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Survivability of Probiotic Bacteria in Model Systems of Non-Fermented and Fermented Coconut and Hemp Milks

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Abstract: This study aimed at determining the survivability of probiotic bacteria cultures in model non-dairy beverages subjected or not to the fermentation and storage processes, representing milk substitutes. The experimental material included milks produced from desiccated coconut and non-dehulled seeds of hemp (*Cannabis sativa* L.). The plant milks were subjected to chemical and microbiological evaluation immediately after preparation as well as on day 7, 14, and 21 of their cold storage. Study results proved that the produced and modified plant non-dairy beverages could be the matrix for probiotic bacteria. The fermentation process contributed to increased survivability of *Lactobacillus casei* subsp. *rhamnosus* in both coconut and hemp milk. During 21-day storage of inoculated milk substitutes, the best survivability of *Lactobacillus casei* was determined in the fermented coconut milk. On day 21 of cold storage, the number of viable *Lactobacillus casei* cells in the fermented coconut and hemp milks ensured meeting the therapeutic criterion. Due to their nutritional composition and cell count of bacteria having a beneficial effect on the human body, the analyzed groceries—offering an alternative to milk—represent a category of novel food products and their manufacture will contribute to the sustainable development of food production and to food security assurance.

Keywords: probiotic; non-dairy beverages; survivability; fermentation; bacteria; coconut; hemp; sustainable food production

1. Introduction

Sustainable food production should be considered through the perspective of a better understanding of food security. In recent years, many researchers and policy makers have focused only on the physical availability of food, owing to the sufficient agricultural production [1,2]. This has partly been driven by widespread claims that we need to boost the global food production to feed the world in 2050 [3]. However according to the FAO (Food and Agriculture Organization of the United

Nations) definition [4]: "food security exists when all people, at all times, have physical and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life". Hence, it needs to be emphasized that the sustainable food production is inevitably related to the food security in its three aspects: food security, food safety, and food quality, without which the development of the food industry sector would not be possible [5,6]. Due to the current dynamic development of sciences related to food and human nutrition, a correlation has been confirmed between the health status and nutritional patterns. A well-balanced diet is the key factor in diseases prevention and treatment. The growing nutritional interests and awareness of consumers have prompted many producers to manufacture functional food [7–9]. A functional food definition covers certain strains of microorganisms being constituents of food of plant and animal origin that contain physiologically active compounds. These compounds are beneficial for human health and help minimizing the risk of chronic diseases development [10]. One of the multiple examples of functional food products are these containing microorganisms endogenous to the human gastrointestinal tract and exhibiting a positive effect on human health [11]. So far, the greatest part of probiotic products has been offered by fermented beverages made of animal milk. Currently, research is underway into other products that may be matrix for probiotic bacteria [12,13]. Consumers avoiding milk because of allergies or lactose intolerance, and consumers following a vegan diet can replace milk with other plant-based substitutes. Beverages derived from soybeans have for many years been the predominant equivalents of milk. Today, coconut, almonds, hemp, and various cereals (e.g., oats, buckwheat, and rice) are also used to produce plant-based beverages [14,15]. A drawback of these products is however their specific taste that does not suit to everyone. A solution to this problem is offered by lactic acid fermentation, which imparts a characteristic, pleasant after-taste to these products and contributes to the improvement of the digestibility [13,16]. Numerous attempts have recently been undertaken to ferment vegan beverages serving as milk substitutes using various strains of probiotic bacteria, which was expected to additionally increase their health value [16,17]. However, most of the study results reported in literature concern the feasibility of producing fermented soybean milk [13–16]. This is related to the fact, that manufacture of high quality plant-based beverages containing probiotic bacteria poses a serious challenge [18,19]. According to Yuliana et al. [20], the production of coconut-based beverages is difficult because of the suppressed growth and survivability of these probiotic microorganisms, compared to dairy beverages. Difficulties in the manufacture and fortification of hemp milk were encountered by Batkiene et al. [21]. The first ones were related to the stability of produced emulsions, whereas the latter ones to the survivability of probiotic bacteria during storage. Worthy of notice is that the production of hemp-based products has increased in recent years due to the confirmed nutritional value and low allergenicity of seeds of this plant [22]. This has been feasible owing to new varieties characterized by a low concentration of a psychoactive compound delta-9-tetrahydrocannabinol (THC) [23] and to cultivations with the use of elite category sowing material [24].

The current definition of a probiotic means those microbial strains that positively affect consumer health when taken in the right amount [25]. Accordingly to FAO/WHO guidelines, the count of probiotic bacteria cannot be less than the value corresponding to 10^6 cfu per 1 mL of a product through the entire period of its storage till the end of its shelf life. This value has been deemed the therapeutic minimum [26–28].

The main problems associated with the fermentation of plant beverages are related to the sensory quality of the final product and to the resistance of probiotic microorganisms. Producers encounter difficulties with the physical stability caused by milk coagulation (it occurs at the beginning or in the course of storage). The appearance of these products resembles that of low-fat yoghurt [29–32]. Additional problems concern the survivability of probiotic bacteria, which is dependent on multiple factors, including e.g., presence of other microorganisms in the product, time and conditions of strains culture and product storage, product processing technology or pH value [13,26,33,34].

Considering the above, the major objective of this study was to determine the survivability of probiotic bacterial cultures in model fermented and non-fermented stored plant beverages being

milk substitutes, because today the plant-based alternative milks provide a huge perspective for the sustainable development of the healthy food market and should therefore be widely scrutinized. Evaluation of the effect of production and processing techniques, and also of fortification techniques, of plant-based beverages may serve to develop a nutritionally complete beverage with a high overall acceptability and health values.

2. Materials and Methods

2.1. Plant Material and Beverages Production

The experimental material included non-dairy beverages produced from the following plant raw materials: desiccated coconut (Bakalland, Warszawa, Poland) and non-dehulled seeds of hemp (*Cannabis sativa* L.; Sante, Warszawa, Poland; Figure 1). Seeds and desiccated coconut were ground in a WZ-1 laboratory mill (Sadkiewicz Instruments, Bydgoszcz, Poland). Chemical composition of analyzed material is presented in Table 1.

Figure 1. Raw materials used for non-dairy beverages production: (**a**) hemp seeds and (**b**) desiccated coconut.

Table 1. Contents of protein, lipids, and sugars of desiccated coconut and non-dehulled hemp seeds (nutritional information available on respective product labels).

Product	Protein	Lipids	Sugars
	(%)		
Desiccated coconut	5.6	63.2	5.9
Non-dehulled seeds of hemp	25.2	36.1	5.4

Milk substitutes to be analyzed were produced according to the schemes presented in block diagrams in Figures 2 and 3.

