ORIGINAL ARTICLE

Assessment of Antioxidant and Anti-inflammatory Activities of Probiotic *Lactiplantibacillus plantarum* Strains Isolated from Traditional Fermented Food Products Ngari and Soibum

Sowmya Shankar[1], Shankar Jayarama[2], Shubha Gopal[1]

[1]Department of Microbiology, University of Mysuru, Mysuru, Karnataka, India, [2]Department of Food Technology, Davangere University, Shivagangotri, Davangere, Karnataka, India

Abstract

Introduction and Aim: Lactic acid bacteria (LAB) with remarkable functional properties have been isolated from different fermented food products. Ngari and soibum are fermented fish and fermented bamboo shoot products produced in Manipur, India. The present study was conducted to isolate potential probiotic strains from ngari and soibum also to evaluate their antioxidant and anti-inflammatory activities. **Materials and Methods:** LAB strains were isolated from ngari and soibum samples, characterized, and evaluated for probiotic properties including acid and bile tolerance, antimicrobial activity, antibiotic susceptibility, and hemolytic activity. The strains were identified by 16S rRNA gene sequencing. Two strains with the best probiotic potential were selected and evaluated for antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging assays and anti-inflammatory activity by real-time polymerase chain reaction. **Results:** The isolates were capable of surviving in an environment with low pH (pH 2) and with bile salts (0.3% oxgall). The cell-free supernatant of the isolates showed high antibacterial activity against the tested pathogens. The isolates were sensitive to most of the test antibiotics and none of the isolates showed hemolytic activity. By 16S rRNA gene sequencing, the isolates were identified as *Lactiplantibacillus plantarum* (five strains) and *Enterococcus faecium* (one strain). *L. plantarum* strains NG28 and SB15 exhibited high antioxidative capacity in terms of DPPH and ABTS radical scavenging assays. These two strains were also capable of reducing the production of nitric oxide as well as the expression of nitric oxide synthase and cyclooxygenase-2 as well as pro-inflammatory cytokines, such as tumor necrosis factor-α, interleukin (IL)-1β, and IL-6 in lipopolysaccharide-induced RAW 264.7 cells. **Conclusion:** The selected LAB strains possess the potential probiotic and beneficial functional properties. Hence, they could be used in functional foods and pharmaceutical applications.

Key words: Anti-inflammatory, antioxidant, lactic acid bacteria, *Lactiplantibacillus plantarum*, probiotics

INTRODUCTION

Fermented foods are the products made by microorganisms or enzymatic conversions of food components. These foods have been produced and consumed since the dawn of civilization around the world. These foods are categorized according to the substrate they employ, which includes dairy, legumes, cereals, vegetables, fruits, meat, and fish.[1] They have advantages over non-fermented foods in terms of shelf life, cooking time, nutritional value, taste, and flavor. Nowadays fermented foods have been attracted for its health benefits. Because of this fermentation process, fermented products and microorganisms involved in fermentation have become scientific interest.[2]

Manipur, one of the seven states in North-east India is characterized by its rich culture and indigenous food habits.

Address for correspondence:
Shubha Gopal, Department of Microbiology, University of Mysore, Manasagangotri, Mysuru, Karnataka, India.
E-mail: shubhagopal_mysore@yahoo.com

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Ngari and soibum are the most popular fermented foods in Manipur. Ngari is the most popular traditional fermented fish product of Manipur, produced exclusively from sun-dried small cyprinid fish *Puntius sophore* (Ham) which is locally known as phabou. It is produced by age-old practice of yearlong natural fermentation.[3] Soibum is one of the most common fermented bamboo shoot product in Manipur. It is prepared exclusively from the young succulent shoot sprouts of *Dendrocalamus hamiltonii, Dendrocalamus giganteus, Bambusa balcooa, Bambusa tulda,* and *Bambusa pallid*.[4]

Probiotics have received a lot of interest recently in the fields of microbiology and human health. Probiotics are live microorganisms when taken in sufficient quantity, improve the balance of the intestinal microbial community, and boost the host's health. Human gut is an extremely complex ecosystem where microbiota, nutrients, and host cells interact extensively.[5] However, gut microbiota can be altered by various factors such as lifestyle, diet, and antibiotics. The alteration of gut microbiota has been implicated in the pathogenesis of various diseases. Probiotics have been proved to treat and prevent a variety of intestinal disorders.[6] Probiotics exert their beneficial effects through various mechanisms, including lowering intestinal pH, decreasing colonization of pathogenic organisms, and modifying the host immune response.[7] Most probiotics belong to the lactic acid bacteria (LAB) which produce lactic acid as the major end product of carbohydrate fermentation. Lactobacilli are Gram-positive, non-pathogenic, and non-spore forming rods. Among lactobacilli, *Lactiplantibacillus plantarum* formerly known as *Lactobacillus plantarum* is one of the most predominant species which produces number of antimicrobial substances and imparts health benefits to host.[8]

Reactive oxygen species (ROS) or free radicals are formed during normal metabolic processes. They are unstable and highly reactive. When there is an excess of ROS in the body's cells, creates oxidative stress. This can damage cells, proteins, and DNA. Persistent oxidative damage of tissue and cellular components can cause several diseases including diabetes, cardiovascular disease, and arthritis.[9]

