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Research Article

Isolation and assessment of potential probiotic microorganisms from modified indigenous foods preparation

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ABSTRACT: **Objective:** The objective of present study is to isolate potential probiotic microorganisms from modified indigenous foods and assessment of its antimicrobial activity against some selected pathogenic strains including *E. coli, E. faecilis, K. pneumonia, S. dysentrae*, *S.epidermidis, S. saprophyticus, S. pyogenes* and *S. aureus. These isolated microorganisms were further characterized using microscopic and biochemical methods.* **Method:** Six different set of probiotic indigenous foods were prepared by Bulgur Wheat and Corn grits. Each probiotic isolate *was grown in Man Rogasa Sharp (MRS) broth for 24 h at 37°C in order to prepare Cell Free Supernatant (CFS)* The CFS obtained from isolated strain was tested for antimicrobial activity by well diffusion method. **Results:** The study finally led us to isolate creamy white, circular, convex elevated and smooth rough textured colonies by using MRS agar containing bromocresol purple. The isolates were Gram positive, rod shaped, catalase negative and shows increase in antimicrobial activity (zone of inhibition) of isolates CFS against *E.coli (22.3 ± 1.2), E. faecalis* (23 ± 0.8), *S. pyogenes* (24.3 ± 0.4), *S. saprophyticus* (18 ± 0.8), *S. aureus* (16.6 ± 0.4), *S.epidermidis (17 ± 0), K. pneumonae* (18 ± 0.8), *S. dysentrae* (17 ± 0.8), which is more than processed probiotic CFS. It was observed that isolates demonstrated good capacity to resist bile salts by showing growth under exposure to 0.3% bile salts and able to grow in pH 2 as well as pH 3. **Conclusion**: These results suggest that these isolates could successfully transit the human stomach and may be capable of reaching the intestinal environment and functioning effectively there.

KEYWORDS: Probiotics, Antimicrobial activity, Pathogenic microorganisms, Probiotic foods

INTRODUCTION

Probiotics are defined as live microbial feed supplement that beneficially affects the host by improving its intestinal balance and are generally added into appropriate foods, usually fermented milks.[1] These bacteria affect the gut microflora positively and decrease the microbial toxic activity.[2-3] Several genera of bacteria and yeast have been proposed as probiotic cultures such as *Lactobacillus, Bifidobactreium*, *Streptococcus, Ruminococcus, Enterococcus, Lactococcus, Leuconostoc*, *Pediococcus, Saccharomyces* and a single strain from *Escherichia*.[4-5] An optimum 'microbial population balance' in our digestive tract is generally associated with good nutrition and health. Stress and improper diet negatively influence the interaction between intestinal microorganisms and lead to detrimental effects in health. Consumption of probiotic microorganisms can help maintain favorable intestinal microbial population and results in several therapeutic benefits, Therefore in recent year's probiotic bacteria have increasingly been incorporated into foods as dietary adjuncts. The 'therapeutic minimum' (10^8 cfu/ml) needs to be consumed regularly for transfer of the 'probiotic' effect to consumers.[6]

The growth of probiotic microorganisms is encourage in presence of prebiotics, which are non-digestible

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substances that provide a beneficial physiological effect for the host by selectively stimulating the favorable growth or activity of a limited number of indigenous bacteria. The appropriate combination of prebiotics and probiotics manifest higher potential for a synergistic effect.[7]

Probiotics have been suggested to be associated with alleviation of lactose intolerance, prevention and cure of viral and bacterial diseases, antibiotic or radiotherapy induced diarrheas, anti-mutagenic and anti-carcinogenic effects, immune modulation and even blood cholesterol reduction.[8-14]

The aim of the present study is to isolate potential probiotic microorganisms from modified indigenous foods (Foods known to be grown in a particular area that can't be grown elsewhere). These isolates were further characterized by using microscopic and biochemical methods such as Gram staining, motility test, indole test, methyl red- voges prosker test, citrate utilization test, carbohydrates fermentation test etc. Antimicrobial activity these isolates were further tested against some selected pathogenic strains.

