Original Article

Isolation of Gram-positive Bacteria from Different Sources and Evaluation of their Probiotic Properties

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Introduction: Probiotics are defined as live non-pathogenic microorganisms which can have beneficial effects on their hosts, if administered in adequate amounts. This study aimed to isolate Gram-positive bacteria from different sources and to evaluate their probiotic properties. **Methods**: We obtained five samples from various sources including yogurt, infant feces, cheese, and soil under aseptic methods. The samples were inoculated on MRS agar and M17 medium. Biochemical assays identified isolated bacteria. The probiotic effect of isolated bacteria was tested by applying acid pH 2.0, 0.3% w/v bile salt concentration and gastric juice in the samples. The antibiotic susceptibility profiling and their antimicrobial effects against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella enterica* and *Pseudomonas aeruginosa* were determined. **Results:** Four Gram-positive bacteria including *Lactobacillus acidophilus*, *Bifidobacterium bifidum, Lactococcus lactis* and *Bacillus subtilis* were isolated from yogurt, infant feces, cheese, and soil, respectively. Except for *B. subtilis,* all the isolated bacteria were catalase-negative and non-motile. The results showed that viable count of *L. acidophilus, B. bifidum,* and *L. lactis* was significantly high ($P<0.05$), and *B. subtilis* was sensitive. The isolates showed an antimicrobial effect against all indicator pathogens except *P. aeruginosa*. The isolated *B. subtilis* showed no antimicrobial effect. The antibiotic susceptibility was determined for ten different antibiotics. All the isolates were susceptible to most of the antibiotics (table 6) and resistant to penicillin G, gentamicin, vancomycin, and kanamycin. **Conclusion:** The species *L. acidophilus, B. bifidum,* and *L. lactis* fulfilled the criteria for probiotic properties and can be used in field conditions as probiotics. *J Med Microbiol Infec Dis, 2017, 5 (1-2): 12-16.*

Keywords: Identification, Gram-Positive Bacteria, Probiotic.

INTRODUCTION

The constant increasing level of bacterial resistance to antibiotics is the highlight of the 21st century. Multi-drug resistant bacteria are creating serious public health problems. Innovative approaches are required to overcome this issue. Probiotics and their metabolites have the potential to be used as antibiotic agents. The word 'probiotic' has a Greek origin, which means 'for life' and is in contrast to the meaning of antibiotics, *i.e.,* 'against life'.

Lilly and Stillwell (1965) used the word "probiotic" for the first time to describe metabolites secreted by some microbes that enhance the growth of other microorganisms. Later in 1989, Fuller defined probiotics as live microbial feed supplements which beneficially affect the health status of their hosts by improving the microbial balance of their gastrointestinal tract [1].

Recently, there has been a growing interest in the usage of probiotics because they have shown benefits in the management of *Helicobacter pylori* infections, inflammatory bowel disease, infantile diarrhea, antibiotic-associated diarrhea, female urogenital infections, and relapsing *Clostridium difficile* colitis [2]. Probiotic bacteria generate a variety of compounds including bacteriocins, reuterin, hydrogen peroxide, acetaldehyde, diacetyl, and organic acid (acetic acid and lactic acid) that inhibit the growth of pathogenic bacteria. The organic acids decrease the environment pH making it fatal for pathogenic bacteria [3].

Probiotics have received much attention and extensive studies have been performed to ensure their safety for the treatment purposes. Also, their functional properties, such as antimicrobial activity and resistance to acidic pH, simulated gastric juice, and bile salt has been evaluated. Many other beneficial effects of probiotics include decreasing serum cholesterol levels, improving lactose intolerance, increasing nutrients utilization, and decreasing the use of antibiotics [4]. Other benefits reported for probiotics include their anti-carcinogenic, antihypertensive, antimutagenic, hypocholesterolemic, immunomodulatory and anti-osteoporotic effects. Probiotics also influence digestion process of living species [6].

The most widely known microorganisms with probiotic properties are *Lactobacilli, Bifidobacteria, Streptococci* and some *Bacillus* spp., all of which produce lactic acid. *Lactobacillus* and *Bifidobacterium* have been widely used for the treatment of intestinal dysfunction. Also, some Gram-negative bacteria like *Escherichia coli* strain Nissle

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1917 (EcN) have shown probiotic effects. For many years, EcN, also known as Mutaflor has been used for the treatment of colitis and chronic constipation [7].

The significant effects of probiotics on their hosts' health status are achieved when an adequate concentration of the bacteria is present in the environment which varies with the species [8]. Likewise, the probiotic properties may be affected by environmental characteristics of the host's gastrointestinal tract. For example, administration of antimicrobial agents leads to the imbalance of normal microbial flora of the intestinal tract and restrain various bacterial groups.

