

Research Article

Coconut Water-Based Probiotic Drink Proposal: Evaluation of Microbiological Stability and Lactic Acid Estimation

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Abstract

Probiotics are in high demand for their role as a health promoter, with lactose-fermented foods being the main source of getting them. However, the options for probiotics are lower for a group of consumers who are allergic or lactose intolerant. As an alternative to a non-dairy product, this work aims to propose the formulation of a low-cost drink with probiotic characteristics based on coconut water, free from lactose and fermented by *Lactobacillus casei shirota*. Two products were made: one with packaged coconut water and the other with fresh coconut water. The inoculum was obtained from a commercial fermented drink (Yakult®). In the first stage (fermentation), the total cultivation time was 48h; however, the most suitable time was 12h at 36°C for both cultures, with monitoring of pH, total acidity and cell concentration. Cultivations were carried out in duplicates with a repetition of the process for each product. After the first stage, the second stage (microbiological stability) at refrigeration temperature (5°C to 8°C) was started. The total refrigeration time was 120h; however, the most suitable time was 72h for both drinks. The estimate of lactic acid production was investigated using infrared spectrometry

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with Attenuated Total Reflectance (FTIR-ATR). It was possible to observe specific bands of carboxylic acids. The results obtained were promising and show potential to produce probiotic non-dairy drinks with inoculum and low-cost substrate.

Keywords: Coconut water; Fermentation; Functional foods; Lactobacillus; Probiotics

Introduction

Chronic-degenerative diseases have been the main cause of death in the world and controlling the risk factors for these diseases is a way to increase life expectancy. Aspects such as the practice of physical activities and a balanced diet are essential for the treatment and prevention of such diseases, contributing to the maintenance of health [1]. In this context, there is an increase in interest in functional foods, to get the beneficial, metabolic and physiological effects that these can cause.

Probiotics are gram-positive bacteria, defined internationally as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” [2]. Probiotics work to maintain healthy intestinal function in the prevention and treatment of diarrhea and food allergies, relief of lactose intolerance and normalization of intestinal transit [3]; decreased serum cholesterol, reduced plasma LDL levels and glucose homeostasis and in the immune function, regulating the defense system [3-5].

Allergies and intolerances related to milk are the main causes of restricting the consumption of dairy products. Allergies and intolerances are manifested through the biochemical incapacity of the organism to digest, absorb or metabolize a specific component. Lactose intolerance is characterized by the inability to digest lactose due to the lack or decrease of the enzyme lactase [6].

Bearing in mind that the most common form of access to probiotics is through fermented milk and yogurts, people who are lactose intolerant cannot consume this food and enjoy its benefits. It is predicted that about 75% of the population, when they reach adulthood, develop this intolerance, because of the loss of the ability to digest lactose [7].

Probiotic foods must meet safety and functionality criteria, which include having no adverse effects and not being associated with infectious diseases, besides containing a microorganism that can survive, maintain metabolic activity and grow at the destination site [5].

Coconut water is the liquid part of the coconut fruit, is low in calories, has a sweet taste and its composition contains mineral salts, vitamins, amino acids and carbohydrates. Coconut water acts as an excellent natural isotonic and among the functional properties attributed is rehydration and electrolyte replacement [8-10].

Some studies show the development of probiotic drinks with coconut water [11-15]. None of these studies show the use of a low-cost

inoculum and using two types of coconut water (industrialized and fresh). The results got were satisfactory and showed the probiotic potential of the planned drinks.

Materials and Methods

Probiotic material and coconut water

The probiotic bacteria used, *Lactobacillus casei shirota*, were obtained from a commercial fermented milk purchased under conditions suitable for consumption and kept under refrigeration (4°C) until the moment of use. The counting of lactic bacteria was performed on Man Rogosa Shape (MRS) Agar (Sigma®) and incubated at 36°C for 72h. The result was expressed in Colony Forming Units per mL of sample (CFU/mL).

The substrate used for fermentation had as raw material only coconut water and for preparing the drink, two formulations were initially used: one with industrialized coconut water and the other with fresh coconut water.

The industrialized coconut water showed sucrose (less than 1%) and antioxidant sodium metabisulfite (manufacturer's data). The commercial condition was sterile (Ultra High Temperature-UHT) in 200mL Tetra Pak® packages. The entire contents were removed from the original packaging and distributed aseptically in sterile glass containers.

