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Probiotification of *Annona reticulata (Ramphal)* juice by using *Lactobacillus rhamnosus*

[1]Kashif A. Shaikh, [2]Shweta J. Raichurkar, [3]Gauri H. Athawale, [4]Mansi.M. Kshirsagar

[1]M.Tech student, [2]Assistant Professor, [3]Assistant Professor, [4]M.Tech student

[1]MIT School of Food Technology, MIT ADT University, Pune, Maharashtra, India

Abstract: The *Annona reticulata* fruit is a highly nutritious fruit and it shows insecticidal properties. The fruit was astringent, anti-dysenteric, anti-diarrheic and anti-helmintic in nature. An attempt was made to study the characterization of microbial strain (*L. rhamnosus*) for probiotification of *Annona reticulata* fruit juice. The *L. rhamnosus* strain showed good tolerance to low pH, High bile concentration and simulated gastrointestinal juice which validates its probiotic nature. The *Annona reticulata* juice was inoculated with *L. rhamnosus* in three different inoculum sizes 2%, 4% and 6% and was stored at refrigerated temperature (4±1°C) for 15 days for probiotification. It was found that *L. rhamnosus* at 6% inoculum was suited excellent for probiotification of *Annona reticulata* juice. The overall acceptability during storage period of probiotic *Annona reticulata* juice was 7.2 on the 9-Point hedonic scale.

Key Words: *Annona reticulata, L. rhamnosus,* bacterial strain, inoculum, Probiotification

1. INTRODUCTION:

When the live microorganisms administrated in satisfactory amount confirms the health benefits to the host are called probiotics (FAO/WHO, 2002). *Lactobacillus rhamnosus* is a rod-shaped facultative anaerobic bacterium that can live in several parts of the human body, including the gastrointestinal tract. *L. rhamnosus* are potential probiotics as they can maintain intestinal homeostasis and alleviate diseases related to dysbiosis. Currently, *L. rhamnosus* is one of the most studied and characterized probiotic strains. In fact, it can provide numerous beneficial effects, as they have show in *in vitro and in vivo* models and in humans. (Martin *et al*., 2019)

When new microbes are introduced, their genera and strains must be found in healthy human intestinal micro flora, and their safety and risk-to-benefit ratio must be thoroughly researched and assessed in accordance with CODEX Alimentarius. GRAS bacteria present in the human gut include LAB and *Bifidobacterium.* For novel products, premarketing product safety determinations are required. (Anadon *et al*., 2014)

The *Annona reticulata* fruit is high in nutrients, with 100 g of the edible portion containing Fat-0.2 g, Vitamin C-5.0 g, Crude fibre-2.1 g, Proteins-1.5 g, Calcium-40 mg, Phosphorus-30 mg, Carbohydrates- 15.8 g Iron-0.5 g, Minerals-07. g, Riboflavin-0.07 mg, Moisture-76.8 g, Energy-71Kcal, Carotene-67 µg, and Iodine-0.64 ppm, Flourine-5.6 ppm, Niacin-0.6 mg, and Ascorbic acid etc.(Chakre *et al.*,1985 ; Rama *et al*., 1989)

Annona reticulata is a good-quality fruit with 72 percent edible portion. Because of its insecticidal properties, the pulp is used to control lice. The flesh of *Annona reticulata* can be eaten plain or with light cream and a sprinkle of sugar after being scooped from the skin. It is frequently sieved and mixed into milkshakes, custards, or ice cream. To make a sauce, combine the seeded flesh, mashed banana, and cream (Orwa *et al*., 2010).

There were several fruit it probiotics products available in the market. Very few researchers had worked on *Annona reticulata* (*Ramphal*) and negligible work has done on the probiotification of *Annona reticulata* (*Ramphal*) juice yet. That's why

the present study entitling 'Characterization of Microbial Strains for Probiotification of *Annona Reticulata* (*Ramphal*) was undertaken.

2. MATERIALS AND METHODS

2.1 Culture collection:

The bacterial strain of *Lactobacillus rhamnosus* (296) was collected from Microbiology department of MIT School of Food Technology, MIT ADT University,Pune.

2.2 Raw material and chemicals:

The fully ripe *Annona reticulata* (*Ramphal*) fruit was purchased fresh from a fruit market in Pune, Maharashtra, India. All of the chemicals used, such as MRS Agar (De Man, Rogosa, and Sharpe agar), Nutrient agar, Agar powder, Bile salts, Xylene etc, were of analytical grade and supplied by Merck, India, as well as Himedia Laboratories and Sigma Chemicals, India.

