PROBIOTIC CASHEW APPLE PULP FERMENTED WITH LACTIC ACID BACTERIA AND BIFIDOBACTERIA

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ABSTRACT Fresh cashew apple (*Anacardium occidentale*) pulp is an agricultural waste from processed cashew nut and contains bioactive compounds with potential health benefits. Cashew apple can be a raw substrate for the production of probiotic cashew apple juice by lactic acid bacteria (*Lactobacillus acidophilus*) and bifidobacteria (*Bifidobacterium longum*). The fermentation process was performed at 37°C for 0, 24 and 48 h. Changes in pH, total titratable acidity, total sugar, reducing sugar, viable cell counts, ascorbic acid, total phenolic, and antioxidant activities (DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) and ABTS$^{\bullet+}$ (2,2-azino-di-[3-ethylbenzthiazoline sulphonate])) of cashew apple during fermentation were investigated. *L. acidophilus* grew well on cashew apple and reached nearly 4.7×10^6 CFU/mL after 48 h of fermentation at 37°C. However, *L. acidophilus* and *B. longum* produced a smaller amount of total titratable acidity expressed as lactic acid which was not different with the control ($p>0.05$). Results showed that pH and total sugar contents were decreased after 48 h of fermentation. However, fermented cashew apple with *L. acidophilus* and *B. longum* in 24 h had the highest vitamin C content. Moreover, fermented cashew apple with *B. longum* had a high antioxidant capacity which did not differ ($p>0.05$) from that of *L. acidophilus*. The DPPH$^{\bullet}$ and ABTS $^{+}$ radical scavenging activities of fermented cashew apple were greater than 60% and 80%, respectively. Finally, *L. acidophilus*, and *B. longum* are optimal probiotics for fermenting cashew apple juice, and could serve as a healthy beverage for vegetarians and lactose-allergic consumers.

1. INTRODUCTION

The cashew nuts represents only 9.5% of the total fruit weight and after harvesting large amounts of cashew apples are left in the field to rot as an agricultural by-product, thus, 90.5% of this production is lost or underutilized (Silveira, et al., 2012). Cashew apples are an excellent source of nutrition include ascorbic acid, vitamins, minerals, organic acid, phenolics (anacardic acids), carbohydrate, reducing sugars (fructose and glucose), and some amino acids (Gyedu-Akoto, 2011). Cashew apple is shown to be suitable for fermentation, because of its unique taste, flavor, availability, high sugar and water content (moisture content), and overall chemical composition (Ward & Ray, 2006).

The use of probiotic microorganisms for fermented foods is traditional. Fermented products may be part of a daily diet, improving the health and quality of life of consumers. Lactic acid fermentation has a well-known role in improving nutritional and functional properties of many vegetable foods (Di Cagno, et al., 2013). Most of the probiotic bacteria used in commercial products today are members of the genera *Lactobacillus* and *Bifidobacterium* (Daly & Davis, 1998). Recently, numerous lactic acid bacteria such as *Lactobacillus acidophilius*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Bifidobacterium longum* and *Bifidobacterium lactis* have been used in fruits to produce probiotic beverage (Gardner, et al., 2001; Yoon, et al., 2006; Kun, et al., 2008). Therefore, the aim of this study was to investigate the chemical composition (pH, total titratable acidity, total sugar, reducing sugar, ascorbic acid, and total phenolic) and antioxidant activity changes in cashew apple pulp fermented with lactic acid bacteria and bifidobacteria.

2. EXPERIMENT

2.1 Materials and Chemicals

Cashew apple was kindly provided by Heritage Grower Corporation Ltd., at the southern part of Thailand. The cashew apple was washed with water and then cut into small pieces. The edible pieces were homogenized using a Waring blender for 5 min and then pasteurized for 15 min at 70°C. Testing chemicals, including 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) were obtained from Sigma-Aldrich (USA).

2.2 Preparation of Probiotic Lactic Acid Bacteria

Lactobacillus acidophilus TISTR 1338, and *Bifidobacterium longum* TISTR 2195 were obtained from Thailand Institute of Scientific and Technological Research (TISTR), Thailand. Cell cultivation was carried out in an incubator until the cell density spectrophotometrically determined reached 1.000 at 600 nm that correspond to 15×10^8 CFU/mL, using the McFarland scale (Chapin & Lauderdale, 2003).

