microbiological characteristics, titratable acidity and sedimentation of the beverage were monitored during refrigerated storage for a month. The probiotic to be added to the formulation was established in a preliminary step. The production of this beverage proved to be a simple technology and the product was suitable for incorporation of the probiotic *Bifidobacterium animalis subsp. lactis*. The probiotic showed good viability and stability during storage. The microbiological quality of the beverage met the Brazilian legal standards. The pH and titratable acidity of the probiotic carbonated beverage remained stable during storage, and slight sedimentation was observed after one week of refrigerated storage.

Keywords: Functional food, whey, byproducts, beverages, probiotics

1. Introduction

Whey is a byproduct of the cheese industry, with a high world production (around 200 million tons/year), and is characterized as an industrial effluent with high biochemical oxygen demand (BOD) due to its high levels of organic compounds, making it the most polluting by-products of food manufacturing [1]. On the other hand, despite the possible polluting effect, whey can also have great applicability as an ingredient in the food industry due to its great nutritional profile.

Whey retains more than half of the nutrient in milk, consisting of salts, vitamins, lactose, enzymes, and proteins rich in essential amino acids with high bioavailability. In addition, whey proteins stand out as precursors of biologically active peptides, which can produce various beneficial physiological effects in the human body, acting on the immune, nervous, and especially on the cardiovascular system [2].

The increase in environmental concern by industries, business groups, government entities and consumers aware of the importance of preserving the environment has led to studies about the use of by-products of the food industry that have functional and biological properties, such as cheese whey, in the production of beverages.

The international beverage market points to a total volume of commercialized beverages (alcoholic and non-alcoholic) of 923 billion liters, of which 74.7% are non-alcoholic beverages, classified into different categories, with an expressive volume of soft drinks and waters [3]. However, although the carbonated beverages represent a high proportion of the non-alcoholic beverages market in Brazil, Mintel [4] carried out a

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study on marketing research and found that 61% of Brazilians stated they would like to consume healthier alternative beverages rather than soft drinks. In this regard, whey-based carbonated beverages can meet this demand.

Carbonated beverages are products with great consumer acceptability, and whey can be one of the raw materials used in the manufacture of this product. The carbonation process is inexpensive, safe, and apparently has no negative effect on dairy products [5]. In addition, the use of probiotics in whey-based products may enhance its functionality. The development of dairy products containing probiotic bacteria is a major focus of the industrial sector, and generally, the production of food containing specific probiotic strains that maintain an adequate concentration of viable cells during the shelf life is a technological challenge [6]. Probiotics have been defined by FAO/UNO (Food and Agriculture Organization/United Nations Organization) and WHO (World Health Organization) [7] as "live microorganisms that, when administered in adequate amounts, confer benefits on their hosts".

Thus, this study aimed to develop a probiotic functional carbonated beverage from cheese whey, and evaluate its microbiological and physicochemical characteristics, soon after manufacture and during the refrigerated storage. The viability and stability of probiotic cultures, the microbiological characteristics, titratable acidity, and sedimentation of the beverage were monitored during the refrigerated storage for a month.

2. Materials and Methods

2.1 Microbial cultures

The experiments were conducted with DVS (direct vat set) cultures of *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* subsp. *lactis* BB-12 (Chr. Hansen/Valinhos/Brazil). The probiotic cultures were suspended separately in 1L sterile milk before use.

2.2 Preliminary study

This study was carried out at the Dairy Technology Center of the Food Technology Institute (ITAL) - Campinas - Brazil. In the initial stage, preliminary tests were done to establish the probiotic culture, additives and process parameters, according to Paula [5], with adaptations. Two types of probiotic cultures were evaluated: *Bifidobacterium animalis* subsp. *lactis BB12* and *Lactobacillus acidophilus La-5*, both purchased from Chr. Hansen.

Based on the results, the parameters were selected as follows: the culture of *Bifidobacterium animalis* subsp. *lactis,* due to the greater viability and less acidification during storage; a blend containing pectin (0.47%) and sodium citrate (0.08%) as a stabilizer; water cooling at 5ºC, and working pressure of 10.5 Kgf / cm^2, due to the higher carbonation rate and lower losses during storage.

2.3. Manufacture of the beverage

The beverage was produced from a premix with subsequent addition of carbonated water, at two different periods of the year, according to the flowchart shown in Figure 1.

