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Probiotication of vegetable juice by *Lactobacillus acidophilus*

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Abstract

The benefits of consuming food with added live microbes (probiotics) on human health are being increasingly promoted by the health professionals. The term Probiotic was technically defined as ''live microorganisms which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition'. In the present study vegetable fruit juice (beet root and bottle guard) are used for the probiotication by *Lactobacillus acidophilus*. The bacteria was isolated from curd sample and characterized on morphological and biochemical basis. The vegeatble juices were probioticated with the isolated bacteria and further analysis of the probioticated juice was done. Increase in percentage acidity and decrease in pH was observed and the nutritonal analysis revealed the presence of vitamins and organic acids. The probioticated juice samples also showed good antagonistic acitivity against the pathogenic bacteria species. The characters of the probioticated substrates were studied and were tested for shelf life. It was evidently proved that immobilized cultures remained viable over a long period of time and the probiotically fermented drinks were potentially inhibiting the pathogenic growth. Prospective studies on mechanisms of the probiotic activities may enable their new medical applications for lactose intolerant and diabetic patients.

Nowadays, healthy foods mean "functional foods", and generally functional food is the food that exerts beneficial effects or more specific body functions, in addition to the traditional nutritional effects. Some of the well known examples of funcional food are those that contain or are prepared with the bioactive compounds, like oligosaccharides, dietary fibers and active "friendly" bacteria maintaining the equillibrium of intestinal bacterial strains. Apart from the well established functional ingredients such as vitamins, minerals and micronutrients, the probiotics belong to the generation of active ingredients including phytonutrients and lipids alonhg with prebiotics[7]. The most interesting area of research and innovation in the food field is covered by the idea of developing functional food, as suggested by the increasing number of scientific papers dealing with this topic since 2007.

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[14]. 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. 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 as healthy foods, designed for young and old people[11].

A study carried out on the effect caused due to the intake of nine micronutrients that is vitamin E, calcium, folate, retinol, nicotin acid, carotene, ribiflavin biotin and pantithenic acid, on the genome damage and repair led to the finding that the juices are rich in these nutrients[5]. Therefore, juice fortification with probiotic microorganisms had become a challenge and a frontier goal, as juices could combine nutritional effects with the added value of a healthy benefit from a probiotic. The most commonly probiotic bacterial genera are *Lactobacillus* and *Bifidobacterium* and in case of yeasts they mainly belong to *Saccharomyces cerevisiae* var. *boulardii*, could be considered as main targets used as probiotics both in dairy and non-dairy functional foods[15].

Maintainance of 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 to the end of shelf-life are two important criteria to be fulfilled in juices, where low pH represents a drawback. This drawback is effectively overcome by several strains of *Lb. plantarum*, *Lb. acidophilus* and *Lb. casei* that can grow in fruit matrices due to their tolerance to acidic environments[13]. In order to improve the survival of probiotics in the juice several techniques have been developed like fortification with prebiotics, adaption and induction of resistance, storage under refrigeration and with antioxidant and microencapsulation of probiotic culture. The technique of microencapsulation has been designed to be applied on various matrices to protect the microbial cells from the damage caused by external environment. Several studies reported that microencapsulation might provide a more favorable anaerobic environment for sensitive probiotic bacteria, as well as a physical barrier from the harsh acidic conditions of the fruit juice[4].

Isolation of bacteria :

For the isolation of *Lactobacillus acidophilus* bacteria selective media de Man, Rogosa and Sharpe (MRS) media was used. Curd sample was used as the inoculum for isolation by the spread plate technique. The sample was serially diluted and inoculated on the sterile media followed by incubation at 37ºC for 24hrs. The strain isolation was based on the colony appearance, at initial stages. For

the Co- fermentation *Saccharomyces boulardii* was isolated from the dietary supplement sachet "Daraorlac" obtained from local drug shop and was maintained as a pure culture slants on YPD media at 4ºC. Further sub culturing was carried out after increasing the volume of the medium for the next 48 hours anaerobically at 37°C [1].