2.3 Analysis of probiotic culture

In vitro tolerance of *L. rhamnosus* strain to simulated gastrointestinal juice, High bile concentration and Low pH was determined before addition of *L. rhamnosus* to *Annona reticulata* (*Ramphal*) juice.

2.4 Preparation of *Annona reticulata* (*Ramphal*) Juice:

The washed *Annona reticulata* fruits were peeled, decorated and deseeded by hand before the pulp was combined in a blender. To dilute the pulp, Sterile distilled water was added in a 1:2 (w/v, pulp/water) ratio. To filter the pulp muslin cloth was used which was previously sterilized. The juice was pasteurised for 10 minutes at 85°C. After chilling, samples were kept at 4°C in the refrigerator until they were utilised for further analysis.

2.5 Preparation of the Probioticated *Annona reticulata* (*Ramphal*) Juice Samples

The *Annona reticulata* (*Ramphal*) juice samples were Probioticated by inoculating 200ml of the pasteurized juice sample with 2%,4% & 6% (v/V) equivalent to 4ml, 8ml & 12ml of the probiotic culture of *Lactobacillus rhamnosus*(296) which had probiotic count 6.1 x10^{11} CFU/ml respectively. The diagram for the preparation of probiotic *Annona reticulata* (*Ramphal*) juice is shown below.

Annona reticulata (*Ramphal*)

↓

Washing

↓

Peeling

↓

Deseeding

↓

Extraction of *Annona reticulata* (*Ramphal*) juice

↓

Pasteurization at 85℃ for 5 minutes

↓

Probiotication of pasteurized *Annona reticulata* (*Ramphal*) juice with 2%, 4% & 6% of bacterial strain

↓

Incubation at 37℃ for 48hr

↓

Analysis of probiotic *Annona reticulata* (*Ramphal*) Juice

↓

Storage at 4°C for 15 days

↓

Analysis of the stored probiotic *Annona reticulata* (*Ramphal*) juice samples

Fig. 2.1 Preparation of Probioticated *Annona reticulata* (*Ramphal*) juice

2.6 Storage of probiotic *Annona reticulata* (*Ramphal*) juice

The inoculated *Annona reticulata* (*Ramphal*) juice samples were incubated at 37°C for 48 hrs and stored at 4°C for 15 days in glass bottles. Analysis such as the Probiotic count, acidity, pH & Total Soluble Solid was done on every 3rd day.

2.7 Determination of the Viability of the Probiotic Strain inside the Stored *Annona reticulata* (*Ramphal*) Juice Samples

A pour plate technique was used to test the viability of bacterial strain in *Annona reticulata* (*Ramphal*) juice samples. At two days of intervals, the samples were poured plated. MRS agar plates were inoculated with 1mL of the probiotic juice, and then incubated overnight at 37°C. The number of distinct colonies was counted then multiplied by the reciprocal of the dilution factor to get the colony forming the unit (CFU). (Akpeji *et al*., 2017)

2.8 Measurement of pH, Titratable acidity and Total soluble solids (TSS)

The pH of all the samples was determined using a pH metre that was correctly calibrated. Titratable acidity was measured by adding 5mL of the sample (juice) diluted with an equal amount of distilled water in a conical flask; 2 to 3 drops of Phenolphthalein were added to designate the end point. The burette's base (0.1N NaOH) was added to the flask drop by drop, with constant mixing, until the ultimate result, a pink colour, was achieved. The results were written down (Sukhvir *et al*., 2016)**.** The total soluble solids (TSS) of all the samples were determined using a hand refractometer (Erma, Japan) in terms of °Bx (°Brix) on every third day of 15 days of storage period. (Maskan *et al*., 2006).All the above parameters of probiotic *Annona reticulata* juice samples was tested at interval of two days.

2.9 Sensory quality evaluation

Training panels assessed the probiotic *Annona reticulata* juice samples for sensory attributes including colour, appearance, taste, consistency, and overall acceptability. With a 9-point hedonic scale, the panellists were asked to complete a sensory sheet (9 and 1 points representing like extremely and dislike extremely). (Sasi *et al*., 2015)

2.10 Statistical Analysis

All of the trials were done in triplicate. The average and standard deviation were used to express the results (SD). The Microsoft Excel software was used to calculate the mean value and standard deviation. ANOVA was used to do statistical analysis on three different probiotic strains. Full Factorial Design of Design Expert Version 7.0.0 was used to optimize pH, acidity, TSS and probiotic count.