For elaboration of the premix, cheese whey from enzymatic coagulation of low-fat *Minas Frescal* cheese (a typical Brazilian cheese) produced at ITAL was used; refined sugar (União); sodium citrate (Synth); fumaric acid (Synth); potassium sorbate (Clariant); natural green lemon flavor (Duas Rodas ref. 405504880001); pectin GENU PECTIN YM-150H (CPKelco); defoamer (Gemacom Tech Tate&Lyle); citric

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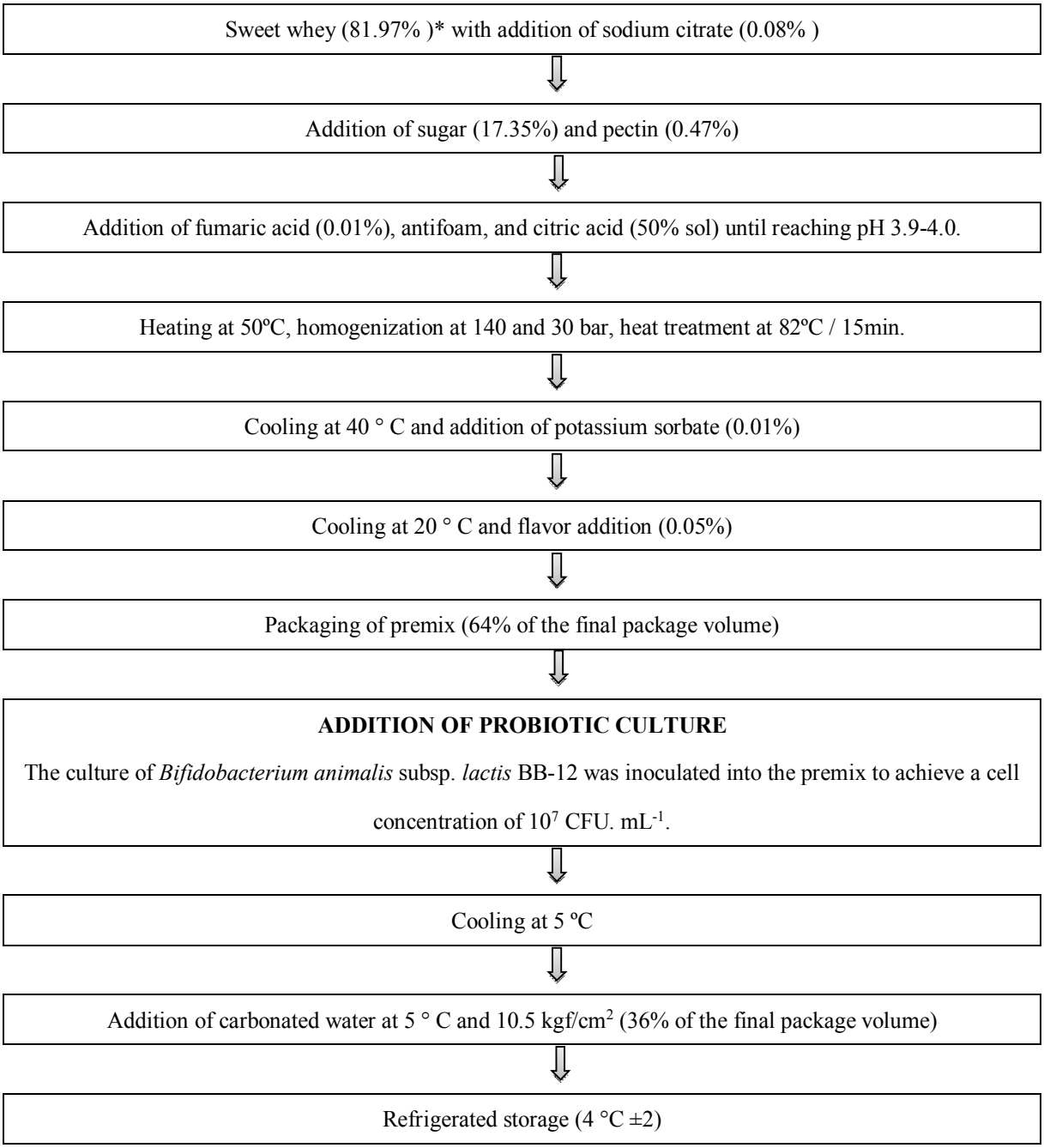
86 acid (Synth); probiotic bacteria *Bifidobacterium animalis* subsp. *lactis* (BB12) (Chr. Hansen). The beverages
87 were packaged in 500 mL Shott Duran bottles.
88
89



90 *The amount of whey and the other constituints of the beverage were calculated as a % w/w of premix

91 **Figure 1.** Manufacture of the probiotic functional carbonated beverage.