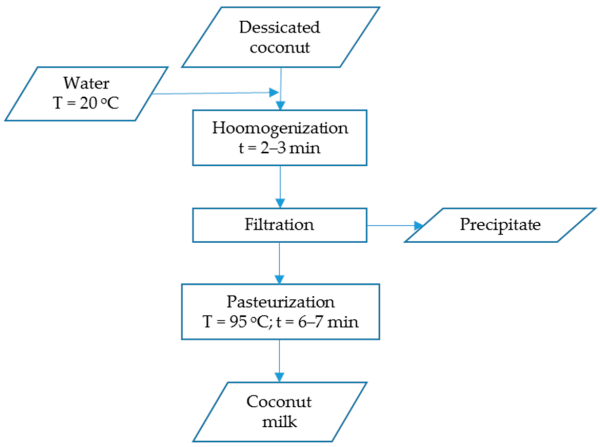

Figure 2. Technological scheme of coconut milk production (own study based on Blasco [35]).

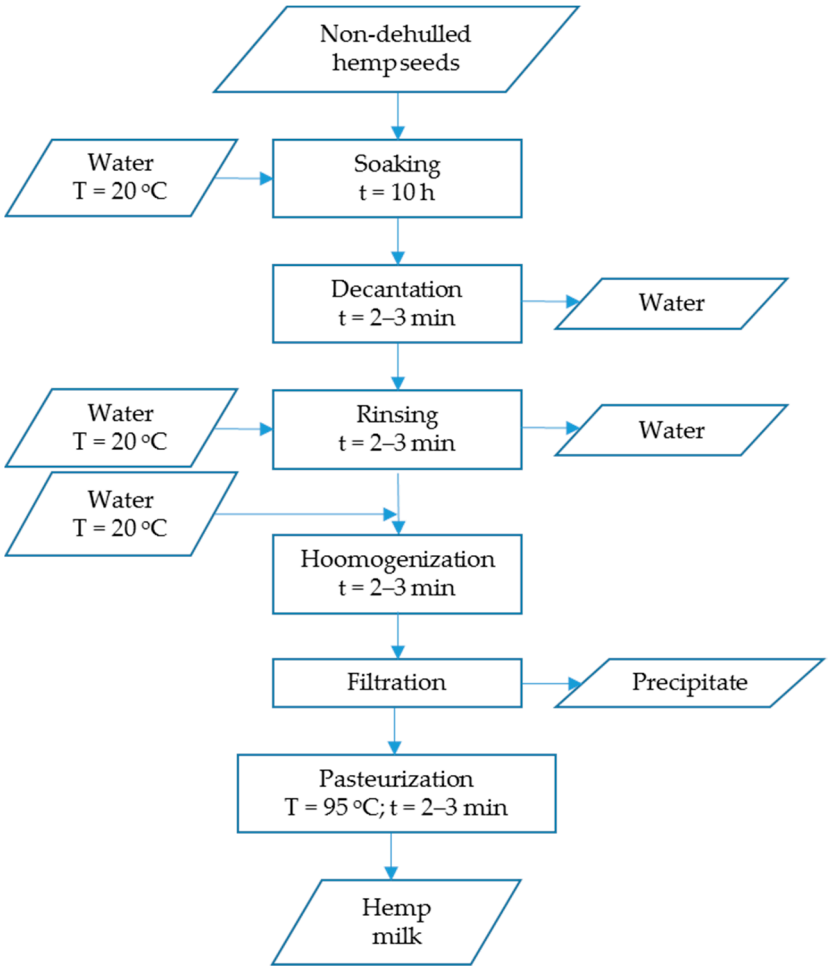

Figure 3. Technological scheme of hemp milk production (own study based on Szakuła [36]).

Immediately after production, the plant-based milks were subjected to the physicochemical analysis to determine content of their protein, lipids, and carbohydrates, and their acidity. Their microbiological status was assessed as well.

Models of non-dairy beverages intended for the determination of counts of viable bacterial cells during storage were divided into four groups, each containing three samples—two groups included beverages with probiotic addition and two groups—beverages without the probiotic. Both, the samples supplemented and not supplemented with a probiotic monoculture were fermented in a laboratory incubator (Binder BD 260) at a temperature of 37 °C for 6 h, and then stored at a temperature of 4 °C. The remaining samples were cold stored. Duration of the fermentation process was chosen based on results of a study conducted by Zielińska et al. [37], who demonstrated that intensive proliferation of *Lactobacillus casei* subsp. *rhamnosus* cells proceeded till the 6th hour of the process. Optimal parameters of the fermentation process, established by Zielińska et al. [38], allow producing plant-based fermented beverages with sensory quality acceptable by consumers [39]. Based these findings, investigations have not assumed own sensory evaluation.

2.2. Probiotic Microorganisms

The study was conducted with probiotic bacterial strain *Lactobacillus casei* subsp. *rhamnosus* LCR 3013 in the form of a lyophilizate (Serowar, Szczecin, Poland). Before analyses, the strain was stored at a temperature of −18 °C to preserve its properties. The bacterial culture was activated by transferring 0.1 g of the lyophilized strain to 5 mL of an MRS broth (Merck, Warszawa, Poland). Next, the suspension was incubated at a temperature of 37 °C for 24 h. The resultant culture was centrifuged at 10,000× *g*

for 5 min. Centrifugation was repeated after rinsing the resultant precipitate with a physiological saline solution. Cell biomass suspended in a physiological saline solution (5 mL) and having the optical density of 1° McF (Densimat, bioMérieux, Grassina FI, Italy) was added to 100 mL of plant milk (5% *v/v*), to achieve bacterial cell count of approximately 10 log (cfu/mL) [21].

2.3. Chemical Evaluation of Non-Dairy Beverages

The produced non-dairy beverages were analyzed for content of: protein with the Kjeldahl's method [40] (Büchi Distilation Unit, K314) and lipids with the Gerber's method [41]. Their active acidity (pH) was measured as well using a VOLTCRAFT KBM-110 m (Conrad Electronic SE, Hirschau, Germany) with a pH electrode [42].

Additional analyses were conducted to determine the content of reducing sugars. They were made with the colorimetric method using 3,5-dinitrosalicilic acid (DNS) [43]. DNS acid (1950 µL) were added to 50 µL of the analyzed non-dairy beverages, and the mixture was incubated in a water bath at a temperature of 99 °C for 10 min. After cooling the mixture, 900 µL of DNS acid were added to 100 µL of mixture sample. Absorbance was measured at λ = 540 nm (UV-vis spectrophotometer, VWR UV-6300PC, USA) and results of these measurements were converted based on the standard curve into equivalents of glucose (g/L) contained in non-dairy beverages.

