CHANGES IN PHYTOCHEMICALS, ANTIOXIDANT PROPERTIES AND MINERAL CONTENT OF TWO PROBIOTIC FRUIT JUICES DURING COLD STORAGE

3.1. Introduction

In the late nineteenth century, microbiologists identified microflora in the gastrointestinal tract of healthy animals that differed from those found in diseased animals. As further research continued into the isolation and characterization of these microorganisms, it was revealed that ingestion of these bacteria could confer a wide range of therapeutic benefits to humans. These beneficial microflora were termed probiotics[1,2]. Since then, the popularity of probiotics has been increasing rapidly worldwide. Food companies are increasingly manufacturing foods with incorporated probiotic bacteria, which fall under the new category of foods called 'Functional Foods' [3,4]. Probiotic dairy products such as yogurts containing *L. acidophilus* and *Bifidobacterium* spp. constitute a significant proportion among the commercially available probiotic foods [5].

Dairy fermented products have been traditionally considered as the best carriers for probiotics; but, nowadays, up to 70% of the world population is affected by lactose-intolerance. Asian diets are relatively low in meat and dairy foods, and plant-based foods contribute the core of the daily intake. Besides dietary habits, lactose intolerance discourages many Asian people from consuming milk. Furthermore, the use of milk-based products may be also limited by allergies, cholesterol diseases, dyslipidemia, and vegetarianism; therefore, several raw materials have been extensively investigated to determine if they are suitable substrates to produce novel non-dairy functional foods [6].

Recently, beverages based on fruits, vegetables, cereals, and soybeans have been proposed as new products containing probiotic strains; particularly, fruit juices have been reported as a novel and appropriate medium for probiotic for their content of essential nutrients. Fruit juices are also extremely healthy, having a high content of antioxidants, vitamins, minerals, dietary fiber and many other beneficial nutrients, and hence could serve as a good medium for cultivating probiotics [7,8].

Fruits are rich sources of phytochemicals, mainly polyphenols and carotenoids that have free radical scavenging capacity as well as antioxidant activities. The phytochemicals include polyphenols or phenolic compounds, dietary fibre, organic acids, carotenoids, micronutrients etc. [9]. The polyphenols present in fruits and vegetables act as antioxidants and have free radical destroying properties. They act as inhibitors of lipid peroxidation, prevent DNA oxidative damage and prevent inhibition of cell communications, all of which are precursors to degenerative diseases [10]. Thus regular consumption of fruits could significantly prevent or reduce the risk of development of degenerative diseases like cancer, cardiovascular heart diseases, diabetes, etc [11,12]. Moreover, they are usually referred to as healthy foods, designed for young and old people [13]. The fruit juices have been suggested as an ideal medium for the functional health ingredients because they inherently contain beneficial nutrients, they have taste profiles that are pleasing to all the age groups, and because they are perceived as being healthy and refreshing [14].

Therefore, in the present research, an attempt was made to test the effect of fortification of fruit juices with probiotic *Lactobacilli* that could serve as a health beverage for consumers who are allergic to dairy products. The objective was to study the changes in the phytochemical and antioxidant property of two probiotic fruit juices after incorporation of selected probiotic lactic acid bacteria during storage at refrigerated condition (4 ±1°C). Two commonly consumed juices *ie*, litchi and pineapple were taken for the study as suitable media (based on the previous experiments) for lactic acid fermentation. Since litchi and pineapple have high acid content and low pH, only two strains of *Lactobacillus* were chosen (from results of Chapter I) for this study on the basis of their greater pH tolerance ability.

3.2. Materials and Methods

3.2.1. Materials

The fruit samples viz. litchi (*Litchi chinensis* Sonn.) and pineapple *(Ananas comosus* L. Merr) were procured from the local fruit market, Tezpur, Assam during the season. The fruits and probiotic strains were selected from previous experiments on the basis of their suitability to develop health promoting functional fruit drinks. Chemicals used in the study were of analytical grade purchased from Sigma-Aldrich, Merck and Himedia. All the standards were purchased from Sigma-Aldrich. *Lactobacillus* isolates, *Lactobacillus*

plantarum MTCC2621 (Lp) and *L. rhamnosus* MTCC1480 (Lr) were obtained from Microbial Type Culture Collection and Gene Bank (MTCC) (IMTECH, CSIR, Chandigarh, India).

3.2.2. Culture preparation

From this culture, stock solution was prepared by adding sterile glycerol (50% v/v) to the activated culture. The glycerol stock culture was stored at frozen (-20 ^{o}C) in sterile screw cap tubes. The identity of all the probiotic bacteria was confirmed using biochemical methods [15]. The probiotic organisms were grown individually by inoculating into 10 mL sterile de Man Rogosa and Sharp (MRS) broth (Himedia Laboratories Pvt. Ltd, Mumbai, India) and incubated at 37 ^{o}C for 2 days under aerobic condition. The cells were harvested by centrifuging (Sigma, Germany) at 1500 x g for 15 min at 4^{o}C. Before inoculation into fruit juices, the harvested cells were washed twice with sterile saline water (0.85% w/v NaCl) to remove any residual MRS.

3.2.3 Fruit juice preparation

Fresh and ripe fruit were collected from the local market, washed and the juice was extracted. The fruits were collected from same lot and all care was taken to collect the fruits of same maturity indices like size, colour and firmness. The samples were washed and the juice was extracted using a household juicer (Philips). The juice was strained through a muslin cloth and pasteurized at 90 ^{o}C for 1 min with constant stirring.

3.2.4. Inoculation of substrates

Pasteurized juice 100 mL were taken into sterile Erlenmeyer flasks. Each flask containing 100 mL juice was inoculated with 1% culture with *Lactobacillus plantarum* MTCC5422 *L. rhamnosus* MTCC1480, and *L. acidophilus* MTCC447 under aseptic conditions and labeled as Lp and Lr, respectively. No culture was added to the flask labeled as Control. The flasks were then incubated at 37 ^{o}C. After 12 h of fermentation at 37 °C, the flasks were kept at refrigerated condition (4 ±1°C) for 6 weeks. At an interval of 7 days, 10 mL of juice was taken out from each flask and used for further analysis.

