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Research Article
In vitro Assessment of Antioxidant, Antimicrobial and Anticancer Properties of Lactic Acid Bacteria

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Abstract

Background and Objectives: Probiotic strains can be utilized as bio-preservatives and functional food supplements. The present study aimed to characterize the 8 lactic acid bacteria (LAB) strains and their probiotic properties and antioxidant and anticancer potential. The selected LAB strains displayed antimicrobial activity against foodborne pathogens. **Materials and Methods:** The *in vitro* probiotic characteristics of these 8 strains demonstrated superior acid and bile tolerance, simulated gastric juice (SGJ) tolerance and an *in vitro* ability to adhere to Caco-2 cells. The antioxidant effects of intracellular and cell-free extracts of LAB strains were assessed by several antioxidant assays including resistance to hydrogen peroxide, DPPH radical scavenging, ABTS radical scavenging and hydroxyl radical scavenging (HRS). **Results:** Findings showed that the bacterial lysate and cell-free supernatant (CFS) of *S. thermophilus* BLM 58, *L. lactis* subsp. *lactis* DSM 20481, *P. acidilactici* ATCC 8042 and *L. Brevis* NRRL B-4527 possessed excellent antioxidant capacities. **Conclusion:** The current study demonstrated for the first time that the high capacity of *S. thermophilus* BLM 58 to have anticancer effects against cancer cells from four types of cancerous cell lines. This may be a promising finding for future cancer treatments and in the prevention of cancer using natural probiotics.

Key words: Natural probiotics, lactic acid bacteria, antioxidant, anticancer, simulated gastric juice, hydroxyl radical scavenging, cancerous cell lines

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Probiotics are living micro-organisms with both nutritional and health benefits[1]. Probiotic research was first conducted by Élie Metchnikoff 100 years ago[2]. Most probiotic micro-organisms are lactic acid bacteria (LAB), such as *Lactobacillus* sp., *Bifidobacterium* sp., *Enterococcus* sp., *Streptococcus thermophilus* and *Pediococcus acidilactici* [3]. Several functional properties of probiotics provide health benefits. These features include resistance to gastric juices and bile salts, adherence to human epithelial cells, antimicrobial activity against pathogenic micro-organisms and bile salt hydrolase activity. The capacity for resistance and growth in the low pH conditions of the stomach and high salt concentrations and pH of bile enable probiotics to survive in different gastrointestinal conditions[4]. Furthermore, probiotics can inhibit microbial growth, particularly foodborne pathogens, such as; *Listeria monocytogenes*, *Staphylococcus aureus*, *Staphylococcus aureus* and *Escherichia coli* [5].

Identification of the antioxidative properties of probiotics against free radicals is an interesting research topic. Oxidative damage contributed to several human diseases (e.g., cancer, atherosclerosis, cirrhosis and inflammatory diseases)[6,7] and oxidative stress caused damage to cellular macromolecules including DNA. Elevated levels of oxidative DNA lesions have been observed in various tumors. The addition of probiotics to food is an exciting alternative to antibiotics and has generated significant public interest due to the emergence of antimicrobial resistance[8]. Artificial antioxidants, such as; butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and n-propyl gallate (PG), have strong antioxidant activities against numerous oxidation systems. However, the use of these antioxidants in food has been hindered in some countries due to their potential risks to human health[9]. Antioxidants from natural sources are likely to be more acceptable than those chemically produced due to the reported side effects of some artificial antioxidants[10]. Consequently, the search for natural, stable, safe, antimicrobial, antioxidant and anticancer agents have become a subject of interest as alternatives to artificial compounds. The present study aimed to characterize probiotic LAB strains and assess their antimicrobial, antioxidant and anticancer efficacies.

MATERIALS AND METHODS

The current study was carried out in the City of Scientific Research and Technological Applications, Alexandria, Egypt from September, 2017-June, 2018.

Table 1: Strains and their growth conditions

Microorganisms	Media and growth temperature
Lactic acid bacteria strains	
Lactobacillus brevis NRRL B-4527	MRS Broth, 30°C*
Streptococcus thermophilus BLM 58	M17 Broth, 37°C
Pediococcus acidilactici ATCC 8042	MRS Broth, 30°C
Lactobacillus rhamnosus CCUG 1452	MRS Broth, 30°C
Lactobacillus curvatus ATCC 51436	MRS Broth, 30°C
Lactococcus lactis subsp. *lactis* DSM 20481	MRS Broth, 30°C
Lactobacillus plantarum DMSZ 20079	MRS Broth, 30°C
Lactobacillus plantarum TF103	MRS Broth, 30°C
Pathogenic strains	
Candida albicans ATCCMYA-2876	YPD Broth, 37°C**
Clostridium botulinum ATCC 3584	TPGY Broth, 37°C#
Escherichia coli BA 12296	LB Broth, 37°C$
Klebsiell apneumoniae ATCC12296	LB Broth, 37°C$
Salmonella enterica subsp. *enterica* *Serovar senftenberg* ATCC 8400	Nutrient Broth, 37°C
Staphylococcus aureus NCTC 10788	Nutrient Broth, 37°C

*MRS Broth: deMan, Rogosa and Sharpe **YPD broth: Yeast peptone dextrose, #TPGY broth: Tryptone-peptone-glucose-yeast extract, $LB broth: Luria-Bertani medium

Micro-organism and growth conditions: The strains used in this study and their growth conditions are listed in Table 1. All strains were maintained as frozen stocks at -20°C in 25% (v/v) glycerol and routinely propagated in MRS[11] broth with a pH of 6.8 for 20 h at 30°C for LAB strains and with a pH of 6.8 for 24 h at 37°C for pathogenic strains.

Evaluation of functional properties of LAB strains

Qualitative and quantitative antimicrobial activity assays: Qualitative antimicrobial activity assays of LAB strains were performed against 7 pathogens (Table 1). Strains were tested using the spot-on-lawn method[12]. Activity was quantified by taking the reciprocal of the highest dilution that exhibited a clear zone of inhibition and was expressed as activity units (AU) per milliliter of culture media. The titre of the antibacterial substance in AU/mL, was calculated as (1000/d) D, where, D is the dilution factor and d is the amount of supernatant[13] in µL.

***In vitro* resistance to simulated gastric juice (SGJ) and bile salt:** Acid tolerance was determined as described by Charteris *et al.*[1], with some modifications. The LAB cells from 100 mL MRS culture were harvested by centrifugation (4300 g, 10 min) and washed three times in phosphate buffered saline, pH 7.0. Washed cell pellets were then suspended in (1/10) cultivation volume in the same buffer, hence, obtaining a 10-fold increase in cell density. To 1 mL of the washed cell suspension, 5 mL of simulated gastric juice and 1.5 mL NaCl (0.5 w/v) were added. Simulated gastric juice was prepared freshly daily by suspending pepsin (3 g L^{-1}) in sterile saline (0.5% w/v) and adjusting the pH to 2.0 with concentrated HCl. The materials were vortexed for 10 sec and

incubated at 37°C for 3 h. Aliquots of 0.1 mL were then removed at constant intervals (0, 1, 2, 3 h) for determination of total viable count. Dilutions were made (up to 10^{-4}) and cells were plated in duplicate on MRS agar. Plates were incubated at 37°C for 72 h before enumeration.

