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Isolation, identification and evaluation of probiotics from nutritive fruit sample

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Abstract
The objective of the present investigation was to isolate, characterize, and to identify the lactobacillus species from fruit samples banana and apple collected from local area Guntur and Mangalagiri. Fruit juices represent a promising carrier for probiotic bacteria; however, there are some drawbacks and limits that could preclude their production at the industrial level, namely the survival of probiotics throughout storage, and the possible impact of bacteria on the sensory traits and overall acceptance. Twenty Four acid producing bacteria were isolated from samples and based on morphological, biochemical and staining techniques these isolates were reduced to 14 samples. Probiotic screening was performed to these isolates acid (pH2, 3, 4) bile (0.3, 0.5%), phenol (0.2, 0.4%) and NaCl tolerance (3, 6, 9, and 12%) which are main criteria for probiotic to grow under Gut conditions. These results suggest that various Lactobacillus species are distributed in banana and apple was most abundant found in this study.

Keywords: fruit samples, probiotic characterization, lactobacillus sp.

Introduction
Micro organisms play a prominent role in fermentation process of foods. Of all the microbes, lactic acid bacteria (LAB) have the major role. LAB utilizations in food fermentations are one of the most ancient preservation techniques and have arisen from 6000 BC, describing the fermentation of milk and fermentation of meat 1500 BC and vegetable products 300 BC [1]. These strains are referred to as Probiotic cultures in scientific literature of Lilley and Stillwell [2]. Probiotics refer to viable microorganismsthose promote or support a beneficial balance of the microbial population of the gut [3]. Today most of the strains of lactic acid producing bacteria- *Lactobacillus* and *Bifidobacterium* are being widely used in food and pharmaceutical industries as commercial probiotics. Lactic acid bacteria confer health benefits by producing nutraceuticals that are helpful for prevention and treatment of diseases [4]. Exopolysaccharide (EPS), a nutraceutical produced by lactic acid bacteria has shown health- beneficiary properties- such as immune stimulation, antiulcer activities, antitumor activities and cholesterol-lowering activity. These LAB probiotic strains compete with other pathogenic strains in the gut and inhibit their growth by producing a wide range of antimicrobial metabolites such as lactic acid, bacteriocin, hydrogen peroxide, acetic acid, other molecular mass peptides and fatty acids. In addition to these compounds, LAB synthesizes the vitamins B1, B2, B6, B12, niacin, folic acid and pantothenic acid and different enzymes that can enhance the digestibility of protein [5]. The contributions of LAB and their metabolites in the field of preservation of food against spoilage have generated interest towards their isolation and identification [6].
Among the LAB species, *Lactobacillus* and *Bifidobacterium* species are most commonly utilized group of microorganisms for their beneficiary effects as probiotics and are the predominant members of the intestinal microflora [7]. The genus *Lactobacillus* is one of the major groups of lactic acid bacteria having great economical importance in food processing industries. *Lactobacillus acidophilus* L1 hasshown the ability to reduce serum cholesterol level indicating the potential of reducing the risk of coronary heart disease by 6% to 10% in hypercholesterolemic humans [8]. *Lactobacillus rhamnosus* GG has exhibited anti-carcinogenic effects by decreasing the activity of β-glucuronidase [9]. Twenty five strains of lactic acid bacteria (LAB) were isolated from South Indian traditional fermented foods ˊKallappamˊ batter, ˊKoozhˊ and ˊMorKuzhambuˊ and further 6 strains including *Lactobacillus plantarum and Lactobacillus fermentum.* Earlier reports have shown that *Lactobacillus plantarum*AS1 possess antimicrobial activity [10]. Studies on *Lactobacillus acidophilus* and *bifidobacteria* suggest that these strains synthesize folic acid, niacin, thiamine, riboflavin, pyridoxine and Vitamin K, which are slowly absorbed by the body [11, 12]. These vitamins can act as co-factors in many biological processes.
Fruits are serving as the best substrates for the isolation of probiotics. Banana and apple are high nutritive fruits containing small amounts of protein and essentially no fat. These fruits serve as good sources of energy-producing nutrients, mainly in the form of fructose- a simple and natural sugar commonly found in other fruits. Apple and banana provide essential vitamins and minerals-especially banana is rich in folate. Research survey on banana suggest that it possess a wide range of health benefits which include- Protection from ulcers, anticancer properties, immune booster, energy booster, reduce risk of stroke, restore normal bowel activity, lowering cholesterol levels [13]. Studies had shown that some groups of probiotic strains were successfully isolated from banana fruit. Banana contains Fructo oligosaccharide, a compound called prebiotic as it nourishes probiotic bacteria in the colon. These bacteria improve the ability of absorbing nutrients by producing digestive enzymes and vitamins [13]. Hence, in the present study banana and apple are used for the isolation of probiotics.