3.2.5. Determination of total phenolic content

Total phenolic content in the sample extracts was assessed using the Folin–Ciocalteau assay [16] with slight modification. For the analysis, 20µL each of filtered juice, gallic acid standard or blank were taken in separate test tubes and to each 1.58 mL of distilled water was

added, followed by 100µL of Folin–Ciocalteau reagent, mixed well and within 8 min, 300 µL of sodium carbonate was added. The samples were vortexed immediately and the tubes were incubated in the dark for 30 min at 40°C. The absorbance was then measured at 765 nm in a UV-Vis spectrophotometer (Cecil, Aquarius7400). The results were expressed in mg GAE/ 100 mL.

3.2.6. Determination of total flavonoid content

The flavonoid content was determined by aluminium trichloride method. [17]. Briefly, 0.5 mL of the filtered juice was mixed with 1.5 mL of 95% ethanol, 0.1mL of 10% aluminium trichloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of deionised water. After incubation at room temperature for 40 min, the reaction mixture absorbance was measured at 415 nm against deionised water blank in a UV-Vis spectrophotometer (Cecil, Aquarius 7400). Results were expressed as quercetin equivalent (mgQE/100 mL) of sample.

3.2.7. Determination of ferric reducing antioxidant property (FRAP)

FRAP activity of the samples was measured by the method of Benzie and Strain [18] Briefly, a 40 µL aliquot of properly diluted sample extract was mixed with 3 mL of FRAP solution. The reaction mixture was incubated at 37°C for 4 min and the absorbance was determined at 593 nm in a UV-Vis spectrophotometer (Cecil, Aquarius 7400) against a blank that was prepared using distilled water. FRAP solution was pre warmed at 37°C and prepared freshly by mixing 2.5 mL of a 10 mM 2,4,6-TPTZ [2,4,6-tri(2-pyridyl)-1,3,5-triazine] solution in 40 mM hydrochloric acid with 2.5ml of 20mM ferric chloride and 25 mL of 0.3M acetate buffer (pH 3.6). A calibration curve was prepared, using an aqueous solution of ferrous sulfate (1-10 mM). FRAP values were expressed as µM Fe^{2+} equivalent per 100 mL of sample.

3.2.8. Determination of DPPH radical scavenging activity

Radical scavenging activity of the sample extracts was measured by determining the inhibition rate of DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical. [19] Precisely, 100 µL of extracts was added to 1.4 mL DPPH radical methanolic solution (10^{-4} M). The absorbance at 517 nm was measured at 30 min against blank (100 µL methanol in 1.4 mL of DPPH radical solution) using a UV-Vis Spectrophotometer (Cecil Aquarius 7400). The results were expressed in terms of radical scavenging activity (**Eq. 3.1**).

Radical scavenging activity (%) = [(Ao-As)/ Ao] × 100 **Eq. 3.1**

Where, Ao is absorbance of control blank, and As is absorbance of sample extract.

3.2.9. Quantification of polyphenols by HPLC

Reverse-phase high performance liquid chromatography (HPLC) was performed to analyse the major phenolic compounds in the juice. The separation module consisted of a Waters HPLC (Waters) equipped with a C18 Symmetry 300^{TM} C_{18} (5 µm, 4.6 X 250 mm) column with a binary pump (Waters, 1525) and a UV-Vis detector (Waters, 2489). The samples were eluted with a gradient system consisting of solvent A [acidified ultrapure water (0.1% acetic acid, pH 3.2)] and solvent B (methanol), used as mobile phase. The flow rate was maintained at 0.8mL/min and wavelengths used for UV-Vis detector were 254 nm and 325 nm. The temperature of the column was maintained at 25 ^{o}C and the injection volume was 20 µL. The gradient system started at 80 % solvent A (0-8 min), 65 % A (9-12 min), 45 % A (13-16 min), 30 % A (17-20 min), 20 % A (21-30 min), 10 % of A (31-34 min) and then washing of the column with 65 % A (35-39 min) and lastly, 80 % A (40-45 min) was followed. The juice samples were centrifuged at 15000 x g using a Sigma 3-18K centrifuge (Sigma, Germany) for 15 min. The juice supernatant was then filtered through a Whatman membrane filter (0.2 µm) before injection into HPLC. The ethanolic extract was evaporated under vacuum in a rotary vacuum evaporator (Roteva, Medica Equipments) and then redissolved in 1mL methanol. Sample volume of 20 µL was used. The standards (Sigma-Aldrich) used for comparison and identification were (±) catechin, quercetin, gallic acid, coumaric acid, caffeic acid, syringic acid, ferulic acid, chlorogenic acid, kaempferol and rutin hydrate. The peaks of the phenolic compounds were monitored and concentration of phenolic compounds were determined using external calibration curve of standard compound at 0.62, 1.25, 2.5, 5, 10 and 20 mg/L.

3.2.10. HPLC analysis of the organic acids

For direction of the organic acids in probiotic fruit juice, 5 ml aliquots of the juice was taken on a weekly basis and frozen in 50mL Falcon tubes (Tarsons, India). The samples were diluted with 70 µL of 15.5 N HNO_3 and 4930 µL of 0.009% H_2SO_4, and then mixed gently. The samples were then centrifuged at 14,000 x g for 10 min (Sigma, Germany). The supernatant was removed using a 2 mL sterile syringe and then filtered into HPLC vials using

a Whatman 0.2 µm PTFE membrane filter. The analysis of organic acid concentration was performed using method described by Ong *et al*. [20]. The HPLC apparatus used consist of a Waters 1525 binary pump and 2489 UV/Vis detector and Breeze software. The column was a Waters Symmetry ™ C18 5µm 4.6 x 300 mm and was heated to 65°C when used. Mobile phase consisted of 0.009 H_2SO_4 which was filtered through Whatman 0.45 µm membrane filter and degassed with bath sonicator (JSGW, India). The mobile phase was set at a flow rate of 0.6 mL/min. A sample volume of 20µL was used for both standards and samples, and detection was achieved at 220 nm. The organic acid standard kit was obtained from Sigma Aldrich® Inc. All samples were run for 40 min in gradient mode and all analyses were carried out in triplicate. All instrument control, analysis, and data processing was performed via Waters® Breeze® Chromatography Data Software (CDS).

3.2.11. Mineral analysis

For mineral analysis, the samples were digested in Digestion System using mixture of nitric acid and sulphuric acid in 1:1 ratio and the aqueous solutions were injected to Atomic Absorption Spectrophotometer AAS (Thermo, iCE 3500) and analysed in reference to calibration of 3 standard concentrations made from certified single element AA standards (Sigma Aldrich®).