Bile salt tolerance was determined as described by Shehata *et al.*[4]. The MRS broth supplemented with 0.3% bile salt was prepared. A preparation without bile salt was used as a control. The cells from 100 mL (20 h MRS tested culture) were collected by centrifugation (3400 g, 10 min), washed twice in saline (8.5 g L^{-1} NaCl) and resuspended in 10 mL MRS broth. This suspension was inoculated (1%) into MRS broth lacking or containing bile salt. After 0, 1, 2 and 3 h of incubation at 37°C, viable counts on MRS agar plates and absorbance of the culture at 625 nm were determined. Experiments of acid and bile tolerance were repeated three times each with duplicate analysis.

***In vitro* cell adhesion assay:** The *in vitro* adhesion abilities of selected LAB strains were assayed using Caco-2 cells. Human Caucasian colon adenocarcinoma (Caco-2) was obtained from American Type Culture Collection (ATCC, USA). The Caco-2 cells were cultivated in DMEM (Lonza, USA) containing 10% fetal bovine serum (GIBCO, USA), seeded in 6-well plates and incubated in a 5% CO_2 incubator at 37°C for 24 h. After incubation, the Caco-2 cells (300,000 cell mL^{-1}) were incubated with a 1 mL suspension of a LAB strain (1×10^8CFU mL^{-1}) for 2 h in a 5% CO_2 incubator. After washing the cells with sterile 1× phosphate-buffered saline (PBS) (pH 7.4), cells were fixed and stained with 1 mL of Giemsa[14] solution (1×) at 37°C for 20 min. The wells were washed with absolute ethanol to remove excess stain, air dried and investigated under an inverted microscope (Olympus, Japan). To determine the percentage of adhesion of LAB strains to Caco-2 cells, bacterial cells were detached from the monolayer by adding 500 µL of trypsin-EDTA solution. Then, the detached cell suspension was serially diluted, plated onto an MRS agar plate and incubated at 30°C for 48 h. Subsequently, colonies were counted and the percentage of adherent bacterial cells was determined[15]. Experiments of adhesion assay were repeated three times each with duplicate analysis:

$$\text{Adhesion (\%)} = \frac{B_1}{B_0}\times100$$

where, B is the number of viable LAB cells (CFU mL^{-1}) before (B_0) and after (B_1) adhesion.

***In vitro* determination of antioxidant activity of LAB strains**

Preparation of the culture supernatant and bacterial lysate extract: Bacterial lysate extracts were prepared according to a method by Lin and Yen[16]. The LAB strains were grown in MRS broth at 37°C for 18 h. The bacterial cells were harvested by centrifugation (4300×g, 10 min, 4°C), washed twice and resuspended in deionized water. Bacterial counts in cell pellets were adjusted to 10^8, 10^9 and 10^{10} CFU mL^{-1}.

Antioxidant assays

Resistance to hydrogen peroxide: A method by Buchmeier *et al.*[17] was used with some modifications. Overnight cultures of the LAB strains were inoculated at 1% (v/v) into MRS broth and MRS broth containing 0.4, 0.7 or 1.0 mM hydrogen peroxide (30 wt.% solution in water), followed by incubation at 30°C for 8 h. Cell growth (absorbance) was measured spectrophotometrically at 600 nm.

Scavenging of hydroxyl radicals: Hydroxyl radical scavenging (HRS) assays were conducted by a Fenton reaction method[18]. Briefly, a reaction mixture containing 1.0 mL of Brilliant Green (0.435 mM), 2.0 mL of $FeSO_4$ (0.5 mM), 1.5 mL of H_2O_2 (3.0%, w/v) and 1.0 mL of bacterial lysate extract in different concentrations was incubated at room temperature for 20 min and the absorbance was measured at 624 nm. Changes in the absorbance of the reaction mixture indicated the scavenging ability of the LAB strains for hydroxyl radicals. HRS activity was expressed as follows:

$$\text{Scavenging activity (\%)} = \frac{A_S\text{-}A_0}{A\text{-}A_0}\times100$$

where, A_s is the absorbance of the sample, A_0 is the absorbance of the control and A is the absorbance without the sample or Fenton reaction system.

Antioxidant capacity using the $ABTS^{\bullet+}$ method: The antioxidant activity was determined by using the $ABTS^{\bullet+}$ radical cation method with some modifications as described by Rossini *et al.*[19]. All determinations were carried out at least three times. The percentage inhibition of absorbance at 734 nm was calculated by using an ascorbic acid standard curve:

$$\text{ABTS radical scavenging activity (\%)} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}}\times100$$

where, $Abs_{control}$ is the absorbance of ABTS radical+methanol; Ab_{sample} is the absorbance of ABTS radical+sample/standard.

Scavenging ability for DPPH radicals: The DPPH was used at a concentration of 60 mM in methyl alcohol. In the dark, 0.1 mL sample aliquots (culture supernatant or bacterial lysate extract) were transferred to test tubes containing 3.9 mL of DPPH radical solution and were homogenized by shaking. The absorbance of the solution at 517 nm was measured using a spectrophotometer. All of the tests were carried out in triplicates. The inhibition of DPPH radical was calculated as follows:

$$\text{Inhibition (\%)} = \frac{\text{Abs } 517_{\text{Control}} - \text{Abs } 517_{\text{sample}}}{\text{Abs } 517_{\text{Control}}} \times 100$$

where, $A_{control}$ is the absorbance of the control solution, A_{sample} is the absorbance of the test extract.

The results are expressed as IC_{50} (mg mL^{-1}), which is the minimum antioxidant concentration required to reduce 50% of the initial DPPH reaction from the time the extract reached stability[20]. All determinations were carried out at least three times.

Cytotoxicity of probiotic lysates: Normal human dermal fibroblast (HDF) cells were sustained as adherent cells in DMEM containing 10% fetal bovine serum (FBS). The cytotoxicity of bacterial lysate samples toward normal cells was determined using MTT assays as described by Mosmann[21].

Anticancer activity of probiotic lysates: A colon cancer cell line (Caco-2) and a prostate cancer cell line (PC3) were maintained as adherent cell cultures in DMEM. A liver cancer cell line (HepG-2) and a breast cancer cell line (MCF-7) were cultured in RPMI 1640 medium (Lonza, USA). All media were supplemented with 10% FBS. The sensitivity of tumor cells to bacterial lysate samples was assessed using MTT assays as described by Mosmann[21]. Control untreated cancer cells which was incubated with culture medium without any addition of bacterial lysates. The anticancer activities of the bacterial lysates were expressed as IC_{50} values, i.e., values associated with 50% death of cancer cells and were estimated by GraphPad Instat software version 3.10. The selectivity index (SI) was estimated as the ratio of the IC_{50} of normal PBMCs to that of cancer cells. The most effective anticancer bacterial lysates were also investigated by phase contrast inverted microscopy (Olympus, Japan). All determinations were carried out at least three times.

Flow cytometry analysis of the anticancer activity of probiotic lysates: Human cancer cell lines (MCF-7, HepG-2 and Caco-2) were treated with IC_{50} doses of the most potent anticancer bacterial lysates and incubated in a 5% CO_2 incubator at 37°C for 72 h. Untreated and treated cancer cells were harvested by trypsinization, washed and resuspended in PBS. Then, the untreated and treated cells were stained with annexin V-biotin (Molecular Probes™, USA) and propidium iodide (PI) in dark for 15 min. Cell death rates were detected by Partec flow cytometry (ex = 488 nm, em = 530 nm) using a FITC signal detector (FL1) for annexin-stained apoptotic cells and a phycoerythrin signal detector (FL2) for PI-stained necrotic cells.

