VIABILITY AND STABILITY OF PROBIOTICS IN FRUIT JUICES DURING STORAGE

2.1. Introduction

Foods are consumed with the aim to satisfy hunger and provide the necessary nutrients for humans, promote a state of physical and mental well-being, improve health, prevent and/or reducing diet-related diseases. Consumers are increasingly becoming aware of the relationship between food and health which has led to an explosion of interest in "healthy foods"; this phenomenon could be partly attributed to the increasing cost of healthcare, the steady increase in life expectancy, the increase in population with diet-related chronic diseases, and the desire of older people for an improved quality of their later years [1].

Nowadays, healthy foods mean "functional foods", and functional foods are those containing or prepared with bioactive compounds, such as dietary fibre, oligosaccharides, and active "friendly" bacteria that promote the equilibrium of intestinal bacterial strains. Probiotics belong to an emerging generation of active ingredients, which includes prebiotics, phytonutrients, and lipids [2]. The label "functional food" was introduced in 1980 in Japan, which was the first country that stated a specific regulatory approval process for functional foods, known as Foods for Specified Health Use (FOSHU) [3]. On the other hand, in Europe, the interest in functional foods started in the 1990s, when the European Commission created a commission called Functional Food Science in Europe (FuFoSE) to explore the concept of functional foods through a science-based approach [4]. The market of functional foods is characterized by an increasing trend, and some researchers reported that probiotic foods represent around 60%–70% of functional foods [5].

The word probiotic comes from the Greek word "προ-βίος" that means "for life"; thus, probiotics are live microorganisms (mainly bacteria but also yeasts) that confer a beneficial effect on the host if administered in proper amounts [6]. 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. 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. Moreover, they are usually referred to as healthy foods, designed for young and old people [7]. Many authors reported on the effects of juices on health; for example, Sutton [8] demonstrated that aqueous extracts of kiwifruit and avocado had very low cytotoxicity and high anti-inflammatory activity in a Crohn's gene-specific assay. Non-aqueous extracts of kiwifruit, blueberry and avocado had similarly high anti-inflammatory activity, with slightly higher cytotoxicity than the aqueous extracts. Fenech *et al.* [9] studied the effect of the intake of nine micronutrients (vitamin E, calcium, folate, retinol, nicotin acid, β-carotene, riboflavin, pantothenic acid and biotin) on genome damage and repair; these compounds can be easily found in juices. Furthermore, fruit juices have shown negative effects on some pathogenic microorganisms, while improving the growth of probiotics because berries, such as blueberry, blackberry and raspberry, possess antimicrobial effects towards many pathogens [10].

Therefore, juice fortification with probiotic microorganisms is a challenge and a frontier goal, as juices could combine nutritional effects with the added value of a healthy benefit from a probiotic. Maintaining the viability (the recent trend is to have one billion viable cells per portion—*i.e.*, 100 g of product) and the activity of probiotics in foods till the end of shelf-life are two important criteria to be fulfilled in juices, where low pH represents a drawback. The most commonly used probiotic bacterial genera are *Lactobacillus* and *Bifidobacterium*, while *Saccharomyces cerevisiae* var. *boulardii* is the yeast used and these serve as probiotics both in dairy and non-dairy functional foods [11]. Several strains of *L. plantarum, L. rhamonsus, L. acidophilus* and *L. casei* can grow in fruit matrices due to their tolerance to acidic environments [12].

Assam, the North- Eastern state of India grows a variety of fruits. As composition and biochemical activity differs according to climatic conditions, it is imperative that fruits

grown in Assam are also studied for suitability as probiotic juice. The viability and stability of probiotic bacteria in juices from fruits of Assam are not reported. Therefore, based on the above aspects, a study was carried out to examine the viability of some established probiotic strains of *Lactobacillus* to make probiotic juices from four fruits of Assam, and determine the suitability of these fruit juices for production of probiotic juices.

2.2. Materials and Methods

All the chemicals used were of analytical grade and supplied by Merck, India and Himedia Laboratories and Sigma chemicals, India.

2.2.1. *Lactobacillus* strains

Three *Lactobacillus* isolates were collected from Microbial Type Culture Collection and Gene Bank (MTCC) (IMTECH, CSIR, Chandigarh, India).

(i) *Lactobacillus plantarum* MTCC2621 (Lp)

(ii) *Lactobacillus rhamnosus* MTCC1408 (Lr)

(iii) *Lactobacillus acidophilus* MTCC447 (La)

2.2.2. Fruit samples

Four different fruits viz. litchi (*Litchi chinensis* Sonn.), pineapple (*Ananas comosus* L. Merr), guava (*Psidium guajava*), Khasi mandarin orange (*Citrus reticulate* Blanco) were procured from the local fruit market, Tezpur, Assam during the season.

2.2.3. Inoculum preparation

The freeze dried *Lactobacillus* isolates, *Lactobacillus plantarum* MTCC2621, *L. rhamnosus* MTCC1480, and *L. acidophilus* MTCC447 were coded as Lp, Lr and La, respectively. The freeze dried cultures were activated in sterile glycerol (50% v/v). The glycerol stock culture was stored at -20 ^{o}C in sterile screw cap tubes. The cultures were grown at 37 ^{o}C for 24 h in sterile de Man Rogosa and Sharp (MRS) broth (dextrose 20.0 g/L; meat peptone 10.0 g/L; beef extract 10.0 g/L; yeast extract 5.0 g/L; sodium acetate 5.0 g/L; disodium phosphate 2.0 g/L; ammonium citrate 2.0 g/L; tween 80 1.0 g/L; magnesium sulfate 0.1 g/L, manganese sulfate 0.05 g/L) 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 sodium chloride) to remove any residual MRS.

2.2.4. Characterization of probiotic cultures

Preliminary probiotic properties such as acid and bile tolerance and antibiotic sensitivity were studied [13].

(a) Acid tolerance assay

A five mL sample of cultures was taken during the late log phase and centrifuged at 3500 rpm for 1 to 2 min and this step was repeated again with neutral phosphate buffer saline (pH 7.2, PBS). The supernatant was decanted and the pellet was resuspended in phosphate buffer saline (PBS) at either pH 1.5 or 3 separately. The buffer was added until the turbidity of the solution was reached. One hundred µL of this solution was inoculated on MRS agar at 0^{th}, 2^{nd} and 4^{th} h of incubation without any dilution. Both the cultures were tested individually at both the pH values. CFUs (colony forming unit) were enumerated after 36-72 h of incubation at 37°C using formula given in Eq 2.1.

$$\text{Log (CFU/mL)} = \text{Log } \{(\text{Number of colonies} \times \text{Dilution factor})/0.1\} \qquad \textbf{Eq.2.1}$$

(b) Bile tolerance assay

Five mL of cultures were taken at the late log phase and centrifuged at 3500 rpm for 1 to 2 min and this step was repeated again with neutral PBS (pH 7.2). The supernatant was decanted and the pellet was resuspended in 10 mL of 0.3% Oxgall prepared in MRS. One hundred µL of this mixture was spread on MRS agar at 0^{th}, 2^{nd} and 4^{th} hour without any dilution. CFUs were enumerated after 36-72 h of incubation at 37°C using Eq 2.1.

(c) Antibiotic sensitivity test

Antibiotic disks diffusion method was used to assess the antibiotic sensitivity of the *Lactobacilli*. The MRS agar plate was spread with 100 µL active late log phase culture and antibiotic disks were placed on the agar with sterile forceps. To diversify the selection of different classes of antibiotics, six different broad spectrum standard antibiotics were used. Antibiotics that are commonly used to treat various infections in humans were used: Ampicillin (10 µg), chloramphenicol (30 µg), penicillin G (10 units), streptomycin (10 µg), sulfamethoxazole-trimethoprim (25 µg), and tetracycline (30 µg). The diameters of zones of inhibitions were measured and compared with previous data from literature to classify them as resistant, intermediate or sensitive [14].