3.2.12. Method validation

The methods for analysis of organic acid and mineral content were validated for this kind of matrix. All validation parameters are shown in **Table 3.1**. Linearity was assessed from the calibration curves obtained at five concentration levels of each compound. The correlation coefficient and linearity coefficient were also evaluated and good linearity is indicated in the working range ($r \geq 0.990$ and $LC \geq 95\%$). The sensitivity of the method was evaluated by the limits of detection (LOD) and quantitation (LOQ). The methods and analytical techniques used were very sensitive for the compounds studied. Accuracy was measured by spiking a sample with each of the analytes of interest at three different concentration levels (low, medium and high in the range calibration) in triplicate. Reference materials were also used to assess accuracy in the analysis of mineral elements. The results are the average of all the measurements.

Table 3.1. Validation parameters of the instrumental methods used (organic acids and minerals)

Analyte	Linearity			Sensitivity		Accuracy	
	Linear range	R^2	LC %	LOD (mg/L)	LOQ (mg/L)	Spiked amount (mg/L)	Accuracy (%)
Citric acid	30-1000	0.993	98	10	30	50-500	101
Malic acid	25-1000	0.994	98	10	20	25-100	109
Tartaric acid	30-500	0.995	97	10	25	25-100	108
Oxalic acid	1-20	0.991	95	0.5	1	25-100	106
Lactic acid	30-1500	0.996	95	10	30	30-500	102
Sodium	10-500	0.966	99	NC	10	100-250	98
Potassium	50-500	0.998	98	NC	50	100-250	92
Calcium	1-500	0991	98	NC	1	100-250	102
Magnesium	1-500	0.999	97	NC	1	100-250	98
Iron	0.2-10	0.989	99	NC	0.2	0.05-10	97
Copper	0.05-10	0.992	98	NC	0.05	0.05-10	100
Zinc	0.2-5	0.988	99	NC	0.2	0.05-5	108
Manganese	0.005-5	0.990	98	NC	0.005	0.01-0.05	98

Regression coefficient (R^2), linearity coefficient (LC), limit of detection (LOD), limit of quantification (LOQ), not calculated (NC).

3.2.13. Statistical analyses

All experiments were carried out at least in triplicate and reported as mean ± standard deviation of mean (S.E.M). The data were statistically analyzed by Duncan's multiple range test at $p \leq 0.05$ significant levels using SPSS (SPSS Statistical Software Inc.) version 11.5.

3.3. Results and Discussion

3.3.1. Phytochemical and antioxidant changes

The total phenolic content (TPC) was very high in litchi juice (2420 mg GAE/100mL) in comparison to pineapple juice (92 mg GAE/100mL), as seen from **Table 3.2** . TPC was observed to vary depending on storage period and the type of bacterial strain used for probiotication (**Table 3.2**). A significant ($p \leq 0.05$) decrease in TPC was observed in the control litchi juice with storage period. TPC in control litchi juice at the end of 6^{th} week was

79.7 % of 0 day value. Similarly, TPC also decreased significantly on probiotication and at the end of 6th week the fall was 34.2 % for Lp treated juice and 42.9 % for Lr treated juice against their 0 day values.

There was significant decrease of TPC in control pineapple juice with a fall of 34.2 % from 0 day. Further, there was significant decrease of 58.8 % and 36.5 % of TPC after 6th week in Lp and Lr treated pineapple juices, respectively. The extent of % decrease on probiotication varied between juices and probiotic species used.

No significant change in TFC was observed in control juices. The total flavonoid content (TFC) in fermented litchi and pineapple juice lowered from 11.58 to 3.88 mg QE/100mL and 4.25 to 1.85 mg QE/100mL, respectively after addition of Lr (**Table 3.3**). TFC was also found to decrease from 12.56 to 9.58 mg QE/100mL and 4.12 to 2.12mg QE/100mL in litchi and pineapple juice fortified with Lp in 6 weeks refrigerated storage.

The FRAP value of probiotic litchi and pineapple juices showed a declining trend (**Fig.3.1** and **Fig. 3.2**.) for both strains on storage. However, highest decrease in litchi was observed in Lp fortified juice while in pineapple, Lr fortified juice showed the highest decrease.

The DPPH radical scavenging activity was higher in litchi juice than pineapple juice and the scavenging activity also varied on storage among the probiotic strain used (**Fig.3.1** and **Fig. 3.2**). The DPPH activity was lowest in litchi juice with Lr, it reduced from 88.71 % to 13.17% after 6th week. In probiotic pineapple juice with Lr, DPPH activity ranged reduced from 24.8 % to 7.8 %. Probiotication, therefore had an adverse effect on radical scavenging activity.

The antioxidant capacity depends on the structural conformation of phenolic compounds. The decreasing trend of polyphenols and antioxidant capacity could be understood by the fact that antioxidant capacity of the food depends on the synergistic and redox inter-actions among the different compounds present in the fruits [21]. The reduction in one group of compounds may lead to the loss in functionality against certain type of free radicals.

Table 3.2. Total phenolic content (TPC) of the probiotic juice during storage at refrigerated condition (4 ±1°C)

Week	Total phenolic content (mg GAE/100mL)					
	Litchi			Pineapple		
	Control	Lp	Lr	Control	Lp	Lr
0	2420.00^{b} ±12.80	2400.00^{f} ±17.00	2360.00^{f} ±38.23	92.00^{c} ±7.09	89.15^{f} ±6.40	80.55^{g} ±4.40
1	2365.00^{b} ±45.60	2298.00^{e} ±51.08	2300.00^{e} ±41.34	85.47^{b} ±4.17	87.68^{e} ±5.29	67.18^{f} ±6.20
2	2255.00^{b} ±26.48	2100.00^{d} ±56.18	2010.00^{d} ±28.21	84.78^{b} ±5.25	85.74^{e} ±5.08	55.55^{e} ±7.48
3	2158.50^{b} ±13.71	1958.50^{d} ±17.13	1750.00^{c} ±33.28	82.37^{a} ±4.29	78.47^{d}±4.04	68.17^{d} ±4.04
4	2020.00^{a} ±41.18	1720.00^{c} ±31.61	1400.00^{b} ±18.35	82.58^{a} ±3.19	52.11^{c}±2.30	42.81^{c} ±2.25
5	1952.50^{a} ±11.60	1623.00^{b} ±46.29	1457.00^{b} ±25.15	81.51^{a} ±3.25	48.61^{b} ±3.24	38.44^{b} ±3.09
6	1927.00^{a} ±32.26	1580.00^{a} ±19.41	1348.00^{a} ±17.46	80.26^{a} ±3.40	36.76^{a} ±2.16	26.15^{a} ±3.11

*results are Mean ± SD for n = 3: Lp: *L. plantarum*, and Lr: *L. rhamnosus*; Same letter within the column means no significant difference at p≤0.05 by DMRT.