Inflammation refers to the body's normal physiological defense against a variety of factors including pathogens, damaged cells, and toxic compounds. Therefore, inflammation is vital to health.[10] Limiting the inflammatory mediators is just as important as producing it. Normal inflammatory responses have a regulatory process during which the production of pro-inflammatory mediators decreases over time. However, prolonged inflammation can damage tissue and contributes to the pathogenesis of many diseases including arthritis, asthma, and cancer.[11] The long-term usage of steroidal antioxidant and anti-inflammatory drugs causes adverse side effects and damage human biological system. Hence, there is a need for the safe, potent, and non-toxic drug. LAB are known to have prominent antioxidant and anti-inflammatory properties. Therefore, the purpose of this study was to isolate potent LAB from fermented food products and to assess their antioxidant and anti-inflammatory activities.

MATERIALS AND METHODS

Chemicals and reagents

MRS, oxgall, ascorbic acid, chloroform, Tween 80, Triton X-100, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, dimethyl sulfoxide, Dulbecco's Modified Eagle's Medium (DMEM), RPMI1640, antibiotics, fetal bovine serum (FBS), phosphate-buffered saline (PBS), and 1% streptomycin/penicillin solution were purchased from Hi-Media (India). The RNeasy Mini Kit, cDNA Synthesis Kit, SYBR Green, and RT-PCR kit purchased from Thermo Fisher Scientific (India). Specific primers used for performing the real-time polymerase chain reaction (RT-PCR) and 16S rRNA universal primers were purchased from Eurofins Scientific (India).

Isolation of LAB

Three samples of each ngari and soibum were purchased from the local markets of Imphal in Manipur, India. For isolation of potential LAB, about 10 g of each sample was homogenized in 90 mL of 0.85% (w/v) sterile saline. Thereafter, 1 mL of homogenized samples was serially diluted into 9 mL of saline and plated on MRS (de Man, Rogosa, and Sharpe) agar (Hi Media, India). The inoculated plates were incubated under anaerobic condition at 37°C for 24–48 h. The representative colonies that grew on MRS agar were isolated and repeatedly sub-cultured for purity.[12] The glycerol stocks of the isolates were maintained at −20°C for further analysis.

Preliminary identification of LAB

Preliminary identification of LAB was made by Gram staining, cell morphology, and catalase reaction. Physiological properties such as growth at different temperatures and different NaCl concentrations, gas production from glucose, and carbohydrate fermentation were also studied.[13]

Characterization of LAB

Acid and bile tolerance

Acid resistance and bile tolerance were determined as described by Gu *et al*.[14] with some modifications. Overnight cultures of LAB isolates were inoculated into MRS broth adjusted to pH 2.0 using 1N hydrochloric acid and MRS broth containing 0.3% (w/v) oxgall. The isolates were incubated for 0, 2, and 4 h, respectively, at 37°C, and plating was done

at respective time intervals. After anaerobic incubation for 24 h at 37°C, viability of the isolates was assessed by plate count method and expressed as log CFU/ml. Survival rate was calculated by following formula.

% survival rate = (log CFU at 2^{nd}h, 4^{th}h/log CFU 0^{th}h) × 100

Antibacterial activity

Antibacterial activity of the isolates was determined by agar well diffusion method.[15,16] *Staphylococcus aureus* MTCC 737, *Escherichia coli* MTCC 1687, *Bacillus subtilis* MTCC 736, *Pseudomanas aeruginosa* MTCC 1688, and *Listeria monocytogenes* MTCC 1143 were used as indicator strains. The isolates grown in MRS broth for 18 h at 37°C were centrifuged at 4000 rpm at 4°C for 30 min. The supernatants were filtered using 0.22 µm syringe filters. Half part of the each cell-free supernatant (CFS) was adjusted to pH 6.5 with 5M NaOH and designated as neutralized CFS (nCFS). The 18 h cultures of pathogenic bacteria were swabbed on Muller Hinton agar (High-Media, India). Then, 6 mm diameter wells were created and 50µl of CFS and nCFS of the isolates were loaded to the respective wells. After 24 h of incubation at 37°C, antibacterial ability of the isolates was evaluated by measuring the zone of inhibition.

Antibiotic susceptibility test

Antibiotic susceptibility test was performed by disk diffusion method.[17] The antibiotic discs used included ampicillin (10 mg), vancomycin (30 mg), tetracycline (30 mg), kanamycin (30 mg), and Streptomycin (10 mg) (Hi-Media, Mumbai, India). Overnight culture of isolates was swabbed on MRS agar plates and allowed to dry. The antibiotic discs were placed on the surface of the inoculated plates and incubated at 37°C for 24 h. The diameter of the zone of inhibition was measured and the results were interpreted as susceptible (S), intermediate (I), and resistant (R). The test was performed according to the Clinical Laboratory Standard Institute standard procedure.

Hemolytic activity

The hemolytic activity of isolated strains was examined by streaking the isolates onto blood agar plates containing 5% sheep blood.[18] After 48 h of incubation at 37°C, the plates were observed for hemolytic patterns as clean zone (β-hemolysis), greenish zone (α-hemolysis), or no such zone (γ-hemolysis).