2.3 Fermentation of Probiotic Cashew Apple

Fermentation experiments were conducted in sealed Erlenmeyer flask, each containing 20 g of pasteurized cashew apple, without supplementary nutrient or water and inoculated *L. acidophilus* and *B. longum* (2.0 mL of inoculum). The fermentation process was performed at 37°C for 48 h. Samples were taken at 0, 24, and 48 h for biological and chemical analyses.

2.3.1 Viable Cell Counts Determination: Viable cell counts were obtained by serial dilution with 0.7% NaCl solution until 10^{-6} dilution. Aliquots of 0.1 mL of dilution were plated, in triplicate in plates containing MRS Agar (spread plate method). The plates were incubated for 72 h at 37°C. Plates containing 20-350 colonies were measured and recorded as colony forming units (CFU) per mL of solution.

2.3.2 pH, Total Titratable Acidity, and Sugar Determination: The pH of probiotic cashew apple was measured using a pH meter. Total titratable acidity, expressed as lactic acid, was determined by titration with 0.1M NaOH using the method of AOAC (2000). Reducing sugar content was analyzed as glucose equivalents by Nelson-Somogyi method (Somogyi, 1952). Total sugar content was analyzed as glucose equivalents by the phenol sulfuric acid method of Dubois, et al. (1956).

2.3.3 Vitamin C Content: Ascorbic acid analysis of sample was done with 2,6-dichloroindolphenol according to the Official titrimetric method (AOAC, 2000). Vitamin C content was expressed as mg ascorbic acid equivalents (AAE)/ 100g dry weight (DW).

2.3.4 Total Phenolic Content: Total phenolic content was measured using the Folin–Ciocalteu colorimetric method (Huang, et al., 2005). Sample (20 µL) was added to the test tube and mixed with 80 µL of distilled water and 2 mL of 2% (w/v) Na_2CO_3 solution. After incubation for 3 min, 100 µL of Folin-Ciocalteu reagent was added. After standing for 30 min at room temperature, absorbance was measured at 750 nm, using distilled water as a blank. Results were expressed as mg gallic acid equivalents (GAE)/ 100g dry weight (DW).

2.3.5 Estimate Antioxidant Capacity: Two methods were used to evaluate the antioxidant capacities of fermented cashew apple using $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays.

1) $DPPH^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) Radical Scavenging Efficiency: The scavenging effect was measured according to the modified method of Bersuder, et al. (1998). A 10 µL aliquot of the sample extract was added to the test tube and mixed with 30 µL of distilled water. Two milliliter of 0.1 mM $DPPH^{\bullet}$ dissolved in 95% ethanol was added to the sample. The mixture was shaken and left for 30 min in the dark at room temperature, and was read the absorbance at 517 nm. The percent DPPH scavenging effect (%) was calculated by the following equation:

$$DPPH^{\bullet}\text{ scavenging effect (\%)} = \frac{A_{Blank} - (A_{Sample} - A_{Control})}{A_{Blank}} \times 100$$

where A_{Blank} = absorbance at 517 nm of 40 µL distilled water + 2 mL of 0.1 mM ethanolic $DPPH^{\bullet}$ solution, A_{Sample} = absorbance at 517 nm of 40 µL aqueous sample + 2 mL of 0.1 mM ethanolic $DPPH^{\bullet}$ solution, and $A_{Control}$ = absorbance at 517 nm of 40 µL aqueous sample + 2 mL of ethanol.

2) $ABTS^{\bullet+}$ (2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic) Radical Scavenging Activity: The scavenging activity was measured according to Shan, et al. (2005). $ABTS^{\bullet+}$ radical cation was generated by reacting 7 mM $ABTS^{\bullet+}$ and 2.45 mM potassium persulfate in the dark for 24 h at 20°C. Before analysis, the $ABTS^{\bullet+}$ solution was diluted to obtain an absorbance of 0.700 ± 0.020 at 734 nm with ethanol. An aliquot of the sample (10µL) was added to the test tube and mixed with 70 µL of distilled water. After adding 4 mL of the diluted $ABTS^{\bullet+}$ solutions, which was prepared daily to the sample, the absorbance was measured at 734 nm after exactly 6 min of initial mixing, using distilled water as blank. The antioxidant activity of the sample was calculated by the same equation as $DPPH^{\bullet}$.

