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ISOLATION OF PROBIOTIC BACTERIA FROM *Panchamirtham*: ETHINIC SOUTHERN INDIAN FERMENTED FRUIT MIX

S. UMA MAHESHWARI[1]*, S. AMUTHA[1], G. HEMALATHA[1], N. SENTHIL[2],R. ANANDHAM[3] AND K. KUMUTHA[3]
[1]Department of Food Science and Nutrition, Home Science College and Research Institute, Madurai -625 104.
[2]Department of Biotechnology, [3]Department of Microbiology,Agriculture College and Research Institute, Madurai - 625 104.
e-mail: ?

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***Corresponding author**

ABSTRACT
Probiotics are beneficial microbes that confer a realistic health benefit on the host, which in combination with prebiotics, (indigestible dietary fibre/carbohydrate), increases health benefit. Hence, the present study was directed towards the isolation of probiotic bacteria from Panchamirtham which is fermented fruit mix. A total of three samples of Panchamirtham were collected from three different locations for the isolation of probiotic bacteria by using MRS agar medium. Fifteen isolates were selected based on colour, size and shape. Isolates were tested for pH and sucrose tolerance. The 58 and 61% of the isolates tolerated pH 5 and 8 respectively. Among the bacterial isolates, five percent, seventy percent, seventy five percent and eighty percent showed 100, 80, 67 and 47% sucrose tolerance, respectively. Among the isolated bacteria, ninety three percent were Gram positive which exhibited negative for catalase, citrate, H2S and gelatin hydrolysis. Based on the biochemical and morphological tests isolated bacteria were tentatively identified as Lacococcus spp.,

INTRODUCTION

Probiotics are deûned by the World Health Organization (WHO) as ''living microorganisms which, when administered in adequate amounts, confer a health beneûts to the host'' (FAO/WHO, 2002). Lactic acid bacteria (LAB) together with biûdobacteria are classiûed as the generally recognized as safe (GRAS) and have become widely used worldwide as probiotics to improve health. Fermented foods and beverages are staples of the human diet and have been produced and consumed since the development of human civilizations (Hutkins, 2008). Various studies have indicated that probiotics may alleviate lactose intolerance; have a positive influence on the intestinal flora of the host; stimulate/modulate mucosal immunity; reduce inflammatory or allergic reactions; reduce blood cholesterol; possess anti-colon cancer effects; reduce the clinical manifestations of a topic dermatitis, Crohn's disease, diarrhea, constipation, candidiasis, and urinary tract infections; and competitively exclude pathogens (Gill and Guarner, 2004). Considering this impressive list of potential health-promoting benefits, it is not surprising that there continues to be considerable interest in the use of probiotics as biotherapeutic agents (Reid *et al*., 2003). Furthermore, given a heightened awareness among consumers of the link between diet and health and the fact that probiotic-containing foods are generally perceived as "safe" and "natural," the global market for such foods is on the increase, particularly dairy-based products marketed for the prophylaxis or alleviation of gastrointestinal disorders(Stanton *et al*.,2001). Panchamirtham, is a fermented fruit mixture which contains five types of foods used in hindu worship and pooja to divinity. Panchamirtham encapsules "astonishing" properties which keep in conditions suitable for consumption over a long period without refrigeration. It is prepared with "sirumalai or virupakshi plantains", a special variety which has "very little water content" that ensures a longer shelf-life. Panchamirtham is prepared from crushed plantains, seedless dates, sugar candy, kismis and jaggery are mixed together and allowed for fermentation for 12-15 hours. Plantains contain a higher amount of dietary fibre with folates, niacin, riboflavin and thiamin; among the different vitamins folates are essential for healthy pregnancy. Plantains are rich in vitamin-C helps the body to develop resistance against infectious agents and act as a powerful antioxidant. However to the best of our knowledge probiotic microbial composition of Panchamirtham has not yet been elucidated. Hence, in this study we report the presence of potential probiotic bacteria in Panchamirtham.