Characterization of the isolate :

The sub-cultured isolate was subjected to the characterization by gram's staining to determine morphology and several biochemical tests to determine its metabolic activities. The biochemical test carried out for the isolate were selected according to the Bergey's mannual and they include sugar fermentation test, Citrate utilization test, MR-VP test, Starch hydrolysis test, Casein Hydrolysis test, Urease test, and Catalse test. The activecultures were grown on the slants and plates of the modified or specified substrate to check the response of the bacillus cultures. The cultures were maintained at 37°C [2].

Preparation of substrate and Probiotic culture :

The vegetables (Bottle guard and Beet root) taken as substrate were purchased from the local market and stored in a box at Rt for further maturation. The vegetable were washed with tap water to re-move soil and other impurities, air dried at room temperature prior to use, and blanched in water bath for 20 min at 60 ºC. Juice was extracted by using a grinder and filtered through a muslin cloth with a sieve (0.8 to 1.1 mm pore size) to get a clear juice. For the preparation of probiotic culture isolated pure culture was activated by two successive subculturing in MRS broth culture at 37 ºC for 24 h. For co-fermentation, lactic acid bacteria and *S. boulardii* were mixed (1:1 ratio) and use as inoculum for probiotication of vegetable juice.

Harvesting and Micro-encapsulation of Probiotic culture :

The activated probiotic culture was harvested by centrifuging the culture at 3000rpm for 15 minutes at 25ºC and the obtained pellet was washed two times by sterlie saline. The cells obtained were divided in two parts, one part to be used as free cell suspension and second part used for the immobilization by microencapsulation. For the immobilization process the cells were suspended in 5ml of sterile water and mixed with 20ml of sterile 2% sodium alginate solution. This suspension was taken in sterile syringe and was added drop wise into sterile 0.05M $CaCl_2$ solution.Alginatejellification occurred, entrapping lactic acid bacteria in the form of solid beads of 2mm in diameter. Beads remained in $CaCl_2$ for one hour to permit hardening. $CaCl_2$ was then removed by decantation and 100 ml of cold (7°C) MRS broth was added. Beads were stored at 4°C until utilized[8].

Probiotication of Vegetable Juice :

The probiotication of both juice samples was done in three sets with both free cell and immobilized probiotic culture. The set includes the individual culture of *L. acidophilus* and *S.boulardii*and one with their combination in ratio 1:1.Ten grams of microencapsulated beads or 10 mL of free-cell suspension of each probiotic culture was added aseptically into 100 mL of vegetable

juices separately. A high proportion of culture to juice was added in order to provide a high number of probiotic vegetable juices and to increase the sensitivity of the test. Inoculated juice wasincubated at 37ºC for 80 h. Bacterial countswere immediately taken following the separationof immobilized cells in the fermented productson a comparison basis with the juice inoculated with starter cultures on classical methods.

Analysis of Probioticated Juice :

The probioticated juice was analysed two parameters (Physio-chemical and Nutrientional) for determining its characteristics. The physio-chemical analysis involved the monitoring the pH of juice sample by using a pH meter (Cyberscan–Eutech Instruments), while the acidity was determined by the titration against 0.1N sodium hydroxide solution. The nutritional analysis parameter includes the determination of Reducing sugar by DNS method[6], Protein content by Lowry's Assay[10], Crabohydrate estimation by Phenol-Sulphuric acid method[12], Vitamins content by titration against iodine solution and the organic acids by HPLC method.

Determination of Antagonistic activity :

The antagonistic activity of probioticated substrate was determined by the agar well diffusion method against two pathogenic bacteria, *Escherichia coli* and *Staphylococcus aureus*. These pathogenic organism were grown in a fresh nutrient culture prior to use. Actively growing culture of the test organisms were mixed 2.5% (2.5 x 10 7cfu/ml) with melted nutrient agar and poured in sterile petri dishes and allowed to solidify. A 1 cm wide ditch was cut in the agar across the centre of the dish. The probioticated substrate was pipeted out into the ditch and was allowed to diffuse in the agar and then incubated at 37°C for 18 h. After incubation, the diameter of the clear zone is measured in centimeters from the centre of the well. Ciplox antibiotic was used as a positive control for comparative test.