Fresh coconut water was obtained from mature coconuts (*Cocos nucifera*) purchased at a fair in Belém-PA (Brazil). The coconuts were washed in running water and punctured. The water was removed, also removing suspended particles. The distribution was carried out in duplicate in glass containers (200mL/each) and autoclaved for 15 minutes at 121°C.

Inoculum addition

The inoculation temperature in fresh coconut water (W_f) and industrialized coconut water (W_i) was stabilized at 38°C. The initial pH was 4.6 and 4.5 for W_i and W_f respectively. The pH was adjusted to 6.0 and subsequent inoculation (3% v/v) of fermented commercial milk was performed concerning the volume of coconut water. Cultivation occurred in static mode for 48h at 36°C.

Determination of fermentation time

During the 48h period of cultivation, microbial growth and physico-chemical characteristics (pH and total acidity) were evaluated, according to the method of IAL [16]. The quantification of lactic acid bacteria was performed as described in APHA [17]. The serial dilution technique with peptone water (0.1% w/v) was used to count cells in Man Rogosa Sharpe (MRS) Agar (Sigma®) and incubated at 36°C at 72h. The result was expressed in Colony Forming Units per mL of sample (CFU/mL). From these results, the best fermentation times for each substrate were defined.

Evaluation of microbiological stability

In the assessment of microbiological stability, the same conditions were repeated for W_i and W_f and now the fermentation time was established as a function of the minimum cell concentration values, as previously described. After the fermentation step, the material was stored under refrigeration (5°C to 10°C) for 120 hours. During this period,

analyses of pH, acidity and microbiological count were performed at 24-hour intervals.

Microbiological analysis

Analysis of total coliforms, thermotolerant coliforms (45°C), *Salmonella* sp and mold and yeast count were carried out before fermentation, after fermentation and after refrigeration [17].

Estimation of lactic acid

The presence of lactic acid was estimated by spectroscopy in the infrared region with the Fourier transform and Attenuated Total Reflectance (FTIR-ATR) (Agilent Carry 360) in the spectral region from 4000 to 600cm⁻¹ with a measurement of 4cm⁻¹ and 32 scans. A liquid sample (fermented medium) was used without previous treatment. The spectrometric baseline fitting was performed by subtracting the effects of the water bands.

Results and Discussion

Bacterial growth and fermentation time

Quantification of lactic acid bacteria in commercial fermented milk was performed using quantification on MRS agar. The result obtained was in the order of 10⁹ CFU/mL. An inoculum of 3% (v/v) was added, corresponding to 6mL of fermented milk concerning coconut waters (200mL), where a theoretical concentration of 10⁷ CFU/mL was obtained. Figure 1 shows an enlargement of the lactobacillus (1000 X) present in fermented milk.



Figure 1: Microscopic aspect of the inoculum (lactobacillus).

The International Scientific Association for Probiotics predicts that to be considered a probiotic, organisms must survive the conditions of the human digestive tract and the beneficial action must be proven in studies that define the quantity and strain used for the suggested result. Therefore, there is currently no ideal probiotic concentration range. However, some countries adopt a concentration range around 10⁹ CFU/mL for strains of Lactobacillus [2]. Other studies use similar concentration ranges around 10⁶ to 10⁹ [12,14,18]. The planned drink presented a concentration of *Lactobacillus* between 10⁸ to 10⁹ CFU/mL.

The results got concerning cell growth were adequate and showed the viability of the product. In the two types of coconut water (W_i and

W_f), values in the order of 10^8 of cell concentration were reached, which shows the probiotic potential of the product in both types of substrates (Table 1).

After analyzing cell growth, it was observed that the fermentation time for probiotic viability in W_i was 12h, while in W_f it was 6h. In the time after 12h, only W_i did not reach the value of 10^9 CFU/mL until 48h of culture. Thermal treatment was carried out in fresh coconut water to eliminate fermentative micro-organisms, thus guaranteeing a better evaluation of growth with the inoculum, despite the possibility of nutritional loss because of the heating of the raw material. Even with the heat treatment, a better adaptation of the lactobacillus in W_f was observed in principle. The response of microbial growth in W_f was considered satisfactory, considering that W_i undergoes heat treatment at high temperatures (130°C to 150°C) for a short time (2s to 4s) and rapid cooling (below 32°C). It is believed that preservatives in W_i may have caused some inhibitory effect concerning the growth of lactic acid bacteria. The beverages planned preliminarily showed similar sensory characteristics. Both had an odor like fermented milk and a whitish color (Figure 2).