MATERIALS AND METHODS

Sample Collection

Panchamirtham sample was collected from three different locations viz.,Thiruparankundram (9º 52È 51½ N latitude and 78º 04È17½ E longitude), Thiruchendur (8º 29È45½ N latitude and 78º 07È45½ E longitude) and Pazhani (10º 59È34½ N latitude and 77º 06È09½ E longitude), Tamil Nadu, India.

Isolation and Purification

Ten grams of Panchamirtham sample was aseptically transferred into a 250 mL flask containing 90 mL sterile saline

solution (0.85 % NaCl) and the contents were mixed thoroughly for 30 min at 150 rpm in a incubator cum shaker. Serial dilutions (10-1 to 10-6) were made for each sample and 100 μL of each dilution was spread onto each deMan Rogosa Sharpe agar (MRS). The plates were incubated at 30ºC and bacterial counts were observed for three days continuously. After isolation, bacteria were purified by four streak method to get a pure single colony. Purified cultures were stored in 50% glycerol at -20ºC for further use (Anisha et al., 2015).

Biochemical characterization

Bacterial isolates were tested for growth at different intestinal pH of 3.0, 4.0, 5.0 and 8.0 (Son *et al*., 2018). Tolerance to sucrose (5%, 70%, 75% and 80%) was tested using MRS broth (Eman *et al*., 2010; Anisha *et al*., 2015). Then the growth of the bacterial cultures was observed at 600 nm. Sucrose concentration was fixed based on the sugar content in Panchamirtham. 75º brix reading of panchamirtham indicated 75% of sugar. An assay for gelatin hydrolysis was performed in according to Harrigan *et al*. (1970). Catalase activity was tested by spotting colonies with 3% hydrogen peroxide. The release of free oxygen bubbles indicated a positive test (Kushwaha *et al*., 2013).

Acid production test was carried out for the bacteria in MRS broth supplemented with one percent bromo cresol blue as indicator. After twenty four hours of incubation, the acid production was ascertained through the change of colour from blue to yellow.

Citrate utilization

The ability to utilize citrate as sole source of carbon and energy can be used to distinguish certain Gram negative bacteria. Simmon's citrate agar medium was inoculated with 48 hours old cultures and incubated at 37ºC for 48 hours. The alkaline pH turns indicator (bromothymol blue) from green to blue is a positive result (Majumder *et al*., 2017).

Hydrogen sulfide production

Kligler Iron Agar (KIA) test was used to aid in the differentiation of enteric Gram-negative bacilli on the basis of H2S production. Briefly, 55gm of the KIA medium was suspended in 1 l of deionized water, mixed well and sterilized at 121ºC for 20 min. The tubes were cooled in a slanted position to obtain a butt of 1.5 - 2.0 cm depth. Then the medium was inoculated by stabbing the butt and streaking the surface of the tube. After that, the tubes were incubated at 37ºC for 24 hours and the results were recorded (Ali *et al*., 2017).

Morphological characterization

The morphological characterization of isolated probiotic bacteria were performed by following methods of Gerhardt *et al*. (1994). The colony characters viz., colour, shape size and Gram's reaction of the bacterial isolates were observed.

RESULTS AND DISCUSSION

pH and sucrose tolerance

Generally the probiotic bacteria were isolated from fermented food products. Most of the studies documented the fermented dairy products as the source for potential probiotics. However, in this study we demonstrated the fermented fruit mix also a novel source for isolation of probiotic bacteria. The ability of the Panchamirtham to compete with the natural microbiota of the raw material and to undertake the metabolic activities expected is conditioned by its growth rate and survival in the conditions prevailing in the fermented product, *i*.e., an anaerobic atmosphere, rather low to high sugar concentrations and pH. Samples were selected at three different location viz., Thiruparankundram, Thiruchendur and Pazhani. Totally fifteen bacterial isolates were selected based on colony morphology (colour, size and shape). All the isolates were purified by using the four streak method. Table 1 showed the results of pH tolerance of fifteen isolates at pH 3.0, 4.0, 5.0 and 8.0 and sucrose tolerance (5%, 70%, 75% and 80%). All the test isolates showed the highest acid tolerance and hence taken for further characterization. A bacterial strain to serve as potential probiotic should survive the pH stress of gastric acid. Following this criteria, acid tolerance of isolates was determined at different pH for different time intervals simulating human gastric passage. Strains capable of tolerating the pH stress for studied period are recognized as acid tolerant. Hwanhlem *et al*. (2011) reported that lactic acid produced by LAB is an essential compound for food preservation because it maintains the acidity conditions of the fermented foods and antagonistic against food spoilage and poisoning bacteria.