2.4. Microbiological Status of Plant Beverages

The produced plant beverages were pasteurized and afterwards subjected to a microbiological analysis based on the pour-plate Koch's method [44] and the sterile serial dilutions method (from 10^{-1} to 10^{-8}). For this purpose, 1 mL of each of the two subsequent dilutions (10^{-6} and 10^{-8}) and 9 mL of each of the appropriately selected medium were transferred onto Petri dishes and left to solidify [16]. Nutrient agar (BTL, Łódź, Poland) was used to isolate mesophilic and psychrophilic bacteria, whereas Sabouraud agar enriched with chloramphenicol (BTL, Łódź, Poland) was used for fungi and yeast isolation from the beverages. Samples were incubated at a temperature of 30 °C for 48 h (mesophiles) and at 20 °C for 72 h (psychrophiles), and at 20 °C for 5 days (fungi and yeast) [44]. Total bacterial count (TBC) was determined as well. Bacterial cultures were inoculated with the pour-plate method in three replications for each sample. Plates with inoculates were incubated at a temperature of 37 °C for 48 h under anaerobic conditions using anaerostats with anaerocult A inserts (Merck, Darmstadt, Germany). Nutrient broth agar (BTL, Łódź, Poland) was used for inoculations. After completed incubation, results were converted into the number of colony forming units per 1 mL of product (cfu/mL). Dilutions of 10^{-6} and 10^{-8} were used for analyses and for TBC determination in each sample. The above analyses were carried out for plant beverages without probiotic strain addition.

2.5. Microbiological Analyses of Counts of Viable Bacterial Cells During Storage of Fermented and Non-Fermented Non-Dairy Beverages

Analyses of the survivability of the probiotic strain *Lactobacillus casei* subsp. *rhamnosus* in fermented and non-fermented models of coconut and hemp beverages (with added starter monoculture of probiotic bacteria) were conducted immediately after their preparation, after their fermentation as well as on day 7, 14, and 21 of their storage at a temperature of 4 °C. The maximal cold storage time assumed in the study was selected based on results of a research conducted by Gustaw et al. [45] into the survivability of *Lactobacillus casei* strain in fermented beverages with the addition of selected protein preparations.

Having been diluted in sterile water, the analyzed samples were transferred onto sterile Petri dishes (1 mL of sample from each dilution of 10^{-6} and 10^{-8}), to which 9 mL of the selective MRS Agar medium (by de Man, Rogosa and Sharpe) [46] (BTL, Poland) were added afterwards. Next, the

samples were incubated at a temperature of 30 °C for 72 h. The total count of viable lactic acid bacteria per 1 mL of the sample was computed according to the following formula:

$$N = n_c \cdot d_r, \quad (1)$$

where: N—number of viable bacterial cells (cfu/mL), n_c—number of bacterial colonies, and d_r—dilution rate.

2.6. Statistical Analysis

All experiments were carried out in three replications. Results were expressed as arithmetic means. The Shapiro–Wilk test was used to evaluate the normal distribution of data. Results were analyzed using a one-way analysis of variance (ANOVA). The significance of differences between mean values was estimated based on Tukey confidence intervals, at a $p < 0.05$. Values followed by different small letters are significantly different at $p < 0.05$ (effect of storage). Values followed by different big letters are significantly different at $p < 0.05$ (effect of treatment). The standard deviation (±SD) value was determined for all reported mean values. The statistical analysis was performed using Statistica 13.3 software (StatSoft, Kraków, Poland).

3. Results and Discussion

3.1. Evaluation of the Produced Non-Dairy Beverages

One of the key technological aspects in probiotic food production is to maintain optimal conditions that would ensure the proper growth and viability of potentially probiotic bacteria during fermentation and storage [47]. This may be accomplished through, i.e., the appropriate choice of a carrier and product supplementation with nutrients [48]. Hence, raw materials of plant origin need to be analyzed for the content of nutrients indispensable for probiotic bacteria metabolism, and for the effect of environment on their survivability [27,49].

In order to identify factors that affect probiotics survivability, a study with non-dairy beverages produced from desiccated coconut and hemp seeds under laboratory conditions was conducted. It needs to be emphasized that the nutritional value of non-dairy beverages is largely determined by their protein content [28,50]. In the study, protein content was determined at 3.23 g/100g in coconut beverage and at 6.96 g/100 g in hemp beverage (Table 2).

Table 2. Contents of protein, lipids, and glucose, and active acidity of coconut and hemp milks.

Non-Dairy Beverage	Protein	Lipids	Reducing Sugars	Active Acidity
	(% ± SD)		(g glucose/L ± SD)	(pH ± SD)
Coconut milk	3.23 ± 0.28	21.08 ± 0.41	34.53 ± 0.39	6.15 ± 0.15
Hemp milk	6.96 ± 0.19	18.02 ± 0.54	30.21 ± 0.33	6.81 ± 0.11

Differences in protein content were determined depending on the plant raw material used for beverages production. Discrepancies were also noted when comparing protein content determined in the study and these declared by selected producers of plant beverages intended for the European market [13], i.e., obtained results were higher than protein content declared by producers of coconut and hemp beverages. These differences might be due to the various quality of raw materials used for beverages production and to treatments applied in the production process (e.g., heat treatment) that contribute to a decrease in total protein concentration.

Hoffman and Kostyra [15] evaluated plant-based milk substitutes in terms of their nutritional value and demonstrated that only the beverage made of soybean seeds equaled milk in this respect. The other plant materials had significantly lower content of protein, i.e., two-fold lower—quinoa,

and three-fold lower—a mixed beverage made of soybean, rice, and oats. Beverages made of coconuts and almonds were characterized by trace amounts of protein.

This indicates that although coconut-based milk substitutes provide very low amounts of protein, they may offer an alternative to consumers who seek for gluten-free food.

Results of study confirm findings reported by Sethi et al. [13], who demonstrated that plant beverages are inexpensive substitutes of milk, especially for consumers allergic to milk, but are not comparable nor equal with it in terms of their nutritional value, as hemp milk (living harvest) provides barely 2 g and coconut milk less than 1 g of protein in 240 mL of the product.

Coconut beverages are food products characterized by a high content of lipids, including significant content of the following saturated fatty acids: lauric (50%) and myristic (6%–7%). The unsaturated fatty acids of coconut include oleic acid (monounsaturated) and linolic acid (polyunsaturated) [51]. In turn, the hemp beverage contains approximately 80% of essential unsaturated fatty acids (EFAs), including linolic acid (56%) and α-linolenic acid (19%). According to dieticians, the optimal ratio of these acids should reach 3:1, as is the case with the hemp beverage [52]. Figure 4 presents content of lipids in the analyzed raw beverages made of coconut and hemp seeds. As it results from our study, the coconut beverage contained 21% and the hemp beverage contained 18% of lipids.

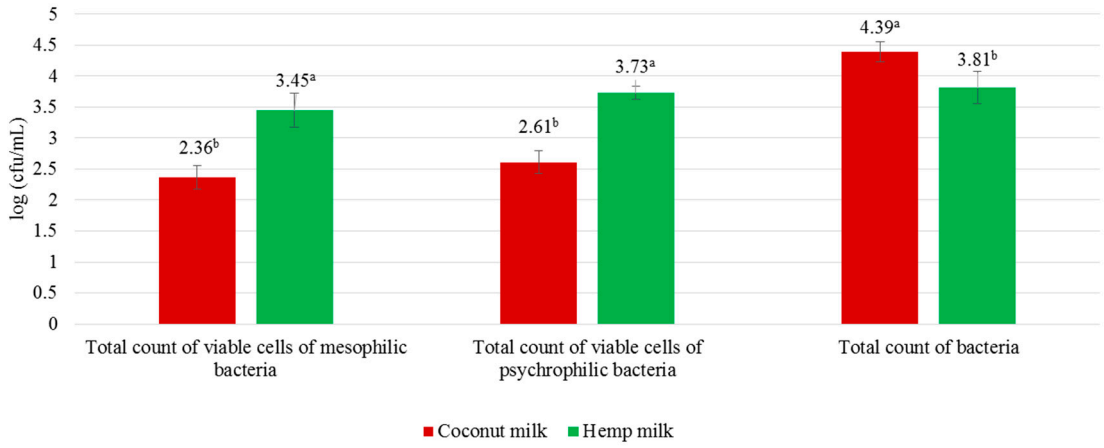

Figure 4. Total count of microorganisms isolated from pasteurized non-diary beverages (a, b—statistically significant differences, $p \leq 0.05$).