3. RESULTS AND DISCUSSION

3.1 Proximate composition of raw material

Acidity, pH, moisture and TSS of fruit juice

The acidity and pH of the freshly prepared *Annona reticulata* juice is 1.1% and 5 respectively. Moisture content of fresh pulp is around 68%-72%. The TSS is 10 °Bx of the freshly prepared *Annona reticulata* juice as shown in table 3.1.

Table.3.1. Composition *Annona reticulata* juice

Sr. No.	Parameter	Values
1	Moisture (%)	72% ± 2
2	pH	5.2 ± 0.3
3	Acidity (%)	1.14% ± 0.04
4	TSS (°Bx)	10 °Bx

3.2 Effect of inoculums size on viability of bacterial strains in *Annona reticulata* juice

The concentration of probiotics in food products must be high in order to have any health benefits. Suggested probiotic levels also known as therapeutic minimums, ranged from 10^6 (Kurmann *et al.*, 1991) to more than 10^7 or 10^8 CFU/mL. (Lourens-Hattingh *et al.*, 2001)

Table: 3.2.Effect of inoculums size on viability of bacterial strains in *Annona reticulata* juice

Bacterial strain	Size of inoculums	Storage period (probiotics count log cfu/ml)					
		Day 0	Day3	Day 6	Day 9	Day 12	Day 15
L. rhamnosus(296)	2%	10.92±0.04	10.74±0.02	10.54±0.05	10.41±0.1	9.55±0.08	8.81±0.1
	4%	12.66±0.08	12.41±0.1	12.3±0.03	11.34±0.08	10.74±0.1	9.44±0.06
	6%	12.85±0.02	12.79±0.3	11.62±0.1	10.95±0.01	10.64±0.02	9.97±0.1

Values (Mean ± SD, n=3)

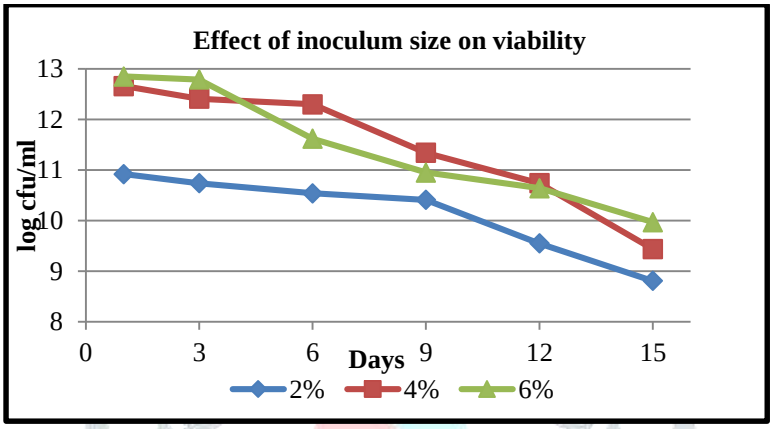

Fig. 3.3 Effect of inoculum size on viability during storage

Consequently, due to the viability of all the bacterial strains in probiotic *Annona reticulata* juice during storage period of 15 days it was observed without any nutrient supplementation it could be considered as a probiotic beverage.

These findings was in line with the observations of Yáez *et al.*, (2008), who found that some factors, like the fermentation process caused increased in acidity due to which it can reduced the survival and viability of probiotic stains in the product. Hossain *et al.*, (2017) also found the same kind of results in the study of probiotic Pineapple juice. The viability of bacterial strains reduced and probiotics count of juice reduced to 10^6 cfu/ml.

3.4 Effect of inoculums size on pH during storage

All the samples showed dropped in the pH values as their size of inoculums increased from 2% to 6% in the Annona reticulata juice during storage of 15 days. The reduction in pH also observed with time in all the samples of different inoculum sizes as shown in table 3.4.