Table 3.3. Total flavonoid content (TFC) of the probiotic juice during storage at refrigerated condition (4 ±1°C)

Week	Total flavonoid content (mg QE/100mL)					
	Litchi			Pineapple		
	Control	Lp	Lr	Control	Lp	Lr
0	13.13^{b}±0.13	12.56^{d} ±0.15	11.58^{h} ±0.05	4.30^{b} ±0.07	4.12^{d} ±0.09	4.25^{f} ±0.31
1	13.11^{b}±0.15	11.25^{c} ±0.17	9.88^{f} ±0.15	4.00^{b} ±0.05	3.87^{d} ±0.15	4.01^{e} ±0.18
2	13.00^{b} ±0.06	11.14^{c} ±0.10	8.17^{e} ±0.15	3.84^{b} ±0.23	3.12^{c} ±0.27	3.78^{d} ±0.19
3	12.70^{b} ±0.03	11.07^{c} ±0.05	6.88^{d} ±0.25	3.70^{b} ±0.13	3.00^{c} ±0.21	3.58^{d} ±0.28
4	12.30^{b} ±0.08	10.71^{c} ±0.15	5.00^{c} ±0.05	3.41^{a} ±0.10	2.89^{c} ±0.47	3.12^{c} ±0.06
5	11.54^{a} ±0.10	10.15^{b} ±0.18	4.75^{b} ±0.28	3.12^{a} ±0.14	2.50^{b} ±0.13	2.96^{b} ±0.10
6	11.00^{a} ±0.45	9.58^{a} ±0.02	3.88^{a} ±0.17	3.10^{a} ±0.15	2.12^{a} ±0.10	1.85^{a} ±0.15

* results are Mean ± SD for n = 3: Lp: *L. plantarum*, and Lr: *L. rhamnosus*; Same letter within the column means no significant difference at p≤0.05 by DMRT

Phenolic compounds have a tendency to undergo some kind of structural rearrangement that could lead to either increased or decreased antioxidant activities. But mainly, the increase or decrease in phenolic content depends on the overall composition and types of individual phenolic acid present in maximum in the concerned fruit juice.

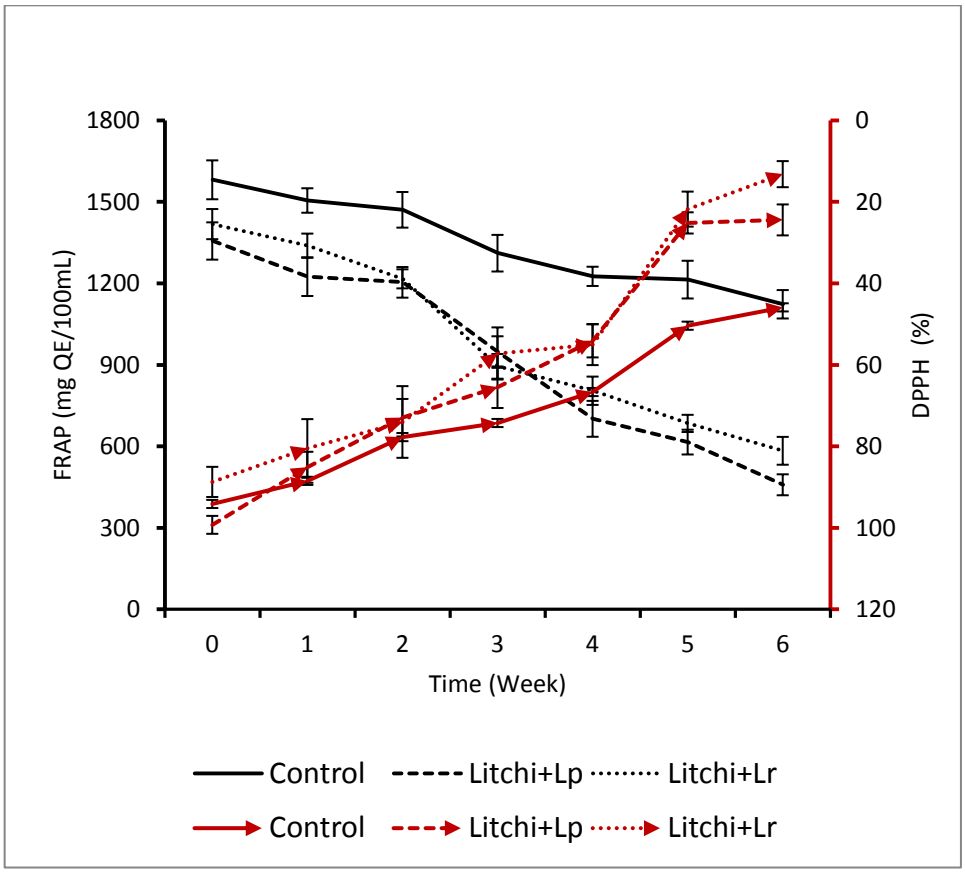


Fig. 3.1. Changes in ferric reducing antioxidant property (FRAP) and DPPH radical scavenging activity of the probiotic litchi juice during storage at refrigerated condition (4 ±1°C)