Determination of Viable cell count of Probiotics :

Viable cell count of bacteria from the probioticated substrate were determined in duplicate by using the pour plate method using MRS agar medium supplemented with 2.5 mg/ L Amphotericin B to inhibit the yeast growth, and viable cell count of yeast *S. boulardii* was determined by the spread plate method on YPD agar medium. Probioticated vegetable juice (10 g of each) samples were mixed with 90 mL of sterile 0.85% saline and vortexed for 30 seconds. The resulting suspension was serially diluted in 9 mL saline and 1 mL of the appropriate dilution was used for selective enumeration by pour plate technique. The cell growth of each organism was assessed by enumerating bacterial population after 12, 24, 48 and 72 h of probiotication of beet root and bottle guard juice on MRS agar. Plates containing 25 to 250 colonies shall count and recorded as colony forming units (CFU) per gram sample.

The colonies obtained on the MRS media after incubation were white, shiny, and raised colonies with circular margins depicting the colony characteristics of Lactic acid bacteria. The pure culture was prepared from it to carry the characterization protocols. The isolated pure culture was found to be gram positive bacteria with bacilli morphology. The

results of biochemical test were similar with that of *Lactobacillus acidophilus* and are summarized in table-1.

Table-1 showing the results of biochemical test for the isolated LAB

S.No.	Biochemical test	Result
1	Citrate utilization test	-
2	Methyl red test	+
3	Voges-Proskaeur test	-
4	Urease test	+
5	Starch Hydrolysis test	-
6	Casein Hydrolysis test	+
7	Catalase test	+
	Sugar fermentaion test	-
	Glucose	+
8	Sucrose	+
	Lactose	+

For the probiotication of the both juice samples that is beet root and bottle gourd three different sets were prepared, one probioticated with only *Lactobacillus acidophilus*, second only with *Sacchromyces boulardii* and third combination of both organism in 1:1 ratio. In overall 6 different samples, 3 for each juice was obtained and the analysis was carried out for all these samples separately. In case of the physio-chemical analysis all the juice samples showed a significant increase in percentage of titrable acid, with increase in time period, leading to decreased pH and increased turbidity, results for the same is given in table-2. The nutritional analysis of the juice sample was carried to determine the concentration of important nutreints in all the juice samples, the result for the quantitative estimation of nutrients is summarized in table-2 and table-3. The HPLC analysis of all probioticated samples for determination of organic acids revealed the presence of acetic acid, tartaric acid, oxalic acid and ascorbic acid. The HPLC detection of organic acid was done with the help of the standard organic acid solutions applied under the same condition.

Table-2 showing the result for the physio-chemical analysis of the different probioticated juice samples

Substrate Name	Type of Probiotic Culture	Time interval (hour)	% acidity	pH
Beet Root Juice	*L. acidophilus*	48 h	0.40	5.70
		72 h	0.60	5.00
	S. boulardii	48 h	0.20	5.08
		72 h	0.30	4.01
	L. acidophilus+ *S.boulardii*	48 h	0.17	5.45
		72 h	0.42	4.30
Bottle gourd Juice	*L. acidophilus*	48 h	0.46	4.96
		72 h	0.49	4.20
	S. boulardii	48 h	0.80	4.70
		72 h	0.85	4.00
	L. acidophilus + *S.boulardii*	48 h	0.04	4.74
		72 h	0.01	4.10

Table-2 showing the result for the nutritional analysis of the different probioticated juice samples