2.2.5. Preparation of probiotic juice

Four different fruits viz. litchi (*Litchi chinensis* Sonn.), pineapple (*Ananas comosus* L. Merr**),** guava (*Psidium guajava*), Khasi mandarin orange (*Citrus reticulate* Blanco) were procured from the local fruit market, Tezpur, Assam during the season. 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. Juice was extracted using a household juicer (Make: Philips) and mixed thoroughly and before exposed to pasteurization. The quantity of juice recovered from 1 Kg of fruit was, litchi: 180 mL, orange: 335 mL, pineapple: 278 mL, guava: 165 mL. No enzyme was used during the extraction to keep the juice natural as much as possible. The juice was strained through a muslin cloth and pasteurized at 90 ^{o}C for 1 min with constant stirring [35]. This temperature time combination was able to give 5-log reduction of *Lactococcus plantarum* as per FDA guidelines [36]. Pasteurized juice in 100 mL lots were taken into sterile Erlenmeyer flasks was inoculated with 1% culture each of Lp, Lr and La under aseptic conditions. 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 stored at refrigerated condition (4 ±1°C) for shelf life study.

2.2.6. Enumeration of bacteria

The enumeration of free probiotic cells was performed using method described by Yoon et al. [15] and expressed in CFU/mL (colony forming unit). Enumeration of the probiotic bacteria in fruit juice was performed using same formula presented in Eq 2.1. on weekly basis over a period of 6 weeks, using MRS agar after incubation at 37 °C for 24 h under aerobic conditions.

2.2.7. Biochemical analysis of the probiotic fruit juice during storage

At an interval of 7 days, 10 mL of juice was taken out from each flask and tested for biochemical parameters viz. pH, titratable acidity and total soluble sugar and color change. The pH of each juice sample was measured in a pH meter (Eutech, Germany) after proper calibration. Titratable acidity, expressed as g lactic acid/100 g was determined by titration against 0.1N sodium hydroxide using phenolphthalein as an end point indicator. The total soluble sugar was estimated in terms of °Brix by hand held refractometer (Erma, Japan). All experiments were performed in triplicate to determine mean and standard error of the mean.

2.2.8. Colour analysis

The colour of the fermented litchi juice was determined by a Hunter ColorLab UltraScan-Vis colorimeter (USA). The colorimeter was calibrated and measurements were made through a 0.375 inch port/ viewing area. The reflectance instruments determined three colour parameters: lightness (*L*), redness (*a*), and yellowness (*b*). Numerical values of *L* (light/dark), *a* (*a*+ redness/green *a*-) and *b* (*b* + yellowness/blueness *b* -) were converted into ΔE (total colour difference) which was calculated using the equation at **Eq 2.2**.

$$\Delta E = \{(\Delta L^2) + (\Delta a^2) + (\Delta b^2)\}^{1/2} \qquad \textbf{Eq.2.2}$$

0.2.2.9. Sensory evaluation

Sensory attributes of colour, flavour, taste, odor, mouth feel, after taste and over all acceptability of probiotic juices were evaluated by a trained panel of 10 members. A 9-point Hedonic scale reading (1-4 dislike extremely to dislike slightly, 5-neither like nor dislike, 6-9 like slightly to like extremely) was performed on day 28 of product storage.

2.2.10. Statistical analysis

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

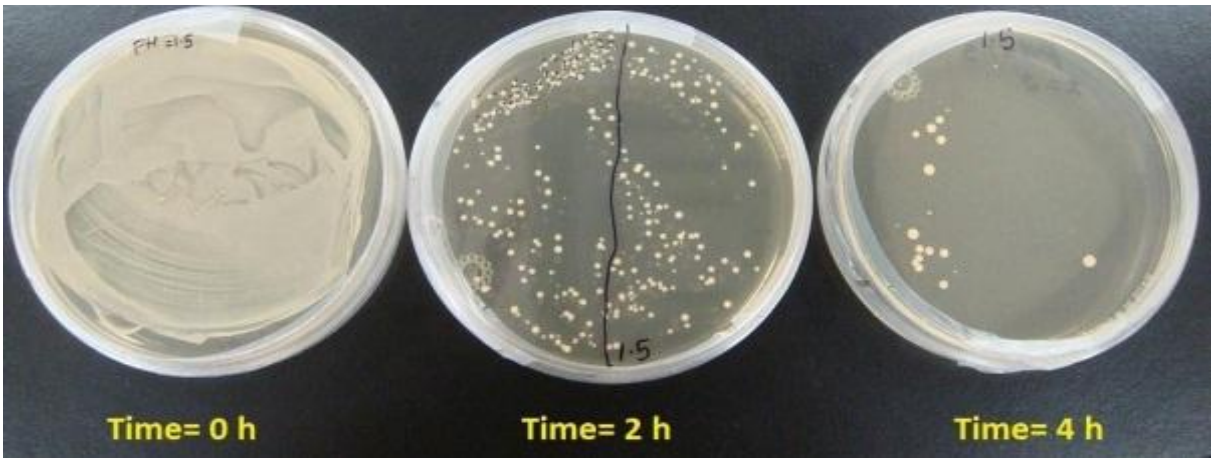
2.3. Results and Discussion

2.3.1. Characterization of probiotic cultures

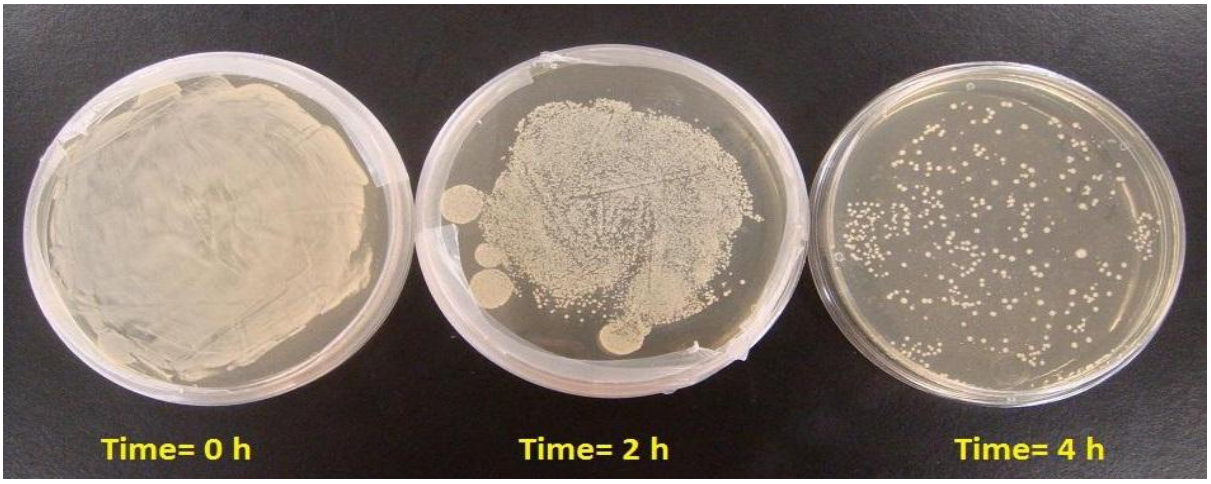
The growth characteristics for each bacterial species were measured and preliminary probiotic tests were done.

(a) Acid tolerance assay

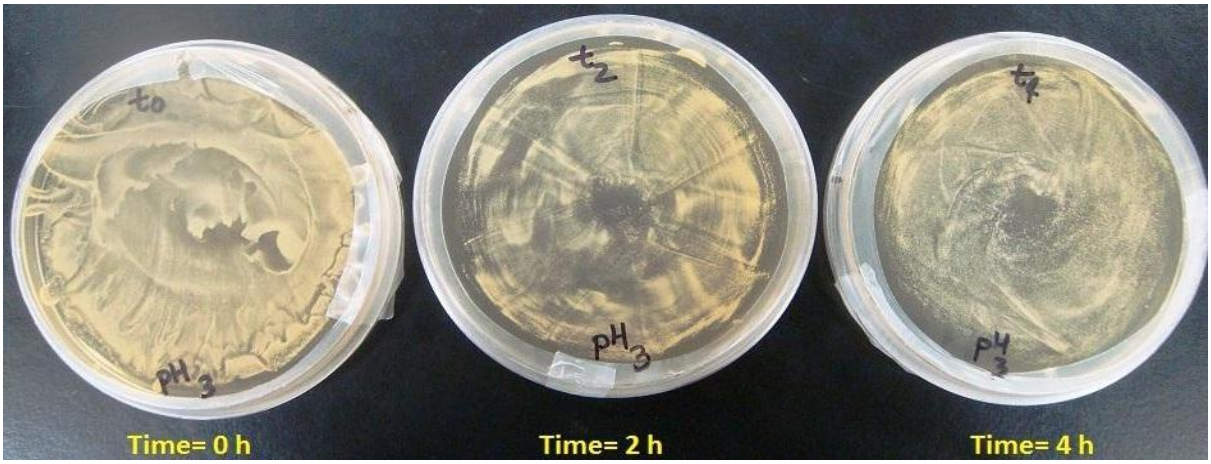
The pH of stomach, due to gastric juice is usually between 2 to 3 and during fasting it decreases to as low as 1.5 [13]. Most fresh meal diets around the world comprise many acidic foods such as fruits, vegetables and fermented dairy products. So after ingestion into the gastro intestinal tract the probiotics must be able to withstand the high acidic environment and still retain the function for which they are consumed. All the three species of *Lactobacillus* showed tolerance at pH 1.5 although the bacterial density was reduced by the end of 4 h (**Fig 2.1**). At pH 3, incubation of species produced a dense lawn growth on MRS agar plates indicating their resistance to acid. Since similar growth pattern was observed for all the time intervals, images of plates at pH 1.5 for *L. plantarum* and *L. rhamnosus* only and at pH 3 for *L. acidophilus* only are shown in **Fig 2.1**.