Statistical analysis: Data are expressed as the means±standard errors of the means (SEM) by multiple comparison tests and one-way analysis of variance (ANOVA) using the SPSS16 software package. Probability (p) values<0.05 were considered significant. The correlation coefficient between antioxidant and anticancer activity was calculated by Pearson correlation using the SPSS16 software package.

RESULTS

Antimicrobial properties of LAB: As shown in Table 2, the antimicrobial activity results of the LAB strain isolates in terms of activity units (AU mL^{-1}) illustrated. The selected LAB strains showed antimicrobial activities against these pathogenic micro-organisms on several levels (Table 2). *Lactobacillus brevis, Pediococcus acidilactici* and *Lactobacillus plantarum* TF103 exerted strong inhibitory activity against *Klebsiella pneumoniae* ATCC12296 (6400 AU mL^{-1}), whereas, *Lactobacillus rhamnosus* CCUG 1452 showed slightly better inhibitory effects against *Salmonella enterica* subsp. *enterica serovar* senftenberg ATCC 8400 (3200 AU mL^{-1}). *Pediococcus acidilactici ATCC 8042* showed moderate antimicrobial activities against *Escherichia coli* BA 12296 and *Staphylococcus aureus* NCTC 10788 (1600 AU mL^{-1}).

Tolerance to acid and bile: Expectation of the strain resistance to the gut conditions where in vitro to measure their tolerance to low pH, bile predicated and pancreatic fluid. In the present study, all selected LAB strains survived in SGJ at pH 2 and 0.3% bile after 3 h of incubation. *Pediococcus acidilactici* ATCC 8042, *L. plantarum* TF103 and *L. brevis* NRRL B-4527 exhibited acid resistance with 78.94±0.7, 78.94±0.7 and 76.58±1.55% of viability, respectively (Table 3). In case of bile tolerance *L. brevis NRRL B-4527*, *L. lactis* subsp. *lactis* DSM 20481, *L. plantarum* TF103, *P. acidilactici* ATCC 8042 showed a high surviving percentage 75.24±2.48, 75.21±2.18, 74.38±2.1 and 71.72±3.23%, respectively (Table 4).

Table 2: Antimicrobial activities of cell-free supernatants of 8 selected LAB strains against various pathogens

	Antimicrobial activity against indicator strain expressed (AU mL^{-1})*					
Lactic acid bacteria strains	*Staphylococcus aureus* NCTC 10788	*Candida albicans* ATCC MYA-2876	*Salmonella senftenberg* ATCC 8400	*Escherichia coli* BA 12296	*Klebsiella pneumoniae* ATCC 12296	*Clostridium botulinum* ATCC 3584
L. brevis NRRL B-4527	400	200	1600	800	6400	400
S. thermophilus BLM 58	400	200	800	800	400	200
P. acidilactici ATCC 8042	1600	400	800	1600	6400	200
L. rhamnosus CCUG 1452	200	0	3200	400	200	0
L. curvatus ATCC 51436	200	0	400	200	200	0
L. lactis subsp. *lactis* DSM 20481	200	200	400	800	400	800
L. plantarum DMSZ 20079	800	200	200	200	800	0
L. plantarum TF103	800	200	800	800	6400	200

*Activity units/mL cell-free supernatant were calculated according to the following equation: (1000/d)×D, where, D is the two-fold dilution factor and d is the amount of supernatant used

Table 3: Survival of LAB strains under simulated gastric juice pH 2 conditions at 37°C

	Mean of viable count (log_{10} CFU mL^{-1})±SD*				
	Time of exposure (h)				
Strains	0	1	2	3	Surviving (%)
P. acidilactici ATCC 8042	8.12±0.09^{b}	7.77±0.22^{a}	6.93±0.11^{a}	6.41±0.05^{a}	78.94±0.7^{a}
L. lactis subsp. *lactis* DSM 20481	8.06±0.05^{b}	7.32±0.04^{bc}	6.12±0.11^{b}	5.75±0.19^{b}	71.33±2.07^{b}
S. thermophilus BLM 58	8.15±0.15^{b}	7.24±0.07^{c}	6.29±0.03^{b}	5.38±0.05^{c}	66.01±0.77^{c}
L. plantarum TF103	8.25±0.08^{ab}	7.73±0.19^{ab}	7.10±0.04^{a}	6.42±0.04^{a}	78.94±0.7^{a}
L. brevis NRRL B-4527	8.20±0.06^{ab}	7.18±0.10^{c}	6.80±0.25^{a}	6.28±0.15^{a}	76.58±1.55^{a}
L. rhamnosus CCUG 1452	8.18±0.13^{ab}	6.95±0.37^{c}	5.04±0.42^{d}	3.78±0.23^{e}	46.21±2.6^{e}
L. curvatus ATCC 51436	8.37±0.08^{a}	6.90±0.32^{c}	4.93±0.25^{d}	4.00±0.19^{e}	47.78±1.87^{e}
L. plantarum DMSZ20079	8.26±0.10^{ab}	6.96±0.34^{c}	5.71±0.19^{c}	4.81±0.20^{d}	58.23±2.36^{d}

^{abcd}Mean in the same column followed by different superscript letters are *significantly different ($p<0.05$)

Table 4: Survival of LAB strains in MRS broth supplemented with 0.3% bile salts after 0, 1, 2 and 3 h at 37°C

	Mean of viable count (log_{10} CFU mL^{-1})±SD*				
	Time of exposure (h)				
Strains	0	1	2	3	Surviving (%)
P. acidilactici ATCC 8042	8.31±0.04^{a}	7.14±0.12^{bc}	6.44±0.07^{b}	5.96±0.25^{a}	71.72±3.23^{a}
L. lactis subsp. *lactis* DSM 20481	8.19±0.18^{a}	7.81±0.22^{a}	6.91±0.18^{a}	6.16±0.06^{a}	75.21±2.18^{a}
S. thermophilus BLM 58	8.24±0.07^{a}	6.98±0.07^{c}	5.95±0.073^{cd}	5.35±0.035^{b}	64.92±0.99^{b}
L. plantarum TF103	8.12±0.05^{a}	6.89±0.17^{c}	6.15±0.08^{c}	6.04±0.14^{a}	74.38±2.1^{a}
L. brevis NRRL B-4527	8.12±0.14^{a}	7.07±0.14^{bc}	6.87±0.20^{a}	6.11±0.09^{a}	75.24±2.48^{a}
L. rhamnosus CCUG 1452	8.14±0.16^{a}	7.26±0.07^{b}	6.06±0.07^{cd}	4.93±0.22^{c}	60.56±2.7^{c}
L. curvatus ATCC 51436	8.22±0.15^{a}	6.65±0.14^{d}	5.33±0.07^{e}	4.11±0.09^{e}	50.00±1.98^{d}
L. plantarum DMSZ 20079	8.16±0.11^{a}	7.09±0.03^{bc}	5.81±0.21^{d}	4.63±0.16^{d}	56.74±2.25^{c}

^{abcd}Mean in the same column followed by different superscript letters are *significantly different ($p<0.05$)

***In vitro* cell adherence of LAB strains:** The adhesion ability of LAB strains with Caco-2 cell line were observed by inverted microscope after Giemsa staining shown in Fig. 1. Data of adhesion (%) were summarized in Fig. 2. *Pediococcus acidilactici* ATCC 8042, *L. brevis* NRRL B-4527 and *S. thermophilus* BLM 58 showed significant (p-0.05) adhesion percentages of 17.2±2.11, 15.23±2.14 and 12.9±2.51%, respectively. Adherence of LAB strains to gastrointestinal tract and their ability to resist the elimination by peristalsis are required factors to maintain their probiotic efficacy, therefore, strains *P. acidilactici* ATCC 8042, *L. brevis* NRRL B-4527 and *S. thermophilus* BLM 58 can potentially used as commercial probiotics. Further future investigation will required to determine the complex cell adhesion between the LAB membrane and interacting cell surface of mammalian cell line.