Substrate Name	Type of Probiotic Culture	Reducing Sugar (mg/ml)	Carbohy-drates (mg/ml)	Protein (mg/ml)	Vitamin (gm/l)
Beet Root Juice	*L. acidophilus*	1.4	3.59	0.20	12.50
	S. boulardii	0.07	2.32	0.40	12.50
	L. acidophilus+ *S.boulardii*	2.19	1.22	0.17	25.00
Bottle gourd Juice	*L. acidophilus*	2.07	0.81	0.03	18.75
	S. boulardii	2.07	1.35	0.18	12.50
	L. acidophilus+ *S. boulardii*	0.15	2.18	0.47	18.75

Table-3 showing the result for the organic acid analysis of different probioticated juice samples by HPLC

Substrate Name	Type of Probiotic Culture	Organic Acid	Retention time (min)	Area (mAus)	Height (mAu)
Beet Root Juice	*L. acidophilus*	Oxalic acid	0.777	92.374	3.879
		Ascorbic acid	3.283	845.375	28.817
		Tartaric acid	2.513	4529.7	208.553
	S. boulardii	Tartaric acid	2.246	274.352	25.488
	L. acidophilus + *S. boulardii*	Tartaric acid	2.124	61.790	6.620
		Acetic acid	3.423	40.444	2.713
Bottle gourd Juice	*L. acidophilus*	Tartaric acid	2.498	5523.0	232.195
		Acetic acid	3.305	2134.333	128.910
	S. boulardii	Tartaric acid	2.746	5.527	32.290
		Ascorbic acid	3.263	312.038	15.446
		Oxalic acid	0.109	7.720	0.825
	L. acidophilus + *S. boulardii*	Oxalic acid	0.193	17.276	0.853
		Tartaric acid	2.72	210.877	23.880

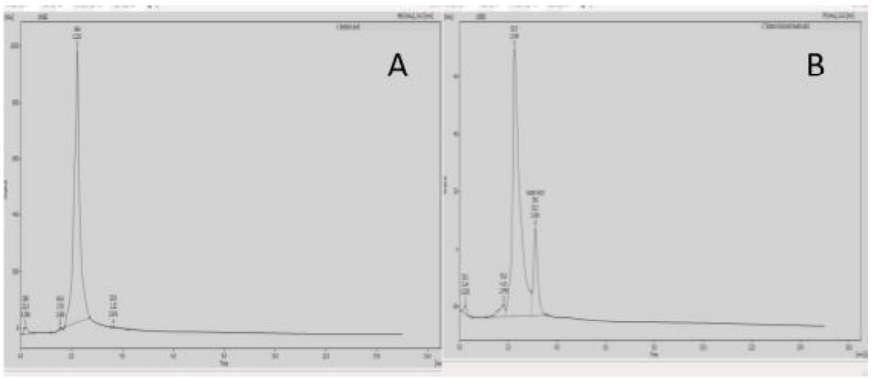


Fig. 1. HPLC chromatogram of standard A) tartaric acid and B) Acetic acid at UV spectrum of 210 nm

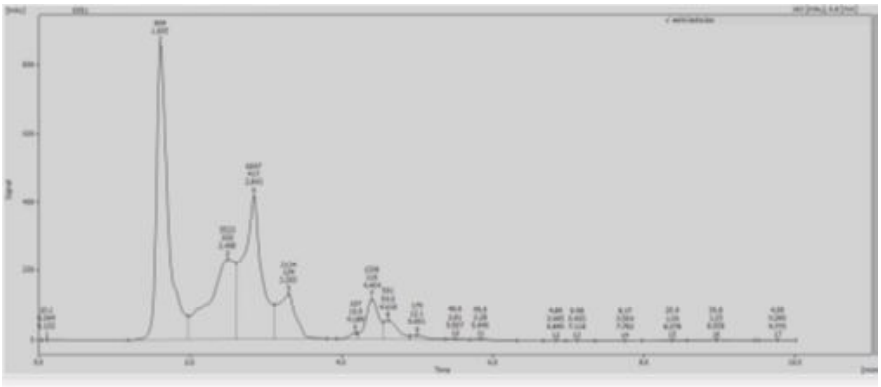


Fig. 1. HPLC chromatogram of Bottle gourd juice probioticated with *L. acidophilus* at UV spectrum of 210 nm