Table.3.4. Effect of size of inoculums on pH of Annona reticulata juice

Bacterial strain	Size of inoculum (%)	Storage period (pH)					
		Day 0	Day3	Day 6	Day 9	Day 12	Day 15
L. rhamnosus(296)	*2%*	4.7±0.1	4.6±0.1	4.5±0.1	4.4±0.2	4.3±0.3	4.1±0.1
	4%	4.6±0.3	4.4±0.2	4.2±0.2	4.2±0.4	4.0±0.1	3.8±0.3
	6%	4.4±0.2	4.3±0.1	4.2±0.1	4±0.3	3.9±0.2	3.6±0.2

Values (Mean ± SD, n=3)

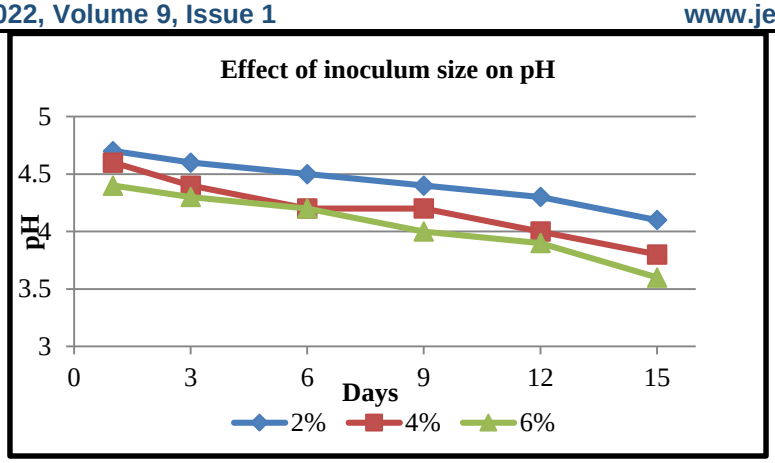

Fig.3.4. Effect of inoculum size on pH during storage

Similar observations were also recorded by Panghal *et al.*, (2017). They prepared probiotics Beetroot juice the storage study of the probiotics beetroot juice revealed that the pH of probiotic juice was decreased to 4.75±0.03 in 24 hr from 6.5±0.03 at the beginning. The pH was dropped due to *Lactobacillus* produces lactic acid during fermentation.

Similar trend was reported by several workers for probiotic fermented milks (Hughes *et al.*, 1995; Shah *et al*., 2001). Our findings are consistent with those of (Panesar *et al*., 2012), who also observed a continuous drop in pH during probiotic yoghurt storage.

3.5 Effect of inoculum size on titratable acidity during storage

In the present study also all the inoculated samples showed increase in their lactic acid content .There was increased in lactic acid quantity observed as the size of inoculums increased from 2% to 6% as shown in the table 3.5.

Table: 3.5 Effect of size of inoculums titratable acidity of *Annona reticulata* juice

Bacterial strain	Size of inoculums	Storage period (Titratable Acidity %)					
		Day 0	Day3	Day 6	Day 9	Day 12	Day 15
L .rhamnosus(296)	2%	0.162±0.01	0.189±007	0.207±0.02	0.225±0.03	0.243±0.09	0.279±0.04
	4%	0.189±0.08	0.207±0.05	0.243±0.02	0.252±0.07	0.270±0.06	0.315±0.08
	6%	0.198±0.02	0.234±0.08	0.270±0.04	0.279±0.2	0.306±0.03	0.342±0.07

Values (Mean ± SD, n=3)

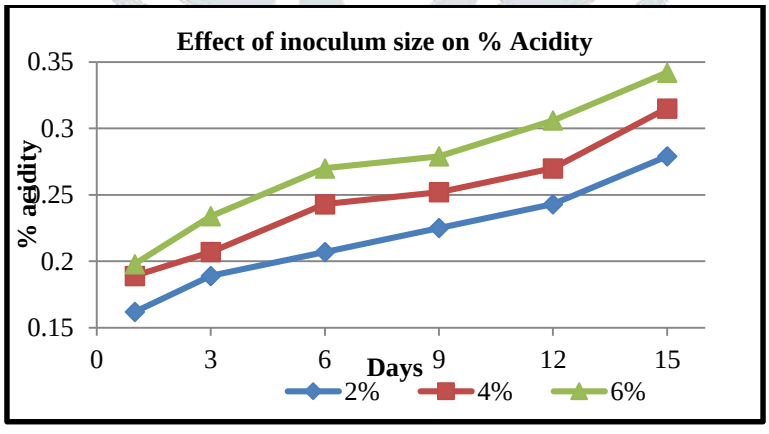

Fig. 3.5 Effect of inoculum size on titratable acidity during storage

Akpeji *et al*., (2017) also found that the Probiotics Soursop juice sample showed an increase in lactic acid during storage period from 1621.44 mg/l and 2450.176 mg/l, the Soursop juice sample produced the most lactic acid in the second and third weeks of storage. According to Yoon *et al*., (2004), tomato juice becomes more acidic when inoculated with *L. delbrueckii*, *L. acidophilus*, *L. plantarum*, and *L. casei.*