Due to the composition of the plant milks, their fatty acid profile differed significantly from that of milk. Milk contains approximately 1.2% of saturated fatty acids, whereas their content in its substitutes does not exceed 0.7%. Similar conclusions were formulated by Belewu and Belewu [53], who determined lipids content in the produced coconut milk at 24.10%. In turn, Sethi et al. [13] demonstrated that the analyzed plant-derived milks were characterized by a similar concentration of lipids reaching 6 g in hemp milk (living harvest) and 5 g in coconut milk per 240 mL of the product.

Content and types of carbohydrates in fermented beverages have a significant impact on the development and activity of health-promoting bacteria. Reducing sugars present in the medium may be good sources of carbon necessary for probiotics metabolism [54]. This was confirmed by Jurkowski and Błaszczyk [55], who demonstrated monosaccharides to be indispensable substrates during the fermentation process by lactic acid bacteria. Hence, control of their content seems necessary in the production of probiotic foods. In the study, the content of reducing sugars was expressed as glucose concentration in the produced non-dairy beverages (Figure 4), and reached 34.52 g glucose/L in coconut milk and 30.21 g glucose/L in hemp milk. Data obtained demonstrate that the coconut milk is a better raw material for the production of probiotic non-dairy beverages because it is richer in compounds necessary for fermentation (e.g., glucose). This is in line with results reported by Quasem et al. [17] who analyzed a sesame beverage as a bacteria matrix. Differences in the content of reducing sugars are one of the factors, which determine the possibility of using plant-based milk as a natural medium for

lactic acid bacteria development. According to Sethi et al. [13], however, the choice of a matrix for these beneficial microorganisms may also be driven by the total content of carbohydrates. In hemp milk, their concentration reaches barely 1 g, whereas in coconut milk it is high and reaches 7 g/240 mL of product.

The content of acids is a factor that determines food freshness, but at the same time it largely affects its color and taste. Acids in beverages and other food products, contribute to control microflora growth [56]. In our study, the active acidity of coconut beverage (before fermentation) was determined at pH 6.15 (Figure 4). This value is similar to results reported by other authors. For instance, when investigating the effect of strawberry beverage supplementation with a soybean protein isolate, Dłużewska et al. [57] observed changes in the real acidity, values of which ranged from pH 5.82 to pH 6.61. In turn, acidity of a coconut beverage analyzed by Belewu and Belewu [53] reached pH 6.23. A negligibly higher active acidity (pH 6.81) was determined in our study for the hemp seed beverage. Many scientists have reported on the decreased acidity of the medium in the case of fermented products, which appeared to result from the accumulation of lactic acid caused by the activity of microorganisms.

3.2. Microbiological Purity of Non-Dairy Beverages

From the perspective of health safety, pasteurization is an indispensable process during the manufacture of plant-based beverages. It contributes to eradication of pathogenic microorganisms and to inactivation of certain spore-forms. Therefore, in the present study, we evaluated its effectiveness (Figure 4), which is consistent with provisions of Commission Regulations (EC) 2073/2005 and 229/2019 [58], according to which the safety of food is mainly ensured by a preventive approach including, e.g., control of heat treatment effectiveness.

It was demonstrated that the type of raw material influenced on the presence of bacteria, both the mesophilic and psychrophilic ones, in the produced non-dairy beverages. The determined count of mesophilic bacteria ranged from 2.36 log (cfu/mL; coconut milk) to 3.45 log (cfu/mL; hemp milk). A more numerous group of the isolated microorganisms turned out to be the psychrophilic bacteria, with counts ranging from 2.6 to 3.7 log (cfu/mL). In contrast, no fungi or yeast were detected. The total bacterial count in the produced non-dairy beverages reached 3.81 log (cfu/mL) in hemp milk and 4.39 cfu/mL in coconut milk. It was, therefore, concluded that the short-term temperature increase to 95 °C did not contribute to the complete neutralization of the microflora of the non-dairy beverages. Thus, it seems necessary to develop some other method that would be more effective in ensuring the appropriate microbiological purity of plant-based beverages. Lee et al. [59] evaluated the effect of increased hydrostatic pressure coupled with high temperature on counts of viable, spore-forming cells of pathogenic microorganisms. They reported a significant decrease in the number of active resting spores to a negligible level (below 1 cfu/mL) upon the coupled use of pressure of 207 MPa and temperature of 90 °C.

3.3. Evaluation of the Effect of Fermentation Process on Chemical and Microbiological Properties of Non-Dairy Beverages

Immediately after the addition of the inoculum from *Lactobacillus casei* subsp. *rhamnosus* lyophilizate, the total count of viable lactic acid bacteria (LAB) cells reached 11.72 log (cfu/mL) in coconut beverage and 8.41 log (cfu/mL) in hemp beverage. The fermentation process (37 °C/6 h) caused an increase in bacteria count in the samples to 13.26 and 10.92 log (cfu/mL), respectively (Figure 5). Initially, the difference in the count of viable LAB cells could be due to the viability of probiotics themselves in the food matrix, which may be affected by pH (initial pH values were at 6.12 for coconut milk and 6.79 for hemp milk), oxygen level, and presence of competing microorganisms (bacterial cells and their resting spores undamaged during pasteurization: 4.39 log (cfu/mL) in coconut milk and 3.81 log (cfu/mL) in hemp milk) [60–62]. Therefore, LABs resistance to inconvenient conditions appears to be an important technological trait, which enables selecting strains for untypical food matrix like, e.g., non-dairy beverages [63]. Initial differences in the count of probiotic bacterial cells in both

analyzed types of plant milks could be due to the fact that viable, metabolically active cells may rapidly lose their capability for growth, and this dormancy state of a part of the population may occur especially when the cells are exposed to unbeneficial factors. For explicit confirmation of these assumptions, fluorescent techniques should be employed that allow monitoring subtle changes in the dynamics of proliferation and decay of microorganisms that may be in the viable but non-culturable (VBNC) or active but non-culturable (ABNC) state, i.e., in the state of bacteria transition to the dormancy state under unfavorable colonization conditions [63–66].