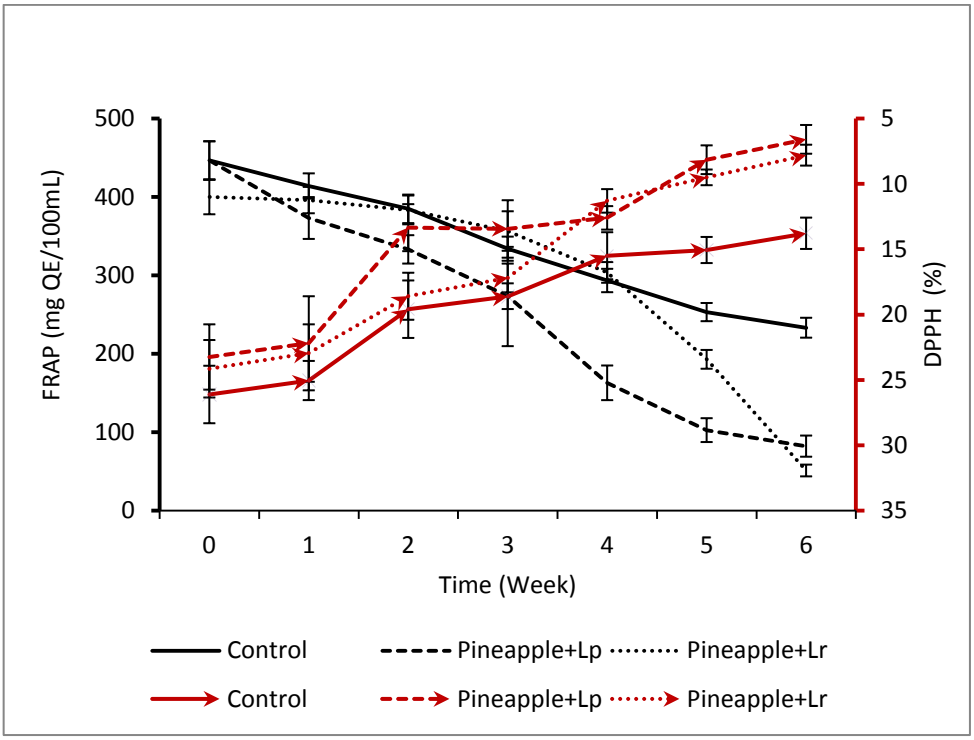


Fig. 3.2. Changes in ferric reducing antioxidant property (FRAP) and DPPH radical scavenging activity of the probiotic pineapple juice during storage at refrigerated condition (4 ±1°C)

3.3.2. HPLC determination of the phenolic acids content in the probiotic juice samples

The phenolic acids detected are given in **Table 3.4**. The following phenolic acids were identified at 254 nm by comparing their known standards in the probiotic fruit juice samples at 0 week and after 6 weeks of storages. Gallic acid (RT=3.23 min), catechin (RT= 11.89 min), chlorogenic acid (RT= 13.54 min), caffeic acid (RT=14.49 min), syringic acid (RT= 14.73 min), ferulic acid (RT= 16.55), coumaric acid (RT= 16.72 min), rutin (RT= 17.31 min), kaempferol (RT= 19.61 min) and quercetin (RT=19.89 min) were detected. The phenolic acids in both probiotic juice samples showed decrease or complete destruction with storage time while, in some cases, an increase or appearance of new phenolic acid originally not detected in the fresh juice was observed. In litchi juice, addition of probiotic bacteria changed the phenolic acid composition as compared to the control one. The HPLC chromatograms (**Fig. 3.3**) showed the presence of gallic acid, catechin, quercetin in both control and fermented litchi juice. On probiotication of the litchi juice with Lp, presence of new phenolic compounds like syringic acid and coumaric acid were detected which were not present in the control juice. Moreover, fermentation of

litchi juice with Lr did not show any major change in the phenolic acid profile. Similar results were also observed in case of pineapple juice with Lr (**Fig. 3.4**). Storage time also affected the phenolic profile of both the probiotic juices by destruction of some phenolic acids and development of new phenolic compounds. In litchi juice, addition of probiotic bacteria caused decrease in gallic acid and quercetin content and increase in catechin and coumaric acid. Similar trend was also observed for gallic acid and ferulic acid in litchi fermented with Lr.

The destruction of phenolics in most of the cases could be due to oxidation of the phenolic acid due to other factors like light and oxygen [22]. Similarly, the increase and detection of new phenolic acids originally absent in the fresh and control samples could be the result of release of the bound phenolics. The phenolic acids comprise of both free and bound phenolic acids. The bound phenolic acids remain bound to some structural carbohydrate and protein either through ester linkage with carboxylic groups or ether linkages with lignin through their hydroxyl groups in the aromatic ring or acetal bonds. [23,24,25,26]. Increase in the content of some phenolic acid and their antioxidant activity after addition of probiotics has been reported by Jaiswal et al. [27]. Similarly, Kusznierewicz et al. [28] and Othman et al. [29] reported an increase in some phenolic acids such as catechin and caffeic acid in some cases after probiocation. They also reported the decrease in the phenolic acid content with application of heating, storage time and storage temperature. This might be the result of the cleavage of the esterified bond between sugar glycoside and phenolic acids.

Another probable reason for increase in phenolic content could be due to degradation and molecular rearrangements of the existing phenolic acids during processing like pasteurization of juices [30]. Application of heat may break these bonds and cause their release due to cell disruption and rupture of the food matrix which in turn facilitates their release in to the liquid medium [30].

Overall, probiotic litchi juice showed good content of ferulic acid. Also, probiotic pineapple juices are rich in gallic acid as well as rutin and coumaric acid. Depending on the juice type and phenolic acid compositions, it was found that addition of probiotic bacteria had both positive as well negative impacts on the phenolic acid composition of the fruit

juice. Moreover, the degradation of large polymeric phenolic by some enzymes of fermented fruit juices could increase the content of total phenolics [31] (Rodríguez et al., 2009). Further investigation of the enzymes related to these biochemical changes would be the focus of future studies.

Even though antioxidant activity lowered on storage, there is formation of new phenolic acids which also have antioxidant properties. Further, even though the quantity of phenolic acids is less, their regular consumption is expected to provide health benefits.

