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Potential Probiotic Tiger Nut-Cashew Nut-Milk Production by Fermentation with Two Lactic Bacteria Isolated from Ivorian Staple Foods

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Abstract

Probiotification of plant milk can improve its sensory and health-promoting properties. As traditional fermented foods where lactic acid bacteria (LAB) are present have been associated with beneficial effects on human health, the beneficial effects of two LAB recently isolated from two current Ivorian staple foods (a pepper and a traditional beer) were screened. These two strains LAC 1 (*Lactobacillus plantarum*) and LAC 2 (*Pediococcus acidilactici*) which presented probiotic, exopolysaccharides, inflammatory and anti-oxidant activities, were used to ferment a composite plant milk of tiger-nut and cashew (80/20) compared to two starters of a commercial yogourt. The obtained plant milks SCT 2 and SCT 3 with a significant increase in their antioxidant and/or anti-inflammatory activities and lactic bacteria contents were more preferred by consumers than SCT 1 obtained by fermentation of the commercial yogourt starters. The mixing of LAC 1 and LAC 2 was not beneficial. SCT 2 (with an anti-inflammatory activity of 31.38% and an anti-oxidant activity of 17.30%) and SCT 3 (with an anti-oxidant activity of 22.28) could be further tested in animal models to verify their nutrition-health claims.

Keywords

Probiotic, Staple Food, Lactic Acid, Anti-Oxidant, Anti-Inflammatory

1. Introduction

Probiotics which are intestinal microorganisms regarded as a metabolic "organ" due to their beneficial effects on human health are now clinically exploited to prevent and treat many diseases (diarrhoea, colon cancer, hypertension, diabetes, acute pancreatitis, *Helicobacter pylori* infection, ventilator-associated pneumonia, migraine and autism…). In contrast to antibiotics which do not differentiate between good and bad microflora, probiotics selectively modify immunological response (innate and adaptive) wich altering microbial habitat in the intestine, gut barrier function, competitive adherence to the mucosa and epithelium, and antimicrobial potentiality of intestinal flora against intestinal bad germs [1]. Indeed, as with other diseases, over the past twenty years, scientific discoveries have shown that metabolic diseases are linked to a decrease in beneficial bacteria in the intestinal flora [1]. This dysbiotic microbiota leads to hyperpermeability of the intestinal barrier, an inflammatory state and oxidative stress is associated with various disorders [2]. Thus, the use of probiotics in synergy with current conventional treatments is therefore more considered. Regular consumption of probiotics through yogourts, kefir, cheeses, etc., could help improve intestinal health and reduce metabolic diseases, unlike foods that are too fatty, sweet, salty. Certain lactic acid bacteria such as *Lactobacillus*, *Bifidobacterium* and *Streptococcus* widely used as probiotics also synthesize highly sought-after antioxidant and/or anti-inflammatory molecules [3]. For the selection of potential probiotic micoorganism, decisive important criteria are resistance to gastrointestinal passage (e.g., resistance to acid pH and bile salts), adhesion to the gut, and a beneficial effect on host health. Among the beneficial properties attributed to probiotic strains, their anti-inflammatory and anti-oxidant effects are increasingly listed. Currently, there is a great interest in the isolation of new probiotic LAB strains from unconventional sources, such as fermented staple foods [4] [5]. While vegetables including red pepper are traditionally fermented in Côte d'Ivoire for the preparation of culinary broths, Tchapalo is an Ivorian fermented millet or sorghum beer which also consumed in all West-Africain countries. These traditional foods, which are neglected but widely consumed by modest backgrounds, are increasingly the subject of scientific research in order to improve and standardize their quality. In this sense, potential probiotic LAB with antioxidant and anti-inflammatory aptitudes have been recent, isolated from these two traditional foods by our research team. Their wide use in the food industry will be an asset for their industrial development. Plant milk is often presented as a healthy, sustainable and animal welfare-friendly alternative [6], which offers many development opportunities in Africa where many raw materials for its production exist. While innovation such supplementation of functional ingredients is at the center of the design of plant milks in the West, their sophistication by expensive ingredients should however limit their purchase cost, their accessibility by the less disadvantaged strata and their sustainable development contrary to the effects of the nutritional transition against which these products are called upon to fight. Developing a new product by mixing two or more types of fruit or vegetables in regard to their functional properties can lead to achieving an excellent product as well as economical process and the market popularity. Resembling slightly soft berries, with a mild sweet, milky taste with a hazelnut flavor, Tiger nut (*Cyperus esculentus*) milk has been recognized as a functional food which can prevent the risk of several metabolic diseases (cardiovascular diseases, gastrointestinal disorders, obesity, diabetes, cancers, …). It has been reported that tiger nut tuber consists of starch (29.90%), lipids (24.49%), carbohydrates (43.30%), protein (5.04%), and ash (1.70%) [7]. Tiger nut tuber is known a rich source of high-quality oil and some bioactive ingredients such minerals (calcium, phosphorus, potassium, …), sterols, flavonoids, vitamins, (C and E), … [7] [8] [9]. Just like other dried fruits, cashews (Anacardium occidental) nuts are healthy and riches in minerals and nutrients such as phosphorus, copper, and magnesium, micronutrients that are rare in other foods [6]. Cashew nuts and pistachios are the nuts with the lowest lipid content with almost 80% unsaturation, maintaining healthy cholesterol levels. They are also rich in tocopherols and phytosterols, and contain active ingredients that give it health benefits [10]. In fact, several studies link regular consumption of tree nuts to various health benefits (lower blood cholesterol, reduced risks of cardiovascular disease and type 2 diabetes, gallstones and gallbladder removal, colon cancer in women, respectively). Cashew nut milk, is low in calories and sugar, and has tryptophan (essential amino acid) precursor of well-being hormones [10] [11] [12]. Although they are abundant and available locally and despite their functional potential, these two fruits are not sufficiently valued. Thus, this work leads to the formulation of a functional fermented plant milk of tiger nut/cashew nut of good nutritional quality, accessible and available everywhere.