MATERIALS AND METHODS

Probiotic indigenous foods preparation

Six different set of probiotic indigenous foods were prepared by using two cereals, Bulgur Wheat (*Triticum aestivum*) used as substrate 1, Corn grits (*Zea mays*) used as substrate 2 in a 100 ml screw cap plastic box. Each set contain 5 gm of cereal and labeled as sample 1 to 6. Each set of cereal was taken aseptically and rinsed with autoclaved warm water. Sample 3 (containing substrate 1) and sample 4 (containing substrate 2) were partially boiled at 100°C for 10 minutes and samples 5 and 6 were mixed with equal amount of yoghurt. Each sample was inoculated with probiotic microorganisms ($2x10^8$cells/ml) obtained from probiotic capsules (Aristo pharmaceutical pvt ltd. each containing *Lactobacillus acidophilus, Lactobacillus rhamnous, Bifidobacterium longum, Saccharomyces boulardii*). Different combinations of indigenous foods prepared with probiotic micro organism were given in Table 3.

Isolation of Bacteria

Sample was taken aseptically and enriched with MRS broth (Himedia) and incubated an-aerobically at 37°C for 24 h. These MRS broth cultures were serially dilute and inoculated on a selective MRS agar media containing 3% bromocresol purple (Himedia laboratories Pvt. Ltd., Mumbai) at 37°C. After incubation, colonies surrounded by a yellow-color zone were isolated from MRS agar plate.[15]

Table 1: Probiotic foods and there components

S.No.	Probiotic foods	components
1	Sample 1	Substrate 1 + probiotic bacteria
2	Sample 2	Substrate 2 + probiotic bacteria
3	Sample 3	Boil Substrate 1 + probiotic bacteria
4	Sample 4	Boil Substrate 2 + probiotic bacteria
5	Sample 5	Substrate 1 + Yogurt + probiotic bacteria
6	Sample 6	Substrate 2 + Yogurt + probiotic bacteria

Microscopic characterization of isolates

Identification of isolates was performed by microscopic tests including Gram staining and motility testing. Morphological characteristics were observed directly.[16]

Biochemical characterization of isolates

The isolates were characterized by biochemical methods. The biochemical tests includes indole test, methyl red-voges prosker test, citrate utilization test, catalase test, starch, gelatin and casein hydrolysis test, triple sugar iron test, arginine hydrolysis test and carbohydrate fermentation test.[17-19]

Selection of potential probiotics

Probiotic bacterial cultures must be capable of surviving and growing in the intestine and maintaining their viability and activity.[20] Potential probiotic bacteria have been selected on the basis of following characteristics.

Acid tolerance

The tolerance of isolates to acids was evaluated in MRS containing 3% bromocresol purple with pH 1, 2 and 3. Above media were inoculated with overnight culture and incubated an-aerobically at 37°C for 2–4 d, followed by change of the color from purple to yellow.[21]

Bile tolerance

The tolerance of isolates to bile salts (Rankem) was evaluated in MRS containing 3% bromocresol purple supplemented with bile salts using a modified method.[22] The control medium was MRSB without bile salt thereafter both media were inoculated with overnight culture and incubated an-aerobically at 37°C for 2–4 d, followed by change of the color from purple to yellow in presence of acid production, which are characteristics of probiotic

microorganisms as they are produce organic acids such as lactic acid, propionic acid, acetic acid etc.

Growth at different temperatures

MRS containing 3% bromocresol purple indicator were incubated with overnight culture for 7 d at 10°C, 15°C, and 45°C and cells growth at different temperatures was observed.

Growth at different NaCl concentrations

MRS containing 3% bromocresol purple were prepared with two different concentrations of NaCl (4% and 6.5%) and inoculated with 1% overnight cultures of isolates and then incubated an-aerobically at 37°C for 7 d.

Determination of antimicrobial activity by well diffusion method

Antimicrobial activity of the selected probiotic isolates was checked by using the agar well diffusion technique.[23]

Preparation of cell free extract (CFS or sample filtrate)

Each probiotic isolate was grown in MRS broth for 24 h at 37°C in order to prepare CFS. CFS was obtained by centrifuging the culture (10000 r/min, 10 min) followed by filtration of the supernatant through a 0.2 µm pore size filter.[24]

Preparation of pathogenic strains

Seven gastric pathogenic bacteria were chosen as test organisms. The test organisms were revived in growth medium as shown in Table 2 from glycerol stocks and incubated at 37°C for 16–18 h.