Selection of microorganisms as probiotic agents requires their accurate identification followed by *in vitro* evaluation of their probiotic properties by standard assays. Probiotics have been found in dairy products, infant feces, and intestinal tract. The main features of probiotic bacteria are resistance to acidic pH, bile salt, and gastric juice as well as the production of antimicrobial agents. The included probiotic bacteria should be able to attach and colonize in the epithelial lining of the GIT [9]. In this study, we aimed to isolate the bacteria from various sources to determine their probiotic properties.

MATERIAL AND METHODS

Sample collection and processing. Samples were obtained from yogurt, cheese, soil, and infant feces and sent to the laboratory in sterile plastic bags and screw cap microtubes. The samples were immediately stored at 4°C for 3-4 h and then were inoculated on the Man-Rogosa-Sharpe (MRS) agar and nutrient agar.

Bacterial isolation and identification. The selected Gram-positive bacteria, including *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Lactococcus lactis* and *Bacillus subtilis*, were isolated from the samples. The samples were inoculated on MRS agar and M 17 medium. The MRS agar was used for isolation of *L. acidophilus* and *B. bifidum* while *L. lactis* and *B. subtilis* were isolated by M 17 medium and Nutrient agar, respectively. Each sample was diluted serially 10-fold in phosphate buffer saline and then was inoculated on required medium [10]. The inoculated plates were incubated at 37°C for 48 h. The macroscopic appearance of the isolated colonies including colonies' shape, color, and texture was examined. For microscopic examination, slide smears were prepared, and morphological characteristics were studied after Gram staining. For isolation of pure bacterial colonies, single colonies were subcultured by a streaked method on selective media. These evaluations were followed by biochemical tests, following the Bergey's Manual of Systematic Bacteriology.

Assessing probiotic properties of isolated bacteria

Acid tolerance. Acid tolerance was determined by cultivating the bacteria in acidic MRS and nutrient broth. The broth was poured into test tubes, and acidic pH was adjusted with 1M HCl and 0.5M NaOH. The pH of the trial and control MRS agars was set at 2 and 7.0, respectively. For each isolated bacterial species, 5 log10 CFU (10^5CFU) of culture product was poured into each broth and was incubated for 2 h at 37°C. The plate count method was used to evaluate the survival of the isolated bacterial species [10].

Bile salts resistance. The ability of isolated bacterial species to grow in the presence of bile salts was determined in MRS and nutrient broth. Briefly, for each bacterial culture, 10^5 CFU of culture product was inoculated in selective broths supplemented with 0.0% and 0.3% (w/v) concentrations of bile salts. The cultures were incubated at 37°C for 24 h. Plate count method was used to evaluate the survival of isolated bacterial species [10].

Gastric juice tolerance. The tolerance of the isolated bacteria to simulated gastric juice was examined. The gastric juice was prepared in the laboratory by mixing water with 3% Pepsin and 0.5% NaCl (w/v). The pH 3.0 was adjusted with 1M HCl and 0.5 M NaOH. After the preparation of gastric juice, 10^5CFU of each isolated bacterial culture was mixed with 3 ml of gastric juice and 1 ml of phosphate buffer saline and incubated at 37°C for 90 min or 2 h. Then, the bacterial survival was evaluated by a plate count method [1].

Antibiotic sensitivity. The antibiotic susceptibility profiling of the isolated bacteria species was determined with the commonly used antibiotics by using disc diffusion method using solid Mueller-Hinton (MH) agar plates. The bacteria cultures were added to bottles containing 100 ml melted agar and mixed gently. Equal amounts of agar were poured into Petri dishes. Once agar solidified, different antibiotic discs were placed aseptically on the agar surface, and plates were incubated at 37°C for 24 h. The diameter of inhibition zone was then measured.

Antimicrobial activity. Antimicrobial activity of the isolated Gram-positive bacteria against pathogenic *E. coli* ATCC 29922, *Staphylococcus aureus* ATCC 29923, *Salmonella enterica* ATCC 13311 and *Pseudomonas aeruginosa* ATCC 27853 was determined by the agar well diffusion method. Nutrient agar was prepared and poured into the Petri dishes. By the use of sterilized swabs lawn cultures with the fresh pathogenic bacteria culture were made on MH agar plates. Wells with diameters of 6-8 mm was made on the MH agar, and 100 µl from the each isolated bacteria was transferred into the wells. The plates were stored for two hours in the refrigerator followed by incubation at 37°C for 24 h. Finally, the diameters of inhibition zone were measured in millimeters using calipers [1].