Materials and Methods

Collection of Samples

Four varieties of Banana and One variety of Apple were collected from nearby market, Guntur, Andhra Pradesh due to wide consuming of people of Andhra Pradesh. Immediately after the collection, the samples were kept in -4ºC refrigerator under aseptic conditions to reduce the risk of contamination.

Media Used

To isolate the lactic acid producing bacteria from fruits MRS media was opted. In addition to this 0.05% cysteine was included to the MRS to increasethe selectivity of this medium for isolation of lactobacillus species. pH of the media is 6.5

LAB Isolation

Lactic acid producing bacteria were screened from apple and banana by using specific MRS medium. One gram of sample was diluted in 9ml of saline solution, after homogenization sample were serially diluted and using spread plate technique plating was done. Plates were incubated at 37ºC for 24-48h under anaerobic condition. After the incubation period plates were observed for colony growth and morphological studies. Single colonies were identified and repeated colonies were not included. The selected colonies were sub cultured by using streaking method. This purified isolates were stored at 4ºC and used for further analysis.

Morphological Identification

Cell Shape and Size

Isolated colonies were simple stained with crystal violet and identified under 45X of compound microscope to observe cell shape and the size.

Gram Staining

Gram staining was conducted to all the isolates for the identification of morphological structure of lactic acid bacterial isolates as per method of (14). Bacterial smear were made with all the isolates on autoclaved clean slide and air dried. Next to the heat fixing, one drop of crystal violet was added after 30sec excess stain was washed with distilled water. After that grams iodine solution was added to the smear for 30sec, then the iodine solution was washed off with 95% ethyl alcohol by adding drop by drop continuously to destain iodine solution. Again, slides were washed with distilled water; finally, safranin stain was applied for 30 sec excess stain was removed with distilled water after 30 sec. The slides were air dried and observed under compound microscope.

Biochemical Characterization

Catalase Test

Bacterial isolates grown on MRS agar for 48hr were used to conduct this test. 1ml of 3% hydrogen peroxide was added to culture. Rapid effervescence showed positive results for catalase activity.

Carbohydrate Utilization test

Utilization of carbohydrates using sugars and fermentation broth were done as per (15). Bacterial isolates were inoculated separately into 10ml of fermentation broth with different carbon source which include glucose, fructose, sucrose, lactose and galactose. Durham tube was inserted in inverted position in test tube and tubes were incubated for 48hr at 37ºC. After incubation period change in the colour of the broth and formation of gas bubbles were observed and results were noted.

Probiotic Screening

Acid tolerance

Initial screening of the isolated microorganisms for their probiotic features were evaluated by determining the tolerance of cultures against various pH based (16). Tolerance for pH was studied by incubating the isolates in appropriate medium adjusted to pH 2.0 and 3.0. One mL of overnight bacterial suspension was adjusted to 0.6 OD at 620 nm in UV Visible spectrophotometer, was inoculated in 10 mL sterile medium and incubated at 37 °C. Samples were withdrawn periodically at 0, 30, 60, 90 and 120 min to determine the cell concentration by measuring OD at 620 nm. The best pH tolerable isolates were further studied for tolerance to bile salt concentration.

Bile tolerance

The tolerance of the isolates for bile salt was determined based on (17).BHI and MRS medium were prepared for aerobes and anaerobes by adding various concentrations of bile salt. The medium was prepared at 0.3, 0.5 and 0.8% concentration of bile. One mL of overnight bacterial suspension was adjusted to 0.6 OD at 620 nm in UV-Visible spectrophotometer, was inoculated in 10 mL sterile medium and incubated at 37 °C. Samples were withdrawn periodically at 0, 30, 60, 90 and 120 min to determine the cell concentration by measuring OD at 620 nm. The best bile tolerable isolates were further studied for other probiotic properties.

Tolerance to NaCl, Temperature, Phenol

Probiotic characterization was evaluated by checking the resistance of the isolates to different concentrations of salt, phenol and temperature. MRS broth was opted to study the above-mentioned inhibitory substances. Growth at an incubation period in different NaCl concentration [3, 5, 9 and 12% (w/v)], phenol level (0.2–0.4 g phenol/100 ml), and temperature (15, 28, 37 and45ºC) were monitored at 620 nm after 24 h (17).

Results and Discussion

Isolation and Identification of Probiotic isolates

From Apple and Banana, a total of 34 strains were isolated from two different types of media MRS agar and MRS cysteine of fermented samples of Banana and Apple. On screening total 14 isolates were short listed on basis of morphological, biochemical and potential characterization. The screened 12 isolates from 4 varieties of Banana were MBa2, MBa9, MBa14, MBb19, MBb23, MBb31, MBb34, MBc42, MBc49, MBd56, MBd62, MBd73, and from Apple MA7, MA11 were observed in various forms (circular/irregular), size (small/medium/punctiform), surface (rough/veined/glistening), texture (moist/mucoid/dry), color (cream/ translucent/ white/ yellow/ pale yellow) elevation (flat/raised) and Gram staining –Gram positive. (Table: 1)