2.3.7 Statistical Analysis: All experiments were carried out in triplicate, and each sample was analyzed in duplicate. Results were expressed as mean ± S.D. (standard deviation). The SAS program (SAS Institute, 2004, USA) was used to analyze the experimental data. Significant differences ($p<0.05$) between means were determinated by Duncan's Multiple Range Test (DMRT).

3. ANALYSIS

Changes in pH, total titratable acidity, reducing sugar and total sugar of the probiotic cashew apple are shown in Table 1. The pH of fermented cashew apple with *L. acidophilus* and *B. longum* was decreased ($p\leq0.05$) throughout 48 h fermentation period from 3.88 to 3.70 and 3.87 to 3.73, respectively. The profile of total titratable acidity measured throughout fermentation period in fermented cashew apple was in agreement with the pH trend. The total titratable acidity of *L. acidophilus* and *B. longum* fermented cashew apple for 48 h at 37°C slightly increased ($p>0.05$) from 0.44% to 0.56% and 0.50% to 0.52%, respectively. The patterns of pH and acidity exhibited are in agreement with the findings of Ibanoglu, et al. (1995), who have reported that lactic acid bacteria have been responsible for the production of lactic acid during fermentation.

Population changes of the enumerated microbial groups of cashew apple are shown in Fig. 1. The initial cell counts of *L. acidophilus* and *B.longum* were 15.8×10^8 CFU/mL.

Viable cell counts of *L. acidophilus* fermented cashew apple was sharply decreased within 24 h fermentation, and then remained at 4.7×10^6 CFU/mL at 48 h fermentation, while those of 48 h *B.longum* fermented cashew apple was remained at 4.45×10^2 CFU/mL. It was also found that *L. acidophilus* was unable to survive at the low pH and high acidity conditions of fermented cashew apple.

Sugar content of cashew apple has usually been assessed in terms of total sugar or reducing sugar. Total sugar levels of *L. acidophilus* fermented cashew apple were sharply decreased from an initial value of 3.63 to 2.86 g/ 100 g DW ($p>0.05$) (Table 1). Meanwhile, total sugar levels of *B.longum* fermented cashew apple were slightly decreased from an initial value of 3.55 to 3.31 g/ 100 g DW (Table 1). This finding agrees with the changes in reducing sugar content of fermented cashew apple that slightly increased from an initial value ($p>0.05$). Results indicated that *L. acidophilus* and *B.longum* utilized fruit sugar and produced lactic acid without any additional nutrient.

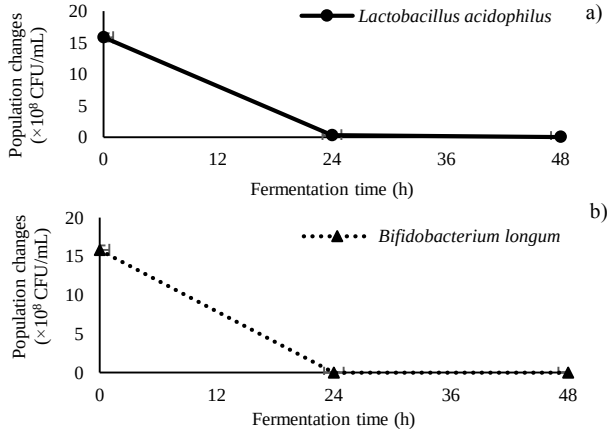

Fig. 1 Changes in population of a) *Lactobacillus acidophilus* and b) *Bifidobacterium longum* cashew apple for 0, 24 and 48 h, at 37°C.

Antioxidant contents (ascorbic acid and total phenolic) of fermented cashew apple during fermentation are presented in Table 2. It was observed that vitamin C content sharply increased after ferment for 24 h, and then decreased of 48 h of fermentation. The highest ascorbic acid of fermented cashew apple with *L. acidophilus*, and *B. longum* was at 24 h of 18.10 and 17.79 g AAE/100 g DW, respectively. Total phenolic content of fermented cashew apple with *L. acidophilus* and *B. longum* at the fermentation time of 0 to 24 h decreased slightly and then increased at 48 h, but they were not different compared to the control ($p>0.05$). The decrease in antioxidant activity of fermented cashew apple with *B. longum* did not differ ($p>0.05$) from that with *L. acidophilus* during 48 h of fermentation. A decrease in antioxidant activity throughout fermentation (48 h) could be due to oxidation or degradation of compounds with antioxidant (Johnson, et al., 2011).