2. Materials and Methods

2.1. Microbial Strains Used in This Study

Two LAB of our Laboratory microbial collection LAC 1 isolated from the traditional sorghum fermented beer "tchapalo" and identified as *Lactobacillus plantarum* and LAC 2 isolated from fermented piper and identified as *Pediococcus acidilactici* by their ARN 16S sequencing (article submitted accepted for publication) were selected as starters comparatively to two LAB starters of an international commercial yoghourt sold in Côte d'Ivoire.

2.2. Screening of Technological Properties of LAC 1 and LAC 2

For probiotic properties, the acidity resistance (for 3 hours in MRS broth adjusted to pH 2 using hydrochloric acid according to Hyronimus *et al.* (2000) [13], bile salt resistances (in MRS broth supplemented with 0.3% of a mixture of bile salts (Oxgall Powder B-3883 Sigma)) and Cell surface hydrophobicity aptitude) of the strains were screened. Diluted pellet OD_{600nm} = 0.5 (after centrifugation at 8000 rpm for 15 min and washed twice with PBS (pH 7.4)) of 18 hours cultures (in MRS broth of each strain) were used. After acidity and bile salt resistance tests, successive dilutions of tested microbial suspension were done to inoculate MRS agar which was then incubated at 37˚C for 48 and the survival rate was evaluated comparatively to their controls. Adhesion of each strain to xylene (as hydrophobic substance) was used to screen their hydrophobicity aptitude [14]. One milliliter of each pellet (OD_{600nm} = 0.5) was resuspended in PBS and the OD_{600nm} value (A_0) was determined. An egual proportion of xylene was again added and the mixture was then vortexed for 5 minutes, incubated at 37˚C for 1 hour. The OD_{600nm} of organic matter (A_1) was read. The Bacterial Adhesion to Solvents (BATS) was calculated as follows:

$$BATH\% = [(A_0 - A_1)/A_0] \times 100.$$

Exopolysaccharides (EPS) synthesis by each strain was screened on MRS agar containing 40 g/L sucrose addition [15]. The 18 h bacterial pellet was inoculated on the Petri dishes by streaking and incubated at 37˚C for 48 h. EPS producers strains showed a slimy appearance with large, sticky colonies [15]. Antimicrobial activity of each strain against pathogens (*Staphylococcus*, *Pseudomonas*, *Escherichia coli*, *Salmonella*, *Aspergillus*) was aseptically done by the well (6 mm wells done by a Pasteur pipette) diffusion method [16] on MRS agar for 18 h at 37˚C. After solidification of the MRS agar containing the pathogens, each well was filled with 10 μL of every tested strains ((OD_{600nm} = 0.5) and was refrigerated at 4˚C for 2 hours to allow a good diffusion of the substance. Inhibition zones diameters were measured at 37˚C after 24 h of incubation. Anti-inflammatory aptitude of each strain was done by its culture supernatant against bovine serum albumin (BSA) as protein denaturation inhibition method [17]. The reaction mixture (0.5 mL) consisted of 0.45 mL of BSA (5% aqueous solution) and 0.05 mL of each strain culture supernatant. The pH was adjusted to 6.3 using hydrochloric acid (1 N and samples were incubated at 37˚C for 20 min, then heated to 57˚C for 3 min. After cooling, 2.5 mL of phosphate buffer solution (pH = 6.3) was added. Absorbance was measured at 614 nm. For the control, distilled water replaced the microbial supernatant. Inhibition of protein denaturation (%) was calculated as follows:

$$I(\%) = 100 - [(\text{OD Sample} - \text{OD Control}/\text{OD Control})] \times 100$$

Antioxidant activity of every strain was obtained by the DPPH (2,2 diphenyl-1-picryl hydrazyl (DPPH•) scavenging test. One (1) mL of 48 h bacterial culture supernatants was added in 2 mL of ethanol (70%) and centrifuged (6000 rpm for 10 min) and the supernatant collected. 50 μL was added to 1950 μL of DPPH (0.6 mg/mL). The negative control was prepared by mixing 50 μL of 70% ethanol with 1950 μL of DPPH at the same concentration. After incubation in the dark for 30 min, OD_{517nm} was read and the percentage of DPPH decolorization was determined by the formula below:

$$I(\%) = ((\text{OD control} - \text{OD sample})/\text{OD control}) \times 100$$

For the fermentative type of every strain, the presence or absence of gas in the Durham bell after 48 h of incubation at 37˚C in MRS broth indicates the heterofermentative or homofermentative metabolism. Sugars fermentation profile of each strain was studied by replaced dextrose by the tested sugar in MRS agar supplemented with bromocresol purple at 0.04% (pH indicator) at 37˚C for 48 h. Change from purple to yellow of the pH indicator due to the acidification of the medium reflects the fermentation of the tested sugar.