Molecular identification

Molecular identification was performed by 16S rRNA sequencing.[19] Genomic DNA was extracted from the isolates and subjected to PCR analysis. In brief, PCR amplification was performed using a set of universal primers 27F-5′ AGAGTTTGATCCTGGCTCAG3′, and 1495R-5′CTACGGCTACCTTGTTACGA3′. A total volume of 25 µl reaction mixture contained 0.2 mM of dNTP, 3.0 unit Taq DNA polymerase, 100 ng DNA, 2.5 µl 10 × PCR buffer with 1.5 mM $MgCl_2$, and 10 pmol of each primer. The PCR condition was as follows: An initial denaturation at 94°C for 2 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 2 min, and a final extension of 72°C for 10 min. The PCR products were resolved by electrophoresis in a 1.5% (w/v) agarose gel and visualized by ethidium bromide staining. The PCR products were sequenced. The sequences were analyzed with BLAST program and deposited in NCBI GenBank.

Adhesion ability to HT-29 cells

The adhesion ability of LAB strains was examined using HT-29 cells (human colon adenocarcinoma cell line).[20] HT-29 (1×10^5 cells/mL) cells were seeded onto a 24-well cell culture plate and incubated at 37°C for 24 h. Overnight cultures of LAB strains were centrifuged at 10,000 rpm at 4°C for 10 min, washed twice with PBS, and resuspended in PBS. The bacterial suspension (1×10^7 CFU/mL) was inoculated onto HT-29 cells and incubated at 37°C under 5% CO_2 for 2 h. After incubation, non-adherent bacterial cells were removed by washing with PBS 3 times. To detach the adherent bacteria, 1% (v/v) Triton X-100 solution was added into each well and incubated for 10 min at 37°C. The number of adherent bacterial cells was determined by counting viable cells on MRS agar plates.

Antioxidant activity of selected LAB strains

Preparation of bacterial sample

Selected LAB strains were cultured in MRS broth for 18 h at 37°C. After incubation, the bacterial cells were harvested by centrifugation at 10,000 rpm for 10 min and washed 3 times with PBS. The washed bacterial cells were resuspended in PBS to a final concentration of 10^7 CFU/ml.[21]

Scavenging analysis of DPPH radical

The DPPH radical scavenging activity of the isolated LAB strains was determined according to Yang *et al.*[22] with some modifications. Briefly, 0.4 mM DPPH solution was prepared in methanol. Two milliliters of the bacterial sample were added to 2 mL of methanolic DPPH radical solution. The reaction solution was mixed and incubated in the dark at 37°C for 30 min. The control group contained equal amount of distilled water and methanolic DPPH solution. The resulting solution was centrifuged at 8000 rpm for 10 min and absorbance of the supernatant was measured at 517 nm. The scavenging activity was calculated according to the following formula.

DPPH radical scavenging activity (%)
$= (1–[A_{sample}/A_{control}])\times 100$

A_{Sample} and $A_{Control}$ were the absorbance of bacterial sample with DPPH solution and absorbance of distilled water with DPPH solution, respectively.

Scavenging analysis of ABTS radical

The ABTS radical scavenging activity of the isolated LAB strains was determined according to the method of Yang *et al*.[22] and Han *et al*.[23] with some modifications. The ABTS radical cation ($ABTS^+$) was produced by allowing ABTS (High Media, India) solution with 5.5 mM potassium persulfate (High Media, India) to stand in the dark for at least 6 h. The $ABTS^+$ solution was diluted by 20 mM sodium acetate buffer to an absorbance of 0.7 at 734 nm. One hundred and fifty microliters of bacterial samples and 1.5 mL of $ABTS^+$ solution were mixed and incubated in dark at 37°C for 10 min and centrifuged at 8000 rpm for 10 min at 4°C. The absorbance of the resulting solution was measured at 734 nm and the ABTS radical scavenging activity was calculated according to the following formula.

ABTS radical scavenging activity (%) =
$(1–[A_{sample}/A_{control}])\times 100$

A_{Sample} and $A_{Control}$ were the absorbance of bacterial sample with $ABTS^+$ solution and absorbance of distilled water with $ABTS^+$ solution, respectively.

Anti-inflammatory activities of selected strains

Cell culture condition

RAW 264.7 cells were purchased from. These cells were grown at 37°C in 5% CO_2 in fully humidified air. For routine sub-cultivation, DMEM (Himedia, India) supplemented with 10% of FBS and 1% of penicillin–streptomycin was used and sub-cultured every 2 days to 80–90% confluence.

Nitric Oxide (NO) Production in RAW 264.7 cells

The production of NO in LPS-induced murine macrophage RAW 264.7 cells was determined according to the methods of Lee *et al*.[24] with some modifications. RAW 264.7 cells were seeded at a concentration of 2×10^5 cells/well in 96-well plates and incubated in a differentiation medium at 37°C in 5% CO_2 for 24 h. Then, the RAW 264.7 cells were treated with LAB strains (10^7 CFU/mL) and lipopolysaccharide (LPS, 1 µg/mL) for 24 h. After centrifugation at 10,000 rpm for 3 min, 100 µL of the supernatants was added to the equal volume of Griess reagent in 96-well plates and allowed to react for 10 min at room temperature. The absorbance of the mixture was measured at 540 nm using a microplate reader. The amount of NO was estimated from a calibration curve constructed using sodium nitrate as the standard.