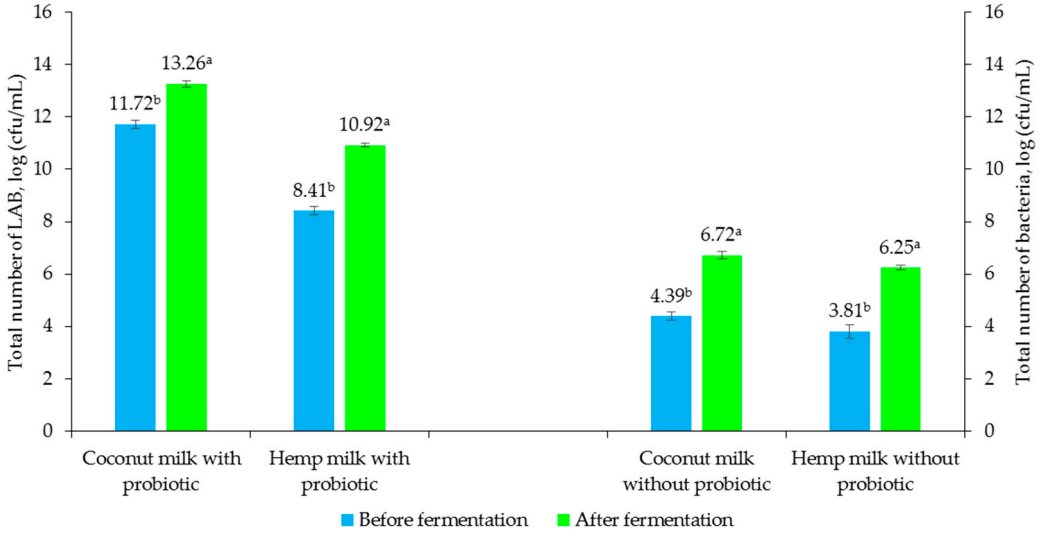

Figure 5. Total count of viable bacterial cells in the analyzed non-dairy beverages before and after fermentation (a, b—statistically significant differences, $p \leq 0.05$).

A similar dependency was demonstrated in the study conducted by Zaręba [67], who noted that 4-h fermentation process of soybean milk contributed to the proliferation of *Lactobacillus* species bacteria by approximately 0.5 log (cfu/mL). A study conducted by Bartkiene et al. [21] also showed that *L. casei* cell count in fermented hemp milk reached 8.78 log (cfu/mL) and was higher compared to the count determined before the fermentation process (8 log (cfu/mL)).

The analyzed models of non-dairy beverages differed in terms of the total bacteria count. TBC was also determined in the control samples (without the probiotic). Before fermentation it reached 4.39 log (cfu/mL) in coconut milk and 3.81 log (cfu/mL) in hemp milk; whereas after fermentation for the respective values were at 6.72 and 6.25 log (cfu/mL; Figure 5). Presumably, these were resting spores that had survived pasteurization and whose proliferation was promoted by fermentation temperature (37 °C) being optimal for their growth. These speculations may be confirmed by results reported earlier by Czaczyk et al. [68], who noticed the greatest growth of *Bacillus* ssp. bacilli under these conditions. Similar observations were made by Huy et al. [69]. The activity of microorganisms during incubation is also affected by the type and amount of nutrients available in the medium. However, considering the "Microbiological Limits for Assessment of Microbiological Quality of Ready-to-eat Foods" [70], the criterion related to the microbiological quality did not exceed the maximum value of 7 log (cfu/mL), set by the International Commission for Microbiological Specification of Food.

Monosaccharides, including mainly glucose, present in plant beverages represent a good source of carbon to bacteria. The physicochemical analysis conducted in the study allowed concluding that coconut milk (34.53 g glucose/L) was a better source of these compounds compared to hemp beverage (30.21 g glucose/L). The content of reducing sugars decreased significantly after the fermentation process (Figure 6).

The non-dairy beverages with probiotic addition were characterized by a greater reduction in glucose content after fermentation, i.e., to 23.05 g glucose/L in coconut milk and to 19.79 g glucose/L

in hemp milk. In turn, milks without the probiotic bacteria were characterized by noticeably higher glucose content, which decreased due to the activity of undesirable microorganisms.

Figure 7 presents results of measurements of active acidity of the non-dairy beverages before and after fermentation.

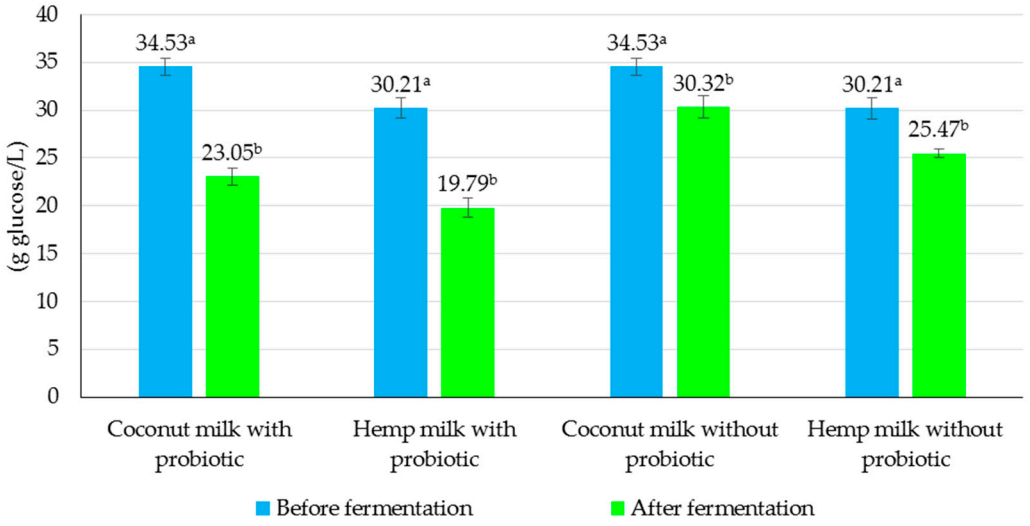

Figure 6. Reducing sugars concentration in the analyzed non-dairy beverages before and after fermentation (a, b—statistically significant differences, $p \leq 0.05$).