Table 1: pH and sucrose tolerance of probiotic bacteria

S. No	Isolates	pH tolerance (OD600nm)					Sucrose tolerance (OD600nm)		
		3	4	5	8	5%	70%	75%	80%
1	M3S1B1	0.41±0.00	0.41±0.01	1.61±0.03	1.59±0.02	1.90±0.01	0.64±0.00	0.35±0.01	0.29±0.00
2	M3S1B2	0.10±0.00	0.30±0.00	1.63±0.03	1.38±0.04	1.86±0.05	0.62±0.00	0.44±0.00	0.46±0.01
3	M3S1B3	0.51±0.00	0.48±0.01	1.55±0.05	1.63±0.02	2.21±0.09	0.67±0.01	0.63±0.01	0.56±0.01
4	M3S1B4	0.11±0.00	0.11±0.00	1.23±0.00	1.27±0.00	1.82±0.08	0.63±0.02	0.64±0.02	0.60±0.02
5	M3S1B5	0.19±0.00	0.49±0.00	0.50±0.02	0.56±0.01	1.87±0.03	0.79±0.02	0.53±0.02	0.49±0.01
6	M3S1B6	0.19±0.00	0.45±0.01	0.72±0.02	1.30±0.02	1.23±0.04	0.33±0.01	0.25±0.00	0.24±0.00
7	M3S1B7	0.58±0.00	1.63±0.02	1.53±0.06	1.60±0.02	1.98±0.02	0.64±0.01	0.56±0.00	0.50±0.01
8	M3S1B8	0.09±0.00	1.43±0.01	1.66±0.03	1.55±0.09	1.92±0.08	0.53±0.00	0.75±0.00	0.44±0.01
9	M3S2B1	0.34±0.00	1.42±0.00	1.39±0.05	1.51±0.05	1.76±0.04	0.80±0.03	0.51±0.01	0.92±0.02
10	M3S2B2	0.61±0.02	0.52±0.01	0.67±0.01	0.54±0.01	1.98±0.06	0.61±0.02	0.58±0.00	0.46±0.01
11	M3S2B3	0.41±0.00	0.37±0.01	1.58±0.01	1.73±0.04	1.88±0.05	0.63±0.01	0.57±0.01	0.59±0.00
12	M3S2B4	0.32±0.00	1.24±0.02	1.44±0.04	1.56±0.03	1.84±0.02	0.75±0.01	0.65±0.02	0.65±0.00
13	M3S2B5	0.21±0.00	0.38±0.01	1.32±0.01	1.37±0.00	1.02±0.03	0.34±0.00	0.30±0.00	0.20±0.00
14	M3S3B1	0.12±0.00	0.17±0.00	1.37±0.05	1.28±0.05	1.86±0.01	0.81±0.01	0.65±0.01	0.62±0.02
15	M3S3B4	0.10±0.00	0.08±0.00	0.07±0.00	1.54±0.02	0.65±0.01	0.13±0.00	0.10±0.00	0.09±0.00