Anti-inflammatory effect of LAB strains by semi-quantitative real-time PCR analysis

The anti-inflammatory effect of LAB strains by RT-PCR analysis was determined as described by Han *et al*.[20] RAW 264.7 cells were seeded at a concentration of 2×10^5 cells/well in 96-well plates and incubated in a differentiation medium at 37°C in 5% CO_2 for 24 h. Then, the RAW 264.7 cells are treated with LAB strains (10^7 CFU/mL) and LPS (1 µg/mL) for 24 h. Total RNA was extracted from treated RAW 264.7 cells using the RNeasy Mini Kit (Thermo Fisher Scientific) according to the manufacturer's recommendations and cDNA was synthesized using the First strand cDNA Synthesis Kit (Thermo Fisher Scientific). The expression levels of nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor-α (TNF-α), IL-1β, and IL-6 were measured using synthesized cDNA as a template and a PCR mixture containing SYBR Green PCR Master Mix using qRT-PCR (Thermo Fisher Scientific). The primers are listed in Table 1. Semi-quantitative real-time PCR was performed as follows: 95°C for 2 min for polymerase activation, followed by 40 cycles of 95°C for 5 s for denaturation, and 60°C for 15 s for annealing/extension. The results were analyzed using the delta–delta Cq method. The melting curve was used to analyze the measurement of reaction specificity.

Table 1: Primer sequences related to anti-inflammatory potential used in real-time PCR

Primer*	Primer sequence (5'–3')
TNF-α	
Sense	5'-TTG ACC TCA GCG CTG AGT TG-3'
Antisense	5'-CCT GTA GCC CAC GTC GTA GC-3'
iNOS	
Sense	5'-CCC TTC CGA AGT TTC TGG CAG CAG C-3'
Antisense	5'-GGC TGT CAG AGC CTC GTG GCT TTG G-3'
COX-2	
Sense	5'-CAC TAC ATC CTG ACC CAC TT-3'
Antisense	5'-ATG CTC CTG CTT GAG TAT GT-3'
IL-1β	
Sense	5'-CAG GAT GAG GAC ATG AGC ACC-3'
Antisense	5'-CTC TGC AGA CTC AAA CTC CAC-3'
IL-6	
Sense	5'-GTA CTC CAG AAG ACC AGA GG-3'
Antisense	5'-TGC TGG TGA CAA CCA CGG CC-3'
GAPDH	
Sense	5'-CCA TGG AGA AGG CTG GGG-3'
Antisense	5'-CAA AGT TGT CAT GGA TGA CC-3'

*TNF-α: Tumor necrosis factor-α, iNOS: Inducible nitric oxide synthase, COX-2: Cyclooxygenase-2, IL-1β: Interleukin-1β, IL-6: Interleukin-6

Table 2: Morphological, physiological, and biochemical characterization of the isolated LAB strains

Tests	Isolated strains					
	NG06	NG18	NG21	NG28	SB09	SB15
Grams staining	+	+	+	+	+	+
Morphology	Rods	Rods	Cocci	Rods	Rods	Rods
Catalase test	-	-	-	-	-	-
Growth at different temperature						
10°C	-	+	+	+	-	+
37°C	+	+	+	+	+	+
45°C	+	-	+	+	-	-
Growth at different NaCl concentrations						
3%	+	+	+	+	+	+
5%	+	+	-	+	-	+
7%	+	-	-	+	-	+
Fermentation type	Homo	Homo	Homo	Homo	Homo	Homo
Carbohydrates fermentation						
Glucose	+	+	+	+	+	+
Fructose	+	+	-	+	+	+
Lactose	+	+	-	+	+	+
Sucrose	+	+	+	+	+	+
Arabinose	-	+	-	+	-	+
Maltose	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+
Xylose	-	-	+	-	-	-
Raffinose	-	-	+	-	-	+
Sorbital	-	-	+	-	-	-

+, positive; -, negative; homo, homofermentative

Statistical analysis

Statistical analysis was performed using SPSS for windows program package. Values were expressed as the mean ± standard deviation for the control as well as the experimented subjects.

RESULTS AND DISCUSSION

Isolation and preliminary identification of LAB isolates

Ngari and soibum are unique traditional fermented food products in Manipur. Ngari has got immense nutritional value with respect to essential fatty acids and antioxidative potential.[25] Soibum is a very good reservoir of carbohydrates, proteins, fibers, folic acid, Vitamin C, and low fat content.[26] They are found to be predominated by LAB species.[27,28] A total of 64 colonies were isolated from both ngari and soibum samples. The isolates were subjected to morphological, microscopic, and biochemical characterizations. Thirty-six isolates that were Gram-positive and catalase-negative were tested for ability to grow at different temperature, different saline (NaCl) concentrations, and ability to ferment different carbohydrates. On the basis of biochemical characteristics 6 isolates NG06, NG18, NG21, NG28 (from ngari), SB09 and SB15 (from soibum) have been considered for further studies. These isolates were found to be rods except for NG21 which was coccus. The optimum growth was observed at 37oC and 3% NaCl concentration in MRS broth. All the six isolates were homofermentative and able to ferment different carbohydrates [Table 2].