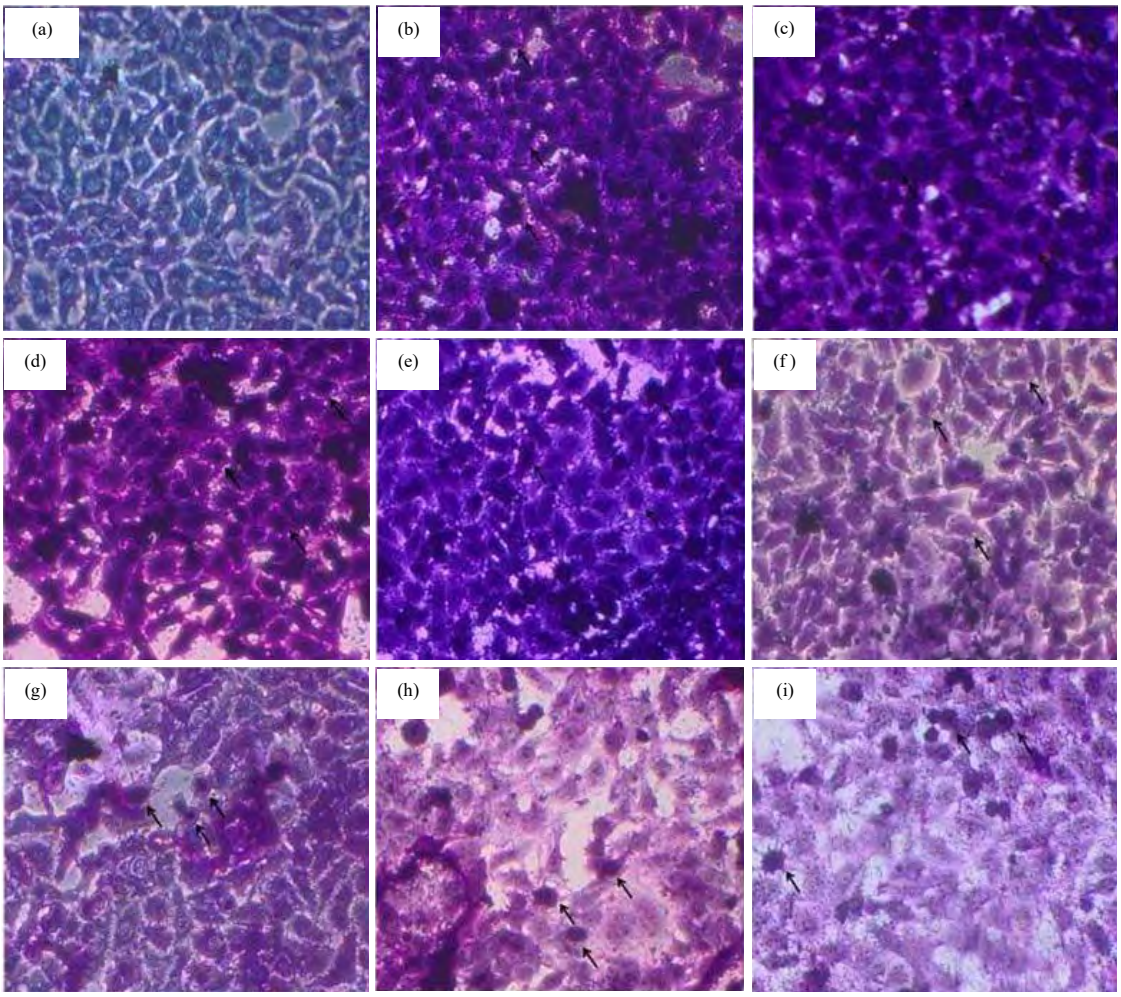

Fig. 1(a-d): Adhesion of selected LAB strains on Caco-2 cell line observed under inverted microscope (200 x) after Giemsa staining. (a) Control Caco-2 cell line, treated Caco-2cells with (b) *P. acidilactici* ATCC 8042, (c) *L. plantarum* TF103, (d) *L. brevis* NRRL B-4527, (e) *S. thermophilus* BLM 58, (f) *L. plantarum* DMSZ20079, (g) *L. Lactis* subsp. *lactis* DSM 20481, (h) *L. curvatus* ATCC 51436 and (i) *L. rhamnosus* CCUG 1452 for 2 h at 37°C
The arrows indicate the attachment of bacterial cell to the mammalian Caco-2cells

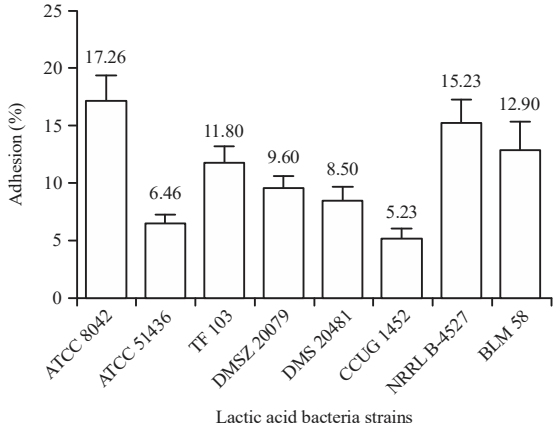

Fig. 2: Adhesion (%) of LAB strains on Caco-2 cell line

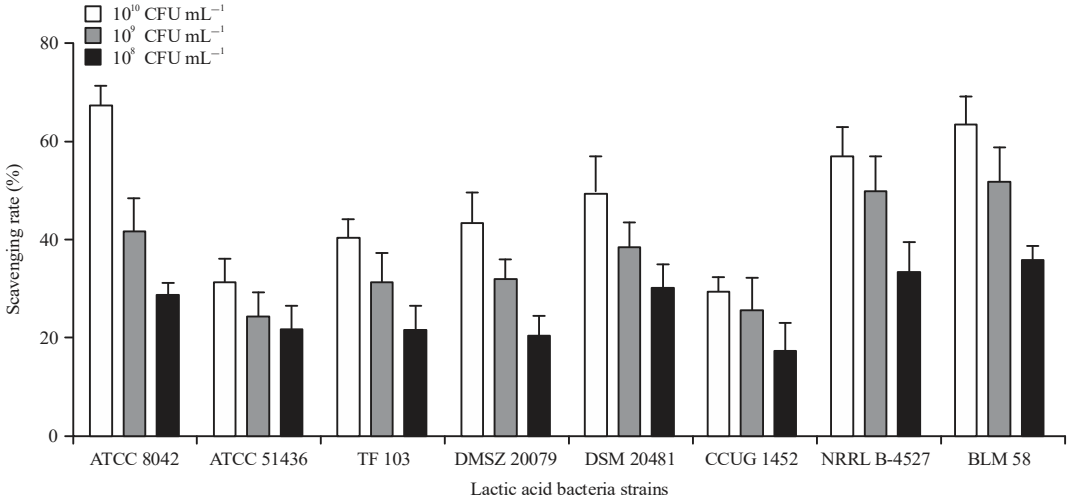

Fig. 3: Scavenging activity on DPPH free radicals by intact cells of LAB strains incubated at 30°C for 24 h
Each value is expressed as Mean±SD (n = 3)