RESULTS

Bacterial isolation and identification. All the isolated bacteria were Gram-positive and except for *B. subtilis* were catalase negative, non-motile and non-spore forming. The Gram-positive bacteria isolated in this study were *L. acidophilus*, *L. lactis*, *B. bifidum* and *B. subtilis*, isolated from yogurt, cheese, infant feces, and soil, respectively. Table 1 shows cultural characteristics and morphological details of the isolated bacteria.

Screening probiotic properties

Acid tolerance. The tolerance of the isolated bacteria to acidic environments significantly varied against the acidic

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pH 2.0 and neutral pH 7.0 as a control (P<0.05) (Table 2). The species *L. acidophilus*, *B. bifidum* and *L. lactis* were resistant, and their viable count increased with time, while *B. subtilis* could not survive at acidic pH and its viability count decreased with time.

Bile salt resistance. All the isolated bacterial species showed a significant tolerance to the bile salt at a concentration of 0.3 w/v %. The resistance level was found significantly (P<0.05) variable among all tested species including *L. acidophilus*, *L. lactis*, *B. bifidum* and *B. subtilis.* At 0.0 w/v % concentration of oxgall, *L. acidophilus* and *B. subtilis* reached maximum count ($2.35\times10^5\pm 0.20$) while *B. bifidum* and *L. lactis* gave their viable count CFU/ml *(*$2.10\times10^5\pm 0.50$) and ($2.32\times10^5\pm 0.15$), respectively. At 0.3 w/v % concentration of oxgall, the viable count of *B. bifidum* increased while the viable count of all other bacterial species decreased. The results for bile salt resistance of investigated bacteria isolates are given in Table 3.

Gastric juice tolerance. After incubation, the viable count of the isolated bacteria varied significantly (P<0.05). When incubation time was 90 min, *B. subtilis* gave the maximum viable microbial count as compared to other species. However, after 120 min of incubation, *B. subtilis* showed sensitivity to gastric juice with a significant decrease in its viable count ($1.02\times10^3\pm 0.22$). This pattern was reversed for the rest of bacterial isolates tested in our study, *i.e.*, their viable count increased significantly with time (Table 4).

Table 1. Cultural and morphological characterization of the isolated bacteria

Isolates	Cultural characteristics	Morphological characteristics
L. acidophilus	Rough and smooth, typical grayish-white colonies	Gram +ve, Rod Shape, straight single and chain form
B. bifidum	Small circular creamy-white color colonies	Gram +ve, Club-shaped Rods, V or Y form shape
L. lactis	Creamy white, small circular, rough colonies	Gram +ve, Cocci, singly as well as chains
B. subtilis	Round shape, large white/pale color colonies, Dull surface and irregular margin	Gram +ve, Straight Rods, short chains or single cell arrangement

Table 2. Plate count of isolated bacteria in acidic and normal pH environments

	Group		*P* value
	Test (pH 2)	**Control (pH 7)**	
Isolated bacteria	Mean (±SE)	Mean (±SE)	
L. acidophilus	$2.45\times10^6\pm 0.20$*	$2.50\times10^6\pm 0.10$*	P<0.05
B. bifidum	$2.80\times10^6\pm 0.35$*	$2.65\times10^6\pm 0.10$*	P<0.05
L. lactis	$2.60\times10^6\pm 0.24$*	$2.30\times10^6\pm 0.15$*	P<0.05
B. subtilis	$1.00\times10^3\pm 0.15$	$2.20\times10^6\pm 0.05$*	P<0.05

* shows that result is significant at 0.05 level of significance

Table 3. Plate count of isolated bacteria against two concentrations of oxgall

	Group		*P* value
	Test (Bile salt) 0.3 w/v % (conc)	**Control (Bile salt) 0.0 w/v % (conc)**	
Isolated bacteria	Mean (±SE)	Mean (±SE)	
L. acidophilus	$2.12\times10^5\pm 0.10$*	$2.35\times10^5\pm 0.20$*	P<0.05
B. bifidum	$2.23\times10^5\pm 0.24$*	$2.10\times10^5\pm 0.50$*	P<0.05
L. lactis	$1.10\times10^3\pm 0.50$	$2.32\times10^5\pm 0.15$*	P<0.05
B. subtilis	$2.01\times10^5\pm 0.15$*	$2.35\times10^5\pm 0.20$*	P<0.05

* shows that result is significant at 0.05 level of significance

Table 4. Plate count of isolated bacteria after 90 and 120 min of incubation in gastric juice