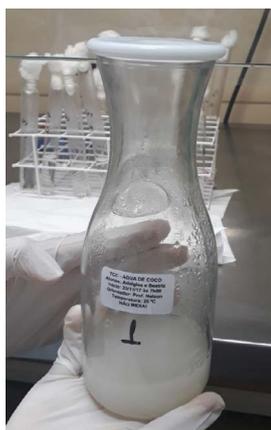


Figure 2: Probiotic drink based on coconut water after 12 hours of fermentation at 36°C .

Analysis of total coliforms, *Salmonella* sp. and molds and yeasts

Microbiological analysis carried out in coconut waters before the fermentation showed the absence of total and thermotolerant coliforms in MPN/mL. Microbiological analysis performed after fermentation showed the absence of total coliforms and thermotolerant coliforms and the absence of *Salmonella* sp. Regarding the product after refrigeration (storage), the absence of molds and yeasts was observed. These results showed that the entire process remained within microbiological standards.

Total acidity and pH analysis

The initial pH of coconut waters was corrected to 6.0. This procedure aimed to promote the most suitable pH range for the development of lactic acid bacteria, which can preferably grow at slightly acidic to neutral pH (5.5 to 6.2) and at temperatures between 30°C to 40°C [19,20].

The results of pH in W_i showed a variation between 5.4 and 5.7 in the 48h period. The acidity value decreased approximately 25% after 6 hours of cultivation and remained stable until 36 hours. After 48 hours, a return to initial acidity was observed. The pH and acidity values show important information about hydrogen concentration and organic acids in the medium. In this case, it is possible to deduce that the reduction in pH and the increase in acidity may be mostly related to the presence of lactic acid. Ramos et al. [21], formulated three fermented milk drinks with cajá flavor (with 40% whey and 15%, 20% and 25% of fruit pulp) with an inoculum of *Lactobacillus acidophilus*, *Bifidobacterium* and *Streptococcus thermophilus*; fermented at 42°C for 4h. Thus, as in this work, practically constant results of pH and acidity were observed (Figure 3).

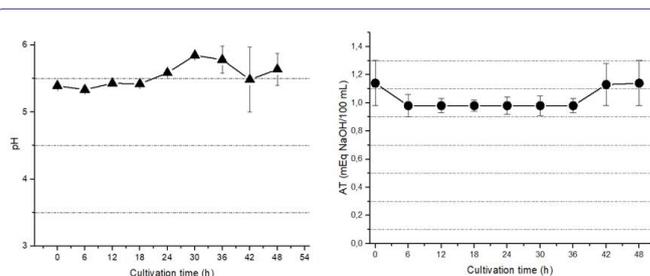


Figure 3: Profile of pH and total acidity with industrialized coconut water (W_i).

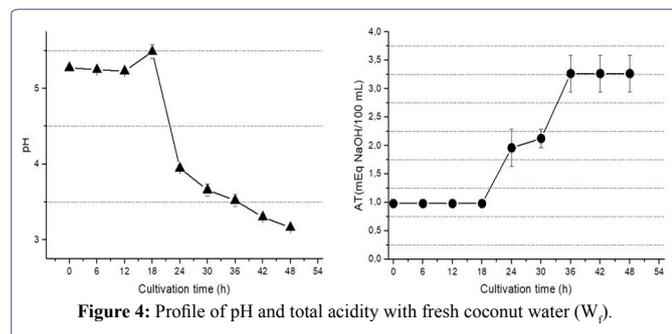
Otherwise, W_f showed different results. A marked decrease in pH was observed after the initial 24h of fermentation and a more expressive increase in acidity. The total acidity value at the end of the fermentation was 3.2 times higher when compared to the beginning. Based on this observation, it is possible to infer that cultivation in W_f has a greater potential for lactic acid production when compared to W_i (Figure 4). Giri et al. [11], showed similar results in preparing a coconut water drink fermented with *Lactobacillus casei* L4. In this study, a decrease in pH from 6.5 to 3.32 was observed after 48 hours of fermentation, with a consequent increase in acidity.

The two coconut water substrates had different profiles in terms of pH and acidity. The increase in acidity and consequent decrease in pH observed in the W_f suggests a better adaptation of lactic acid bacteria in this condition. It is believed that this occurs because of the fresh coconut water presenting nutritional characteristics more suitable for microbiological growth.