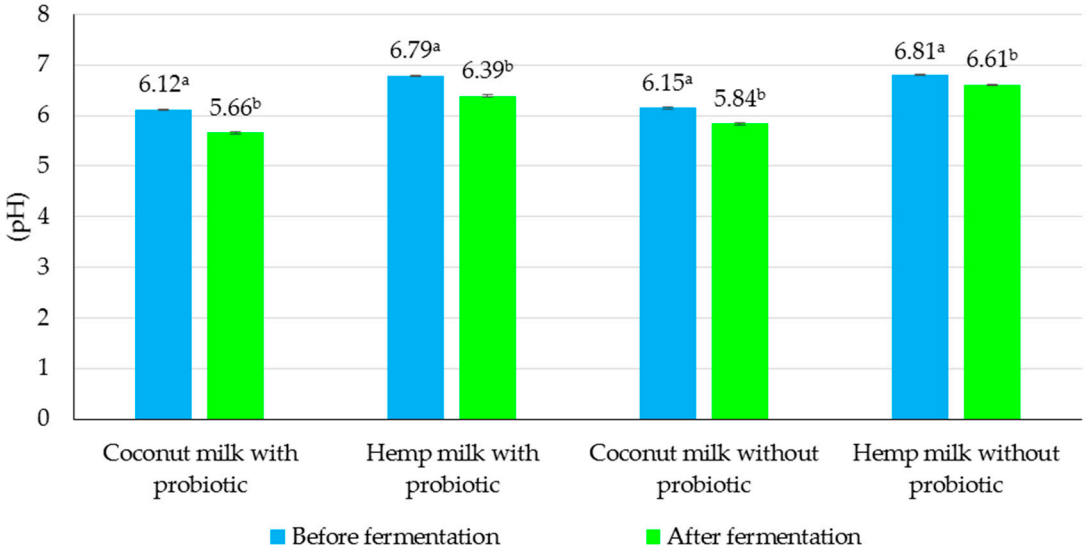

Figure 7. Active acidity of the analyzed non-dairy beverages before and after fermentation (a, b—statistically significant differences, $p \leq 0.05$).

The greatest differences in the active acidity, before and after fermentation, were demonstrated in the fermented probiotic beverage made of coconut, i.e., pH 6.12 and pH 5.66, respectively. In turn, the smallest decrease in the pH value (by 0.21) was noted in the fermented hemp seed milk without the probiotic. It may, therefore, be concluded that the acidity level is determined by the accumulation of organic acids caused by monosaccharides metabolism. So negligible pH changes may be due to the fact that *Lactobacillus casei* subsp. *rhamnosus* strain used in the study is a representative of facultatively heterofermentative bacteria. Apart from lactic acid, these bacteria are capable of producing CO_2, acetic acid (aerobic conditions), acetic aldehyde, and/or ethanol (anaerobic conditions) [71–73]. During the heterofermentation process, glucose degradation proceeds accordingly to the pentose

phosphate pathway, and the capability of lactic acid bacteria for this fermentation results from a lack of certain enzymes like, e.g., triphosphate isomerase and aldolase [74].

3.4. Assessment of the Quality of the Produced Non-Dairy Beverages During Cold Storage

Changes of the active acidity of the non-dairy beverages during 21-day cold storage were presented in Table 3. As expected, storage time had a significant effect on the acidity level of milk substitutes, causing vast differences in pH values of the fermented samples of coconut and hemp milks between day 1 and 21 of storage.

Table 3. Changes in the active acidity of fermented and non-fermented non-dairy beverages during 21-day storage.

Treatment	Storage Time			
	Day 1	Day 7	Day 14	Day 21
	Fermented			
CP	5.61 ± 0.02^{aF}	5.11 ± 0.02^{bG}	4.93 ± 0.02^{cB}	4.81 ± 0.02^{dB}
HP	6.47 ± 0.01^{aC}	6.08 ± 0.02^{bA}	5.91 ± 0.02^{cA}	5.78 ± 0.02^{dA}
C	5.84 ± 0.01^{aE}	4.85 ± 0.01^{bH}	4.08 ± 0.01^{cG}	3.58 ± 0.02^{dG}
H	6.62 ± 0.02^{aB}	5.49 ± 0.02^{bD}	4.39 ± 0.02^{cF}	3.41 ± 0.01^{dH}
	Non-Fermented			
CP	6.15 ± 0.02^{aD}	5.31 ± 0.02^{bE}	4.88 ± 0.02^{cC}	4.21 ± 0.01^{dC}
HP	6.81 ± 0.02^{aA}	5.87 ± 0.02^{bB}	4.65 ± 0.01^{cD}	3.95 ± 0.02^{dE}
C	6.15 ± 0.01^{aD}	5.25 ± 0.02^{bF}	4.53 ± 0.02^{cE}	4.01 ± 0.02^{dD}
H	6.81 ± 0.02^{aA}	5.71 ± 0.01^{bC}	4.35 ± 0.02^{cF}	3.70 ± 0.02^{dF}

CP—Coconut beverage with probiotic; HP—Hemp beverage with a probiotic; C—Coconut beverage without probiotic; H—Hemp beverage without probiotic. Means in the rows, followed by different small letters (a–d) are significantly different at $p < 0.05$ (effect of storage). Means in the columns, followed by different big letters (A–F) are significantly different at $p < 0.05$ (effect of treatment).

Usually, the active acidity (pH) of fermented plant-based beverages should not be lower than 4.0 throughout the storage period [75]. Results presented in Table 3 indicate that the pH value remained above 4.0 within fourteen days of cold storage of the analyzed milk substitutes. However, a pH decline was recorded at the end of the storage period in the case of the samples not inoculated with the probiotic monoculture. This is in agreement with results reported by Paseephol and Sherkat [76] and by Colakoglu and Gursoy [77]. Guo et al. [78] reported that the pH value of fermented buffalo milk containing *Lactobacillus casei* decreased from 5.02 (day 1) to 4.00 (day 30 of storage) [78]. In turn, Akalin et al. [79] demonstrated that the probiotic bacteria decreased the pH value of various yoghurts from 4.51 to 4.40 after 28 days of their cold storage [79].

Data obtained in the study indicate that pH values of all beverage samples decreased during cold storage. This dependency may be explained by the persistent metabolic activity of the probiotic monoculture, which was also noticed by Bonczar et al. [80] during cold storage of fermented beverages. When comparing pH values over the storage period, these researchers observed decrease in all samples. Similar conclusions were drawn by Bartkiene et al. [21], who analyzed hemp milk in a 15-day storage model. The pH value of the fermented hemp milk with the addition of a probiotic culture of *L. casei* decreased slightly from 5.15 in the first day to 4.77 in the last day of cold storage.

The study demonstrated also a decrease in protein content of the produced non-dairy beverages along with storage time (Table 4).

The analysis of the nutritional value of non-fermented milk substitutes demonstrated that, after 21 days of storage, the total protein content was higher in the non-dairy beverages supplemented with probiotic bacteria.

A similar tendency was noted during storage of fermented non-dairy beverages (i.e., decreased protein concentration). However, the conducted analyses showed a higher total protein content in the

fermented than in the non-fermented beverages, and lower in the non-dairy beverages fortified with the probiotic monoculture.

Table 4. Protein content of fermented and non-fermented non-dairy beverages during 21-day storage.