Table 5: Resistance of LAB strains at different hydrogen peroxide concentrations

	Hydrogen peroxide concentration			
Strains	Control	0.4 mM	0.7 mM	1 mM
S. thermophilus BLM 58	1.79±0.176^{a}	1.13±0.10^{cd}	0.76±0.06^{cd}	0.69±0.09^{a}
L. plantarum TF103	1.50±0.28^{bc}	1.19±0.1422^{bcd}	1.11±0.166^{a}	0.88±0.118^{a}
L. plantarum DMSZ 20079	1.41±0.60^{c}	1.09±0.13^{d}	0.87±0.12^{bc}	0.43±0.11^{b}
P. acidilactici ATCC 8042	1.85±0.07^{a}	1.35±0.13^{abc}	1.25±0.06^{a}	0.80±0.09^{a}
L. rhamnosus CCUG 1452	1.43±0.06^{c}	1.15±0.08^{cd}	0.75±0.03^{cd}	0.27±0.05^{b}
L. brevis NRRL B-4527	1.69±0.09^{ab}	1.24±0.10^{bcd}	1.06±0.04^{ab}	0.76±0.16^{a}
L. lactis subsp. *lactis* DSM 20481	1.87±0.09^{a}	1.46±0.12^{a}	1.23±0.22^{a}	0.85±0.06^{a}
L. curvatus ATCC 51436	1.70±0.06^{ab}	1.39±0.07^{ab}	0.64±0.11^{d}	0.37±0.06^{b}

Result are expressed as Mean±SD, each value is the average of three experiments each was carried out in duplicate. The absorbance was measured at 624 nm.
^{abcd}Mean in the same column followed by different superscript letters are significantly different ($p<0.05$)

Evaluation of antioxidant activity of LAB strains

Resistance of intact cells to hydrogen peroxide: All eight strains showed tolerance to 0.4 mM hydrogen peroxide for 8 h with variations in viability. The *L. plantarum* TF103, *L. brevis* NRRL B-4527, *P. acidilactici* ATCC 8042 and *L. lactis* subsp. *Lactis* DSM 20481 showed maximum resistance against hydrogen peroxide with optical densities of more than 0.7 after incubation for 8 h with 1.0 mM hydrogen peroxide, whereas, strains, *L. rhamnosus* CCUG 1452 and *L. plantarum* DMSZ 20079 showed the highest sensitivity. While *L. curvatus* ATCC 51436 showed resistance at 0.4 mM while sensitive at 1.0 mM hydrogen peroxide (Table 5).

Scavenging activities of intact LAB strains against DPPH: The DPPH free radical scavenging activities of the LAB strains measured at 10^{10}, 10^{9} and 10^{8} CFU mL^{-1} is shown in Fig. 3. *Pediococcus acidilactici* ATCC 8042 showed the highest radical scavenging activity (67.5%), followed by *S. thermophilus* BLM 58, *L. brevis* NRRL B-4527 and *L. lactis* subsp. *Lactis* DSM 20481 (63.46, 57.05 and 49.35%, respectively, at a concentration of 10^{10} CFU mL^{-1}). However, all strains showed lower DPPH radical scavenging activities at 10^{8} CFU mL^{-1}.

Scavenging activities of bacterial lysate and supernatant of LAB strains against DPPH, ABTS and hydroxyl radicals: The bacterial lysates (BL) and supernatants of the tested LAB (CFS) showed remarkably stronger scavenging activity for ABTS than for DPPH. Overall, the bacterial lysates (BL) and supernatants of the tested LAB (CFS) showed remarkably stronger scavenging activity for hydroxyl radical than for DPPH. The BL and CFS of *S. thermophilus* BLM 58 exhibited the highest antioxidant activity among all tested strains ($p>0.05$) (Table 6). The bacterial lysate of *S. thermophilus* BLM 58 had the lowest IC_{50} values of 1.18±0.05 μg mL^{-1} in ABTS scavenging and its CFS had the lowest IC_{50} values of 5.57±0.19 μg mL^{-1} in DPPH.

Table 6: Scavenging activities of bacterial lysate and supernatant of LAB strains against DPPH, ABTS and hydroxyl radicals

	DPPH (IC_{50} μg mL^{-1})		ABTS (IC_{50} μg mL^{-1})		Hydroxyl radical (IC_{50} μg mL^{-1})	
Lactic acid bacteria strains	BL	CFS	BL	CFS	BL	CFS
P. acidilactici ATCC 8042	7.25±0.39^{b}	8.60±0.70^{b}	1.66±0.07^{c}	7.28±0.48^{d}	4.92±0.24^{c}	6.42±0.17^{c}
L. plantarum TF103	8.89±0.75^{a}	9.70±1.08^{b}	3.83±0.20^{b}	9.57±0.56^{b}	6.40±0.188^{a}	11.06±1.51^{ab}
L. plantarum DMSZ20079	9.32±0.84^{a}	12.99±2.06^{a}	7.63±0.57^{a}	12.04±0.45^{a}	6.32±0.48^{a}	11.72±1.82^{a}
L. rhamnosus CCUG 1452	9.64±0.71^{a}	11.96±1.64^{a}	7.85±0.49^{a}	11.78±0.86^{a}	5.71±0.16^{b}	12.56±1.18^{a}
S. thermophilus BLM 58	5.38±0.37^{c}	5.57±0.19^{c}	1.18±0.05^{c}	7.03±0.38^{d}	3.99±0.18^{d}	5.63±0.27^{c}
L. Lactis subsp. *lactis* DSM 20481	5.78±0.42^{c}	5.71±0.24^{c}	1.22±0.02^{c}	7.66±0.45^{cd}	4.78±0.22^{c}	6.15±0.33^{c}
L. curvatus ATCC 51436	9.38±0.73^{a}	12.55±1.81^{a}	8.33±0.80^{a}	11.11±0.81^{a}	6.44±0.20^{a}	9.33±0.86^{b}
L. brevis NRRL B-4527	7.02±0.47^{b}	6.09±0.37^{c}	1.40±0.04^{c}	8.41±0.33^{c}	4.91±0.25^{c}	7.07±0.39^{c}

CFS: Cell free supernatant, BL: Bacterial lysate. Result are expressed as mean±SD, each value is the average of three experiments each was carried out in duplicate. ^{abcd}Mean in the same column followed by different superscript letters are significantly different ($p<0.05$)

Table 7: IC_{50} and EC_{100} doses (μg mL^{-1}) of each investigated probiotic lysates toward HDF cells

Lactic acid bacteria strains	IC_{50}	EC_{100}
P. acidilactici ATCC 8042	198.07±1.45^{a}	103.56±7.7^{ace}
L. curvatus ATCC 51436	151.98±6.03^{b}	60.28±2.9^{b}
L. plantarum TF103	185.55±3.05^{a}	119.30±3.6^{cd}
L. plantarum DMSZ20079	228.45±0.59^{c}	127.08±2.1^{d}
L. Lactis subsp. *lactis* DSM 20481	194.34±2.6^{a}	94.31±9.2^{e}
L. rhamnosus CCUG 1452	185.57±1.34^{a}	93.31±7.3^{e}
L. brevis NRRL B-4527	190.47±8.8^{a}	101.63±8.2^{e}
S. thermophilus BLM 58	467.89±9.6^{d}	228.60±8.5^{f}