3.6 Effect of inoculums on TSS during storage

The Total Soluble Solid (TSS) content of freshly prepared *Annona reticulata* juice samples was 10 °Bx. There is immediate reduction in TSS observed in all the inoculated samples of *Annona reticulata* juice. The TSS of all the samples was reduced to 9°Bx on 3rd day as shown in the table 3.6.

Table 3.6 Effect of inoculum size on Total Soluble Solids of *Annona reticulata* juice

Bacterial strain	Size of inoculums	Storage period Total soluble solids (°Bx)					
		Day 0	Day3	Day 6	Day 9	Day 12	Day 15
L .rhamnosus(296)	2%	9.6±0.5	9.3±05	9±0.0	8±0.0	8±0.0	7.6±0.5
	4%	9±0.0	9±0.0	8.6±0.5	8.3±0.5	8±0.0	7.6±0.5
	6%	9±0.0	8.6±0.5	8±0.0	7.6±0.5	7±0.0	7±0.0

Values (Mean ± SD, n=3)

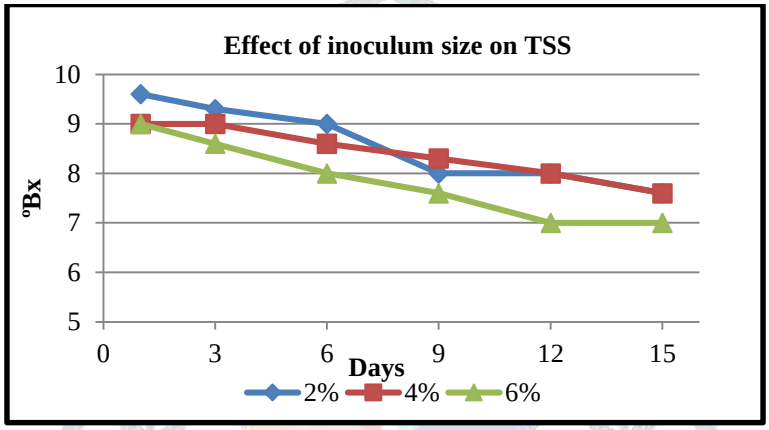

Fig. 3.6 Effect of inoculum size on TSS during storage

Hossain *et al*., (2019) developed probiotics mango juice by using *Lactobacillus plantarum* and *Lactobacillus fermentum* and they found that the Total soluble solid (TSS) content was almost same among the sample but as the retention time increases the value was decreased. The TSS of probiotics Mango juice was reduced from 15.2°Bx to 12.3°Bx during 21 days of storage period.

3.7 Sensory Analysis of *Annona reticulata* juice

Sensory characteristics of any food product are the ultimate criterion for its acceptability by the consumers. The sensory quality of a food was generally considers its attributes related to color and appearance, aroma, flavor and textural properties (Majchrzak *et al*., 2010).