(a) *L. plantarum*



(b) *L. rhamnosus*



(c) *L. acidophilus*

Fig. 2.1. Acid tolerance test at different time intervals of (a) *L. plantarum* at pH 1.5, (b) *L. rhamnosus* at pH 1.5, and (c) *L. acidophilus* at pH 3.0.

Acid tolerance is supposed to be mediated by membrane H+-ATPases although there may be other uncharacterized proteins involved [15]. Acids passively diffuse through the cell

membrane to enter the cytoplasm and dissociate into protons. This affects the transmembrane pH and proton motive force. It may also reduce the activity of enzymes and denature proteins and DNA [16].

(b) Bile tolerance assay

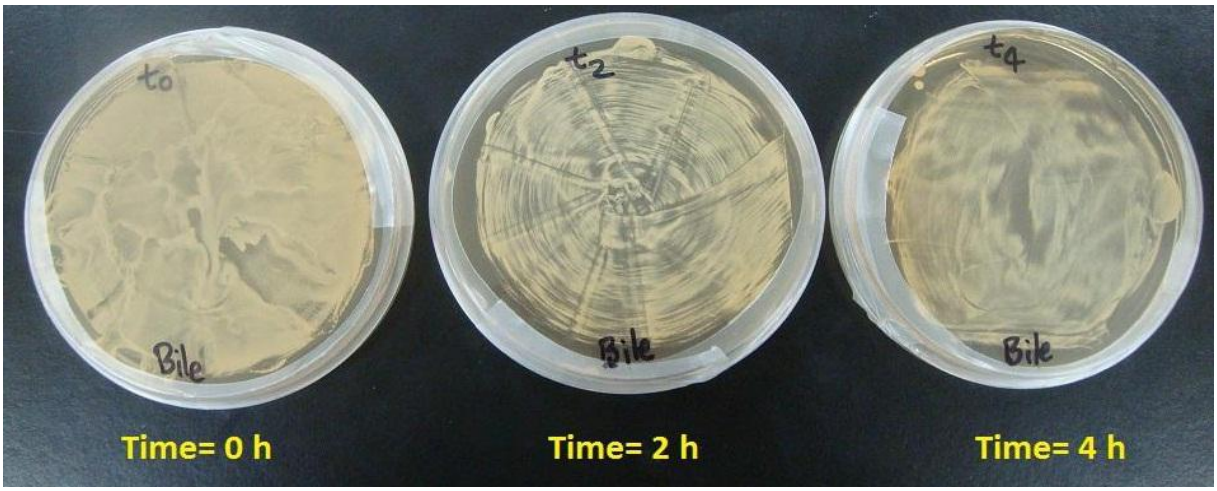
The dense lawn culture growth on MRS agar plates, as seen in **Fig 2.2**, indicated that all the cultures were strongly resistant to the bile salt Oxgall (0.3%) even after 4 h of exposure. Bile salts act as detergents and antimicrobial agents and disassemble the biological membranes. However *Lactobacilli* and *Bifidobacteria* are able to metabolize bile salts into amino acids and steroid derivatives by hydrolysis.

This notably reduces the bile's solubility at low pH as well as its detergent property and hence permits a better survival. But the actual physiological response, regulatory pathways and molecular mechanisms are still obscure [16].

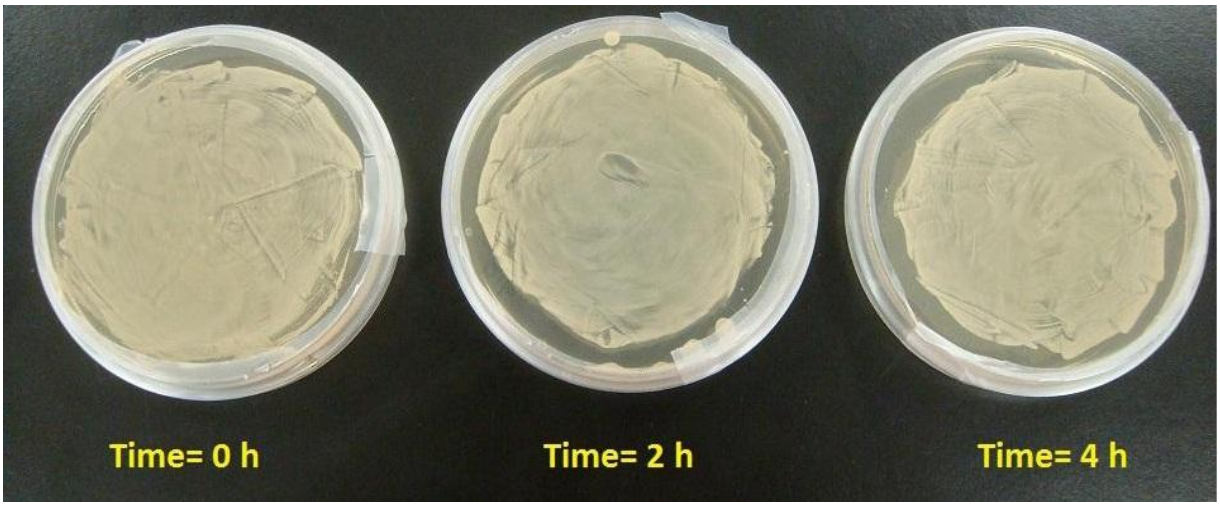
(c) Antibiotic sensitivity assay

Human large intestine contains more than 10^{11} bacterial cells as native microflora with more than 500 different species of bacteria (mostly facultative anaerobes like *Enterobacteria, Coliforms, Lactobacillus,* etc.) [17,18]. When patients are under an antibiotic treatment there is a reduction in the microflora numbers. So it is critical that the probiotics are able to withstand these antibiotics for their sustained growth. Antibiotic resistance genes may be acquired due to continuous exposure to antibiotics especially in irritable bowel syndrome (IBS) patients under long term antibiotic therapy. Hence the *Lactobacilli* were tested and compared for their survival against selected common broad spectrum antibiotics and the observations are presented in **Table 2.1.**

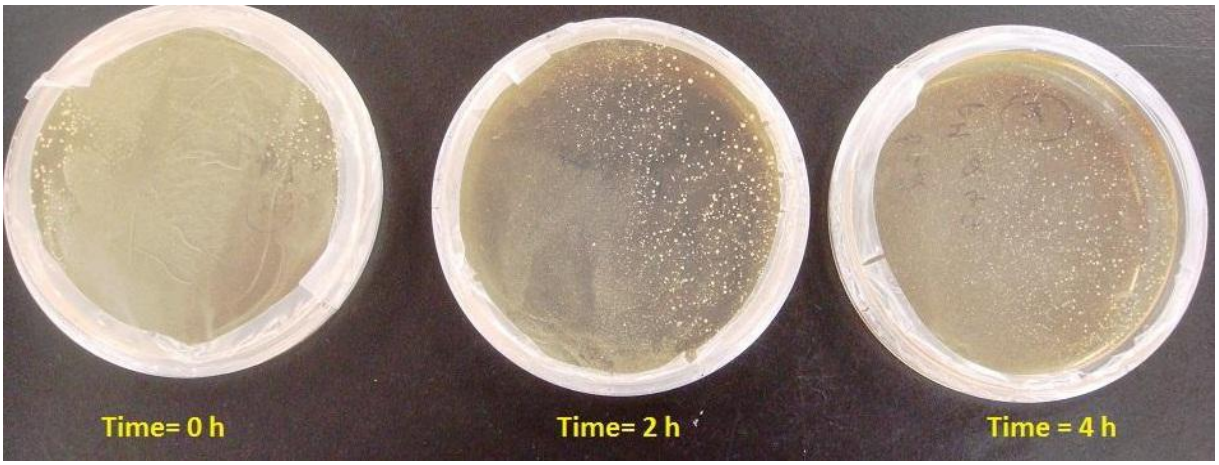
Table 2.1 reveals that the studied *Lactobacillus* strains varied in their sensitivity to antibiotics. *L. acidophilus* was more sensitive than *L. plantarum* and *L. rhamnosus* to some antibiotics (such as streptomycin and ampicillin). Similarly, *L. plantarum* and *L. rhamnosus* were more sensitive to penicillin G than *L. acidophilus*. All the three strains were sensitive to tetracycline and were resistant to sulfamethoxazole-trimethoprim.