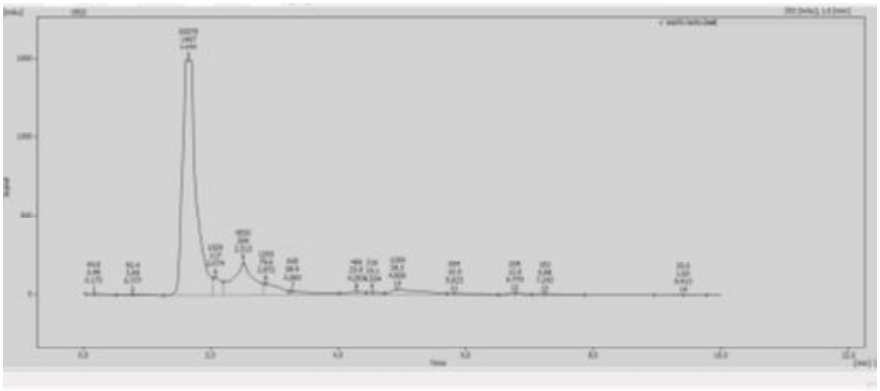
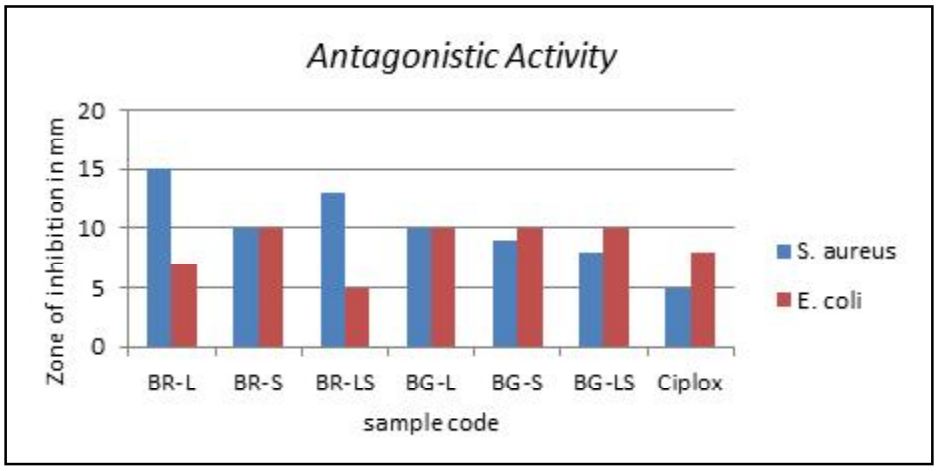


Fig. 1. HPLC chromatogram of beet root juice probioticated with *L. acidophilus* at UV spectrum of 210 nm

After the analysis the probioticated samples were checked for their antagonostic activity against the pathogenic bacterial species that is *S. aureus and E. coli*. All the probioticated juice sample showed antagonistic activity against both pathogens. The best antagonostic activity was shown by beetroot juice sample probioticated with *L. acidophilus* and also with both organisms. The results of antagonostic activity are summarized in table no.4 and also with the graph 1. The antagonistic activity of the juice samples are result of the secondary metabolites of the probioticating agent or the production of their primary metabolism of the juice substrate.

Table-4 showing the results for the antagonistic activity of the probioticated juice samples against pathogenic bacterial species; shows the zone of inhibition measured in mm

Sample Name	Type of Probiotic Culture	Bacterial spp. (ZOI in mm)	
		S. aureus	*E. coli*
Beet Root Juice	*L. acidophilus (BR-L)*	15	7
	S. boulardii (BR-S)	10	10
	L. acidophilus + S. boulardii (BR-LS)	13	5
Bottle gourd Juice	*L. acidophilus (BG-L)*	10	10
	S. boulardii (BG-S)	9	10
	L. acidophilus + S. boulardii (BG-LS)	8	10
Positive Control	*Ciplox (500ppm)*	5	8



Graph 1. showing the results for the antagonistic activity of probioticated juice samples against pathogenic bacterial spp.