	Group		*P* value
	Test 1 (Gastric Juice)/ Time (90 min)	**Test 2 (Gastric Juice)/ Time (120 min)**	
Isolated bacteria	Mean (±SE)	Mean (±SE)	
L. acidophilus	$2.10\times10^5\pm 0.50$*	$2.70\times10^5\pm 0.50$*	P<0.05
B. bifidum	$2.01\times10^5\pm 0.15$*	$2.60\times10^5\pm 0.24$*	P<0.05
L. lactis	$2.10\times10^5\pm 0.50$*	$2.23\times10^5\pm 0.24$*	P<0.05
B. subtilis	$2.35\times10^5\pm 0.20$*	$1.02\times10^3\pm 0.22$	P<0.05

* shows that result is significant at 0.05 level of significance

Table 5. Mean values (±SE) of zone of inhibition (mm) for antimicrobial activity of isolated bacteria against pathogenic bacterial strains

Isolated bacteria/ZOI	Zone of inhibition (mm) for pathogenic bacterial strains			
	E. coli	***S. aureus***	***S. enterica***	***P. aeruginosa***
L. acidophilus	7± 0.1*	5± 0.13*	8± 0.6*	0.0± 0.0
B. bifidum	9± 0.0*	4± 0.2*	9± 0.45	0.0± 0.0
L. lactis	5± 0.4*	7± 0.6*	12± 0.7*	0.0± 0.0
B. subtilis	3± 0.5	0.0± 0.0	4± 0.21*	0.0± 0.0

* shows that result is significant at 0.05 level of significance

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Table 6. Mean value of zone of inhibition (mm) for antibiotic susceptibility test against isolates

Isolates/ZOI	SXT 25 µg	P 10 U	CN 10 µg	AMC 10 µg	CRO 30 µg	TE 30 µg	PIP 100 µg	TOB 10 µg	K 10 µg	V 30 µg
L. acidophilus	S	R	S	I	S	I	S	S	R	R
	18	10	17	22	20	13	18	19	12	8
B. bifidum	S	R	S	I	S	S	S	S	I	R
	19	7	21	23	17	16	21	18	14	9
L. lactis	S	I	I	S	I	S	S	S	S	I
	16	15	13	29	13	15	19	21	13	11
B. subtilis	I	R	S	I	S	S	S	S	R	R
	11	13	15	24	19	18	18	19	7	7

S: Susceptible, I: Intermediate, R: Resistant, ZOI: Zone of Inhibition

Antimicrobial activity. Antimicrobial activity of the isolated bacteria species was checked against some indicator pathogenic bacterial species including *E. coli*, *S. enterica*, *S. aureus* and *P. aeruginosa* by the well diffusion method. The isolated species exhibited different degrees of inhibitory activity against the pathogenic bacterial strains. The isolates showed significant results against *E. coli*, *S. enterica*, and *S. aureus*, but not *P. aeruginosa* ($P<0.05$). The strongest antimicrobial effect was observed by *L. lactis* against *S. enterica*. Similarly, *B. bifidum* showed a maximum antimicrobial effect against *E. coli* and *S. enterica*. The results showed that all the isolates inhibited the growth of pathogenic bacteria species except *P. aeruginosa*, while *B. subtilis* exhibited a very low level of antimicrobial activity (Table 5).

Antibiotic susceptibility profiling. The sensitivity of the isolates against ten different types of antibiotics is given in Table 5. The bacteria species showed variable sensitivities to the tested antibiotics and were sensitive to most of them. They also showed resistance to gentamicin, vancomycin, and kanamycin, the agents that are used against Gram-negative bacteria [11].