92

93 **2.4. Analytical determinations**

94 Immediately after the manufacture, the probiotic functional carbonated beverage was subjected to the
95 following determinations: coliforms at 30 ºC and 45 ºC or thermotolerant coliforms counts, mesophilic and
96 psychotrophic aerobic bacteria counts, molds and yeasts counts, detection of *Salmonella* sp, probiotic culture
97 counts, proximate composition, pH, titratable acidity, and sedimentation test. In addition, further

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determinations were carried out every 7 days during storage (28 days): total mesophilic and psychrotrophic counts, coliforms at 30-35°C, coliforms at 45°C, yeasts and molds counts, the viability of the probiotic culture, pH, titratable acidity, and sedimentation test.

2.4.1 Microbiological characterization

The total aerobic mesophilic counts were performed on standard plate count agar (Difco PCA agar) containing triphenyl tetrazolium chloride (TTC) and incubated at 32±1°C/48h [8]. The most probable number procedure (MPN) was used to determine coliforms at 30-35°C and at 45°C with lauryl sulfate tryptose broth (LST from Difco) and brilliant green bile lactose broth (BGBLB from Difco), incubating at 35±1°C for 24-48h for determination of coliforms at 30-35°C [9] (ISO 4831:2006) and *Escherichia coli* broth (EC from Difco) incubating at 44±1°C for 24 h [10] (ISO 7251:2005) for determination of heat tolerant coliforms. Dichloran rose bengal chloramphenicol agar (DRBC from Difco) was used for the yeast and mold counts, incubating at 25±1°C for 5 days [11] (ISO/IDF, 2004, number ISO6611). PCA (Difco), was used for the aerobic psychrotrophic counts, incubating at 7±1°C for 7 days [8]. The presence of *Salmonella* was determined according to the procedures recommended by Henning *et al*. [12]. The results of the microbial counts were expressed as log $CFU.mL^{-1}$, with the exception of the coliform counts, expressed as $MPN.mL^{-1}$ and the presence of *Salmonella*, expressed as present or absent.

2.4.2 Enumeration of probiotic cultures in selective media

Lactobacillus acidophilus La5 was counted according to the methodology of Technical Bulletin P-10 from Chr-Hansen, with an adaptation of the standard ISO 20128/IDF 192:2006 methodology [13]. MRS agar culture medium (Difco) was used, with 0.5mL of clindamycin stock solution (Sigma) per liter of medium, inoculating using the pour plate technique with anaerobic incubation (Anaerogen, Oxoid) at 37°C/72 hours. The methodology of Technical Bulletin P-12 from Chr-Hansen was used for *Bifidobacterium animalis* subsp. *lactis* counts, with adaptations of the standard IDF No. 411/2007 methodology [14]. An aliquot of 5 mL of dicloxacillin stock solution (Sigma), 10 mL of LiCl stock solution (Merck), and 5mL of CyHCl stock solution (Merck) were added to each liter of medium. The pour plate technique was used, with anaerobic incubation (Anaerogen, Oxoid) at 37°C for 72 hours. Catalase test and Gram staining (LABORCLIN) were performed for confirmation of Gram-positive bacteria, and verification of the typical morphology [15].

2.4.3 Proximate composition, pH, acidity, and sedimentation test

The following parameters were evaluated: total solids (TS) [16], fat (F) [17], ash (A) [18], and total nitrogen content [19]. The total protein content (TP) was calculated by multiplying the total nitrogen content by the conversion factor 6.38. The carbohydrate (CH) content was calculated by difference, according to the Equation 1:

$$(CH = TS - (F+A+TP)) \quad (1)$$

The pH was measured in a Micronal - B-375 digital potentiometer. The acidity was performed by titration with 0.1 N NaOH and expressed as a percentage of lactic acid (% LA) [20].

The sediment deposition was determined through the direct measurement of the sedimented phase, expressed as a percent, according to the methodologies described by Angelucci [21] and Oliveira et al. [22].