The enzymatic profile of every strain was studied on Modified MRS agars containing 2% of a sole carbon source (soluble starch, cellulose, pectin, tannic and phytic acids) which were sterilized, cooled at 50˚C and poured into Petri dishes. After solidification, they were inoculated per well by the microbial colonies, and then incubated at 30˚C for 48 h. According to the tested substrate, enzymatic synthesis was revealed by the appearance of a clear halo around the wells.

2.3. Production of Fermented Vegetable Milk of Tiger/Cashew

After buying at Gouro Market (Abidjan), tiger nuts and cashew nuts were separately used to extraction of their milks according to Udeozor (2012) [18] and Adedokun *et al*. (2013) [19], respectively. For tiger milk obtention, one kilogram of dried tiger nuts was sorted, manually cleaned to remove undesirables, soaked in tap water at a ratio (1:3) for 48 h, then washed. The nuts were then crushed (using a blender) with 3 L of tap water and the obtained slurry was pressed with a muslin cloth to extract the milk. For cashew milk obtention, 450 g of cashew nuts were cleaned to remove undesirable and soaked in 250 mL of drinking water for 6 hours. After, the water was drained and the soaked nuts were crushed (using a blender) by mixing with tap water at a ratio of 1:6. The slurry was pressed with a muslin cloth to extract the milk. The tiger and cashew milks were then mixed at a ratio of 80/20, homogenized to obtain the composite milk in sterile jars for pasteurization (82˚C for 10 min) before the controlled fermentation at room temperature by inoculation with 10^7 CFU/g of viable colonies of the starters.

2.4. Physico-Chemical Characterization of the Plant Milks

pH determination was done with 10 mL of every milk [20]. For titratable acidity, 10 mL of each sample was titrated using a sodium hydroxide solution (NaOH, 0.1 N) with two drops of phenolphthalein. Dry matter and ashes content were evaluated by AOAC (1990) [21] method. The fat content was determined with 10 mL of every sample by the Soxhlet extraction method with 300 mL of hexane according to AFNOR (1982) [22] for 7 hours. After, the solvent was recovered using a HEIDOLPH brand rotary evaporator. The flask (initially tared) containing the fat was weighed to determine the mass of the extracted fat. The reduced sugars content of the milks was obtained with the DNS Bernfeld method (1955) [23] using a dextrose (1 mg/mL) standard curve at 540 nm. Proteins content was determined by the colorimetric assay of Bradford (1976) [24] at 595 nm with a standard curve from bovine serum albumin solution. Antioxidant (on 1 mL sample) and anti-inflammatory activities (on 0.05 mf sample) of the milks were obtained by the DPPH (2,2-diphenyl-1-picryhydrazyl) and the bovine serum albumin protein denaturation inhibition method according to Huang *et al*. (2005) [25] and Anoop *et al*. (2015) [17], respectively.

2.5. Microbiological Analysis of the Plant Milks

Stock solutions (10^{-1} dilution) were aseptically prepared by 10 g of milk which were homogenized with 90 mL of buffered peptone water. Then, after 10^{-2} dilution (one (1) mL of each stock sterilely mixed with 9 mL Tryptone-salt (Bio-Rad) à, several successive decimal dilutions were done to 10^{-10} dilution. Enumeration of Total Mesophilic Aerobic Flora Count was performed by ISO 4833-1 (2013) [26] method on Plate Count and, total coliforms count was obtained on lactose bile agar with crystal violet and neutral red (VRBL) according to ISO 4832 (2006) [27]. Lactic acid bacteria were enumerated on MRS agar (de Man, Rogosa, Sharpe) according to ISO 15214 (1998) [28]. *Bacillus* sp. count were done on nutrient agar. The inoculation consisted of spreading 0.1 mL of the stock solution and its decimal dilutions on the nutrient agar pre-poured in the Petri dishes after solidification. After 24 h incubation at 30˚C, typical colonies (large, smooth or rough) were counted as *Bacillus* species. Yeasts enumeration was carried out on Sabouraud chloramphenicol agar according to ISO 21527-1 (2008) [29].