DISCUSSION

In the present study, Gram-positive bacteria were isolated from four environmental sources, and their probiotic properties were evaluated. We isolated four bacterial species, including *L. acidophilus*, *B. bifidum*, *L. lactis* and *B. subtilis* from yogurt, infant feces, cheese, and soil, respectively. To show metabolic and antimicrobial benefits, it was necessary for the isolated bacteria to pass through the stressful conditions of the stomach and small intestine. Probiotics should be able to resist the environmental pressures of upper GI tract, and can adhere to, and colonize the epithelial tissue of lower GI tract. Therefore, before clinical use of candidate bacteria, it is essential to evaluate their tolerance to the stressful gastrointestinal conditions. The antimicrobial susceptibilities were also determined for the isolated bacteria. Antibiotic susceptibility profiling was assayed for ten commonly used antibiotics by disk diffusion method. The isolated bacteria were sensitive to the most antibiotics tested in this study. Bacterial isolates also showed resistance to gentamicin, vancomycin, and kanamycin, the agents that are used against Gram-negative bacteria (Table 6). The survival rates of the isolates were determined by a plate count method after exposure to acidic pH 2.0. Their tolerance to the acidic environment varied significantly ($P<0.05$). The species *L. acidophilus*, *L. lactis* and *B. bifidum* were found most resistant at pH 2.0 and their variable count increased over time. While *B. subtilis* could not survive the acidic pH and their survival rate decreased with time (Table 2). The bile salt concentration in the human gastrointestinal tract varies, but the mean value of bile concentration is believed to be 0.3% w/v. Due to this fact, all the isolates were screened against bile salt at 0.3% w/v concentration. The tolerance level was found significantly variable ($P<0.05$) among all tested species. All the species were resistant to bile salt with *L. lactis* showing the lowest resistance (Table 3). The resistance of isolated species to gastric juice was also examined for 90 and 120 min. After incubation, viable count of *B. bifidum*, *L. lactis*, and *L. acidophilus* was found significantly *($P<0.05$)* high. Only, *B. subtilis* was found sensitive to gastric juice as its viable count decreased significantly. The results showed that all the isolates could resist the gastric juice except *B. subtilis*, which gave less number of viable counts after incubation (Table 4). With the same objective, a study was conducted to isolate probiotic bacteria from diverse fermented products like yogurt, milk, butter, and cheese. The isolates including *Bifidobacterium spp* and *Lactobacillus spp* were screened against (0.3%) bile salt concentration and acidic pH 3. The isolates were also tested against pathogens to examine their antimicrobial property *in vitro*. The test exhibited different inhibitory action against the human and plant pathogens. Antibacterial inhibition zones ranges were found 8.53-8.74 mm for *S. aureus* and *E. coli*. Similarly, antifungal behavior for *Fusarium oxysporum* and *Rhizoctonia solani* were also tested. The inhibition growth ranges 17.1-51.2%, and 26.3-52.3% were found correspondingly [12].

Antimicrobial activity of isolated bacterial species was checked against indicator pathogenic bacterial species including *E. coli*, *S. enterica*, *S. aureus* and *P. aeruginosa* by the well diffusion method. The isolates showed significant results ($P<0.05$) against *E. coli*, *S. enterica* and *S. aureus*, but not *P. aeruginosa*. *L. lactis* demonestrated the strongest antimicrobial effect against *S. enterica*. Similarly, *B. bifidum* showed a maximum antimicrobial effect against *E. coli* and *S. enterica*. The results showed that all the isolates inhibited the growth of pathogenic bacteria species except *P. aeruginosa*, while *B. subtilis* exhibited a very low level of antimicrobial activity (Table 5). In a similar study, the potential probiotic properties of *lactobacilli* species were investigated. Bacteria were isolated from yogurt and

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intestinal content of healthy chicks. *Lactobacillus delbrucekii* subsp. *bulgaricus* and *L. paracasei* subsp. *paracasei* were isolated from yogurt and *L. acidophilus, L. salivarius,* and *L. rhamnosus* from intestinal contents of chicks. The isolated bacteria were exposed to acidic pH, bile salt and gastric transit and their ability to inhibit the growth of pathogenic bacteria was assayed. Tolerance level was found variable (P<0.05) among all the tested species of *Lactobacillus*. All the isolated *Lactobacilli* species inhibited the growth of *S. aureus* and *E. coli* with *L. delbrucekii* showing the least antimicrobial effect (P<0.05). At last, it was concluded that the isolated *L. rhamnosus, L. acidophilus,* and *L. salivarius* showed good probiotic properties *in vitro* [13]. Another research was carried to characterize that new strain proposed to be used as probiotic particularly rising species is *Bacillus coagulans*. The probiotic properties of isolated bacteria were determined *in vitro.* The isolated bacteria were screened for bile salt resistance, tolerance to acidic pH and their survival in the GIT [14]. One of the important parameter to select bacteria for probiotic usage is their susceptibility to antibiotics. In the present study, the isolated species were sensitive to most of the antibiotics and showed resistance to penicillin G, gentamicin, vancomycin, and kanamycin (Table 6).

Given all study aspects and parameters for evaluating the isolated bacteria for probiotic usage. This study concluded that the locally isolated bacterial species including *L. acidophilus, L. lactis* and *B. bifidum* could survive in stressful condition of the gastrointestinal tract with good antimicrobial effects on pathogenic bacteria compared to *B. subtilis* and can be used as probiotic bacteria.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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