3. Results

3.1 Proximate composition of the beverage

The mean composition (n = 2) of the probiotic whey beverage immediately after manufacture is shown in Table 1.

Table 1. Mean composition (n=2) of probiotic functional carbonated beverage.

Determination	Value*
Total solids (%)	15.87±0.32
Ash (%)	0.29±0.02
Fat (%)	0.20±0.00
Protein (%)	0.53±0.03
Carbohydrates (%)	14.85±0.27

*mean ± standard deviation

Similar protein and ash contents and higher total solids, fat, and carbohydrate contents were observed in the present study, when compared to the results reported by Paula [5], who studied carbonated beverage made with whey from *Minas Padrão* cheese or *Mozzarella* cheese, and found 14.16% total solids; 0.34% ash; 0.10% fat; 0.52% protein; and 13.20% carbohydrates.

Katke and Patil [23] produced carbonated beverages from unclarified, prefiltered, and ultrafiltered shrikhand whey and mango, orange and pineapple juices, using a carbonation pressure of 30psi. The beverages presented 0.25-2.8% protein; 0.42-0.60% ash, 15.9-17.5% total solids, and 0 (not detected) to 0.24% fat.

3.2 Microbiological characterization

The microbiological characterization of the probiotic functional carbonated beverage is presented in Table 2, and the results of culture viability, cell morphology, pH, and titratable acidity are shown in Table 3.

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160 **Table 2**. Microbiological characterization of probiotic functional carbonated beverage immediately after the manufacture,
161 and during the refrigerated storage.

Microorganism	Microbial counts (log CFU.mL^{-1} or MPN.mL^{-1}) during the refrigerated storage (days)				
	0	7	14	21	28
Total aerobic mesophilic bacteria	< 1*	< 1*	< 1*	< 1*	< 1*
Total aerobic psychrotrophic bacteria	< 1*	< 1*	< 1*	< 1*	< 1*
Coliforms at 30°C	< 0.3*	< 0.3*	< 0.3*	< 0.3*	< 0.3*
Coliforms at 45°C	< 0.3*	< 0.3*	< 0.3*	< 0.3*	< 0.3*
Yeasts and molds	< 1*	< 1*	< 1*	< 1*	< 1*
Salmonella* spp*	Absence	-	-	-	-

162 *estimated value, below the detection limit of the method.
163 ** presence/absence in 25 mL sample.
164 - not determined
165
166 **Table 2.** Enumeration of *Bf animalis* subsp. *lactis* Bb-12 and determination of pH and titratable acidity of probiotic
167 functional carbonated beverage immediately after the manufacture, and during the refrigerated storage.

Time (days)	Results (log CFU.mL^{-1})	Cell morphology and Gram staining	Catalase	pH	Titratable acidity (% lactic acid)
0	7.45	Short curved rods with a typical arrangement of bifidobacteria G +	Negative	3.98	0.52
7	6.81	Short curved rods with a typical arrangement of bifidobacteria G +	Negative	3.95	0.51
14	6.85	Short curved rods with a typical arrangement of bifidobacteria G +	Negative	3.95	0.47
21	6.90	Short curved rods with a typical arrangement of bifidobacteria G +	Negative	4.05	0.56
28	6.87	Short curved rods with a typical arrangement of bifidobacteria G +	Negative	4.07	0.58

168 G+ Gram-positive bacteria
169
170 The microbiological quality is adequate for the product since the results in Table 2 are in accordance with
171 the standards required by the Brazilian legislation for milk beverages. The addition of CO_2 may have

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contributed to the inhibition of spoilage microorganisms in the beverage during the storage. In addition, as can be seen in Table 3, the probiotic culture *B. animalis* showed good viability during the storage of the product, with counts between 7.45 and 6.87 log CFU.mL^{-1} (final storage period).

Jardim et al [24] studied four dairy beverages formulations: a control, a fermented beverage, a carbonated beverage, and a carbonated fermented beverage. For the samples subjected to carbonation, the CO_2 dissolved in drinking water was injected into the sample, and the cultures *Lactobacillus acidophilus-LA-5*®, *Bifidobacterium BB-12*® and *Streptococcus thermophilus* (Chr. Hansen) were used for the fermented beverages. According to the authors, only the carbonated fermented beverage was considered to be potentially probiotic during the storage due to the presence of L*actobacillus* spp. in viable counts.