Table 1 Changes in pH, total titratable acidity, reducing sugar and total sugar during fermentation of cashew apple for 0, 24, and 48 h, at 37°C.

Fermentation time		pH	total titratable acidity (% lactic acid) [x]	reducing sugar [y] (g/100g DW)	total sugar [y] (g/100g DW)
Non-microbial	0	3.68±0.01[b]	0.47±0.02	3.66±0.10[a]	3.54±0.24[b]
	24	3.65±0.01[b]	0.47±0.08	3.73±0.09[a]	3.95±0.16[a]
	48	3.67±0.01[b]	0.49±0.06	3.71±0.07[a]	3.60±0.13[ab]
L. acidophilus	0	3.88±0.01[a]	0.44±0.04	2.99±0.04[b]	3.63±0.19[ab]
	24	3.90±0.01[a]	0.52±0.02	2.97±0.06[b]	2.98±0.18[cd]
	48	3.70±0.08[b]	0.56±0.04	3.15±0.10[b]	2.86±0.15[ab]
B. longum	0	3.87±0.04[a]	0.50±0.05	2.98±0.10[b]	3.55±0.13[b]
	24	3.87±0.08[a]	0.48±0.07	3.18±0.06[b]	3.58±0.15[b]
	48	3.73±0.06[b]	0.52±0.06	2.99±0.33[b]	3.31±0.40[bc]
F-test		**	ns	ns	**
LSD		0.08	0.09	0.23	0.36
C.V. (%)		1.26	11.09	4.16	6.04

The results are expressed as means ± standard deviation (SD) with three replications. Values with different superscript letters in a column are significantly different at $p<0.05$.
[x] Expressed as lactic acid (%)
[y] Measured as glucose equivalent and expressed as g/100g dry weight (DW)

Table 2 Changes in total phenolic and vitamin C contents during fermentation of cashew apple for 0, 24, and 48 h, at 37°C.

Fermentation time		Total phenolic content (mg GAE/ 100 g DW)	vitamin C (g/ kg DW)
Non-microbial	0	94.83±6.49[abc]	15.97±0.33[b]
	24	90.96±2.73[bcd]	14.64±0.36[c]
	48	92.12±1.75[abcd]	8.83±0.83[d]
L. acidophilus	0	93.50±3.92[abc]	15.19±0.36[bc]
	24	88.02±3.74[d]	18.10±0.82[a]
	48	97.71±3.57[a]	8.37±1.00[d]
B. longum	0	96.55±7.78[ab]	15.82±0.49[b]
	24	88.44±3.42[cd]	17.79±0.71[a]
	48	95.04±4.84[ab]	8.52±0.80[d]
F-test		ns	**
LSD		6.60	1.16
C.V. (%)		4.13	4.95

The results are expressed as means ± standard deviation (SD) with three replications. Values with different superscript letters in a column are significantly different at $p<0.05$.

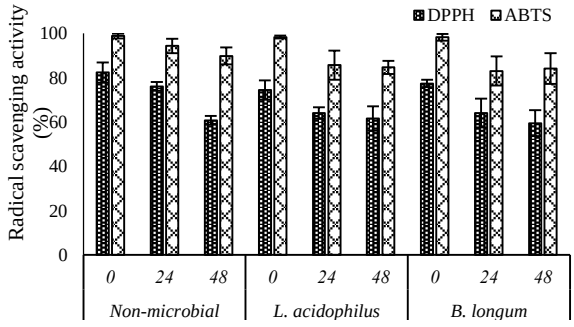

Fig. 2 DPPH and ABTS radical scavenging of fermented cashew apple for 0, 24 and 48h, at 37°C.

CONCLUSION

L. acidophilus retained viability at low pH and under highly acidic conditions, which *B. longum* could not survive at low pH of fermented cashew apple. The decrease in antioxidant activity of fermented cashew apple with *B. longum* did not differ ($p>0.05$) from that with *L. acidophilus* during 48 h of fermentation. Results

obtained that fermented cashew apple with *L. acidophilus* and *B. longum* might be the optimal probiotics for the production of a health beverage.

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