Acid and bile tolerance

Acid and bile tolerance are an essential property for probiotic strains, because most probiotic bacteria are delivered through food. They must go through the process of digestion and survive for a minimum of ninety minutes under low pH conditions of stomach and high bile salt concentration of small intestine in the gastrointestinal tract to exert their beneficial effects.[29] The survival rates of the six isolated LAB strains after 2 h and 4 h of incubation at pH 2 and 0.3% (w/v) concentration of bile salts are shown in Figure 1. All isolates displayed survival rates of more than 50% at low pH

and in high concentrations of bile salts. The survival rates of the NG06, NG28, and SB15 (74%, 80%, and 82%) were higher than other strains NG18, NG21, and SB09 (52%, 59% a,nd 63%) in pH 2 after 4 h exposure [Figure 1a]. In the 0.3% (w/v) concentration of bile salts, NG28 and SB15 (73% and 78%) showed high survival rate compared to NG06, NG18, NG21, and SB09 (60%, 51%, 63%, and 53%) after 4 h exposure [Figure 1b]. Several studies demonstrated the ability of LAB from a wide variety of fermented foods to survive under acidic conditions and high concentrations of bile salts.[30,31]

Antibacterial activity

LAB can exhibit antibacterial activity through several mechanisms such as the production of antimicrobial metabolites (lactic acid, bacteriocin, and hydrogen peroxide), competition for nutrients, inhibition of microbial adhesion to the intestinal mucosa, and immune system modulation.[32,33] To evaluate antibacterial activity, CFS and nCFS of isolates were tested against common enteric pathogens. The CFS of isolates showed good antibacterial activity against all pathogens [Table 3]. NG28 and SB15 strains displayed high antibacterial activity against all the test pathogens ranging from 15 mm to 17 mm diameter of zone of inhibition. Strains NG06 and NG18 exhibited good activity against *S. aureus*, *L. monocytogenes,* and *B. subtilis.* Strain SB09 showed good activity against *B. subtilis* and *L. monocytogenes.* Strain NG21 showed moderate activity against all the pathogens. On the other hand, the antimicrobial activity was significantly reduced in nCFS of the isolates (data not shown). The reduction of antibacterial activity in nCFS suggests that the

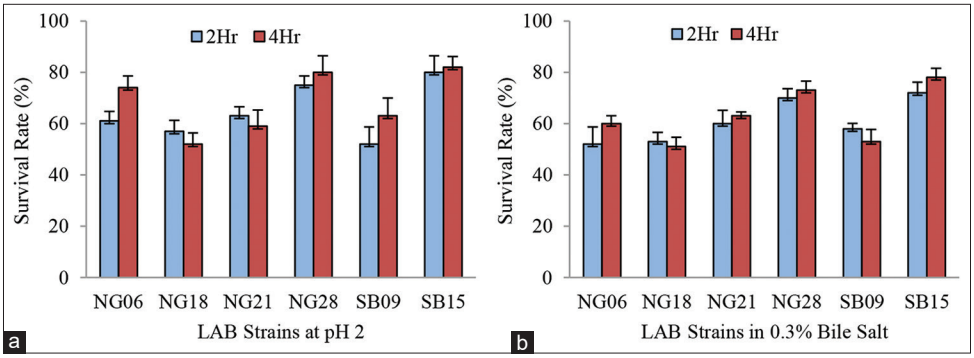

Figure 1: Tolerance of lactic acid bacteria strains under low pH and bile salt conditions

Table 3: Antibacterial activity of the cell-free supernatants of the LAB isolates against pathogenic bacteria

Isolates	Indicator organisms				
	Staphylococcus aureus	***Escherichia coli***	***Bacillus subtilis***	***Pseudomanas aeruginosa***	***Listeria monocytogenes***
NG06	14±1.41	10±1.21	12±1.23	08±1.82	14±1.21
NG18	15±1.71	12±1.51	13±2.82	10 ±2.72	13±2.12
NG21	10±2.48	11±2.41	09±2.82	09±2.12	10±2.41
NG28	17±1.14	16±0.22	15±1.41	15±2.12	16±1.21
SB09	11±2.12	09±2.12	13±2.12	09±1.21	14±1.21
SB15	16±1.23	15±2.82	17±1.21	17±2.18	15±2.68

Data are presented as the mean value±standard deviation of diameter of inhibition zones in mm

Table 4: Antibiotic susceptibility test of the LAB isolates to various antibiotics

Isolates	Antibiotics				
	Ampicillin	**Vancomycin**	**Tetracycline**	**Kanamycin**	**Streptomycin**
NG06	S	R	S	R	I
NG18	I	R	R	I	S
NG21	R	R	S	R	S
NG28	S	I	S	R	I
SB09	R	S	I	R	S
SB15	S	R	S	I	R