2.6. Sensory Analysis of Milks

Hedonic test based on the "pleasure" dimension and the personal feelings of the panelists was carried out according to the ISO 8587 (2006) [30] standard. A tasting, followed by an evaluation of the milk were done by 50 untrained or naive panelists. These panelists were selected to correspond to the product's target and were placed in close conditions. Evaluation of the milk samples was done by assigning them marks on a structured rating scale of 5 levels (from very unpleasant = 1 to very pleasant = 5), expressing the general impression of their preference. Finally, the samples were ranked according to their preference (from the most appreciated to the least appreciated). Descriptive analysis of milks were done by ISO 13299 (2016) [31] standard with 10 panelists which were trained on the basis of the descriptors (sour taste, sweet taste, smell, aroma, shine and color of milk) in order to familiarize themselves with the use of a rating scale, to assess the intensities of these sensory attributes. The intensities of the attributes, on the structured intensity scale of 1 to 9 points, were evaluated by the panel.

2.7. Statistical Analyzes

The tests were carried out in triplicate. The results were expressed as mean ± standard deviation. A one-factor analysis of variance (ANOVA) of the means obtained was carried out using the XLSTAT 2014 software and Duncan's test at the probability threshold ($\alpha = 0.05$) was used.

3. Results and Discussion

In front of the high prevalence of chronic diseases (diabetes, obesity, high blood pressure, cancer, etc.) in the world, the world market for "healthy" plant-based foods, including plant milks, is constantly increasing. However, plant milks are of lower relative quality compared to cow's milk so that their costly dietary adjustments to fully meet nutritional needs are often operated [32]. Although, supplementation with pure functional ingredient such inulin is often operated, mixing various plant milks and/or plants to optimize their nutritional quality is a simple solution [33]. Thus, following the recommendations in favor of the consumption of plants-based-foods (source of prebiotics favorable to the intestinal flora) of the FAO/WHO and in regard to various interesting probiotic benefits [34], five composite plant milks of tigernut enriched in cashew milk have been developed. Tiger nut contributes in Côte d'Ivoire modestly to the income of traders who engage in its marketing, while cashew is essentially exported without any form of processing. For this, the juice extracted from tiger nuts was supplemented with the juice extracted from cashew nuts in a ratio of 80/20. Comparatively to the two strains of an international commercial yogourt which are certainly *Lactobacillus bulgaricus* (YB) and *Streptococcus thermophilus* (YC), these plant milks have been fermented by two LAB identified as *Lactobacillus plantarum* for LAC 1 and *Pediococcus acidilactici* for LAC 2 with probiotic, anti-oxidant, anti-inflammatory and exopolysaccharides (new source of cancer treatment) activities, which were isolated from two local staple foods (a piper and a sorghum beer) (**Table 1**). Generally, the first criteria for a probiotic microorganism are acid and bile salt resistances to ensure their survival through the stomach and intestine, respectively. The resistance to this drastic living condition with high survival rates (more than 50%) suggests that the two selected bacteria LAC 1 and LAC 2 were able to cross stomach acidity and reach the intestinal environment to multiply there. The denaturation of tissue proteins is a well-documented cause of inflammation which may be at the origin of the production of autoantigens in certain diseases (arthritic, rheumatoid, …). Moreover, the ability to trap the free radical DPPH reflects the presence of antioxidant activity. So, the two strains significantly presented high anti-inflammatory (51.75% ± 13.30% for LAC 1 and 47.95% ± 9.50% for LAC 2) and anti-oxidant activities (14.51% for LAC 2 and 30.65% for LAC 1), respectively. Probiotic *Limosilactobacillus reuteri FEEL* 6901 with the abilty to synthetyse anti-inflammatory substances was considered by its intact cells to have an excellent antioxidant activity with 21.6% DPPH inhibition rate (higher than that of WCFS 1 (10.6%) or LGG (17.1%)) [4]. Also, the two strains presented exopolysaccharides synthesis

Table 1. Morphological and healthier activities of the starters used in this work.

Strains	Survival (%) at pH 2	Survival (%) at 0.3% Bile salts	Hydrophobicity (%)	Exopolysaccharide Synthesis	Anti-oxidant activity (%)	Anti-inflammatory activity (%)
LAC 2	51.74 ± 1.08ab	72.25 ± 0.81a	78.64 ± 0.06a	+	14.51ab	41.95 ± 2.48ab
LAC 1	52.27 ± 2.07ab	84.67 ± 0.19ab	70.92 ± 0.01a	+	30.65bc	51.75 ± 3.22a
YB	7.57 ± 0.15a	76.34 ± 0.11a	66.56 ± 0.01a	+	9.62a	0
YC	5.52 ± 0.51a	68.43 ± 0.17a	71.63 ± 0.07a	+	7.31a	0

ability by their sticky and shiny colonies forms on MRS sucrose (40 g/L) agar (**Figure 1**). Comparatively to YB and YC without anti-inflammatory activity on MRS, these characteristics led to use LAC 1 and LAC 2 as probiotic starters.

Also, the antibacterial activity of a probiotic is essential against intestinal pathogenic bacteria. In this sense, LAC 1 and LAC 2 inhibited most of the indicator pathogen strains with good inhibition against *Salmonella* (19.25 - 19.5 mm inhibition diameter) while *Staphylococcus* exhibited the weakest inhibition (13.25 mm inhibition diameter) (**Table 2**). These inhibitions could have several

Table 2. Characteristics of the two starters to environmental conditions.