Table 2: Bacterial strains and there growth medium

S. No.	Accession No.	Strain Name	Growth medium
1	ATCC 9754	*Shigella dysenteriae*	MacConky medium
2	MTCC 6155	*Staphylococcus saprophyticus*	MSA medium
3	MTCC 442	*Streptococcus pyogenes*	MSA medium
4	ATCC 29122	*Enterococcus facecalis*	Nutrient broth medium
5	MTCC 2405	*Klebsiella pneumoniae*	TSB medium
6	MTCC 25922	*Escherichia coli*	Luria Bertani (LB) broth
7	ATCC 25923	*Staphylococcus aureus*	MSA medium

Well diffusion method

Inhibitory activity of CFS of probiotic isolates was investigated by well diffusion method.[25] Pathogenic strains were spread onto Mueller Hinton Agar (MHA) plates to completely cover the surface of the agar. Six mm diameter wells were punched into the agar using sterilized well cutter to obtain a 6 mm diameter bore. 100 µl of each CFS was carefully pipette into each well. The plates were incubated for 24 h at 37°C and diameter of the inhibition zones around the wells were recorded in millimeters.[26]

RESULTS

Isolation and identification of bacteria

Total 6 microbial isolates {Probiotic isolate 1 (P1) to probiotic isolate 6 (P6)} were obtained, one from each sample (sample 1, sample 2, sample 3, sample 4, sample 5 and sample 6) of indigenous food preparation. Gram staining and catalase test was performed for these isolates and processed probiotic microorganisms (Probiotic microorganisms obtained from probiotic capsules were culture in MRS broth), all isolates were Gram positive, rod shaped as shows in Table 3. Colonies appeared creamy white in color, circular in shape, convex elevated and smooth rough textured.

Biochemical characterization

Biochemical tests were performed for all 6 microbial isolates (Table 4). All isolates were unable to utilize casein, citrate and gelatin but were able to ferment sugars used for carbohydrate fermentation test (Table 5).

Table 3: Morphological and gram staining characterization of isolates

S. No.	Isolates from food sample	Gram staining	Shape
1	Processed probiotic microorganisms	+	Rod shape
2	P1	+	Rod shape
3	P2	+	Rod shape
4	P3	+	Rod shape
5	P4	+	Rod shape
6	P5	+	Rod & cocci shape
7	P6	+	Rod & cocci shape

Where P1 to P6 = Probiotic isolate 1 to probiotic isolate 6 obtained from sample 1 to sample 6 respectably, + = Positive result

Acid tolerance and Bile salt tolerance

Resistant towards low pH is one of the major selection criteria for probiotic strains due to significant decrease in the viability at pH 2.0 and below.[3, 27-28] All 6 isolates were able to grow in pH 2 and pH 3. After exposure to acidic conditions, isolates were assayed for bile salt tolerance. Six isolates of indigenous food demonstrated good capacity to resist bile salts by showing growth under exposure to 0.3% bile salts as shown in Table 6. This result indicates that these isolates satisfy the necessary criterion which is used for probiotic microorganisms.

Antimicrobial activity

Antimicrobial activity of P1, P2, P3, P4, P5, and P6 was evaluated by agar well diffusion method against human pathogenic microorganism (*E. coli, E. faecilis, K. pneumonia, S. dysentrae, S. saprophyticus, S. pyogenes and S. aureus*) as shown in Table 7. All the data were

Table 4: Biochemical tests for isolates

Isolates from food sample	Catalase	Indole	Gelatin	Casein	Citrate	MR	VP	Starch	Arginine	TSI		
										GP	H_2S	SF
Probiotics	–	–	–	–	–	–	–	+	+	+	–	+
P1	–	–	–	–	–	–	–	+	+	+	–	+
P2	–	–	–	–	–	–	–	+	+	+	–	+
P3	–	–	–	–	–	–	–	–	+	+	–	+
P4	–	–	–	–	–	–	–	–	+	+	–	+
P5	–	–	–	–	–	–	–	–	+	+	–	+
P6	–	–	–	–	–	–	–	–	+	+	–	+

Where P1 to P6 = Probiotic isolate 1 to probiotic isolate 6 obtained from sample 1 to sample 6 respectably
+ = Positive result, – = Negative result, MR & VP = Methyl–Red & Vogues–Proskauer Test
GP = Gas production, SF = Sugar fermentation