3.3.3. HPLC determination of the organic acids in the probiotic fruit juices

Organic acids play an important role in taste, flavour and consumer acceptance of fruit beverages. The results are shown in **Table 3.5.**

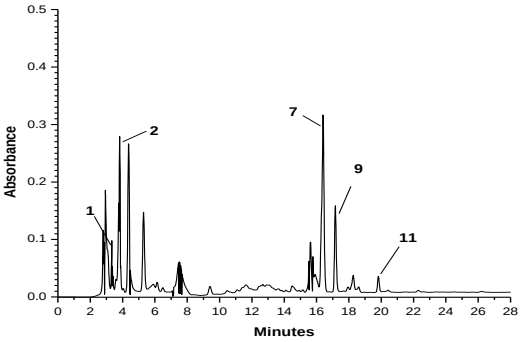
Lactic acid was recognized as the main metabolite produced by both strains of *Lactobacillus*. After 6 weeks of storage, lactic acid concentrations in probiotic litchi plus Lp and litchi plus Lr juices reached 16.8 and 15.2 g/L, respectively. Malic acid in probiotic litchi juice for both strains were completely consumed after 6 weeks. The malolactic fermentation of *Lactobacillus* had been reported in literature [32,33]. After 6 weeks of storage, slight changes in tartaric, acetic and citric acid contents also were observed in probiotic litchi and pineapple juices (**Table 3.5**). In this study, after the storage of 6 weeks at 4±1 °C, both lactic acid contents in fermented litchi and pineapple juices increased significantly, and no significant changes ($p < 0.05$) in the content of tartaric, acetic, and citric acids were observed. The malic acid content was below the detectable limit after fermentation. Similar results were also reported by Zheng et al. [34] in fermented litchi juice using high hydrostatic pressure treatment.

In case of probiotic pineapple juice, increasing trend in lactic acid production was similar to that of litchi juice that reached 46.74 and 51.23 g/L in Lp and Lr fortified pineapple juices, respectively by the end of storage period (**Table 3.5**). No significant change in citric acid, acetic acid and tartaric acid was observed on addition of probiotics but malic acid content decreased significantly when juice was probiocated with LAB. Saradhuldhat et al. [35] and Hong et al. [36] also observed an increase in acid production after fermentation of pineapple juice. There is scant literature on the behaviour of organic acids after addition of probiotics and during storage in fruit juices. Randhawa et al. [37] found that

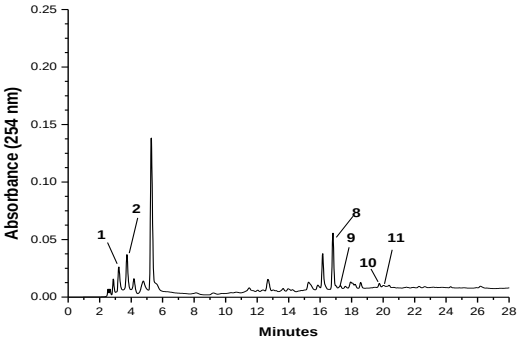
Table 3.4. Phenolic acid content of the probiotic fruit juices stored at refrigerated condition (4 ±1°C) determined by RP-HPLC expressed in mg/100mL

Sample	GA	CTH	CFA	CGA	SA	FA	CMA	RTH	KF	QCT
Litchi										
Co_0week	15.25±0.11	ND	ND	ND	ND	2.15±0.12	ND	3.84±0.14	ND	3.18±0.02
Co_6week	12.45±0.24	4.17±0.08	ND	ND	ND	ND	3.17±0.28	1.49±0.09	1.49±0.05	2.63±0.17
Lp_0week	20.97±0.14	ND	ND	ND	1.16±0.10	ND	ND	ND	0.82±0.02	0.78±0.04
Lp_6week	17.24±0.17	6.77±0.12	ND	ND	ND	ND	5.77±0.12	ND	ND	1.08±0.03
Lr_0week	21.01±0.05	ND	ND	ND	ND	20.08±0.08	ND	ND	ND	ND
Lr_6week	16.18±0.04	6.98±0.04	ND	ND	ND	13.51±0.05	ND	ND	ND	ND
Pineapple										
Co_0week	12.50±0.02	ND	ND	ND	1.12±0.15	0.91±0.07	1.09±0.04	2.12±0.16	2.44±0.06	1.18±0.07
Co_6week	22.61±0.11	1.13±0.02	1.05±0.04	ND	ND	ND	1.13±0.02	ND	ND	ND
Lp_0 week	40.72±0.14	ND	ND	ND	3.86±0.11	ND	0.94±0.03	ND	ND	ND
Lp_6week	15.45±0.07	0.85±0.01	ND	ND	1.78±0.07	ND	0.73±0.11	ND	ND	ND
Lr_0week	19.65±0.23	ND	1.23±0.07	ND	ND	ND	ND	ND	ND	0.55±0.04
Lr_6week	28.91±0.03	1.08±0.10	1.46±0.02	ND	ND	ND	ND	ND	ND	0.46±0.02

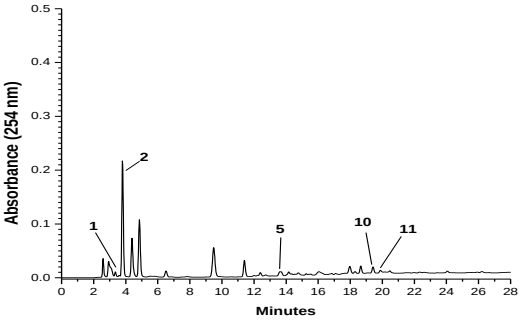
Results (mg/100 mL) are mean ±S.D of triplicate values; Co: Control; Lp: *L. plantarum* and Lr: *L. rhamnosus*; ND: Contents below the detection limit [GA- gallic acid; CTH- catechin; CGA-chlorogenic acid; CFA- caffeic acid; SA- syringic acid; FA- ferulic acid; CMA- coumaric acid; RTH- rutin hydrate; KF- kaempferol; QCT- quercetin]



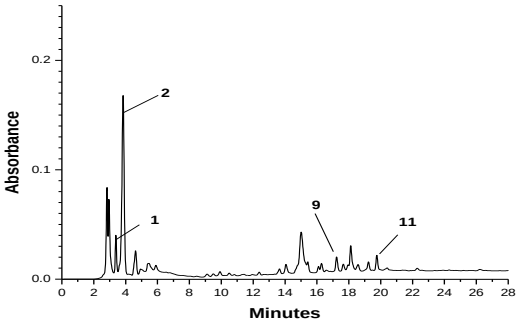
(a) Litchi Juice (Control) at 0 week



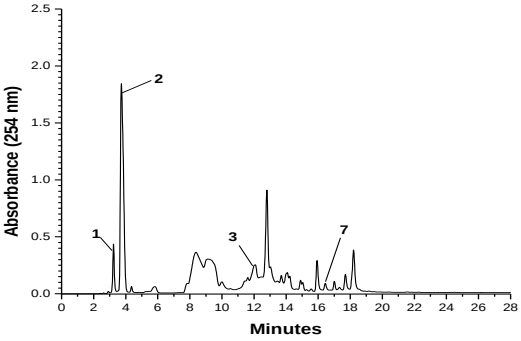
(b) Litchi Juice (Control)) after 6 weeks