Formulation	Incubation Time (h)					
	0	6	12	18	24	48
W_i	$4,15 \times 10^7 \pm 1,63 \times 10^6$	$9,00 \times 10^7 \pm 2,83 \times 10^6$	$1,05 \times 10^8 \pm 4,95 \times 10^7$	$2,03 \times 10^8 \pm 3,54 \times 10^7$	$6,75 \times 10^8 \pm 5,17 \times 10^7$	$7,56 \times 10^8 \pm 4,71 \times 10^7$
W_f	$5,4 \times 10^7 \pm 2,25 \times 10^6$	$7,4 \times 10^8 \pm 1,8 \times 10^7$	$2,5 \times 10^9 \pm 4,5 \times 10^8$	-	-	-

Table 1: Concentration of cells in CFU/mL by incubation time.

Note: W_i : Industrialized coconut water; W_f : Fresh coconut water.



The inverse relationship of pH and total acidity with bacterial growth, suggests a proliferation of lactic acid bacteria, which consume sugars of the production of lactic acid [22]. It is known that *Lactobacillus casei shirota* can ferment different sugars, including galactose, lactose, D-lactose, D-fructose, sorbitol, and others [23].

The bacteria use nutrients from the substrate for fermentation and multiplication. These metabolic activities release more lactic acid, this decreases the pH and increases the acidity of the final product [23]. About industrialized coconut water, bacterial growth occurred, however over a longer time. It is possible that industrial processing has altered the nutritional profile of this substrate. The addition of antioxidants in industrialized coconut water may also have influenced pH stability and acidity.

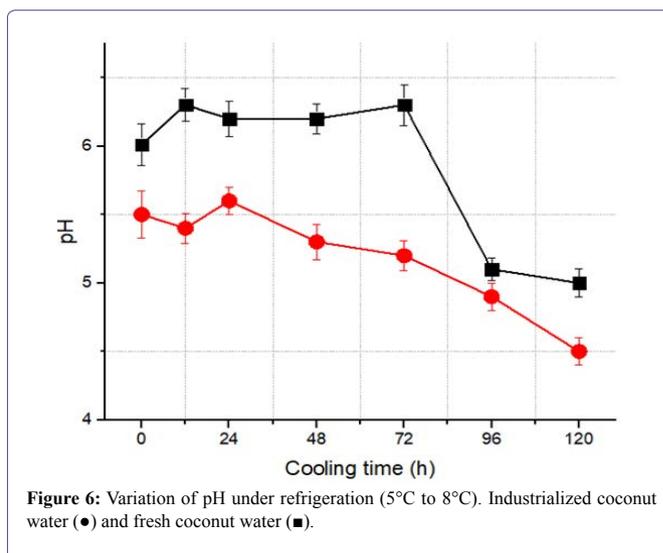
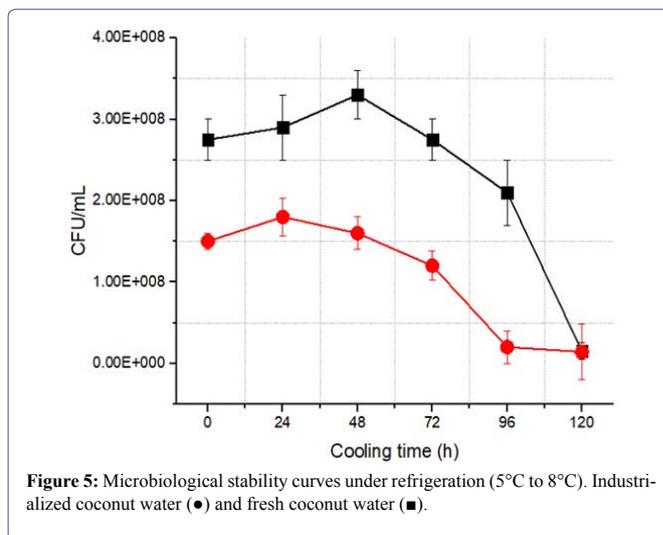
Although the two formulations showed adequate results regarding the probiotic potential (bacterial growth greater than 10^8), the fermentation with W_f presented the desired response more efficiently. The fermentation with W_i reached the appropriate microbial concentration in pH and practically constant acidity. Otherwise, in W_f reached the range of 10^8 - 10^9 CFU/mL (Table 1) and showed increased values of acidity during the 48h of analysis, with a consequent decrease in pH.

Considering that at 12h the two formulations showed probiotic potential, it was decided to use this time as a fermentation standard for both formulations. The establishment of the standard time was important to test the microbiological stability in refrigeration. Thus, there was no need to carry out a long time for the desired microbial growth during the fermentation phase.