(a) *L. plantarum*



(b) *L. rhamnosus*



(c) *L. acidophilus*

Fig. 2.2. Bile tolerance (0.3% Oxgall) of (a) *L. plantarum* (b) *L. rhamnosus* and (c) *L. acidophilus*

Table 2.1. Antibiotic sensitivity profiles of *L. plantarum, L. rhamnosus and L. acidophilus*

Antibiotic	Sensitivity		
	L. plantarum **MTCC2621**	***L. rhamnosus*** **MTCC1408**	***L. acidophilus*** **MTCC447**
Tetracycline	S	S	S
Streptomycin	I	R	S
Penicillin G	S	S	I
Ampicillin	R	I	I
Chloramphenicol	S	S	S
Sulfamethoxazole-Trimethoprim	R	R	R

* S- Sensitive; I- Intermediate; R- Resistant

Since it is well known that the antibiotic resistance genes may be transferred to the host microbes it is essential to study this property. It has been well documented that DNA, ribosome and enzyme melt-down/denaturation occur when the microbes are subjected to high temperature and thus it is expected that the antibiotic resistance might get altered as well [19]. The molecular physiology involved in changing the antibiotic profile due to heat was not assessed as it is beyond the scope of the current study.

2.3.2. Enumeration of bacteria

In order for probiotics to stay alive in the unfavourable conditions of the gastrointestinal tract and reach the intestine in adequate numbers, they must to be present at a concentration of at least 10^7 CFU/mL in the product at the end of shelf-life; this, in the case of fruit juices, corresponds to approximately 10^9 CFU per portion [20]. The bacterial counts of the three strains in probiotic litchi and pineapple juices kept under refrigerated condition are given in **Table 2.2**. The enumeration was done for six weeks against control (pasteurised juice). There was no bacterial colony in the control juice up to six weeks. All the three strains showed good viability up to four weeks in all four juices under refrigerated condition. *L. plantarum* (Lp) and *L. rhamnosus* (Lr) maintained the required CFU/mL upto six weeks in cold storage (**Fig 2.3**). At 0 week the bacterial count of Lp and Lr were 9.5 and 9.1 log CFU/mL in litchi juice which reduced to 8.1 and 8.0 log CFU/mL after six weeks whereas the cell count of La was found below 10 CFU/mL after 4 week in

litchi juice. The reduction of bacterial count of La may be due to presence of secondary metabolites and low pH of the fruit juice. Lp and Lr showed better viability up to 6 weeks compared to La in litchi and pineapple juices. The population of probiotic Lp, Lr and La in orange and guava juices is given in **Table 2.3**. The bacterial count of Lp and Lr was 9.1 and 8.7 log CFU/mL in orange juice at 0 week which was well maintained up to six weeks of storage. On the other hand La had survived up to 4 weeks only. Similar trend was also found in probiotic pineapple juice. The results obtained in this study were in agreement with the results of the study by Sheehan et al. [20], which showed that probiotic *Lactobacillus* and *Bifidobacterium* strains survived better in pineapple juice than in cranberry juice. The cell counts of Lp and Lr in guava juice were 8.8 and 8.5 log CFU/mL at 0 week. The number of bacteria slowly reduced and reached 6.2 and 5.9 log CFU/mL after 5 weeks of storage. The count of L. acidophilus was found to be 8.4 log CFU/mL at 0 week and after that there was sharp decline of the viability that reached below 10 CFU/mL after 4 weeks. Studies were carried out to explore the suitability of juices such as tomato, beetroot and cabbage juices as raw materials for the production of probiotic drinks using L. plantarum, L. acidophilus and L. casei as probiotic bacteria cultures [21, 22]. From the above results it was observed that Lp and Lr grew well as compared to La in all four fruit juices and maintained required cell counts up to 6 weeks. The lesser growth of La may be attributed to the decrease in the pH of the medium and accumulation of lactic acid, diacetyl, and acetaldehyde that causes viability loss of the La. This may be attributed to decrease in the pH of the medium and accumulation of lactic acid, diacetyl, and acetaldehyde from the growth and fermentation are the main factors for viability loss of the probiotics [31]. Results also revealed that litchi and pineapple were more suitable as carriers of the probiotic bacteria than orange and guava. Similar results have been reported by previous researchers, [23] who observed that cells survived well in litchi, blackcurrant and pineapple juices, which can be attributed to the high pH of these juices [24]. Both *L. plantarum* and *L. rhamnosus* were capable of surviving the low pH and high acidic conditions in fermented cabbage juice during cold storage at 4 ^{o}C and also grew well in cabbage juice at 30 ^{o}C with a viable cell count of $10x10^{8}$ CFU/mL after 48 h of fermentation at 30 ^{o}C [25]. Besides this, several other factors that could affect the survival and growth of the probiotics, e.g. accumulation of metabolic end products such as lactic acid and other organic acids, diacetyl, acetyladehyde, acetoin etc., would reduce cell viability in the product [32].

Table 2.2. Enumeration of three strains of *Lactobacillus* (log CFU/mL) in probiotic litchi and pineapple juices during storage at refrigerated condition (4 ± 1°C)

Week	Log CFU/mL							
	Litchi				Pineapple			
	Control	Lp	Lr	La	Control	Lp	Lr	La
0	ND	9.5±0.3^{f}	9.1±0.4^{d}	8.9±0.6^{d}	ND	9.5±0.3^{e}	9.1±0.4^{e}	8.4±0.4^{d}
1	ND	9.1±0.5^{e}	9.0±0.2^{d}	7.0±0.7^{c}	ND	9.3±0.7^{d}	9.2±0.7^{f}	7.1±0.2^{c}
2	ND	8.8±0.6^{d}	8.7±0.2^{c}	6.2±0.2^{b}	ND	8.7±0.5^{c}	8.8±0.2^{d}	6.8±0.7^{b}
3	ND	8.5±0.5^{c}	8.5±0.1^{b}	3.4±0.2^{a}	ND	8.5±0.4^{b}	8.7±0.6^{d}	3.2±0.4^{a}
4	ND	8.3±0.7^{b}	8.3±0.3^{a}	<10CFU/mL	ND	8.3±0.2^{b}	8.2±0.2^{c}	<10CFU/mL
5	ND	8.2±0.4^{a}	8.1±0.6^{a}	<10CFU/mL	ND	8.3±0.4^{b}	7.8±0.4^{b}	<10CFU/mL
6	ND	8.1±0.1^{a}	8.0±0.4^{a}	<10CFU/mL	ND	8.2±0.3^{a}	7.5±0.4^{a}	<10CFU/mL

Results are mean ±S.D of triplicates. Same letter within a row means no significant difference at p≤0.05 by DMRT.
ND: Not detectable, Lp: L. plantarum, Lr: L. rhamnosus and La: L. acidophilus

Table 2.3. Enumeration of three strains of *Lactobacillus* (log_{10} CFU/mL) in probiotic orange and guava juices during storage at refrigerated condition (4 ± 1°C)