S: Sensitive, I: Intermediate, R: Resistance

antibacterial activity was pH-dependent in these strains. In a simple and effective way, creating an unfavorable condition by decreasing pH through the production of organic acids inhibits the growth of other bacteria. A study conducted by Yu *et al*.[34] has reported the pH-dependent antimicrobial activity of *L. plantarum* strains isolated from Chinese and Mongolian traditionally fermented food using agar well diffusion against different enteric pathogenic organisms. Mao *et al*.[35] demonstrated that the organic acids produced by *L. plantarum* DY-6 had excellent antibacterial activity against *E. coli, S. aureus, and Salmonella typhimurium.*

Antibiotic susceptibility test

Antibiotic susceptibility of LAB is one of the important criteria to confirm their safety as probiotics. Transmission of antibiotic-resistance genes in LAB may result in antibiotic-resistant enteropathogenic bacteria. The antibiotic susceptibility of the six isolates against clinically important antibiotics such as ampicillin, vancomycin (inhibitor of cell synthesis) tetracycline, kanamycin, and streptomycin (inhibitors of protein synthesis) was determined. A varied response in terms of susceptibility and resistance was obtained. Results were expressed in terms of resistance, intermediate, or susceptibility by comparing with the diameters of inhibition zones [Table 4]. The isolated strains showed sensitivity to most of the antibiotics used. Strains NG06, NG28, and SB15 were sensitive to ampicillin and tetracycline, while they were intermediate or resistance to other antibiotics used. It was reported in the previous studies, some bacteria were known as intrinsic resistance and they could be considered safe for human consumption.[21,22] Therefore, the isolated strains could be considered as safe probiotics.

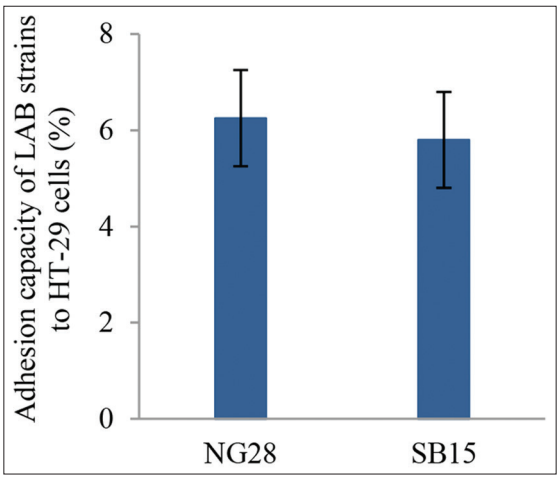

Figure 2: Adhesion of lactic acid bacteria strains to HT-29 cells. *L. plantarum* NG28 and SB15 isolates

Hemolytic activity

Hemolysis of red blood cells indicates the pathogenic potential of bacterial species. Therefore, non-hemolytic activity is regarded as a safety aspect for the selection of probiotic strains.[36] In the study, the hemolytic activity of six isolated LAB strains was evaluated on blood agar plates. None of the isolates had clear transparent or green zone formation on the blood agar plates. Thus, all the isolates were non-hemolytic (γ-hemolysis). Moussaid *et al*.[37] reported similar results regarding LAB strains isolated from Moroccan camel milk.

Molecular identification

The six potential isolates were subjected to BLAST analysis based on their 16S rRNA sequences. BLAST result showed that the five isolated strains (NG06, NG18, NG28, SB09, and SB15) were found to have 99% to 100% sequence similarity to strains of *L. plantarum* and one isolated strain (NG21) with 99% sequence similarity to *Enterococcus faecium* strains. In the present study, *L. plantarum* strains were identified from both of the fermented food samples, confirming that *L. plantarum* was one of the common LAB species in these fermented food samples. This result was in agreement with

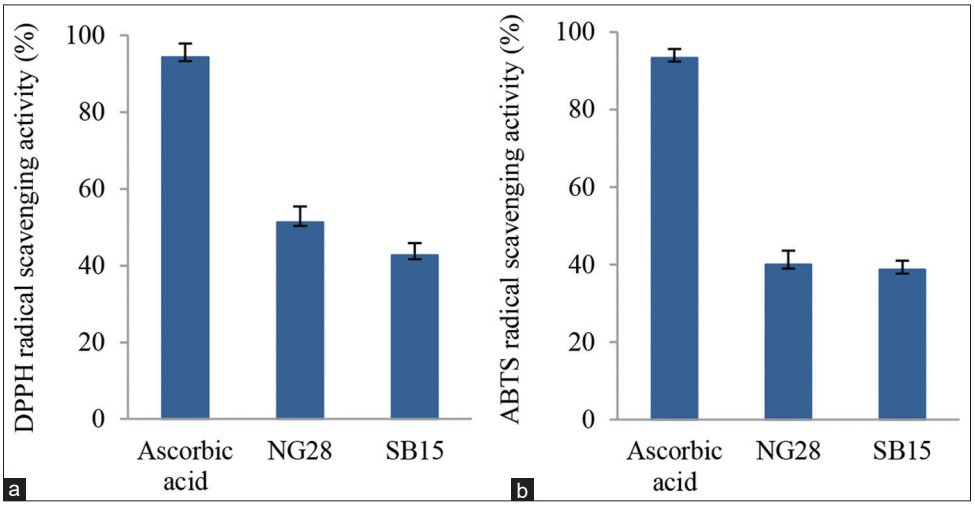

Figure 3: Antioxidant activity of Lactic acid bacteria strains; (a) 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay; (b) 2-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt racial scavenging assay

the study reported by Sonar *et al*.[26] and Thapa *et al*.[27] *L. plantarum* and *E. faecium* have been isolated from different types of fermented food products.[38,39]