All values were expressed as Mean±SEM. Different letters between bacterial lysates within the same column are significantly different at $p<0.05$

Table 8: Anticancer activity of the studied bacterial lysates against human cancer cell lines in the term of IC_{50} (μg mL^{-1})

	Human cancer cell lines			
Lactic acid bacteria strains	Caco-2	MCF-7	HepG-2	PC-3
P. acidilactici ATCC 8042	16.30±5.1^{e}	59.66±9.5^{e}	53.60±5.4^{d}	54.41±2.4^{f}
L. curvatus ATCC 51436	206.60±3.4^{a}	259.79±1.1^{a}	101.10±9.8^{bc}	200.21±5.2^{b}
L. plantarum TF103	77.40±4.5^{b}	275.81±6.8^{a}	111.50±19.9^{b}	308.57±8.6^{a}
L. plantarum DMSZ20079	51.20±4.6^{c}	279.05±19.3^{a}	193.32±9.7^{a}	288.11±9.1^{a}
L. Lactis subsp. *lactis* DSM 20481	29.50±2.5^{d}	136.10±4.8^{c}	56.90±5.12^{d}	137.41±6.4^{d}
L. rhamnosus CCUG 1452	30.40±3.4^{d}	197.80±5.3^{b}	94.94±9.2^{bc}	158.53±8.5^{c}
L. brevis NRRL B-4527	40.86±2.4^{cd}	91.14±6.8^{d}	74.26±3.5^{cd}	92.37±3.4^{e}
S. thermophilus BLM 58	16.90±1.1^{e}	48.54±7.04^{e}	41.18±8.7^{d}	64.81±3.97^{f}

All values were expressed as Mean±SEM. Different letters between bacterial lysates within the same column are significantly different at $p<0.05$

Cytotoxicity and anticancer effect of the LAB lysates: As shown in Table 7, all bacterial lysates were safe in terms of EC_{100} (above 90 μg mL^{-1}), except *L. curvatus* ATCC 51436, which showed the lowest EC_{100} value (60.28 μg mL^{-1}) toward HDF cells. *Streptococcus thermophilus* BLM 58 had the highest IC_{50} value (467.89 μg mL^{-1}), which was significantly different (p-0.05) from the IC_{50} values (<230 μg mL^{-1}) of the other tested bacterial lysates.

Additionally, the bacterial lysates of *S. thermophilus* BLM 58 possessed the lowest IC_{50} values (48.5 and 41.2 μg mL^{-1}) against tested MCF-7 and HepG-2 cancer cell lines as shown in Table 8. There were no significant differences in the IC_{50} values of *S. thermophilus* BLM 58 lysate and *P. acidilactici* ATCC 8042 against the four cancer cell lines (16.3, 59.7, 53.6 and 54.4 μg mL^{-1}). The lowest IC_{50} values of *P. acidilactici* ATCC 8042 and *S. thermophilus* BLM 58 reflect the highest anticancer activity of both lysates (Table 8). The potent anticancer effects of these four bacterial lysates (*P. acidilactici* ATCC 8042, *S. thermophilus* BLM 58, *L. brevis* NRRL B-4527 and *L. lactis* subsp. *lactis* DSM 20481) were evident as shown by severe alterations in the morphology of the treated cancer cell lines, especially the *P. acidilactici* ATCC 8042 and *S. thermophilus* BLM 58-treated cancer cells, compared with the controls (untreated cancer cells, Fig. 4). More importantly, the SI values of *S. thermophilus* BLM 58 for cancer cells were the highest among all the studied bacterial lysates against the four cancer cells, especially Caco-2 cells (SI = 27.8). The next highest SI values were obtained from *P. acidilactici* ATCC 8042; however, the SI value against HepG-2 cells was not significantly different from those of *L. lactis* subsp. *lactis* DSM 20481 and *L. brevis* NRRL B-4527 toward those cells (Fig. 5). The anticancer

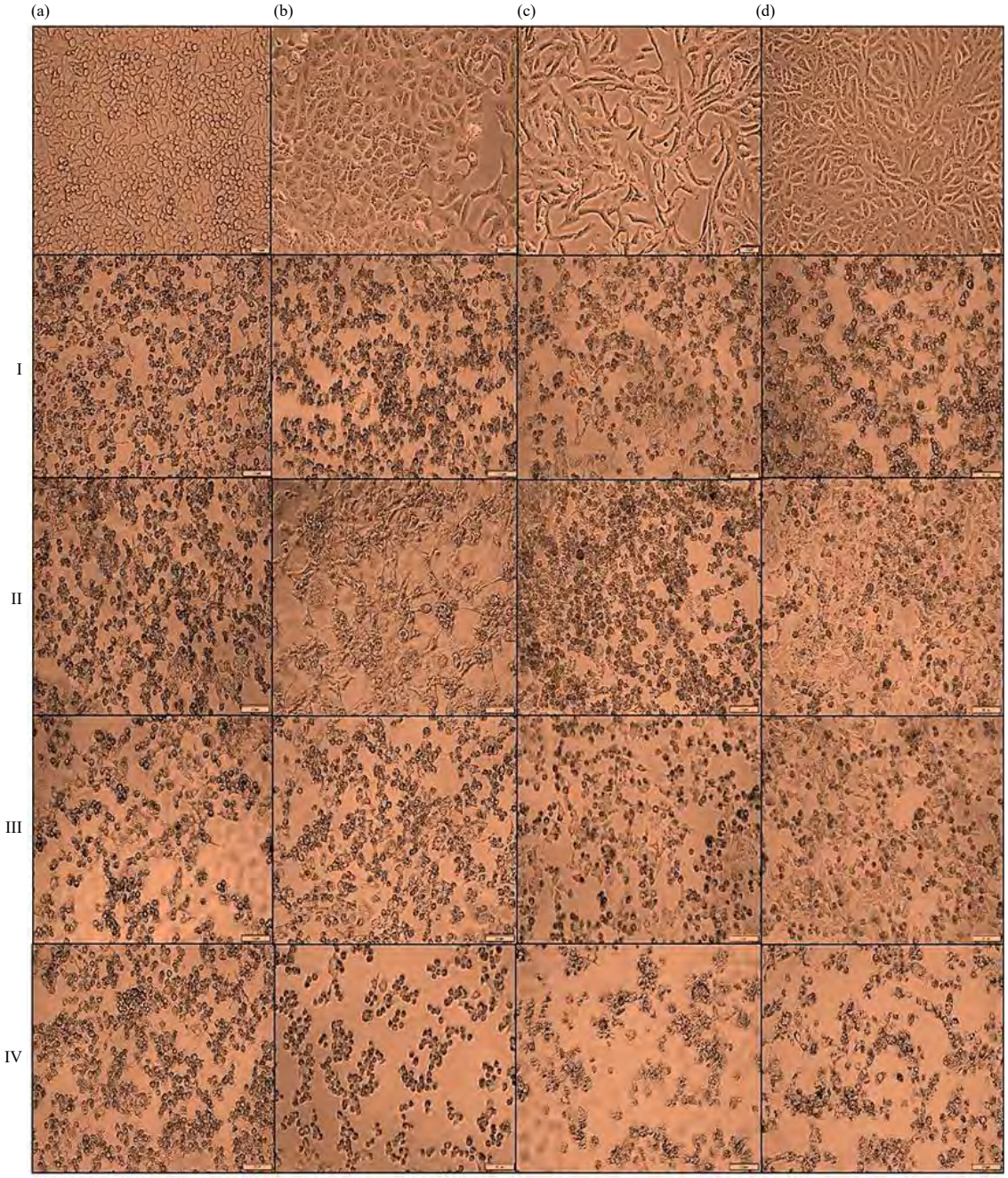

Fig. 4(a-d): Morphological changes of (a) Caco-2, (b) MCF-7, (c) HepG-2 and (d) PC-3 cell lines after exposure for 72 h to (I) *P. acidilactici* ATCC 8042, (II) *L. lactis* subsp. *lactis* DSM 20481, (III) *L. brevis* NRRL B-4527, (IV) *S. thermophilus* BLM 58 (Magnification x200)