Microbiological stability under refrigeration

The quantification of viable cells in W_f showed less variation in the refrigeration period (5°C to 8°C) between times 24 to 96h. In this condition, there was the maintenance in the ideal bacterial growth range for probiotics (10^8 - 10^9) for a longer time (96h) during storage under refrigeration, in comparison with W_i (72h) (Figure 5).

The drink with W_f maintained an average pH equal to 6.2 ± 0.3 up to 72 hours of refrigeration. After this period, there was a decrease to 5.1 ± 0.2 . The W_i drink showed pH stability of up to 24 hours. Between 24 hours and 120 hours, there was a progressive decrease to pH equal to 4.5. It is believed that the pH decrease in both drinks must be related to the accumulation of lactic acid produced during fermentation and later during refrigeration, as has been described in other studies [13,18] (Figure 6).



Lactic acid production

The samples of both drinks were analyzed in the middle infrared region, observing the wavenumbers in 1720 cm^{-1} (C=O stretch of carboxylic acids), 1230 cm^{-1} (C-O stretch of carboxylic acids) and 1128 cm^{-1} (C-O stretch of esters). Figure 7 shows two infrared spectrum profiles for drinks made in 120 hours of storage. For the microbiological characteristic of the inoculum used in this study (*Lactobacillus*), it is possible to infer that the decrease in pH and increase in acidity is because of the production of lactic acid. Spectroscopy in the infrared region with Attenuated Reflectance (FTIR-ATR) is an excellent technique for organic analysis, because it is fast, does not require sample preparation and analytical standard. This mainly facilitates routine analysis in industries with an emphasis on a specific substance as in this study. Figure 7 shows the results of the infrared spectroscopy analysis, resulting from the spectral subtraction of the water molecule in the drinks. This procedure was necessary because of the intensity of the water bands that overlapped the characteristic

bands of the carboxylic acids in the samples of the drinks. Lactic acid is classified as a carboxylic acid, nominated by IUPAC as 2-hydroxypropanoic acid. In this way, it was possible to identify two characteristic bands of carboxylic acids (1720cm^{-1} and 1230cm^{-1}) [24]. A lower transmittance value of 1720cm^{-1} for the W_i drink may show a higher concentration of lactic acid. This result is corroborated by figure 6, which shows a lower pH value in 120h when compared to W_f . In 1128cm^{-1} , it is possible to observe characteristic ester bands, this is because lactic acid undergoes a condensation reaction even at low concentrations [25]. The lower transmittance value for the drink with W_f shows a higher concentration of condensed lactic acid molecules. This is believed to be due to the greater stability of lactic acid polymers at less acidic pH [26].

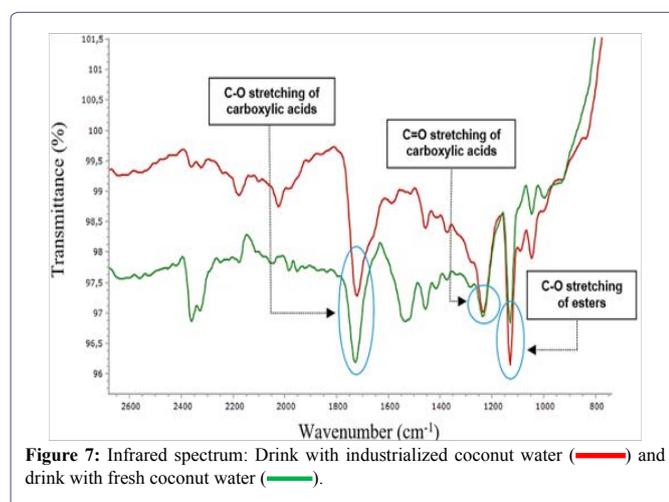


Figure 7: Infrared spectrum: Drink with industrialized coconut water (—) and drink with fresh coconut water (—).

Conclusion

When we compare the two formulations, fresh coconut water substrate with industrial coconut water substrate, we noticed that the two formulations showed adequate results about the probiotic potential after 12 hours of fermentation. Complementary, we can consider that the adequate cooling time is 72h for both formulations at temperatures between 5°C to 8°C .

Coconut water proved to be an excellent substrate, both for its adequate nutritional profile and its ease of acquisition, besides allowing the growth of *L. casei shirota*. Despite being initial data, there is a good prospect of future applicability in the development of a low-cost product that can also be safely prepared in homes, as it is with the production of homemade yogurts.

The drinks proposed in this study can serve an increasingly growing market niche of people who have lactose intolerance or allergy, or even those who do not consume products of animal origin.

Declaration of Conflicting Interests

The authors declare that there is no conflict of interest.

Acknowledgment

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