Adhesion of LAB strains to HT-29 cells

Two strains with the best probiotic potential and antimicrobial activity were selected for further assessments of their functional properties. To provide health benefits to the host, the LAB strains have to adhere to intestinal epithelial cells and colonize in the intestinal tract. Therefore, adhesion ability is an essential property.[21] The *in vitro* adhesion of *L. plantarum* strains to HT-29 cells was examined. The adhesion rates of *L. plantarum* NG28 and *L. plantarum* SB15 to HT-29 were 6.2% and 5.8%, respectively [Figure 2]. In the previous reports Yang *et al*.[22] showed that *L. plantarum* 200655 had a high adhesion ability of 14.03% compared to *L. plantarum* KCTC 31008 with low adhesion ability (1.38%). Han *et al*.[20] reported that *L. plantarum* KU15149 (2.39%) and *Levilactobacillus brevis* KU15176 (2.61%) with an adhesion rate of around 2% could be considered sufficient for a functional probiotic. Therefore, the present study showed that *L. plantarum* NG28 and *L. plantarum* SB15 strains could attach and colonize intestinal epithelial cells.

Antioxidant activity of selected LAB strains

Antioxidant supplements or foods containing anti-oxidants are capable of reducing oxidative damage by neutralizing

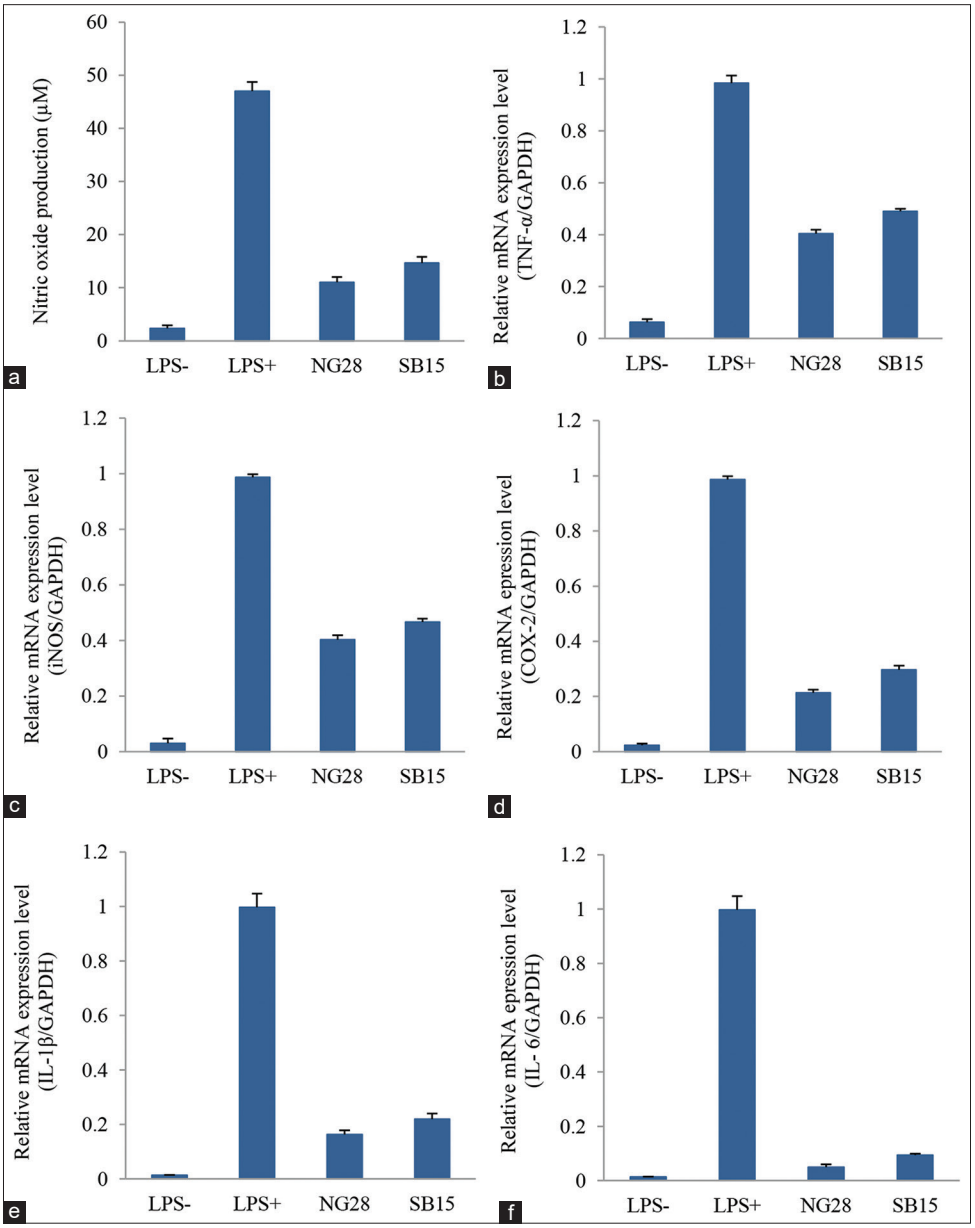

Figure 4: Effect of probiotic strains on mRNA expression levels of pro-inflammatory markers in LPS-stimulated RAW 264.7 cells. (a) production of Nictric oxide; (b) tumor necrosis factor-α; (c) Nitric oxide synthase; (d) Cyclooxygenase-2; (e) interleukin (IL)-1β; and (f) IL-6. Lipopolysaccharide (LPS)-, without LPS; LPS+, with LPS (1 µg/mL); NG28 and SB15 isolates with LPS (1 µg/mL). All values are expressed as the mean ± standard deviation and standardized against the GAPDH internal control

free radicals and terminating free radical chain reaction.[9] Interestingly, probiotics have been considered as effective antioxidants in the reduction of free radicals. Therefore, the ability of the selected LAB isolates to scavenge DPPH and ABTS free radicals was conducted. The DPPH radical scavenging activity of LAB strains is presented in Figure 3a. *L. plantarum* NG28 was exhibited a maximum DPPH scavenging activity of 51.33% compared to *L. plantarum* SB15 with 42.67% of activity. The ABTS radical scavenging activity of LAB strains is presented in Figure 3b. The ABTS radical scavenging activity of *L. plantarum* NG28 and SB15 strains exhibited 40.33% and 38.16% activity, respectively, which were not significantly different. Li *et al*.[40] showed that *L. plantarum* C88 strain isolated from traditional Chinese fermented food exhibited high DPPH radical scavenging activity of 53.05%. In addition, Yang *et al*.[22] reported 30.51% of DPPH and 38.13% of ABTS scavenging activity of *L. plantarum* 200655. The present report showed that both *L. plantarum* NG28 and *L. plantarum* SB15 demonstrated good radical scavenging potential.