mechanisms of the most effective probiotic lysates (*S. thermophilus*, *P. acidilactici*, *L. lactis* subsp. *Lactis* and *L. brevis*) were investigated using flow cytometry. Results in Fig. 6 showed the high percentages of annexin-stained apoptotic HepG-2 cells treated separately with the four most anticancer probiotic lysates compared with untreated HepG-2 cell controls. This result confirmed that the anticancer activity of probiotic lysate samples was mediated through the induction of apoptosis. Table 9 showed that the total percentage of apoptotic *S. thermophilus*-treated HepG-2 cells (68.95±1.53%) was significantly (p-value 0.05) higher than those of *P. acidilactici*, *L. lactis* subsp. *lactis*- and *L. brevis*-treated HepG-2 cells.

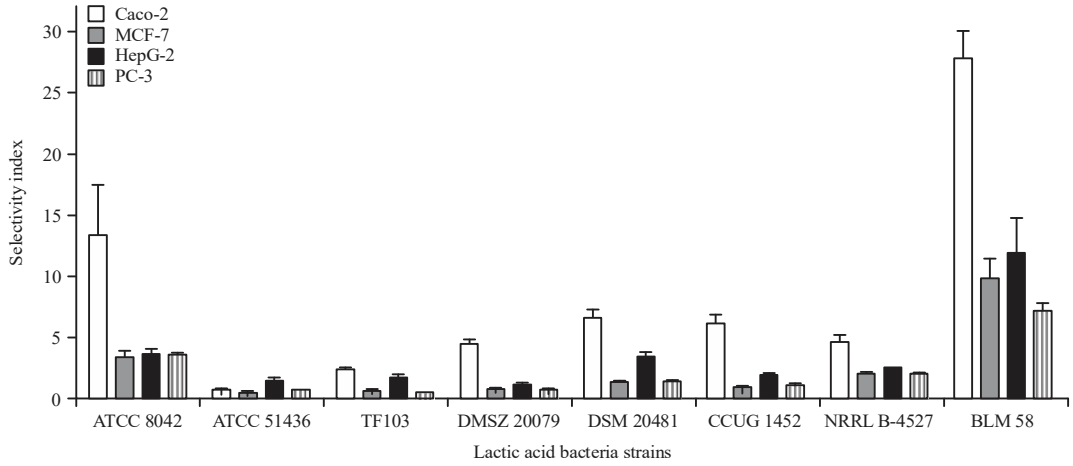

Fig. 5: Selectivity index of the studied bacterial lysates toward human cancer cell lines

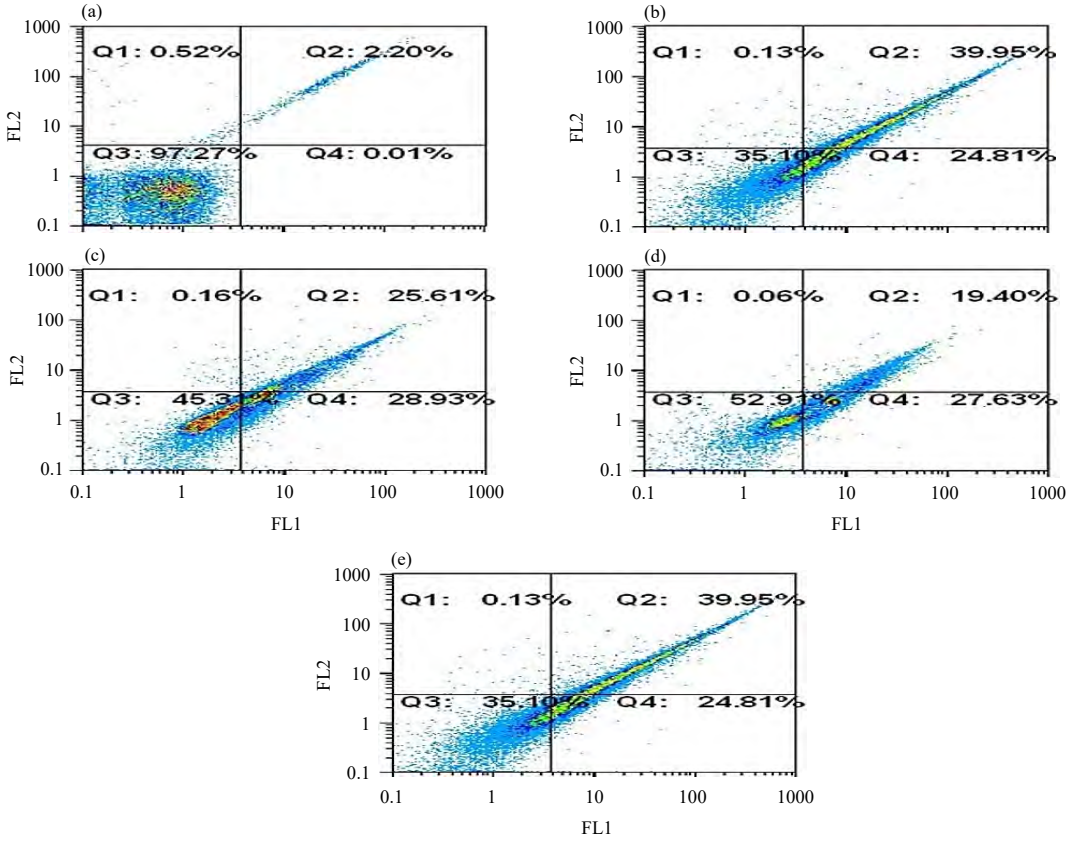

Fig. 6(a-e): Flow cytometric analysis of (a) Control HepG-2 cell line. Treated HepG-2 cell line with (b) *S. thermophilus* BLM 58, (c) *L. lactis* subsp. *lactis* DSM 20481, (d) *P. acidilactici* ATCC 8042 and (e) *L. brevis* NRRL B-4527 for 72 h

Table 9: Apoptosis (%) of HepG-2 cells before and after treatment with selected probiotic lactic acid bacteria

HepG-2	Early apoptosis	Late apoptosis	Total apoptosis
Control	0.015±0.005[a]	2.09±0.1[a]	2.11±0.1[a]
P. acidilactici ATCC8042	23.400±1.4[b]	37.42±2.5[b]	60.82±3.93[b]
L. Lactis subsp. *lactis* DSM20481	24.600±0.69[b]	27.98±0.9[c]	52.59±1.6[c]
L. brevis NRRLB-4527	26.320±1.31[b]	19.70±0.3[d]	46.02±1.01[c]
S. thermophilus BLM58	4.800±3.4[c]	19.70±2.32[d]	68.95±1.53[e]