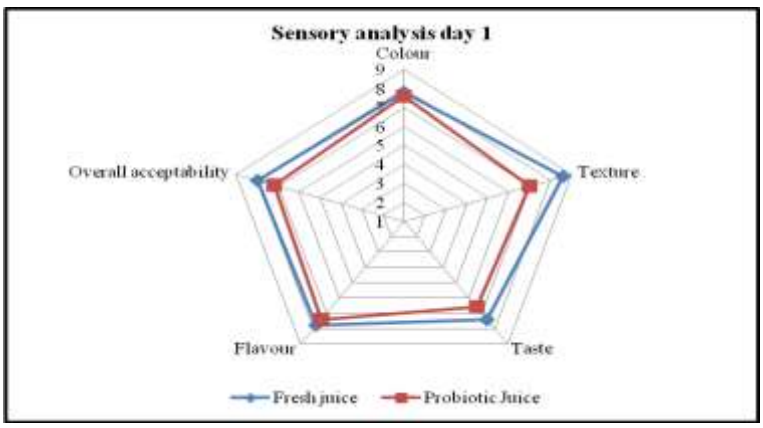

Fig. 3.7.1 Sensory analysis of *Annona reticulata* juice on 1st day

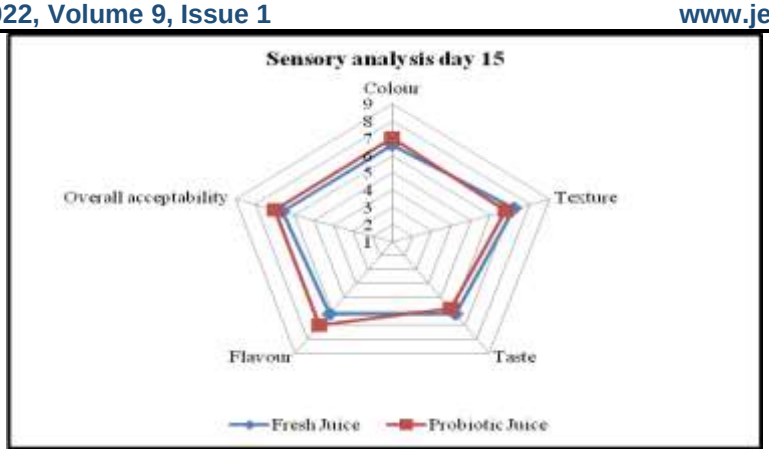

Fig. 3.7.2. Sensory analysis of *Annona reticulata* juice on 15th day

All the sensory parameters were declined from 1st day to 15th day of storage period .As shown in the figure 4.5.1 & figure 4. 5.2 on 1st day the overall acceptability score of fresh juice sample was 8 which was more than probiotic juice (7.2). At 15th day the overall acceptability of probiotic juice was 7 and for the fresh juice samples it were 6.8. From fig 3.7.2 it was revealed that the due to color and flavor retention in the probiotic juice its overall acceptability was more than the fresh juice sample.

Shams *et al.,* (2018) discovered similar sensory attributes when they prepared a Ber fruit probiotic carbonated beverage containing *L. acidophilus*. They discovered that as the storage period progressed, the overall acceptability score decreased. The average overall acceptability score fell from 7.54 to 6.07, as did Taste (7.69 to 5.96), Mouth feel (7.99 to 5.58), and Flavor (7.99 to 5.58). (8.15 to 5.77). Despite the decrease in all sensory characteristics, they concluded that the product was still acceptable on the very last day of storage

3.8 Statistical Analysis

A minimum of three replicates of each experiment were carried out. Results are presented as Mean ± standard deviation (SD). Hermann-Tropsch's Excel software was used to calculate the mean value and standard deviation. Statistical Analysis of three different probiotic strains was done by using ANOVA. The optimization of pH, Acidity, TSS and Probiotic count was done by using Full Factorial Design of Design Expert Version 7.0.0.

Table 4.6.3 Statistical analysis of *L. rhamnosus*

Particulars	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Probiotic count	4.50	1	4.50	41.67	0.0013*	Significant
pH	0.013	1	0.013	271.19	< 0.0001*	Significant
Acidity	1.310E-004	1	1.310E-004	271.19	0.005*	Significant
TSS	0.41	1	0.41	310.50	< 0.0001*	Significant

***Values of "Prob > F" less than 0.0500 indicate model terms are significant.**

4. CONCLUSION

In the above study by observing the effect of inoculum sizes on pH, Acidity, TSS and Probiotic count it was found that the viability of *L. rhamnosus* was more at 6% inoculum the *L. rhamnosus* showed maximum viability (9.9 log cfu/ml) after 15 days of storage period at 6% inoculum.. The probiotic *Annona reticulata* juice sample was found to be more acceptable than the fresh juice. From the above study we have found that the proven probiotic strain of *L. rhamnosus* could survive and capable of rapidly utilizing the nutrients of *Annona reticulata* juice without adding additional nutrients *L. rhamnosus* and was found to be the best at every parameter of characterization of bacterial strains and it also showed viability after 15 days of storage period. Therefore *L. rhamnosus* (6%) should be considered for industrial production of probiotic *Annona reticulata* juice product. Further studies on physicochemical changes in probiotic *Annona reticulata* juice can be done.

Plate 1: ***Annona reticulata***

Plate 2: ***Annona reticulata*** **Juice**

Plate 3:Probiotic ***Annona reticulata*** **Juice**

5. ACKNOWLEDGEMENT

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