Treatment	Storage Time			
	Day 1	Day 7	Day 14	Day 21
	Fermented			
CP	3.02 ± 0.21 ^{aB}	2.95 ± 0.23 ^{aB}	2.84 ± 0.15 ^{aB}	2.67 ± 0.26 ^{aB}
HP	6.68 ± 0.26 ^{aA}	6.41 ± 0.28 ^{aA}	6.33 ± 0.21 ^{aA}	6.19 ± 0.24 ^{aA}
C	3.02 ± 0.19 ^{aB}	2.96 ± 0.17 ^{aB}	2.87 ± 0.18 ^{aB}	2.71 ± 0.18 ^{aB}
H	6.68 ± 0.29 ^{aA}	6.45 ± 0.25 ^{aA}	6.34 ± 0.23 ^{aA}	6.22 ± 0.23 ^{aA}
	Non-Fermented			
CP	3.23 ± 0.22 ^{aB}	3.17 ± 0.19 ^{aB}	3.10 ± 0.21 ^{aB}	3.01 ± 0.24 ^{aB}
HP	6.96 ± 0.27 ^{aA}	6.91 ± 0.25 ^{aA}	6.82 ± 0.23 ^{aA}	6.68 ± 0.26 ^{aA}
C	3.23 ± 0.18 ^{aB}	3.15 ± 0.20 ^{aB}	3.09 ± 0.20 ^{aB}	2.98 ± 0.18 ^{aB}
H	6.96 ± 0.27 ^{aA}	6.89 ± 0.22 ^{aA}	6.78 ± 0.21 ^{aA}	6.63 ± 0.25 ^{aA}

CP—Coconut beverage with probiotic; HP—Hemp beverage with a probiotic; C—Coconut beverage without probiotic; H—Hemp beverage without probiotic. Means in the rows, followed by different small letters (a) are significantly different at $p < 0.05$ (effect of storage). Means in the columns, followed by different big letters (A,B) are significantly different at $p < 0.05$ (effect of treatment).

According to Bernat et al. [81], the significant decrease in protein content is due to the fact that during the fermentation and storage of beverages, the bacterial starters could hydrolyze proteins to synthesize amino acids necessary for their nutrition. Investigations conducted by the aforementioned authors demonstrated also that fermented plant-based substitutes of milk had by approximately 17% lower content of β-glucan than their non-fermented counterparts, while this compound is capable of proteins crosslinking in food [82].

Changes observed in lipids content of the analyzed non-dairy beverages resembled these of protein (Table 5). In the entire period of cold storage, lipids concentration decreased negligibly in all milks. It may, thus, be concluded that the study demonstrated stability of this component in the produced and modified plant-based beverages.

Table 5. Lipids content in fermented and non-fermented non-dairy beverages during 21-day storage.

Treatment	Storage Time			
	Day 1	Day 7	Day 14	Day 21
	Fermented			
CP	21.06 ± 0.06 ^{aA}	20.98 ± 0.06 ^{abA}	20.87 ± 0.06 ^{bcA}	20.75 ± 0.06 ^{cA}
HP	18.01 ± 0.06 ^{aB}	17.95 ± 0.06 ^{abB}	17.81 ± 0.06 ^{bcB}	17.66 ± 0.06 ^{cB}
C	21.06 ± 0.07 ^{aA}	20.96 ± 0.07 ^{aA}	20.89 ± 0.07 ^{abA}	20.73 ± 0.06 ^{bA}
H	18.01 ± 0.06 ^{aB}	17.89 ± 0.05 ^{abB}	17.78 ± 0.06 ^{bB}	17.62 ± 0.06 ^{cB}
	Non-Fermented			
CP	21.08 ± 0.02 ^{aA}	21.01 ± 0.06 ^{aA}	20.94 ± 0.06 ^{aA}	20.84 ± 0.06 ^{bA}
HP	18.02 ± 0.02 ^{aB}	17.97 ± 0.06 ^{aB}	17.92 ± 0.06 ^{aB}	17.69 ± 0.06 ^{bB}
C	21.08 ± 0.02 ^{aA}	21.02 ± 0.06 ^{aA}	20.93 ± 0.06 ^{abA}	20.79 ± 0.06 ^{bA}
H	18.02 ± 0.02 ^{aB}	17.95 ± 0.06 ^{aB}	17.89 ± 0.06 ^{aB}	17.66 ± 0.06 ^{bB}

CP—Coconut beverage with probiotic; HP—Hemp beverage with a probiotic; C—Coconut beverage without probiotic; H—Hemp beverage without probiotic. Means in the rows, followed by different small letters (a–c) are significantly different at $p < 0.05$ (effect of storage). Means in the columns, followed by different big letters (A,B) are significantly different at $p < 0.05$ (effect of treatment).

As reported by Bernat et al. [82], who analyzed the microstructure of oat milk during storage, the similar concentration of lipids may be due to their embedding in a polysaccharide network. Stability of such system is additionally associated with the cross-linking properties of β-glucans [75]. Furthermore, almost all lipid droplets are retained in the polysaccharide-protein matrix, which is responsible for the physical stability of plant milk. It has been demonstrated that certain proteins may be attached to lipid globules, thereby ensuring protection of emulsions against destabilization processes [82].

Changes in concentrations of individual nutrients in plant-based beverages during cold storage may also be due to the fermentation process, which contributes to decreased content of carbohydrates and also of some non-digestible poly- and oligo-saccharides, to the improvement of protein quality, to the facilitated synthesis of selected amino acids, and to the improved availability of vitamins. In addition, it ensures optimal pH conditions for the enzymatic degradation of many compounds being important growth factors for the potentially probiotic bacteria [83].

The *Lactobacillus* strains used in the study are complex microorganisms that need carbohydrates, amino acids, B-group vitamins, nucleic acids, and minerals for their proper growth [84]. For this reason, it can be concluded that the fermentation of plant-based beverages may offer an inexpensive method for the synthesis of substrates in the product that would promote the growth of beneficial microorganisms [85].

3.5. Quantitative Analysis of Viable Bacterial Cells During Storage of Fermented and Non-Fermented Non-Dairy Beverages

An important aspect determining the quality of health-promoting fermented beverages is the analysis of changes in the number of viable lactic acid bacteria in 1 mL of a product over the entire period of its shelf life [86]. Al-Otaibi [87] emphasized that 6 log (cfu/mL) of viable probiotic cells should be consumed every day to ensure health benefits to consumers. Both types of the fermented non-dairy beverages met this requirement regarding viability of *L. casei* till the end of the storage period. On the first day of storage (after fermentation), the number of active bacterial LAB cells ranged from 10.92 log (cfu/mL) in hemp milk to 13.26 log (cfu/mL) in coconut milk (Table 6).

Table 6. Changes in the number of *Lactobacillus casei* subsp. *rhamnosus* during 21-day storage (4 °C) of fermented and non-fermented non-dairy beverages.

Treatment	Storage Time			
	Day 1	**Day 7**	**Day 14**	**Day 21**
	Fermented			
CP	13.26 ± 0.14 ^{aA}	11.46 ± 0.18 ^{bA}	11.26 ± 0.17 ^{bA}	9.41 ± 0.24 ^{cA}
HP	10.92 ± 0.10 ^{aC}	10.31 ± 0.25 ^{aB}	8.28 ± 0.33 ^{bB}	7.35 ± 0.26 ^{cB}
	Non-Fermented			
CP	11.72 ± 0.04 ^{aB}	6.81 ± 0.13 ^{bC}	5.42 ± 0.15 ^{cC}	3.12 ± 0.13 ^{dC}
HP	8.41 ± 0.18 ^{aD}	6.35 ± 0.16 ^{bC}	4.53 ± 0.08 ^{cD}	3.54 ± 0.20 ^{dC}

CP—Coconut beverage with probiotic; HP—Hemp beverage with a probiotic. Means in the rows, followed by different small letters (a–d) are significantly different at $p < 0.05$ (effect of storage). Means in the columns, followed by different big letters (A–D) are significantly different at $p < 0.05$ (effect of treatment).