Week	Log CFU/mL							
	Orange				Guava			
	Control	Lp	Lr	La	Control	Lp	Lr	La
0	ND	9.1±0.7^{e}	8.7±0.3^{e}	8.5±0.4^{d}	ND	8.8±0.3^{f}	8.5±0.6^{e}	8.4±0.7^{e}
1	ND	8.7±0.4^{d}	8.6±0.4^{d}	8.3±0.2^{c}	ND	8.5±0.2^{e}	8.3±0.8^{e}	8.0±0.9^{d}
2	ND	8.5±0.7^{c}	8.5±0.6^{c}	7.2±0.2^{b}	ND	8.1±0.6^{d}	7.9±0.2^{d}	7.6±0.5^{c}
3	ND	8.4±0.6^{c}	8.3±0.8^{c}	7.0±0.1^{b}	ND	7.5±0.7^{c}	7.5±0.9^{c}	5.1±0.8^{b}
4	ND	8.0±0.5^{b}	8.2±0.4^{c}	6.7±0.2^{a}	ND	7.2±0.4^{c}	6.2±0.4^{b}	3.2±0.4^{a}
5	ND	8.0±0.5^{b}	7.7±0.3^{b}	<10CFU/mL	ND	6.2±0.1^{b}	5.9±0.2^{a}	<10CFU/mL
6	ND	8.0±0.4^{a}	7.4±0.2^{a}	<10CFU/mL	ND	5.5±0.4^{a}	<10CFU/mL	<10CFU/mL

Results are mean ±S.D of triplicates. Same letter within a row means no significant difference at p≤0.05 by DMRT.
ND: Not detectable, Lp: *L. plantarum*, Lr: *L. rhamnosus* and La*: L. acidophilus*

2.3.2. Biochemical analysis of the probiotic fruit juice during storage

2.3.2.1. Change in pH

The low pH of fruit juice compared to the fairly neutral pH of milk (6.6–6.7) is likely the most important determinant for the poor probiotic viability in this food matrix. *Bifidobacteria* are generally sensitive to pH values below 4.6 [26]. However, *Lactobacillus* strains are clearly more acid resistant than the strains of other *Bifidobacterium* species [27]. It is known that fruit juices can inhibit the growth of lactic acid bacteria (strain-specific effect) and that the inhibition is mainly due to low of pH of the juices [28]. The changes in pH due to addition of probiotics were studied in four fruit juices for 6 weeks (**Table 2.4 and 2.5**). It is interesting to point out that there were slight changes in pH in probiotic litchi and pineapple juices when fortified with Lp and Lr. The cells survived well in litchi, and pineapple juices; one reason for this was probably the high pH of these juices, which was around pH 4.6-4.7. But change of pH was observed when these juices were fortified with La. The pH of litchi juice changed gradually from 4.60 to 3.93 and in case of pineapple, pH changed from 4.75 to 3.73 after 6 weeks of probiotication (**Table 2.4**). There was significant change of pH in probiotic orange and guava juices when these juices were fermented with Lp and Lr. Moreover, the change of pH was also observed when fortified with La in both the juices during storage at refrigerated condition (**Table 2.5**). Though, all three strains were able to withstand pH of 4.5 and 3.3, only Lp and Lr were able to survive at pH 3.66 for up to 6 weeks. It has been reported that the decrease in the pH is due to the accumulation of lactic acid, diacetyl, and acetaldehyde from the growth and fermentation of the probiotics [31]. The extent of change in pH, depends on the ability of the probiotics to utilize sugars for its growth, the type and quantity of the breakdown products of fermentation that are formed and the cell survivability in the reduced pH levels. Therefore, these two cultures were finally selected for fortification of different fruit juices. Similar results were also found for *L. plantarum* of by other researcher when fruit juices were fortified with LAB [28, 29].

2.3.2.2. Change in total soluble sugars (TSS) and titrable acidity (TA)

Fig. 2.3 illustrates the effect of probiotication using three species of lactic acid bacteria in all four fruit juices. There is a declining trend in TSS content in all the probiotic juices due to addition of probiotics. On the other hand, the TA in all the probiotic juices increased with time of storage. At initial stage (0 week) the TSS of the juice was around 17, 12, 11and 18 ^{o}Brix respectively for litchi, pineapple, orange and guava when fortified with Lp. The TSS of these juices reduced to 11, 7, 5 and 13 ^{o}Brix after 6 weeks of cold

Table 2.4. Change in pH of the probiotic litchi and pineapple juices during storage refrigerated condition (4 ± 1°C)

Week	pH							
	Litchi				Pineapple			
	Control	Lp	Lr	La	Control	Lp	Lr	La
0	4.65±0.09^{c}	4.63±0.04^{d}	4.66±0.11^{d}	4.60±0.15^{f}	4.68±0.02^{b}	4.70±0.05^{f}	4.76±0.10^{f}	4.75±0.05^{g}
1	4.61±0.04^{c}	4.54±0.01^{c}	4.45±0.15^{c}	4.46±0.09^{e}	4.65±0.02^{b}	4.54±0.21^{e}	4.65±0.17^{e}	4.66±0.09^{f}
2	4.59±0.02^{b}	4.47±0.03^{c}	4.31±0.09^{b}	4.42±0.05^{e}	4.61±0.03^{b}	4.49±013^{e}	4.63±0.12^{e}	4.52±0.05^{e}
3	4.58±0.01^{b}	4.40±0.12^{c}	4.27±0.10^{b}	4.28±0.12^{d}	4.58±0.02^{a}	4.41±0.12^{d}	4.55±0.14^{d}	4.28±0.12^{d}
4	4.55±0.03^{b}	4.33±0.11^{b}	4.25±0.07^{b}	4.18±0.08^{c}	4.54±0.04^{a}	4.33±0.07^{c}	4.41±0.04^{c}	4.05±0.08^{c}
5	4.52±0.02^{a}	4.22±0.04^{a}	4.22±0.08^{b}	4.09±0.11^{b}	4.53±0.01^{a}	4.20±0.02^{b}	4.05±0.06^{b}	3.88±0.11^{b}
6	4.51±0.01^{a}	4.18±0.13^{a}	4.15±0.06^{a}	3.93±0.05^{a}	4.50±0.02^{a}	4.08±0.15^{a}	3.86±0.11^{a}	3.73±0.05^{a}

Results are mean ±S.D of triplicates. Same letter within a row means no significant difference at p≤0.05 by DMRT.
Lp: *L. plantarum*, Lr: *L. rhamnosus* and La*: L. acidophilus*

Table 2.5. Change in pH of the probiotic orange and guava juices during at storage refrigerated condition (4 ± 1°C)

Week	pH							
	Orange				Guava			
	Control	Lp	Lr	La	Control	Lp	Lr	La
0	4.19±0.06^{b}	4.63±0.04^{d}	4.66±0.11^{d}	4.60±0.15^{g}	5.32±0.06^{b}	5.22±0.06^{b}	5.28±0.03^{d}	5.27±0.04^{e}
1	4.18±0.03^{b}	4.54±0.01^{d}	4.25±0.15^{c}	4.16±0.09^{f}	5.29±0.03^{b}	5.18±0.03^{b}	5.16±0.02^{c}	5.06±0.16^{d}
2	4.17±0.01^{b}	4.37±0.03^{c}	4.11±0.09^{b}	4.02±0.05^{e}	5.25±0.01^{b}	5.17±0.08^{b}	5.18±0.01^{c}	4.92±0.11^{c}
3	4.13±0.02^{a}	4.10±0.02^{b}	4.07±0.10^{b}	3.88±0.12^{d}	5.23±0.03^{a}	5.13±0.03^{a}	5.15±0.02^{c}	4.88±0.07^{c}
4	4.11±0.01^{a}	4.03±0.01^{b}	4.00±0.07^{b}	3.65±0.08^{c}	5.20±0.02^{a}	5.10±0.03^{a}	5.11±0.02^{c}	4.65±0.10^{b}
5	4.09±0.02^{a}	4.02±0.04^{b}	3.92±0.05^{b}	3.58±0.11^{b}	5.20±0.01^{a}	5.07±0.03^{a}	4.80±0.14^{b}	4.58±0.06^{b}
6	4.08±0.01^{a}	3.88±0.03^{a}	3.66±0.06^{a}	3.33±0.05^{a}	5.18±0.01^{a}	5.06±0.04^{a}	4.66±0.12^{a}	4.33±0.13^{a}

Results are mean ±S.D of triplicates. Same letter within a row means no significant difference at p≤0.05 by DMRT.
Lp: *L. plantarum*, Lr: *L. rhamnosus* and La*: L. acidophilus*