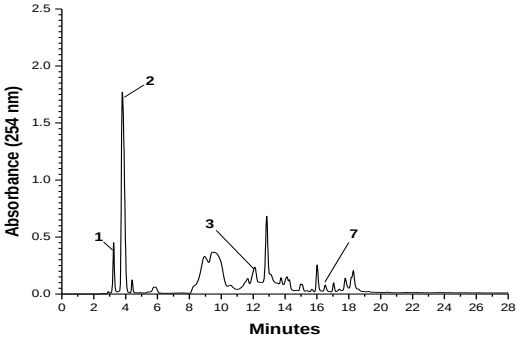
(c) Litchi juice + Lp at 0 week



(d) Litchi juice + Lp after 6 weeks



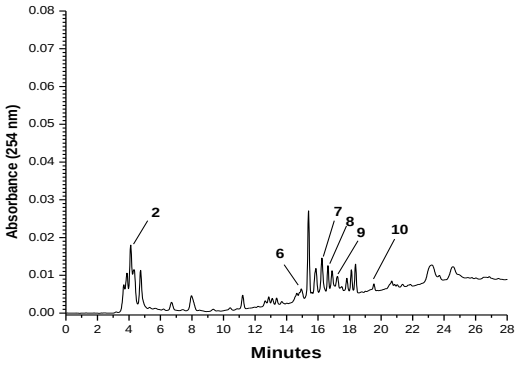
(e) Litchi juice + Lr at 0 week



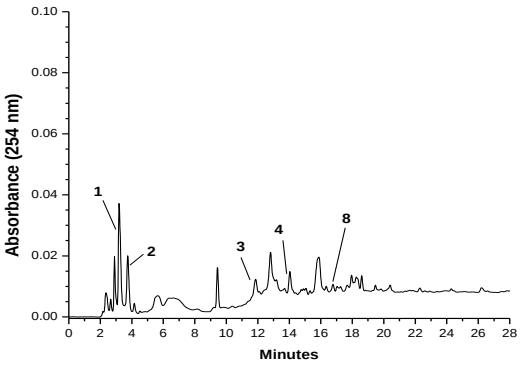
(f) Litchi juice + Lr after 6 weeks

Fig. 3.3. RP-HPLC chromatogram of the phenolic compounds in probiotic litchi juice with *L. plantarum* (Lp) and *L. rhamnosus* (Lr)

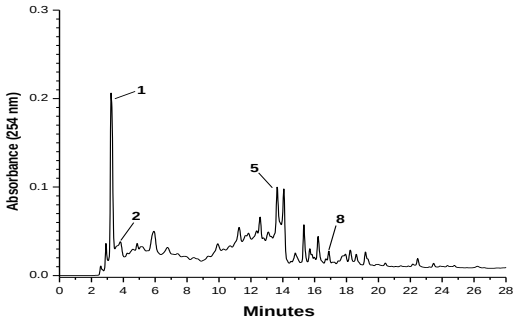
[1=gallic acid (GA); 2= ascorbic acid (AA); 3= catechin (CHT); 4= caffeic acid (CFA); 5=chlorogenic acid (CGA); 6=syringic acid (SA); 7= ferulic acid (FA); 8= coumaric acid (CMA) ; 9= rutin hydrate (RTH); 10= kaempferol(KF) and 11= quercetin (QCT)]



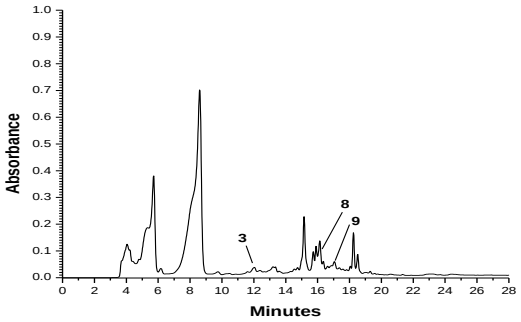
(a) Pineapple juice (Control) at 0 week



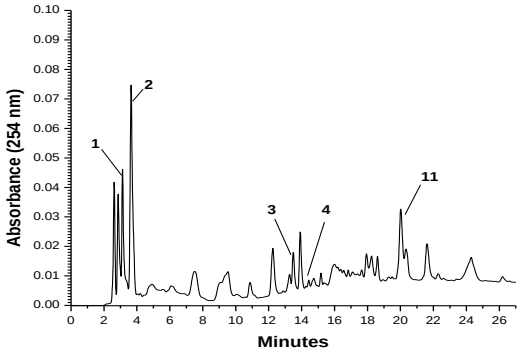
(b) Pineapple juice (Control) after 6 weeks



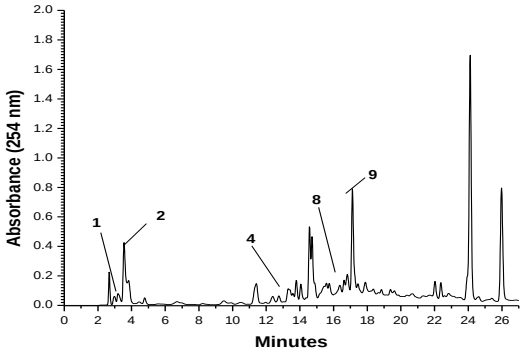
(c) Pineapple juice + Lp at 0 week



(d) Pineapple juice + Lp after 6 weeks



(e) Pineapple juice + Lr at 0 week



(f) Pineapple juice + Lr after 6 weeks storage

Fig. 3.4. RP-HPLC chromatogram of the phenolic compounds in probiotic pineapple juice with *L. plantarum* (Lp) and *L. rhamnosus* (Lr)

[1=gallic acid (GA); 2= ascorbic acid (AA); 3= catechin (CHT); 4= caffeic acid (CFA); 5=chlorogenic acid (CGA); 6=syringic acid (SA); 7= ferulic acid (FA); 8= coumaric acid (CMA) ; 9= rutin hydrate (RTH); 10= kaempferol(KF) and 11= quercetin (QCT)]

citric acid contents decreased and malic acid contents increased in fermented citrus juices throughout the storage period.