It has been suggested that probiotics should be present in the food product in minimal amounts of 10^6 colony forming units (CFU)/g. This minimal count must provide the potential benefits to the host [25, 26]. This amount can be translated into ≥ 10^6 CFU/g/day of probiotics-containing product, given a daily serving portion of 100 g. It is important how many cells are delivered per portion (e.g. total cfu per container consumed) [25].

Such high dosage is required to compensate for the cell loss during the passage through the upper and lower parts of the GIT [25, 27]. For the probiotic beverage in question, a brazilian daily recommendation of 200 mL (1 glass) refers to the consumption of 10^8-10^9 colony forming units (CFU) of *B. animalis*.

Higher pH and lower titratable acidity values were observed in the present study when compared to the findings of Paula [5], who studied a carbonated whey-based beverage without the addition of probiotics, stored at room temperature. The author found pH values ranging from 3.14 to 3.40, and acidity from 0.94 to 1.12% lactic acid. The pH of the beverage of the present study was higher than these values, once a mild acidification was performed in the manufacturing process to allow the addition of the probiotics. Katle and Patil [23] also studied carbonated whey-based beverages and found pH and acidity values ranging from 4.46 to 4.70, and 0.31 to 0.40%, respectively.

3.3 Sedimentation test

Although the immediately processed beverage (1 day after manufacture) did not present this defect, sediments were observed in the beverage in the second evaluation period (7 days). However, the sedimentation rate was only 1%, which is considered low and remained constant until the end of the storage (28 days).

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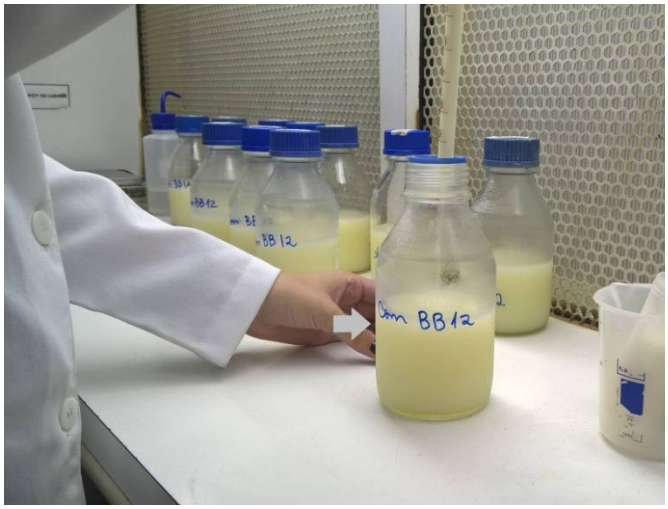

Figure 2. Addition of the probiotic culture *Bifidobacterium animalis* subsp. *lactis* Bb12 to the premix

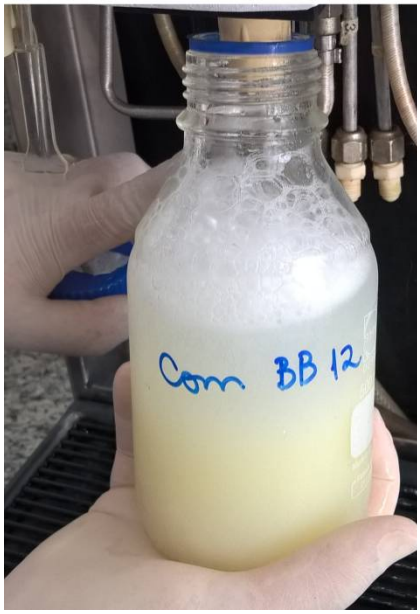

Figure 3. Carbonation step of the probiotic beverage

4. Conclusions

The probiotic culture *Bifidobacterium animalis* subsp. *lactis Bb12* was selected for the pilot-plant scale production of carbonated beverage, once it exhibited good viability and less acidification during the storage, without conferring a strange flavor to the product.

The manufacture of probiotic carbonated whey beverage proved to be a simple technology, and the product was suitable for incorporation of the probiotic culture. The beverage had adequate microbiological quality and stability during the storage.

The conditions and the level of probiotic culture used in the manufacturing process allowed that the viable cells remained between 10^7 and 10^6 CFU mL^{-1} during the refrigerated storage, which meets the values recommended internationally to confer health benefits.

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Conflicts of Interest: The authors declare no conflict of interest

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