Biochemical Characterization

All the cultures isolated from fruits were observed as positive for lactose and lactose gas production, nine isolates

showed positive result for glucose and glucose gas production MBa9, MBa14, MBb23, MBb34, MBc49, MBd56, MBd62, MBd73, MA11 and remaining 5 isolates shows negative only for gas production. And 11 isolates shows positive and 3 isolates shows negative for fructose and fructose gas production. Except one isolate all the isolates show positive color for sucrose utilization and sucrose gas production. All the isolates shows positive for galactose utilization and its gas production except for two isolates are observed as negative (MBb31. MBd62). However, all the isolates shown negative results for catalase assay. (Table: 2)

Probiotic Screening

The 14 cultures isolated from nutritive foods were checked for probiotic properties, all the cultures were observed to be resistance to acidic pH (2, 3, 4) bile (0.3, 0.5) and three isolates at (0.8%), NaCl (3-12%) and phenol (0.2-0.4%). Based on the morphological, biochemical and probiotic parameters eight (MBa9, MBb19, MBb23, MBb31, MBc49, MBd56, MBd73 and MA7) of fourteen potential probiotic isolates observed to be maximum similarities with Lactobacillus species. MBa14 and MBb34 showed maximum similarities with Bifidobacterium where as MBa2, MBc42, MBd62 and MA11 showed maximum similarities with Bacillus spp. Results are listed in the Table: 3.

Table 1: Morphological Studies of Isolates

Isolate	Colony Morphology	Cell morphology	G. Staining
MBa2	Pale yellow, small, round, raised, moist	Long rods	G+
MBa9	Milky white, small, irregular, raised, mucoid	Coccobacilli in chains	G+
MBa14	Creamy white, big, circular, flat, moist	Coccobacilli in singles	G+
MBb19	Creamy white, small, circular, flat, mucoid	Long rods in chains of two	G+
MBb23	Dull white, small, circular, raised, moist	Short rods in chains	G+
MBb31	Milky white, medium, irregular, flat, moist	Long rods	G+
MBb34	Pure white, punctiform, circular, flat, mucoid	Coccobacilli in singles	G+
MBc42	Pale yellow, small, circular, flat, raised, mucoid	Short rods	G+
MBc49	Creamy white, small, circular, flat, moist	Long rods in chains of two	G+
MBd56	White, punctiform, irregular, raised, mucoid	Coccobacilli in chains	G+
MBd62	Dull white, small, circular, raised, mucoid	Coccobacilli in singles	G+
MBd73	Pure white, small, circular, flat, moist	Long rods	G+
MA7	Creamy white, punctiform, circular, flat, moist	Long rods in chain	G+
MA11	Pale Yellow, small, irregular, raised, mucoid	Short rods	G+

Table 2: Biochemical Characterization of Isolates

Culture	Cat	Carbohydrate Utilization									
		Lac	Lac G	Glu	Glu G	Fruc	Fruc G	SUC	Sc G	Gal	Gal G
MBa2	-	+	+	+	-	+	+	+	+	+	+
MBa9	-	+	+	+	+	-	-	+	+	+	+
MBa14	-	+	+	+	+	+	+	+	+	+	+
MBb19	-	+	+	+	-	+	+	+	+	+	+
MBb23	-	+	+	+	+	+	+	+	+	-	-
MBb31	-	+	+	+	-	+	+	+	+	+	+
MBb34	-	+	+	+	+	+	+	+	+	+	+
MBc42	-	+	+	+	-	+	+	+	+	+	+
MBc49	-	+	+	+	+	-	-	+	+	+	+
MBd56	-	+	+	+	+	+	+	-	-	+	+
MBd62	-	+	+	+	+	+	+	+	+	+	+
MBd73	-	+	+	+	+	-	-	+	+	+	+
MA7	-	+	+	+	-	+	+	+	+	-	-
MA11	-	+	+	+	+	+	+	+	+	+	+

Table 3: Probiotic Characterization of Isolated Strains

Isolate	Acid tolerance			Bile Tolerance			NaCl assay	Phenol assay	Temp assay
	pH 2	pH 3	pH4	0.5%	1.0%	1.5%	3-12%	0.2-0.4%	15-45ºC
MBa2	+	+	+	+	+	+	+	+	+
MBa9	+	+	+	+	+	+	+	+	+
MBa14	+	+	+	+	+	+	+	+	+
MBb19	+	+	+	+	+	+	+	+	+
MBb23	+	+	+	+	+	+	+	+	+
MBb31	+	+	+	+	+	+	+	+	+
MBb34	+	+	+	+	+	+	+	+	+
MBc42	+	+	+	+	+	+	+	+	+
MBc49	+	+	+	+	+	+	+	+	+
MBd56	+	+	+	+	+	+	+	+	+
MBd62	+	+	+	+	+	+	+	+	+
MBd73	+	+	+	+	+	+	+	+	+

MA7	+	+	+	+	+	+	+	+	+
MA11	+	+	+	+	+	+	+	+	+

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