All values were expressed as Mean±SEM. Different letters between bacterial lysates within the same column are significantly different at p<0.05

Table 10: Bivariate correlation of antioxidant activities and anti-cancer activity of LAB lysates

Relationship	Correlation coefficient	p-value (2-Tailed)
DPPH and anti-cancer activity (HepG-2 cells)	0.697**	0.000
DPPH and anti-cancer activity (MCF-7cells)	0.788**	0.000
DPPH and anti-cancer activity (PC-3cells)	0.670**	0.000
DPPH and anti-cancer activity (Caco-2cells)	0.490*	0.015

**Correlation is significant at the 0.01 level (2-tailed), *Correlation is significant at the 0.05 level (2-tailed)

Correlation between anticancer activity and antioxidant activity: The Pearson correlation coefficients between the tests for antioxidant and anticancer activity were calculated and are presented in Table 10. Significant relationships between antioxidant activity and anticancer activity (correlation coefficient, r = ~0.697, 0.788, 0.670, 0.490, p<0.01) for the HepG-2, MCF-7, PC-3 and Caco-2 cell lines, respectively, indicated that high antioxidant activity was an important significant factor for determining the anticancer activity of bacterial lysate extracts. The anticancer activity of LAB lysates may be attributed to the antioxidant compounds possessing potent radical scavenging activities.

DISCUSSION

Probiotic strains can be utilized as bio-preservatives and functional food supplements. Consequently, the search for natural, stable, safe, antimicrobial, antioxidant and anticancer agents have become a subject of interest as alternatives to artificial compounds.

The antimicrobial properties of these LAB strains were tested against seven dominant food borne pathogenic bacteria. The observed antimicrobial activity must be due to the presence of active antimicrobial compounds secreted in culture media. The LAB strains are 'recognized as safe' (GRAS). Therefore, their use in food as bio-preservatives has received much interest. These probiotics may be able to resist SGJ (pH 2) and bile in the upper part of the intestine (0.3%), so they can be used as dietary supplements. This property allowed strains to survive for more extended periods of time in high-acid foods without reductions in humans[22].

The adhesion percentages of *Lb. plantarum* strains on HT-29 cells have been reported [23] to be 5-13%. Probiotic strains *L. plantarum* STIII and *L. plantarum* Lp9 showed adhesion percentages of 13±0.2 and 7.4±1.3%, respectively, with Caco-2 cells[24]. These results indicated the ability of *P. acidilactici* ATCC 8042, *L. brevis* NRRL B-4527, *S. thermophilus* BLM 58 and *L. plantarum* TF103 to adhere to the gastrointestinal tract and resist their immediate elimination by peristalsis. Their ability to adhere to colon cells is crucial in maintaining probiotic efficacy. Therefore, these strains can potentially be used as commercial probiotics.

Hydrogen peroxide is a weak oxidative agent that causes oxidative damage, especially to DNA, due to its production of hydroxyl radicals[25]. The *L. plantarum* C88 and *L. fermentum* have been reported to be highly resistant to hydrogen[25,26]. The mechanism responsible for the different degrees of resistance of current tested LAB probiotic strains to hydrogen peroxide remains unclear.

The DPPH free radical scavenging activities indicated that the intact cells of *P. acidilactici* ATCC 8042 showed strong antioxidant activity *in vitro*, which was similar to the antioxidant activity of *Lactobacillus plantarum* C88 described by Li *et al.*[25]. These results confirmed those of Shen *et al.*[27], who showed that proteins extracted from *Bifidobacterium animalis* 01 cells possessed antioxidant activity *in vitro*.

Hydroxyl radicals as well have been considered the most dangerous ROS responsible for oxidative damage to biomolecules. Copper Cu^{2+} and Iron^{2+} ions are critical for hydroxyl radical production through fenton reaction. Antioxidants chelation of these ions prevent the formation of hydroxyl radicals[28]. A few LAB strains, such as *Bifidobacterium longum* 15708, *Streptococcus thermophilus*[16] 821 and *L. casei*[29] KCTC 3260 have been described to possess antioxidant activity in removing transition metal ions that would otherwise participate in hydroxyl radical generation through fenton-type reactions. In the present study, the bacterial lysate and supernatant of *S. thermophilus* BLM 58 exhibited strong HRS activity, likely due to their capability to chelate metal ions, such as Fe^{2+} and Cu^{2+}.

By the same token, the supernatants and bacterial lysates of *S. thermophilus* BLM 58 and *L. lactis* subsp. subsp. *lactis* DSM 20481 demonstrated the strongest scavenging activity toward the DPPH radical. These properties are very important in the food industry.

Previous clinical studies have emphasized the safe administration of LAB to healthy adults and even to immune-compromised patients and premature infants, without any side effects[30]. Several previous results have reported the antitumor activity of many LAB species, such as *Lactobacillus acidophilus*, *Lactobacillus casei*[31] and *P. acidilactici*[32]. The current study reported for the first time, the potent anticancer effects of *S. thermophilus* against colon, breast, liver and prostate cancer cells. These results with agreement with other reports that reviewed that *Lactobacillus acidophilus* has antioxidant properties and is

capable of inducing cytotoxicity in colon cancer cells via the down-regulation of tumor angiogenesis and metastasis[33]. *Lactobacillus* species exhibited remarkable anticancer potency against colon, breast, cervical and gastric cancer cell lines via the induction of apoptosis without affecting normal human endothelial cells[34]. Thus, the anticancer activity of probiotic strains seems to be mediated through the induction of apoptosis in cancer cells. The anticancer activity of these strains may be promising for use in supported therapy or cancer prevention[35]. The present results indicate that *S. thermophilus* BLM 58 had the most effective and safe antioxidant and anticancer probiotic lysate. *Streptococcus thermophilus* BLM 58 exhibited the highest apoptosis-dependent anticancer potency, which may be attributed to its strong antioxidant effects. This deprives cancer cells of free radicals and maintains uncontrolled proliferation. In this study, the bacterial lysate extracts of *S. thermophilus* BLM 58 had a promising antioxidant and the anticancer potent, with a high correlation factor ($r = 0.788$). Further researches will required to confirm the antioxidant and the anticancer potent of functional food application in induced animal models.

CONCLUSION

The present study showed that *S. thermophilus* BLM 58 and *P. acidilactici* ATCC 8042 had the strongest antioxidant potentials. These results indicated that the functional compounds in the cultured broth and bacterial lysates were different. The selected probiotic strains, particularly *S. thermophilus* BLM 58, *L. lactis* subsp. *lactis* DSM 20481, *P. acidilactici* ATCC 8042 and *L. brevis* NRRL B-4527 are considered better strains for functional food applications with potentially antioxidant and anticancer activity.

SIGNIFICANCE STATEMENT

The present study aimed to characterize probiotic LAB strains and assess their antimicrobial, antioxidant and anticancer efficacies. The selected probiotic strains, particularly *S. thermophilus* BLM 58, *L. lactis* subsp. *lactis* DSM 20481, *P. acidilactici* ATCC 8042 and *L. brevis* NRRL B-4527, are considered better strains for functional food applications because they produce antioxidant compounds. The current study demonstrated for the first time the high capacity of *S. thermophilus* BLM 58 to have anticancer effects against cancer cells from four types of cancerous cell lines. This may be a promising finding for future cancer treatments and in the prevention of cancer using natural probiotics.

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