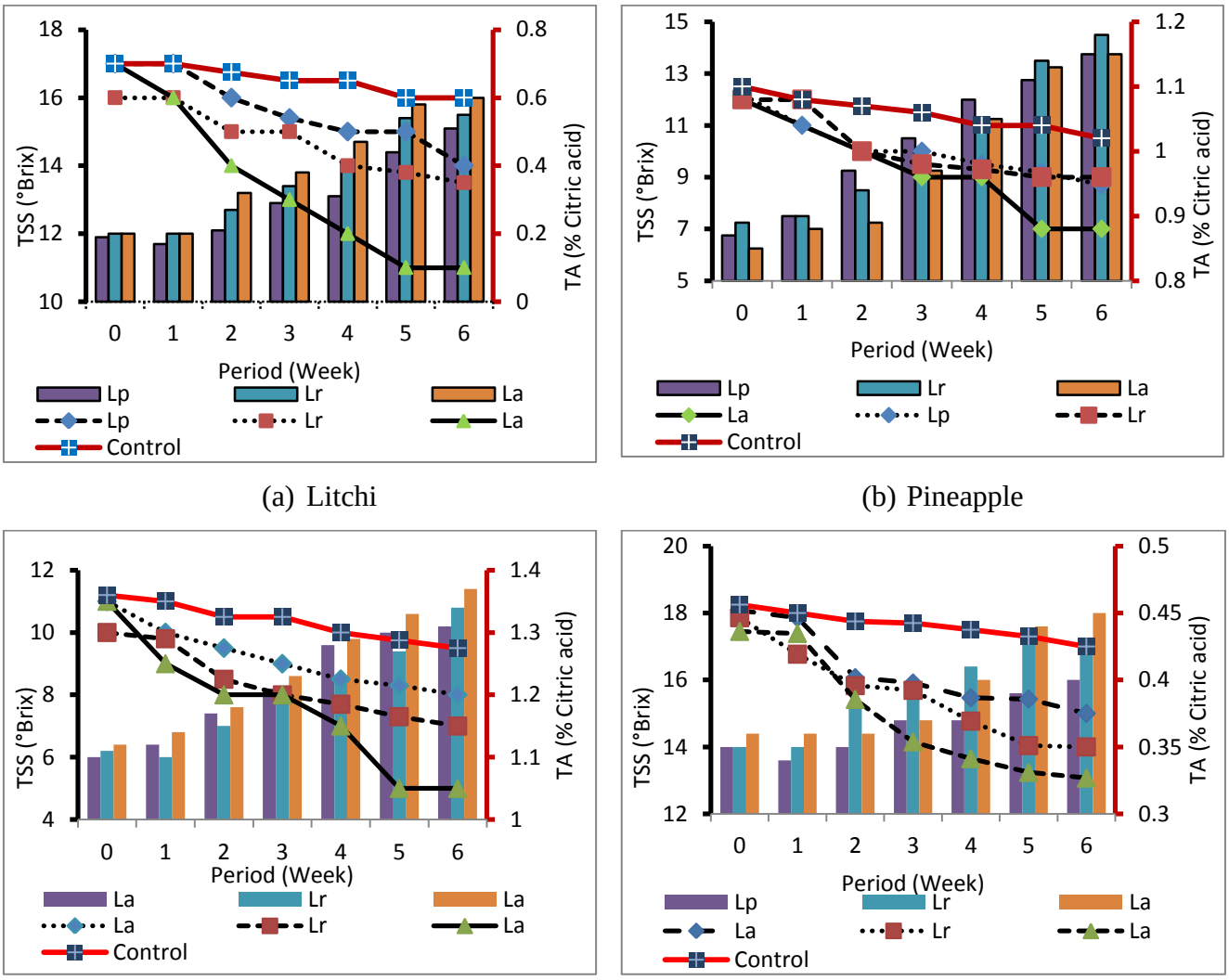


Fig. 2.3. Changes in total soluble sugars and titrable acidity of probiotic juices: (a) litchi, (b) pineapple, (c) orange and (d) guava during storage at refrigerated condition (4 ± 1°C)

storage. Similar trend also found with other two strains of *Lactobacillus*. Highest fall was observed in case of La which sharply declined in all probiotic juices after 1 week and reached as low as °Brix after 6 weeks. TA of the probiotic juices increased from 0.2 to 0.5, 0.9 to 1.15, 1.11 to 1.31 and 0.34 to 0.40 % in litchi, pineapple, orange and guava, respectively when inoculated with Lp. However the increase in TA was more prominent in case of juices fortified with La. The decrease in acidity was concurrent with the decrease in the sugar content of the fruit juices [30]. These results indicated that all the three strains of *Lactobacillus* were not only able to survive but also utilized and fermented the fruit sugars for their cell synthesis and metabolism. Yoon et al. [22] also observed a decrease in sugar

and pH and an increase in acidity when tomato juice was inoculated and incubated with *L. plantarum, L. acidophilus* and *L. casei.*

In the present investigation, the two strains, La (*L. plantarum*) and Lr (*L. rhamnosus*) were observed to not only survive but also utilize the fruit juices for their growth and synthesis of the secondary metabolites, as indicated by the decrease in sugar and pH, and increase in acidity. However La (*L. acidophilus*) was found to consume sugars at a faster rate than other two species. The results of reduced TSS were well supported by the similar trend in increased acidity in the juices inoculated with La (*L. plantarum*) and Lr (*L. rhamnosus*) [30].

2.3.3. Colour analysis

The images of litchi and orange juices at 0day and after 6 weeks of probiotication are presented in **Fig. 2.4**.

Litchi Juice + LAB (0 day)

Litchi Juice + LAB (after 6 weeks)

Orange Juice + LAB (0 day)

Orange Juice + LAB (after 6 weeks)

Fig. 2.4. Probiotic juice with *Lactocacillu*s plantarum at 0day and after 6 weeks at refrigerated condition (4 ± 1°C)

The colour values of the juice samples and their changes upon addition of probiotics are presented in **Table 2.6**. In litchi juice, the '*L*' values decreased when fortified with Lp, while an increase was observed in Lr and La. Similarly, the '*a*' and '*b*' values also significantly decreased in most of the cases with some exceptions. This revealed that the juice became darker due to addition of Lp. When the litchi juice was fortified with Lr and La, increase in brightness was observed due to increase in the '*b*' values.

Table 2.6 Change in colour of the probiotic fruit juices during storage at 4 ± 1°C

Parameters	**0 Day**	**After 6 weeks**		
		L. plantarum	*L. rhamnosus*	*L. acidophilus*
Litchi				
L	31.86±0.09^{b}	29.41±0.14^{a}	36.51±0.07^{c}	38.99±0.18^{d}
a	-1.13±0.04^{b}	-1.03±0.05^{d}	-1.28±0.01^{a}	-1.09±0.03^{c}
b	-1.95±0.07^{a}	-1.38±0.07^{b}	0.71±0.008^{c}	-0.01±0.005^{d}
△E	**--**	**4.66±0.03^{a}**	**5.38±0.03^{b}**	**7.72±0.09^{c}**
Pineapple				
L	24.59±0.16^{a}	23.27±0.10^{a}	22.03±0.04^{a}	27.34±0.12^{a}
a	-1.31±0.06^{a}	-0.18±0.09^{d}	-0.51±0.09^{c}	-0.87±0.03^{b}
b	2.35±0.05^{d}	0.92±0.02^{a}	1.26±0.05^{b}	2.14±0.07^{c}
△E	**--**	**2.45±0.05^{a}**	**3.32±0.01^{b}**	**3.52±0.01^{c}**
Orange				
L	18.28±0.14^{a}	20.57±0.06^{b}	22.19±0.0^{c}	23.65±0.04^{c}
a	1.38±0.03^{d}	0.02±0.006^{a}	0.18±0.02^{c}	0.09±0.01^{b}
b	1.02±0.01^{a}	1.70±0.02^{c}	1.55±0.14^{b}	1.74±0.02^{d}
△E	**--**	**2.06±0.01^{a}**	**4.05±0.03^{b}**	**5.53±0.01^{c}**
Guava				
L	23.86±0.21^{c}	22.36±0.04^{d}	20.59±0.08^{a}	21.84±0.09^{b}
a	0.02±0.001^{b}	-0.03±0.006^{a}	-0.19±0.04^{c}	-0.35±0.02^{d}
b	0.62±0.02^{b}	0.29±0.03^{a}	1.45±0.01^{c}	1.77±0.07^{d}
△E	**--**	**0.34±0.06^{a}**	**1.41±0.07^{b}**	**2.32±0.05^{c}**

Results are mean±S.D of triplicates. Same letter within a row means no significant difference at p≤0.05 by DMRT.