Environmental conditions	LAC 1	LAC 2
Pathogens (Inhibition diameter)		
Staphyloccocus	13.25 ± 0.35a	13.50 ± 2.12a
Pseudomonas	16.25 ± 0.35a	16.25 ± 1.76a
E. coli	15.25 ± 0.35a	16.50 ± 2.12a
Salmonella	19.25 ± 8.13a	19.50 ± 10.60a
Aspergillus	0.00	19.00 ± 2.12a
Sugar (20 g/L) fermentation		
Tréhalose	+	+
Erythritol	–	–
Galactose	+	+
Saccaharose	+	+
Raffinose	+	+
Glucose	+	+
Maltose	+	+
Fructose	+	+
Sorbitol	–	–
Lactose	+	+
Soluble starch	–	–
Arabitol	–	–
Xylitol	–	–
Synthetysed enzymes		
Tannase	–	+
Amylase	+	+
Phytase	+	–
Cellulase	+	+
Produced titratable acidity on MRS	1.44 ± 0.00a	1.40 ± 0.19a

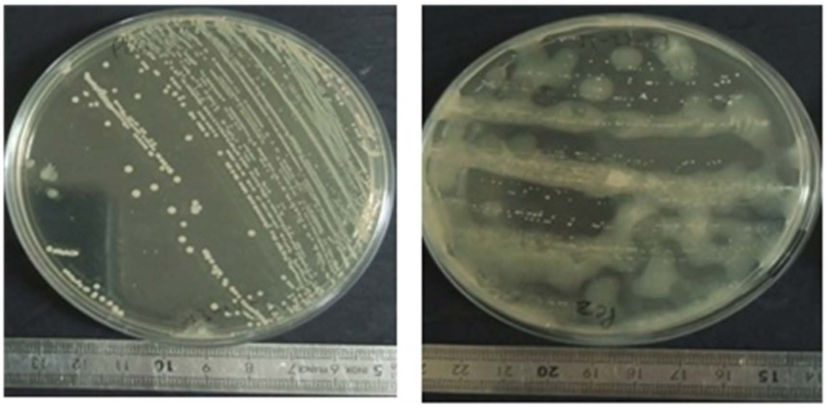

Figure 1. Appearance of Lac 1 colonies on MRS (left) and (on the right) on MRS-sucrose (40 g·L^{-1}) agar with exopolysaccharides synthesis (sticky and shiny colonies forms).

origins among which the production of antimicrobial compounds such as organic acids (lactic acid or acetic acid). They ferment several sugars including raffinose (an indigestible sugar) into lactic acid to prevent indigestion and stomach bloating. Indeed, in humans, these sugars are metabolized by microorganisms in the large intestine, liberating huge amounts of gas, which can then cause gastrointestinal disorders. Also, their acidification ability after 48 hours would lead to decrease their environment pH in order to extend the lag phase of sensitive organisms including foodborne pathogens. In addition to these characteristics, they also synthetize interesting hydrolytic enzymes which are necessary for fermentation. Cellulase and amylase contribute to the softening of foods while tannase and phytase synthesis is useful to eliminate anti-nutritional factors during the fermentation process. In regard to such interesting technological properties, these two isolates were used in further steps to ferment the plant milk of tiger nuts/cashew nuts.

Physico-chemical Characterization of the five milks showed a slight drop in pH of the fermented milks between 3.8 (naturally fermented milk control) to 3.25 (fermented milk SCT 1 with the two strains of commercial yogurt) against 4.00 (for the unfermented milk) and an increase in the titratable acidity from 9.44% (naturally fermented control) to 12.25% (SCT 1) (**Table 3**). The fermentation medium composed of yellow tiger nuts and cashew nut milks (80/20) is a hostile environment where any ferment cannot develop without the ability to tolerate and adapt to a pH ≤ 4. This could indicate that the presence of these starters in these plant milks during fermentation could give them a protective effect on their survival. Despite no significant difference, the pH in the three plant milks fermented by LAC 1 and LAC 2 and their combination drop to 3.45 - 3.50. In regard to these low pH values, the bacteria were clearly able to tolerate acidic environments and grow on these media. The pH essentially did not change, further enhancing food safety during tiger-nut fermentation. These low pH values and their variations are similar to those of other work concerning controlled fermentation of the yellow tiger nut milk and cashew milk and

Table 3. Physico-chemical parameters of the plant milks.