Opposite observations were made in the case of non-fermented models of plant-based beverages. The number of LAB cells isolated from these samples on the first day of storage reached 11.72 log (cfu/mL) in coconut milk and 8.41 log (cfu/mL) in hemp milk. These values decreased to 3.12 log (cfu/mL) and 3.54 log (cfu/mL), respectively, after 21 days of cold storage (Table 6).

Bakirci and Kavaz [88] reported that the total counts of *Lactobacillus acidophilus*, *Bifidobacterium* ssp., and *Streptococcus thermophilus* decreased during cold storage of banana yoghurts, but remained at the required level (above 6 log (cfu/mL)) until day 14. In addition, a few other authors demonstrated that

lactic acid bacteria (*L. debrueckii* spp. *bulgaricus* and *S. thermophilus*) survived well in yoghurt throughout its shelf life [79,89]. Olson and Aryana [90] showed that the number of *Lactobacillus acidophilus* strain cells decreased in natural yoghurt from 6.84 to 4.43 log (cfu/mL) over an 8-week storage period. Results of own study pointed to a higher survivability of potentially probiotic bacteria, compared to that reported by Mousavi et al. [91] for a probiotic juice from pomegranate. These authors observed reductions in counts of *Lactobacillus delbrueckii* and *Lactobacillus plantarum* by three logarithmic cycles after 14 days of cold storage. High survival rates of *Lactobacillus casei* under cold storage conditions were also demonstrated by Pereira et al. [92], who investigated fermentation and survivability of this probiotic in a juice from cashew apple.

The reduced count of *L. casei* subsp. *rhamnosus* during cold storage may be due to the production by these microorganisms of agents exhibiting anti-microbial activity like e.g., organic acid, bacteriocins, and hydrogen peroxide [93]. The concentration of hydrogen peroxide has an important impact, because *L. casei* subsp. *rhamnosus* do not produce the catalase enzyme. Accumulation of metabolism products during storage of non-dairy beverages may lead to the transition of *L. casei* subsp. *rhamnosus* at VBNC (viable but non-culturable). At this stage, the cells have an "unsatisfactory" physiological state, which means that they are alive but do not divide, and as a result do not have the ability to grow and reproduction [63]. Al-Otaibi [87] noticed that the number of bifidobacteria in eight commercial fermented dairy products decreased significantly since the day of manufacture till the end of cold storage (5 °C). This author reported also that the count of viable bacteria maintained at 10^6 cfu/mL till the end of the storage period in only two of the analyzed products. Reduction in the number of viable health-promoting bacteria can also be caused by decreased acidity, presence of post-production acid [94] sensitivity to oxygen [95] and metabolites, i.e., hydrogen peroxide and ethanol, and also to bacteriocins produced by lactic acid bacteria [96].

The determination of the number of active *L. casei* cells in the analyzed non-dairy beverages is difficult due to the presence of other microorganisms [97]. For this reason, simultaneous analyses were carried out for control samples (without probiotic). In their case, the total bacterial count was observed to decrease. In contrast, interesting seem to be changes in the viability of microorganisms (other than LABs) in the non-fermented control beverages, in which acidity approximating the neutral pH contributed to the development of undesirable microorganisms. However, the total bacterial count in the beverages not fortified with the probiotic monoculture, fitted within the range from 3.82 log (cfu/mL) to 3.93 log (cfu/mL) in fermented non-dairy beverages and from 5.92 to 6.67 log (cfu/mL) the non-fermented ones (Table 7). Thus, the criterion related to the microbiological quality did not exceed the maximum value of 7 log (cfu/mL).

Table 7. Changes in the total number of bacteria during 21-day storage (4 °C) of fermented and non-fermented non-dairy beverages.

Treatment	Storage Time			
	Day 1	Day 7	Day 14	Day 21
	Fermented			
C	6.72 ± 0.17^{aA}	6.23 ± 0.30^{aA}	5.54 ± 0.24^{bA}	3.82 ± 0.18^{cB}
H	6.25 ± 0.13^{aB}	5.69 ± 0.29^{aA}	4.37 ± 0.20^{bB}	3.93 ± 0.27^{bB}
	Non-Fermented			
C	4.39 ± 0.17^{bC}	4.41 ± 0.15^{bB}	4.79 ± 0.19^{bB}	5.67 ± 0.18^{aA}
H	3.81 ± 0.14^{bD}	3.86 ± 0.08^{bB}	5.76 ± 0.18^{aA}	5.92 ± 0.16^{aA}

C—Coconut beverage without probiotic; H—Hemp beverage without probiotic. Means in the rows, followed by different small letters (a–c) are significantly different at $p < 0.05$ (effect of storage). Means in the columns, followed by different big letters (A–D) are significantly different at $p < 0.05$ (effect of treatment).

Results of the present study indicate that cell viability was maintained at a satisfactory level throughout the storage period of fermented non-dairy beverages fortified with the probiotic

monoculture. According to Pereira et al. [98], sugars, proteins, and lipids are only some of the factors that may affect the growth of probiotic bacteria and their survival rates in food products. Hence, the relative stability observed in content of nutrients in the plant-based beverages contributed indirectly to ensuring the therapeutic minimum of the analyzed products throughout their storage period.

4. Conclusions

The conducted study proved that the produced and modified plant-based beverages could serve as a food matrix for probiotic bacteria. The growth and survivability of probiotic bacteria in food products was determined by many factors including e.g., storage conditions, medium acidity, and sensitivity of oxygen and metabolites. The fermentation process contributed to the increased survival rates of *Lactobacillus casei* subsp. *rhamnosus* in both coconut and hemp milk. During 21-day storage of inoculated milk substitutes, the highest survivability of *Lactobacillus casei* subsp. *rhamnosus* was demonstrated in the fermented coconut milk (9.41 log (cfu/mL)). On day 21 of cold storage, the number of viable *Lactobacillus casei* subsp. *rhamnosus* cells in fermented coconut and hemp milk ensured meeting the therapeutic minimum (>6 log (cfu/mL)). Due to their nutrients composition and number of bacterial cells exhibiting a positive effect on a human body, the analyzed non-dairy beverages, offering an alternative to milk, represent a category of novel food products, and their manufacture will contribute to the sustainable development of food production and to the assurance of food safety.

Author Contributions: A.S. and S.K. conceived and designed the research. A.S., S.K., B.P. and E.C. performed the experiments. A.S., S.T., S.K., E.C., M.K., and B.P. prepared the materials. A.S., S.T., S.K., M.K. and P.F. analyzed the data. A.S., E.C. and S.K. wrote the paper. A.S., E.C., S.T., S.K., M.K. and P.F. revised the manuscript. All authors read and approved the final manuscript.

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