It is observed that the change in the % TA (Chapter II) has a positive correlation with the change of organic acids as determined by HPLC. The production of the lactic acid due to fermentation of sugar has major contribution to increase in %TA followed by citric acid and acetic acid. Tartaric acid and malic acid do not increase much to have an effect on the acidity of the fermented juice during storage.

Table 3.5. Organic acid content of the probiotic fruit juices stored at refrigerated condition (4 ±1°C) determined by RP-HPLC expressed in g/L

	Organic acid (g/L)				
Sample	**Citric acid**	**Acetic acid**	**Tartaric acid**	**Malic acid**	**Lactic acid**
Litchi					
Co_0week	4.15^{a}±0.06	4.65^{a}±0.28	0.55^{a}±0.12	3.65^{b}±0.21	N.D.^{a}
Co_6week	4.65^{a}±0.21	4.41^{a}±0.13	0.50^{a}±0.10	3.54^{b}±0.14	N.D.^{a}
Lp_0week	4.25^{a}±0.13	4.55^{a}±0.21	0.53^{a}±0.22	3.56^{b}±0.24	2.12^{b}±0.24
Lp_6week	5.98^{b}±0.25	4.18^{a}±0.09	0.48^{a}±0.21	N.D.^{a}	15.20^{c}±0.29
Lr_0week	4.58^{a}±0.14	4.60^{a}±0.15	0.54^{a}±0.15	3.62^{b}±0.15	3.16^{b}±0.15
Lr_6week	6.12^{b}±0.13	4.08^{a}±0.01	0.47^{a}±0.09	N.D.^{a}	16.80^{c}±0.16
Pineapple					
Co_0week	5.18^{a}±0.08	4.72^{a}±0.12	0.57^{a}±0.04	2.76^{c}±0.11	N.D.^{a}
Co_6week	5.23^{a}±0.18	5.07^{a}±0.09	0.42^{b}±0.30	2.41^{c}±0.21	N.D.^{a}
Lp_0 week	5.70^{a}±0.15	5.43^{b}±0.02	0.55^{a}±0.13	1.68^{b}±0.19	10.68^{b}±0.09
Lp_6week	7.65^{b}±0.10	5.66^{b}±0.12	0.45^{b}±0.18	0.14^{a}±0.02	46.74^{c}±0.27
Lr_0week	5.70^{a}±0.07	5.45^{b}±0.10	0.68^{a}±0.06	1.25^{b}±0.08	13.25^{b}±0.08
Lr_6week	6.23^{b}±0.22	5.62^{b}±0.16	0.56^{a}±0.15	0.23^{a}±0.06	51.23^{b}±0.26

N.D. Contents below the detection limit. $^{a, b, c, d}$ Different letters represented a significant difference within the same column ($p < 0.05$).

3.3.4. Mineral analysis of the probiotic fruit juice

Minerals are directly and/or indirectly involved in all aspects of microbial growth, metabolism and differentiation [38] Metals and their compounds interact with microbes in various ways depending on the metal species, organism and environment, while structural

components and metabolic activity also influence metal speciation [38]. The mineral composition of the probiotic litchi and pineapple juices is shown in **Table 3.6.**

Table 3.6. Comparative changes in mineral elements of the probiotic fruit juices during storage (4±1°C)

Sample	Minerals (mg/L)							
	Macroelements				Microelements			
	Na	K	Ca	Mg	Fe	Cu	Zn	Mn
Litchi								
Co_0week	6.8	172.4	4.7	11.5	0.43	0.20	0.16	0.07
Co_6week	6.1	169.6	4.1	13.1	0.36	0.17	0.26	0.05
Lp_0week	7.9	140.2	4.9	15.5	0.41	0.23	0.28	0.11
Lp_6week	7.7	143.3	4.3	13.9	0.33	0.17	0.19	0.06
Lr_0week	4.5	180.6	4.5	16.2	0.38	0.20	0.26	0.10
Lr_6week	3.1	178.7	4.1	13.7	0.28	0.18	0.24	0.05
Pineapple								
Co_0week	6.3	210.4	7.6	15.4	0.49	0.18	0.26	0.38
Co_6week	5.7	174.5	6.8	16.6	0.44	0.17	0.25	0.30
Lp_0 week	8.2	209.0	8.7	17.2	0.53	0.17	0.22	0.19
Lp_6week	6.5	139.2	8.4	16.2	0.41	0.17	0.21	0.07
Lr_0week	6.2	197.6	8.6	13.3	0.42	0.20	0.23	0.16
Lr_6week	5.5	133.5	7.7	12.4	0.40	0.18	0.18	0.12

Potassium was the main macroelement in both probiotic litchi and pineapple juices. There was slight change in the K content of the probiotic fruit juice than the unfermented one. The change in K was also seen with storage period. The K content of the non-fermented litchi and pineapple juices was 172 and 210 mg/L which decreased to 169 and 174 mg/L, respectively**.** After 6 weeks of storage, decrease in K was observed in probiotic pineapple juice. The fall in K content during storage was found in pineapple juice fortified with Lp (209 to 139.2 mg/L**)** than Lr (197.6 to 133.5 mg/L). No major change was observed in case of Ca, Mg, Cu and Mn during the storage period across the juices. Addition of probiotics and storage period did not significantly affect mineral content of the fruit juice. Moreover, refrigerated storage at 4 ^{o}C did not produce any noticeable change in the mineral profile of the probiotic fruit juices, as would be expected given the applied

conditions, meaning that when consumers ingest chilled probiotic fruit juice they receive the same total intake of minerals as in untreated beverage.

3.5. Conclusions

Significant changes occurred in TPC on probiotication of litchi and pineapple juices. Litchi juice with *L. plantarum* showed higher TPC and FRAP activity than with *L.rhamnosus*. Most of the phenolic compounds decreased after incorporation of the probiotics. Phenolic acids like rutin hydrate and kaempferol which were present in normal litchi and pineapple juices reduced to detectable quantities on long storage. Gallic acid and catechin were found in higher quantities in fermented juices whereas the quercetin quantity reduced. Probiotication increased the lactic acid in both juices; the content was higher in probiotic pineapple juice. Content of malic acid in litchi juice decreased while citric acid increased with time on probiotication. Addition of probiotics and storage at refrigerated condition did not significantly affect mineral content of the fruit juice. Therefore, it can be inferred that addition of probiotics has both positive and negative impact on the phytochemical and antioxidant properties litchi and pineapple juices.

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