Plant milks	pH	TA (%)	Fat (%)	Ash (%)	Reducing sugar	DM (%)	% of inhibition	Trapping activity (%)	Protein (%)
Control	3.80 ± 0.10b	9.44 ± 0.26a	35.00 ± 0.00b	2.25 ± 0.35a	2.45 ± 0.83a	10.81 ± 2.65a	0	4.28a	5.23 ± 0.03a
SCT 1	3.25 ± 0.15a	12.25 ± 0.21a	25.00 ± 9.19a	3.50 ± 2.12a	2.34 ± 0.59a	11.8 ± 1.76a	52.77b	16.24b	10.18 ± 0.23a
SCT 2	3.45 ± 015a	11.39 ± 0.18a	34.25 ± 0.35b	2.50 ± 0.00a	3.01 ± 0.15a	11.83 ± 1.26a	31.38a	17.30b	9.26 ± 0.33a
SCT 3	3.50 ± 0.10a	10.15 ± 0.25a	33.75 ± 0.35b	2.75 ± 2.96a	3.13 ± 1.00a	9.38 ± 6.35a	0	22.28bc	7.24 ± 0.008a
SCT 4	3.45 ± 0.05a	11.29 ± 0.25a	34.00 ± 0.70b	2.00 ± 1.41a	2.61 ± 0.16a	8.32 ± 0.66a	0	18.83b	6.14 ± 0.25a

SCT 1: Plant milk fermented with isolates YC and YB from international commercial yoghurt; SCT 2: Plant milk fermented with LAC 1 isolated from tchapalo; SCT 3: Plant milk fermented with the LAC 2 isolated from pepper; SCT 4: Plant milk fermented by LAC 1 and LAC 2, Control: uninoculated milk; TA: titratable acidity; DM: dry matter; % inhibition: anti-inflammatory activity; Trapping activity (%): antioxidant activity. NB: In a column, the average values followed by an alphabetical letter differ are statistically different with a threshold $\alpha = 0.05$.

confirm the capacity for growth and fermentation of LAC 1 and LAC 2. Osho and Sobande (2019) [35] reported a drop of pH from 4.3 to 3.7 during 18 hours of controlled lacto-fermentation of yellow tiger nut mil and Maduka *et al.* (2017) [9] reported a titratable acidity of 0.09% - 0.79%, 0.09% - 0.73%, 0.10% - 0.79% and 0.09% - 0.16% in tigernut drink between 0 - 72 hr of lactofermentation. Otherwise, Mattison *et al.* (2020) [11] reported that commercial cashew milk-based yogurt showed a pH and titratable acidity of 4.46% and 0.53% respectively. These low pH-variations are followed by a significant synthesis of titratable acidity. Among the three plant milks fermented by LAC 1 and LAC 2, the increase in titratable acidity was greater in SCT 2. These activities could be due to the synthesis of organic acids, mainly lactic and acetic acids. LAB is able to dominate other bacteria involved in natural fermentation of tigernut milk drink. They possess adhesional adaptation with ability to survive in diverse food matrices. Dry matter of the five plant milks oscillated between 8.32% ± 0.66% (fermented milk SCT 4 with the two strains of LAC 1 and LAC 2) and 11.83% ± 1.26% (fermented milk SCT 2 with the strain isolated from tchapalo) when their reducing sugar content oscillated between 2.34% ± 0.59% (SCT 1) and 3.13 ± 1.00 (fermented milk SCT 3 with the strain isolated from pepper). Several researchers have confirmed the presence of high sugar content in tiger nuts [9]. The dry matter contents are similar in the two fermented milks SCT 1 and SC 2 while the lowest value is in the milks SCT 4. The ashes of the five plant milks varied from

2.00% to 3.50% with the highest value for the SCT 1 sample (fermented plant milk with the two yogurt strains) and the lowest value for the SCT 4 sample (fermented plant milk with LAC 1 and LAC 2). Their fat content (25.00% - 35.00%) with the low value in the SCT 1 sample were higher compared to the values (3.12%, 2.04%, 2.28% and 3.80%) of cashew, bambara nut, soy milk cow's milk milks respectively [36]. The Codex Alimentarius standard recommended a minimum level (3%) of fat. The protein contents of the milk samples varied between 5.23% (naturally fermented control) and 10.18% with the highest value for the SCT 1 sample (fermented with the commercial yogurt starters). Regarding the functional activities of the five milks, while the naturally fermented control milk (and the unfermented milk) has very low functional properties (without anti-inflammatory and antioxidant activities of 4.85%), the others four milks (SCT 1, SCT 2, SCT 3 and SCT 4) obtained by controlled fermentation, varied from a milk to another depending on the starter used. Concerning the anti-oxidants, the strain LAC 2 (*Pediococcus acidilactici*) isolated from pepper (in single fermentation) gave in SCT 3 the highest activities (22.28%) which was reduced to 18.83% (in SCT 4) with its association with LAC 1 (*Lactobacillus plantarum* isolated from tchapalo) who alone gives in SCT 2 an activity of 17.30% against the mix of yoghurt strains (16.24% in SCT 1). So, the capacity to scavenge the DPPH radical is strain-dependent. It would seem that the synthesis of antioxidant activities in the milks is correlated to the performance of the starter. The values reported here are in agreement with the scavenging capacity and the anti-oxidant potentialities of fruit juices, beverages and hot beverages consumed in Egypt [37] and those reported [38]. The antioxidant effect is linked to the development of reductones which have also been reported as free radical chain reaction terminators [38]. Thus, the antioxidant activity of these bacteria may be related to their reducing capacity and reducing ferric property. Recently, synthetic antioxidants have been used for stabilizing foods from oxidation deterioration [39]. Synthetic oxidants that are being used commonly are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertbutylated hydroxyquinone (TBHQ). Nonetheless, certain of thse synthetic antioxidants (butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertbutylated hydroxyquinone (TBHQ) are carcinogenic and toxic to human health. Hence, natural antioxidants due to their safe statut are preferred over synthetic antioxidants and are readily acceptable by consumers as they. Due to the limitation in the use of synthetic antioxidants, the demand for health-promoting natural antioxidants in foods is increasing. As for the anti-inflammatory activity, the protective effects of the strains against protein denaturation (albumin) caused by heat indicate inhibition percentages of 52.77% with the mix of commercial yogourt starters and 31.38% with the sole strain isolated from tchapalo. However, in milk, the LAC 2 strain isolated from pepper certainly producing substances that inhibit anti-inflammatory activity. This strain presented a fermented milk without anti-inflammatory activity in (SCT 3) similar to its combination with the LAC 1 strain in SCT 4 milk. The anti-inflammatory activity of fermented milks depended on the metabolic products released during the fermentation process.