Table 5: Carbohydrate fermentation test

S. No.	Isolates from food sample	Carbohydrates						
		Glucose	Sucrose	Maltose	Fructose	Lactose	Galactose	Sorbitol
1	Probiotics	+	+	+	+	+	+	+
2	P1	+	+	+	+	+	+	+
3	P2	+	+	+	+	+	+	+
4	P3	+	+	+	+	+	+	+
5	P4	+	+	+	+	+	+	+
6	P5	+	+	+	+	+	+	+
7	P6	+	+	+	+	+	+	+

Where P1 to P6 = Probiotic isolate 1 to probiotic isolate 6 obtained from sample 1 to sample 6 respectably, + = Positive result

Table 6: Growth parameter of isolates

S. No.	Sample	Growth at			NaCl		pH			0.3% bile salt
		10°C	15°C	45°C	2%	6.5%	1	2	3	
1	Probiotics	–	–	+	+	–	–	+	+	+
2	P1	–	–	+	+	–	–	+	+	+
3	P2	–	–	+	+	–	–	+	+	+
4	P3	–	–	+	+	–	–	+	+	+
5	P4	–	–	+	+	–	–	+	+	+
6	P5	–	–	+	+	+	–	+	+	+
7	P6	–	–	+	+	+	–	+	+	+

Where P1 to P6 = Probiotic isolate 1 to probiotic isolate 6 obtained from sample 1 to sample 6 respectably, + = Positive result, – = Negative result

Table 7: Antimicrobial activity of the isolates in terms of ZOI using the agar well diffusion method (mean± SD)

S. No.	Pathogenic strains	Probiotics	Cell free supernatant from food sample (mm)					
			P1	P2	P3	P4	P5	P6
1	*E. coli*	16.6±0.9	18±0	18.3±0.9	17±0.8	18.3±1.2	24.3±0.4	22.3±1.2
2	*S. dysentrae*	13.3±1.2	16.6±0.4	15.6±0.9	14.6±0.4	15.3±0.4	15.3±0.4	17±0.8
3	*E. faecalis*	14.6±0.4	17±0.8	16.6±0.4	16.3±0.4	17.3±0.4	19.3±0.4	23±0.8
4	*K. pneumonae*	12.6±0.4	15±0.8	16.3±0.4	17.3±0.4	19±0.8	19.3±0.4	18±0.8
5	*S. aureus*	12.3±0.4	15±0	14.3±0.4	13.6±0.4	12.6±0.4	12.6±0.4	16.6±0.4
6	*S. pyogenes*	15.3±0.4	16.3±0.4	15.6±0.4	14.6±0.4	16.6±0.4	23.6±0.4	24.3±0.4
7	*S. saprophyticus*	12.6±0.4	14.6±0.4	14.6±0.4	15.3±0.9	15.6±0.4	18±0	18 ± 0.8

represented as mean±standard deviation. CFS from processed indigenous food isolates (P5 and P6) shows enhanced antimicrobial activity (17±0 to 24.3±0.4) as compared to probiotic supernatant (12.3±0.4 to 16.6±0.9). A maximum zone of inhibition was seen against *S. pyogenes* (24.3±0.4) by cell free supernatant obtained from isolate P6.

DISCUSSION/CONCLUSION

Antimicrobial activity of cell free supernatant was measured at a high concentration in the present study as this concentration would be indicative of the amount ingested in a crude food and would therefore be in indication of the efficacy of the probiotic microorganisms in that food. However, to allow for comparison with other antimicrobial agents, MIC's would need to be determined in future studies. Results of above study also shown that probiotic microorganism in its activated form (Processed probiotic microorganisms that is probiotic microorganisms obtained from probiotic capsules were culture in MRS broth) were more beneficial regarding their therapeutic potential. Also, modified indigenous foods (Traditional Indian food like Meheri etc) supplemented with probiotic microorganism good source for taking probiotics in its activated form for effective in shaping and maintaining the population of the gut microbiota and could be used as alternative source than probiotic capsules for treatment of various health problems. Since this is preliminary study, no attempt was undertaken to extract the antimicrobial compound(s). On the basis of this study it may be conclude that these isolates may have high potential to produce antimicrobial compound(s).

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