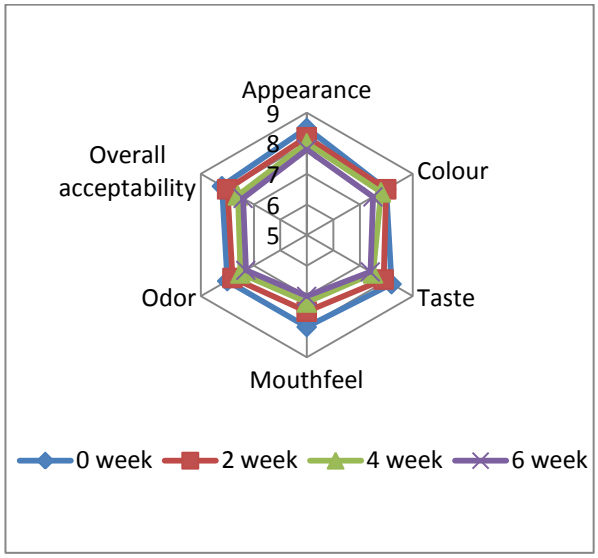
In case of pineapple, no significant change was observed in '*L*' value but the redness increased in all the juices irrespective of the probiotic strains inoculated. When the orange juice was inoculated with probiotics, the brightness of the juice was increased due to increase of the '*b*' values whereas in case of guava, decrease in lightness was observed along with increase in '*b*' values. The total colour change △E (reference value was 0 day of storage) increased in the fermented fruit juices during storage. This increase might be attributed to the increase in the yellow intensity in the fermented samples. The human eye cannot perceive small colour variations and therefore, instrumental colour measurement is helpful. △E values measure the overall colour change [33]. It was observed that the fermented fruit juices did not show a perceptive colour change during storage.

2.3.4. Sensory evaluation

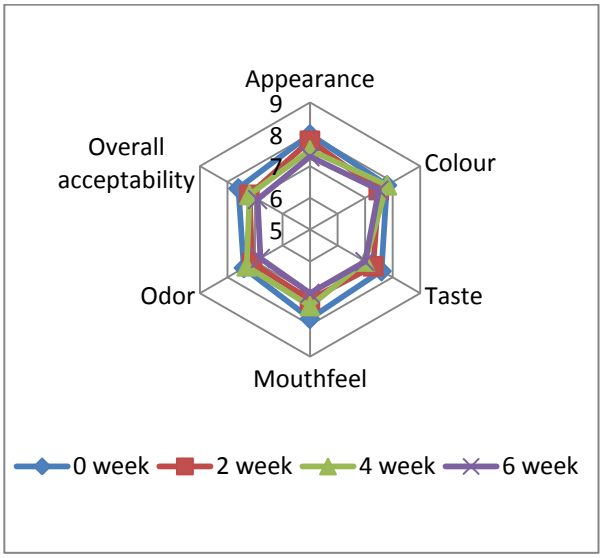
The liking/disliking of the panellists on probiotic fruit juices containing probiotic bacteria are shown in **Fig. 2.5- Fig. 2.7.** Fresh litchi juice incorporated with Lp was liked very much for all the sensory attributes over 6 weeks of storage; the scores gradually decreased with time of storage and after 6 weeks of storage the probiotic juice was evaluated between 'liked moderately' and 'liked very much'. Lp affected the sensory scores of pineapple, orange and guava comparatively more than litchi as it was noticed that after 6 weeks, these juices were evaluated between 'liked slightly' and 'liked moderately' for overall acceptability. Further, taste and overall acceptability were affected more than the other attributes. Incoporation of Lr also was found affect the sensory attributes of the four juices. Scores were lowest for guava followed by orange, pineapple and litchi, in that order. However, all juices were evaluated to have attributes that were either liked slightly or above after 6 weeks of storage. Effect of La was almost similar to that of Lr with litchi getting highest scores and guava getting lowest scores.

The overall acceptability of any fruit juice is mainly influenced by the quality of the product, in which the most important attribute is product's taste, followed by nutritional value, odor and price [34]. This implied that most consumers buy fruit juice due to taste rather than other qualities. According to Granato et al. [35] the food industry takes into consideration many variables to develop or reengineer non-dairy probiotic products, such as sensory acceptance, stability, price, and functional properties. Preliminary acceptance tests showed good acceptability of the product. However, deeper sensory analysis,

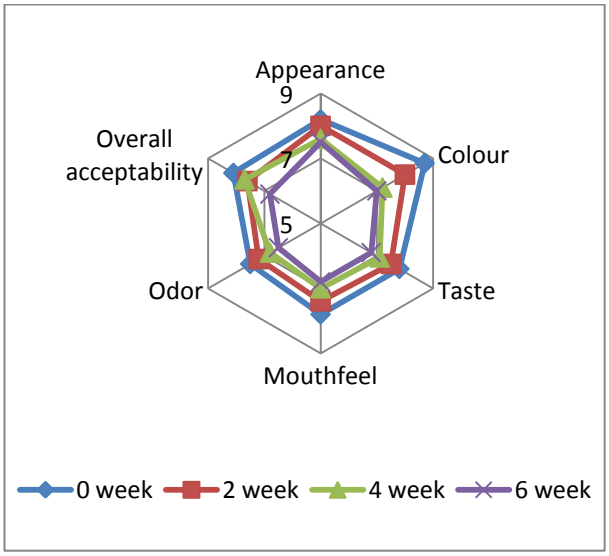
including preference and aroma analysis would have added another dimension for evaluating preference based on sensory properties. Such parameters can be subject of future studies.