Microbiological analysis showed absence of Total aerobic mesophilic flora (TAMF), total coliforms and yeast and a presence of *Bacillus* in these milks (**Table 4**). The presence of *Bacillus* could be attributed to the source of the raw materials purchased on the open market under conditions favoring these organism's growth. The presence of some of these organisms is not surprising as most of them have been isolated from tiger nut [40]. The treatment (pasteurization) eliminated some of the microorganisms, but not the *Bacillus*. However, pasteurization is sufficient to obtain tiger nut milk without any real danger for humans [41]. The presence of LAB in the other milks and its absence in the control could be explained by the fact that lactic isolates were added to the milks except the control. The absence of pathogenic germs unsuitable for consumption leads to healthy, hygienic products without risks of human consumption. Lacto-fermentation makes food last longer; this method is through acid production to decrease pH and create an environment unsuitable for harmful bacteria to survive in. intense interest in fermented tiger nut milk has arisen due to its nutritional, sensory, and probiotic potentials, and because fermentation could be a convenient way to produce microbially steady agents with an extended shelf-life. Tiger nut milk is an energizing and nutritive plant milk rich in bioactive coupounds and would be a good alternative to bovine milk, with a natural sweet flavor, particularly for lactose-intolerant individuals who avoid cow's milk and other dairy products [42]. One well-known application of tiger nut tubers in the food industry is its beverrage "horchata de chufa". "Horchata" is a sweetened beverage of milky appearance made from tiger nut tubers blended with water and sugar. It is used as an aroma agent in ice cream. Cashew milk despite its very low pH near of 4 can be supported lactic fermentation which can even continue during storage [10]. To assure the health benefits of fermented plant-based milk products, probiotics should meet the minimum level requirement for probiotic bacteria between 10^6 and 10^7 cfu/mL until the expiry date [10]. This finding was reconfirmed in the present study. Viability reached more than 10^7 cfu/mL. However, the total

Table 4. Microbiological characteristics of the plant milks.

Plant milks	Yeasts (UFC/g)	Lactic bacteria (UFC/g)	*Bacillus* (UFC/g)	TAMF (UFC/g)	Total coliforms (UFC/g)
Control	0	0	$2.35 \times 10^1 \pm 2.37$a	0	0
SCT 1	0	$3.50 \times 10^7 \pm 1.31$ab	$2.20 \times 10^1 \pm 2.71$a	0	0
SCT 2	0	$1.85 \times 10^7 \pm 5.35$b	$1.70 \times 10^2 \pm 2 \times 10$a	0	0
SCT 3	0	$9.85 \times 10^7 \pm 1.43$ab	$2.70 \times 10^1 \pm 2.71$a	0	0
SCT 4	0	$9.20 \times 10^7 \pm 2.91$ab	$1.80 \times 10^2 \pm 1.28$a	0	0

Lactobacilli count in commercial cashew milk-based yogurt was 7.73 log10 cfu/mL [11]. These differences could be due to several factors such as milk composition, dry matter content, temperature and time of milk heat pre-treatment, type and quantity of starter culture employed to inoculate the milk, fermentation temperature, and storage conditions of the final product.