Anti-inflammatory activities of selected LAB strains

Anti-inflammatory activity is another beneficial characteristic of LAB. The most commonly proposed mechanism of probiotics with anti-inflammatory effects is through immune modulation and regulation of inflammatory mediators. Excessive production of NO and pro-inflammatory mediators can cause tissue damage and contributes to pathogenesis of many disease states.

Therefore, regulation of production of NO and pro-inflammatory mediators is an important factor in biological maintenance. The anti-inflammatory effects of strains NG28 and SB15 were demonstrated by the evaluation of decrease of NO production and pro-inflammatory cytokines in LPS-stimulated RAW 264.7 cells.

NO production was significantly reduced in LPS-induced RAW 264.7 cells treated with strains NG28 (11 μM) and SB15 (14.66 μM) compared to LPS-induced RAW 264.7 cells which were untreated with LAB strains [Figure 4a]. Both *L. plantarum* NG28 and SB15 strains exhibited the high ability to reduce NO production. In the previous report, Han *et al*.[41] reported the reduced level of NO production by *L. lactis* NK34. In addition, Lee *et al*.[42] investigated the ability of *L. plantarum* in decrease of NO production in LPS-stimulated RAW 264.7 cells.

Semi quantitative RT-PCR was used to investigate the inhibitory activity of the selected strains on mRNA expression levels of pro- inflammatory mediators TNF-α, iNOS, COX-2, interleukin (IL)-1β, and IL-6 in LPS-induced RAW 264.7 cells. The results showed that the expression levels of these mediators were increased in LPS-induced RAW 264.7 cells and were reduced by treatment with *L. plantarum* strains NG28 and SB15 as shown in the Figure 4b-f. *L. plantarum* NG28 exhibited high activity in down-regulating the pro-inflammatory mediators compared to *L. plantarum* SB15 strain. Han *et al*.[20] reported that *L. plantarum* KU15149 strain exhibited the inhibition of NO production as well as inflammatory mediators in LPS stimulated RAW 264.7 cells. Thus, our study demonstrated the anti-inflammatory potential of the isolated *L. plantarum* strains NG28 and NG15.

CONCLUSION

Both *L. plantarum* NG28 and SB15 strains isolated from ngari and soibum were found to have potential probiotic properties including low pH tolerance and bile salt tolerance, antimicrobial activity, antibiotic sensitivity, and adhesion to intestinal cells. Both *L. plantarum* NG28 and SB15 possess a high abundance of antioxidant potential. In addition, *L. plantarum* NG28 and SB15 also demonstrated the anti-inflammatory activity in LPS stimulated RAW 264.7 cells by inhibiting the production of NO and inflammatory mediators such as TNF-α, iNOS, COX-2, IL-1β, and IL-6. Therefore, the strains *L. plantarum* NG28 and SB15 could be potentially used in functional and pharmaceutical applications.

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