Table 2: Morphological and biochemical characteristics of bacterial isolates

Isolates	Cell morphology	Gram's reaction	Sugar fermentation test						Cat alase	Citrate	H2S production	Hydrolysis of gelatin
			Glucose		Sucrose		Lactose					
			C	G	C	G	C	G				
M3S1B1	Cocci	+ve	+	-	+	-	+	-	-	-	-	-
M3S1B2	Cocci	+ve	+	-	+	-	+	-	-	-	-	-
M3S1B3	Cocci	+ve	+	-	+	-	+	-	-	-	-	-
M3S1B4	Cocci	+ve	+	-	+	-	+	-	-	-	-	-
M3S1B5	Cocci	+ve	+	-	+	-	+	-	-	-	-	-
M3S1B6	Cocci	+ve	+	-	+	-	+	-	-	-	-	-
M3S1B7	Cocci	+ve	+	-	+	-	+	-	-	-	-	-
M3S1B8	Cocci	+ve	+	-	+	-	+	-	-	-	-	-
M3S2B1	Cocci	+ve	+	-	+	-	-	-	-	-	-	
M3S2B2	Cocci	-ve	+	-	+	-	+	-	-	-	-	-
M3S2B3	Cocci	+ve	+	-	+	-	+	-	-	-	-	-
M3S2B4	Cocci	+ve	+	-	+	-	+	-	-	-	-	-
M3S2B5	Cocci	+ve	+	-	+	-	+	-	-	-	-	-
M3S3B1	Cocci	+ve	+	-	+	-	+	-	-	-	-	-
M3S3B4	Cocci	+ve	+	-	+	-	+	-	-	-	-	-
Tentative identification		Lactococcus spp.										

LAB ferment sugars via a number of different pathways, resulting in homo or mixed acid fermentation. Tolerances to acid and bile stresses are important properties for any potential probiotic bacteria. The ability of probiotic bacteria to survive in adequate numbers after subjected to gastric acidity (low pH) and intestine condition (bile salts) is important to be used in the food industry (Chalas *et al*., 2016; Kotzamanidis *et al*., 2010).

All the tested isolates growth was drastically reduced at higher concentration of sucrose. Traditionally, jaggery was prepared by the concentration of a sugarcane juice extract, made from locally cultivated sugarcane, without addition of any chemicals. Jaggery is the major ingredient in the Panchamirtham, and can constitute upto 75% of the product. Sucrose is one of the major sugars present in jaggery, and its concentration ranged between 66-77% (Chand *et al*., 2012). All the bacterial isolates tolerated 5%, 70%, 75% and 80% sucrose content. Overall, ten isolates showed higher sucrose tolerance which was indicated by OD600nm. Based on sucrose tolerance, minimum growth was observed for the four isolates (OD600nm0.10 to 0.44). Acid and bile tolerances of all the isolates varied significantly due to strain or species specificity. The resistant mechanism towards low pH or bile concentration is strain and species dependent (Montville and Matthews, 2013). Similar results have also been reported by Angmo *et al*. (2016) and Das *et al*. (2016) for lactic acid bacteria isolated from Ladakh beverages and marine samples.

Acid production test

Among the tested bacteria, isolate M3S2B1, the changed the colour from blue to yellow after twenty four hours of incubation. Leite *et al*. (2015) reported that survival at different pH and bile salts concentrations is mandatory for probiotic cultures, since this is related to survival of these bacteria in the passage through the gastrointestinal tract.

Biochemical test

All the isolates were stained with Gram's reagents for detection of their Gram's reaction. Violet colored cocci shaped cells were observed under microscope (Table 2). To identify these isolates biochemically, various biochemical tests like sugar fermentation viz., glucose, sucrose and lactose, cell morphology, Gram's reaction, catalase, citrate, hydrogen sulfide production and gelatin hydrolysis were carried out. The results of various biochemical tests are summarized in Table 2 which showed that all the isolated bacteria are Gram positive. Resulted Gram's staining reaction was further ascertained through KOH string test. Briefly, 3% KOH was placed on glass slide and a loop of the inoculum was suspended in KOH. If bacterial isolates are Gram negative, bacterial cell wall is dissolved in 3% KOH, forms a string between glass slide and inoculation loop. In case of Gram positive bacterium, bacterial cell wall is not dissolved in KOH and string is not formed (Gram,1884; Jayadev and Navamani, 2013). All the tested isolates were found to be catalase negative, citrate utilization negative, hydrogen sulfide negative and hydrolysis of gelatin negative. In the present study, all the isolates were found an ability to ferment glucose, sucrose and lactose and produced gas. Lactococci are generally isolated on rich media such as MRS (De Man *et al*., 1960), which is routinely used for the isolation and counting of lactobacilli from the most of (fermented) food products. Hence lactococcal cultures were confirmed based on phenotypic characteristics by under a light microscope, lactococci are generally regularly shaped, non-motile, non-spore-forming, Gram-positive rods (Coeuret *et al*., 2003).

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