Sensory evaluation is decisive in the quality control and marketing of food products; thus, it is essential for assessing the development of novel food products. The characteristics (texture, taste, flavor, and overall acceptability) of the four-promoter fermented plant milks were evaluated. These plant milks were affected by fermentation. In terms of sensory profiles, these milks showed significant differences at the 5% threshold (**Figure 2**). SCT 1 milk (fermented with the two yogurt strains) was less aromatic, less sweet, very astringent and bitter, very white, and less fragrant. SCT 2 (milk fermented with tchapalo strain *Lactobacillus plantarum*) was less aromatic, less sweet, less bitter and less astringent, strong smell, less white. SCT 3 (fermented with strain isolated from pepper) was aromatic, sweet, mild, very astringent, less fragrant, less white. SCT 4 (fermented with the mix isolated from pepper and tchapalo) was very aromatic, sweet, mild, less astringent, less fragrant, very white. The classification done by consumers (**Table 5**) indicates that the SCT 3 sample (milk fermented with LAC 2 *Pediococcus acidilactici* isolated from pepper) had the highest average of 3.40 ± 0.20 followed by SCT 2 (milk fermented with LAC 1 *Lactobacillus plantarum* isolated from tchapalo strain) with an average of 2.90 ± 0.20. The SCT 4 sample (milk fermented with LAC 1 and LA 2) was also appreciated by the panelists with an average of 2.70 ± 0.20 against an average of 2.40 ± 0 for the SCT 1 sample (fermented with both strains of international commercial yogurt). In term of sensorial studies, it has been recognized that *Pediococcus* strain surprisingly outclassed the other strains of LAB (*Streptococcus thermophilus*, *Lactobacillus bulgaricus and Lactobacillus plantarum*) which are usually used and marketed as probiotics. Strain *Pediococcus acidilactici* produced the best aromatic plant milk and a pronounced typical taste, resulting from its pungent character (spicy) which is characteristic of pepper, offering new unique flavors clearly perceptible in the milk, very appreciated by the panelists. The pungency of the pepper can reach up to 350,000 Scoville heat units and lead to its typical flavor and aroma due to its volatile composition (aldehydes, esters, alcohols, terpenoids, …) [43]. Fermented pepper is very consumed in Asia. It is a good source for aromatic starter isolation but this potential is under exploited. While the source of the starter isolation had a significant impact on the organosensorial characteristics of the fermented plant milk, *Lactobacillus plantarum* isolated from tchapalo gave a rather mild or hidden and mellow flavor and aroma. Also, the product SCT 1 obtained by co-fermentation of the two international commercial yogurt starters YB and YC was of lower relative quality compared to the products obtained by fermentation of LAC 1 or LAC 2 alone. It was as if LAC 1 (*Lactobacillus plantarum*) and LAC 2 (*Pediococcus acidilactici*) express themselves better on their own, unlike their combination.

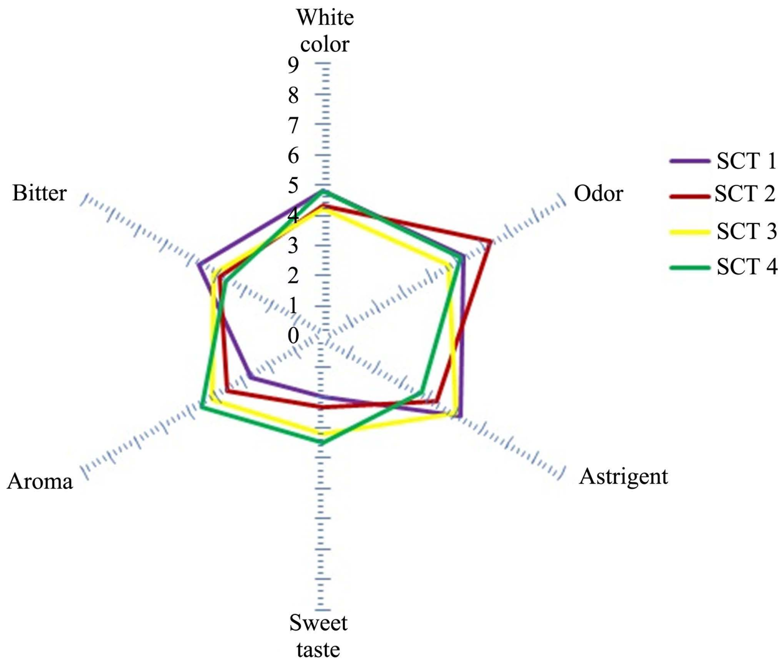

Figure 2. Sensorial profiles of the plant milks.

Table 5. Averages and ranks of plant milks.

Plant milks	Average scores	Ranks
SCT 1	2.40 ± 0.20c	4^{th}
SCT 2	2.90 ± 0.20b	2^{nd}
SCT 3	3.40 ± 0.20a	1^{st}
SCT 4	2.70 ± 0.20bc	3^{rd}

In a column, the average values followed by a different alphabetical letter are statistically different with a threshold $\alpha = 0.05$.

4. Conclusion

The study demonstrated that LAC 1 (*Lactobacillus plantarum*) and LAC 2 (*Pediococcus acidilactici*) isolated from neglected local staple foods would be valid as probiotics to give to the new composite plant milks nutrition-health claims that deserve to be confirmed in animal models and by clinical evaluations in diabetics or hypertensives humans. The GRAS status "Generally Recognized As Safe" of these two lactic acid bacteria could not be in doubt since their isolation matrices are usually consumed without any danger. However, additional data are necessary about these plant milks such as their storage behavior and the chemical nature of their antioxidant and anti-inflammatory substances.

Authors' Contributions

This work was carried out in collaboration among all authors. Authors read and approved the manuscript.

Conflicts of Interest

All the authors declare that they have no conflict of interest.

Ethical Approval

Ethics approval was not required for this research.

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