Finally the viable cell count at different time interval, of the period of incubtaion, was performed. The viable cell counting was done in a separate set for both type of probiotication culture that free cell and micro-encapsulated. The results showed that the viable count was more after 48 hrs of incubation and the viable cells count was less in case of encapsulated sample as compared with the free cell probiotication cultutre. The results obatined for this set of experiment is presented in table-5.

Table-4. showing the results for the viable cells counts of the probioticated juice samples after 24 hrs and 48 hrs of incubation period

Substrate Name	Type of Probiotic Culture	Type of cell	Cell viability CFU/ml	
			24 hrs	48 hrs
Beet Root Juice	*L. acidophilus*	Free cell	75	190
		Micro-encapsulated	90	130
	S. boulardii	Free cell	375	490
		Micro-encapsulated	190	255
	L. acidophilus+ *S.boulardii*	Free cell	140	140
		Micro-encapsulated	190	225
Bottle gourd Juice	*L. acidophilus*	Free cell	155	320
		Micro-encapsulated	125	245
	S. boulardii	Free cell	350	480
		Micro-encapsulated	160	285
	L. acidophilus + *S.boulardii*	Free cell	135	140
		Micro-encapsulated	215	250

The encapsulation of probiotics in alginate beadscan protect the cells inside from the inhibiting compounds, for example acid and flavonoids, in fruit juices. The micro organisms that are encapsulated survive the digestive system of the host and colonize at the place where they can provide the benefits to the host. A rapid decrease in pH in the beginning of fermentation is of great importance for the quality of the end product[18] and the same observation was seen for both the juice samples. The inhibitory action of probiotic bacteriaagainst the pathogens may be due to the accumulation of main primary metabolites such as lactic acid, acetic acids, ethanol and carbon dioxide. They are capable of producing the secondary metabolites including anti microbial compounds such as formic acid, benzoic acid, hydrogen peroxide, diacetylacetin, and bacteriocin. The Probiotics have shown

to process inhibitory activities mostly towards G+ve pathogens and closely selected bacteria due to the bactericidal effect of protease sensitive bacteriocins,[16]. The results of our present study also agree with the findings of Soleimani *et al.*,[17] who evaluated that the antimicrobial substances produced by *Lactobacillus* have a great potential for inhibiting the growth of pathogenic microorganisms.To be more useful in the body, the living probiotic bacterianumber should be at least greater then 107 CFU per gram. The results indicated that the juices served as a good medium for growing Probiotics. According to the Codex Alimentarius standard, a commercial probiotic beverage should possess a minimum viable count of 106 CFU/ ml at the time of consumption[3] and all the samples juice were related with this CFU unit.

Most of the probiotic juices available in the market are milk based while there is an increased demand for the vegetarian consumer and cholestrol free probiotics and this has led the research to find new matrices for probiotic preparation. The Advance technology have made it possible to use the fruit and vegetable juices as the substrate for the probiotic preparation. The results obtained in this study will be helpful for developing an appropriate probiotic juice from beet root and bottle guard having more health benefits which could be served as a health beverage for vegetarians and consumers who are allergic to dairy products. The probioticated juice can differ in their antagonistic property against the pathogens due to the different metabolite secreted by the lactic acid bacteria specially type of organic acids. Although the results from the study shows that encapsulation method increases the survival of probiotics in fruit juices, the effect of probiotic beads on the sensory characteristic and consumers' conception should be analyzed further.There are some challenges to overcome, *i.e.*, the survival of probiotics, and the effects on the sensory traits; however, there are some possible solutions that show that there is a promising way.

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