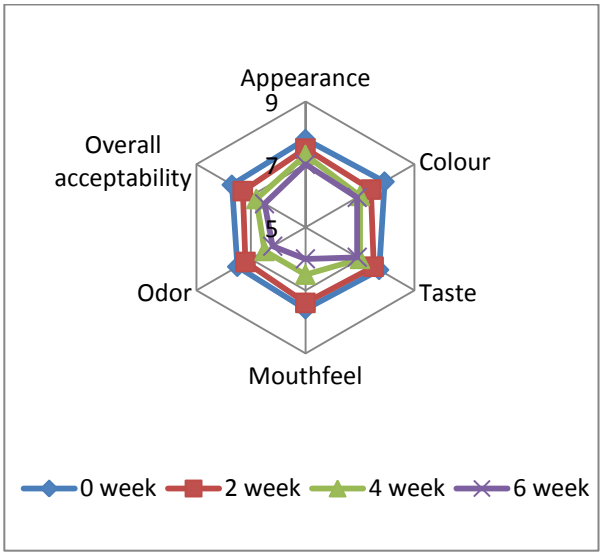
(a) Litchi juice



(b) Pineapple juice

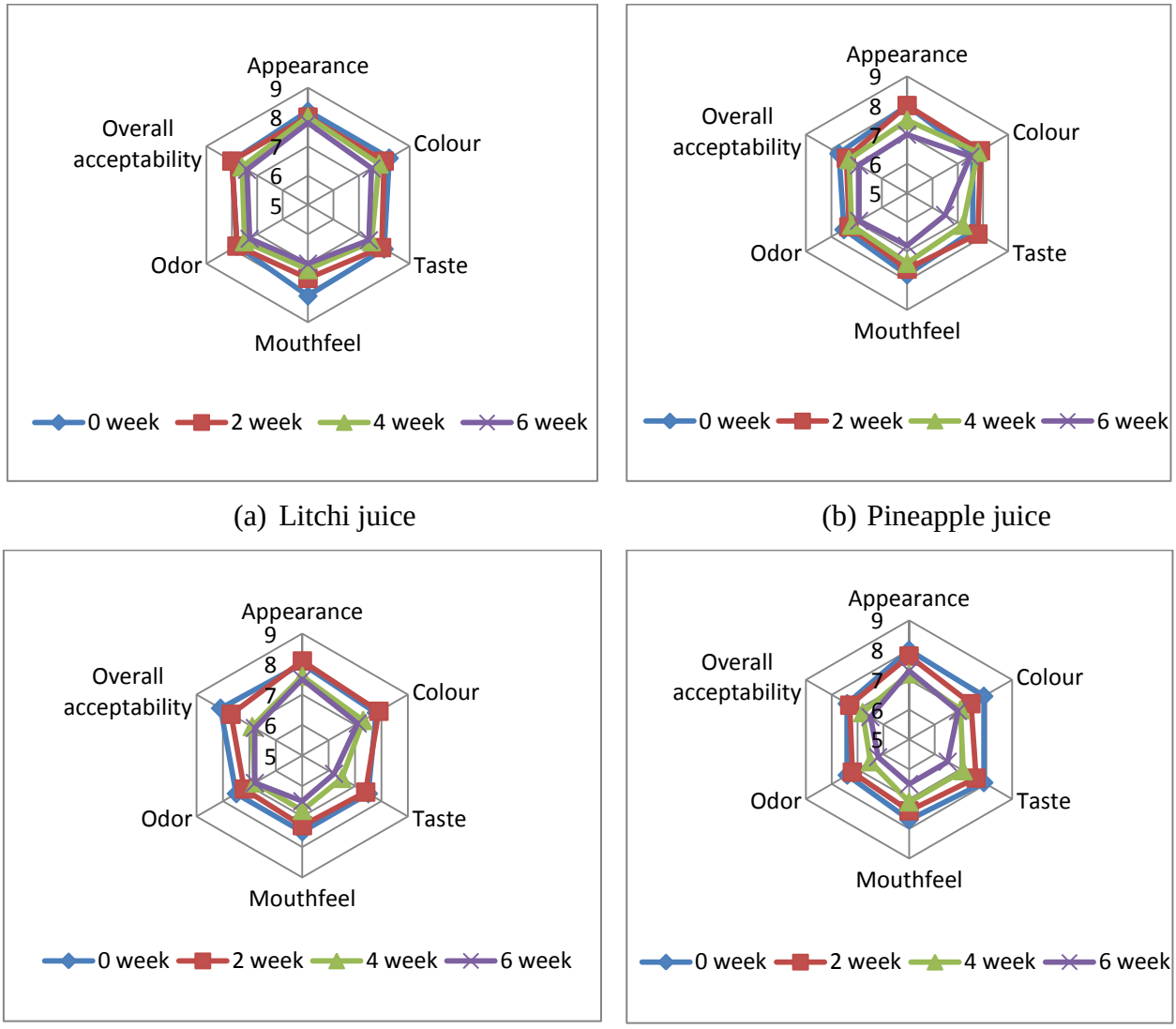


(c) Orange juice



(d) Guava juice

Fig. 2.5. Sensory score for probiotic fruit juices juice inoculated with *L. plantarum* during 6 weeks of storage



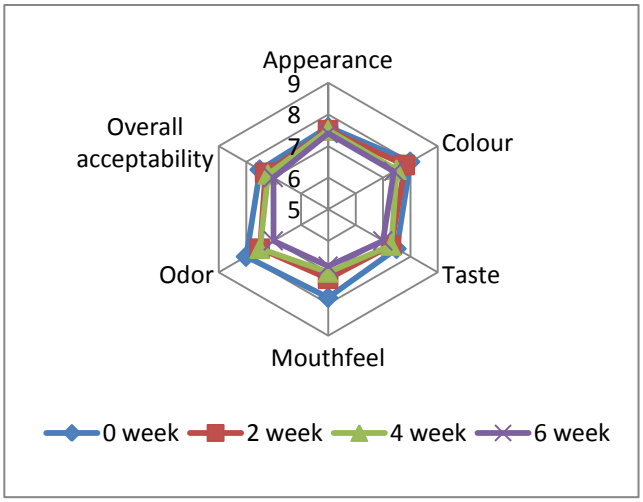
(a) Litchi juice

(b) Pineapple juice

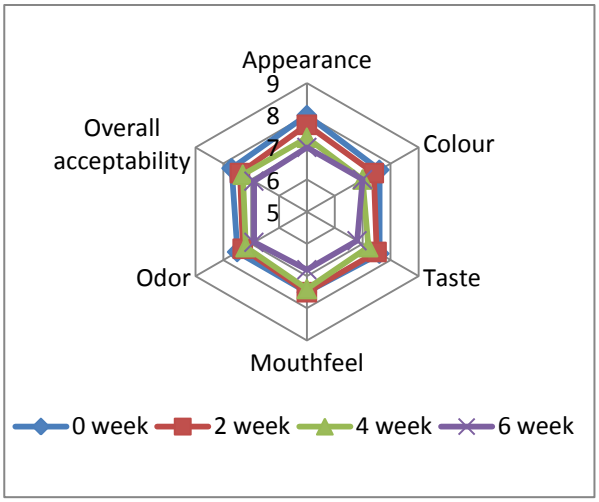
(c) Orange juice

(d) Guava juice

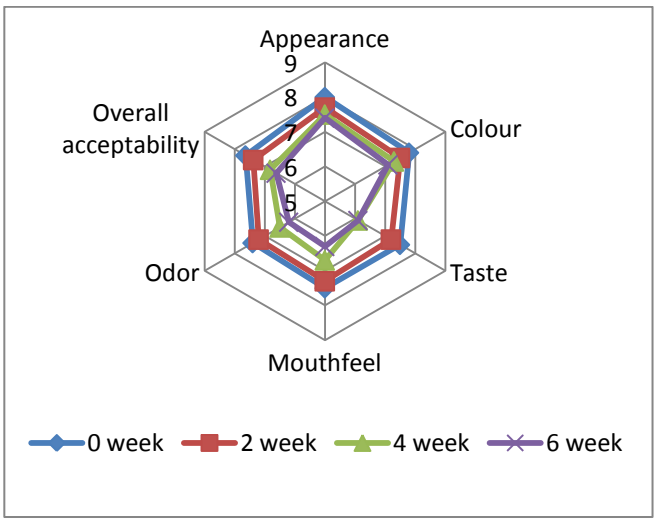
Fig. 2.6. Sensory score for probiotic fruit juices juice inoculated with *L. rhamnosus* during 6 weeks of storage



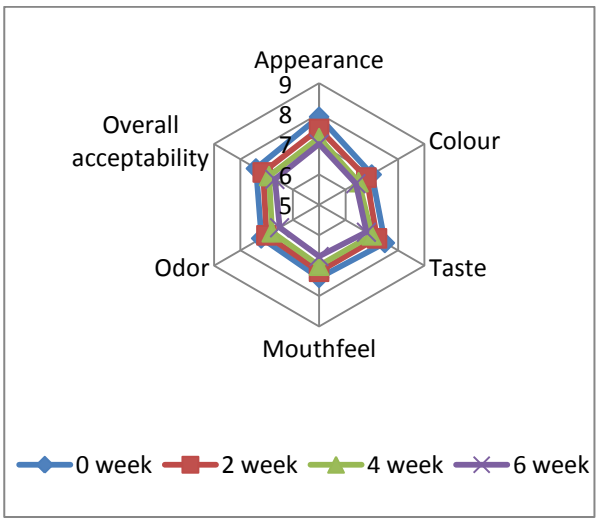
(a) Litchi juice



(b) Pineapple juice



(c) Orange juice



(d) Guava juice

Fig. 2.7. Sensory score for probiotic fruit juices juice inoculated with *L. acidophilus* during 6 weeks of storage

2.4. Conclusion

Preliminary probiotic characteristics (acid and bile tolerance and antibiotic sensitivity) were found superior in *L. rhamnosus* followed by *L. plantarum* and *L. acidophilus.* All the three strains of *Lactobacillus* ie, *L. plantarum* (Lp), *L. rhamnosus* (Lr) and *L. acidophilus* (La) have good capacity to survive in the fruit juices studied. *L. plantarum* (Lp) had maintained the required $\log 10^8$ count in litchi and pineapple juices up to 6 weeks in refrigerated condition (4 ± 1°C). The change in the total soluble sugar, pH and titratable

acidity was minimum in fruit juices fortified with *L. plantarum* (Lp) than other fortified juices. Juice of litchi and pineapple appeared to be better carriers compared to orange and guava for delivery of probiotics due to acceptable changes in their pH, TSS and titrtable acidity on probiotication. The color of the two fermented juices viz. litchi and pineapple were found to be stable during refrigerated storage up to 6 weeks. Overall acceptability of juices was found to be in the range between 7.4 and 7.6. All other sensory parameters were also found to be in the acceptable range between 5.5 and 7.8. After 6 weeks of storage the fruit juices was found to have lower acceptability due to changes in appearance, colour, taste, mouthfeel. *L. plantarum* (Lp) was found to be the superior species and litchi juice was found to be the suitable carrier for probiotic bacteria for developing health promoting functional fruit drink.

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