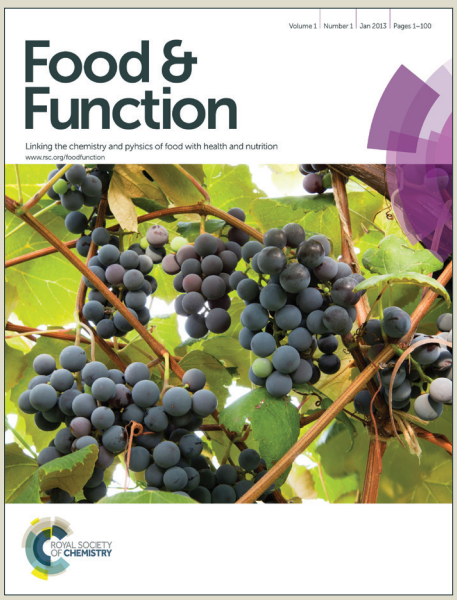
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

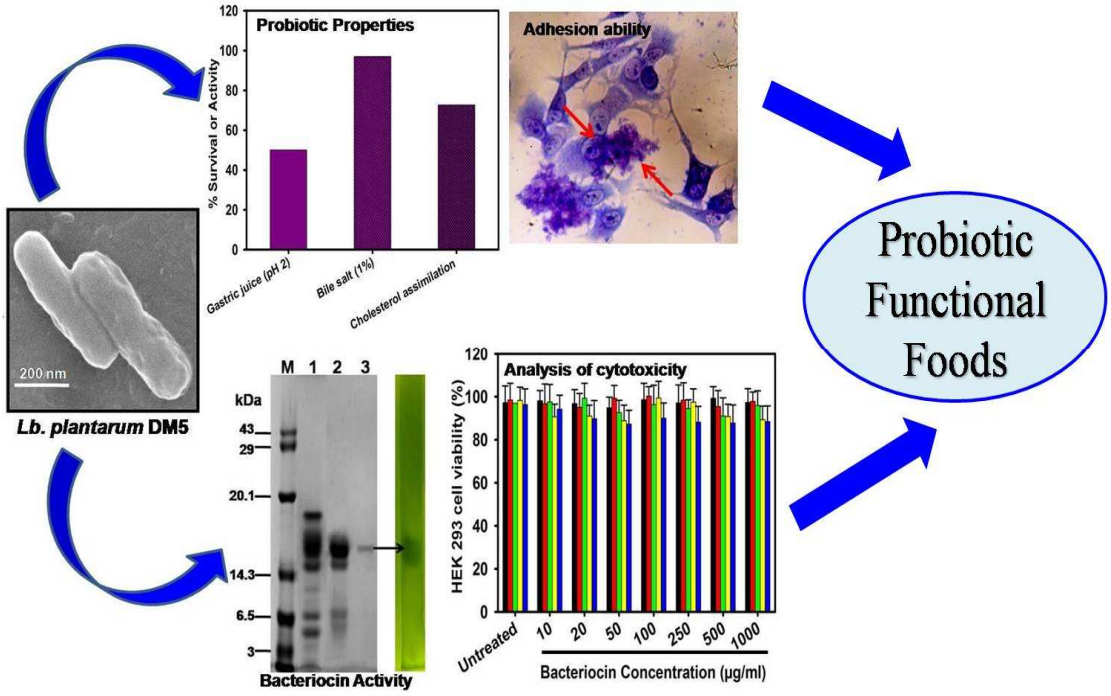
You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

Graphical Abstract:



Highlights:

- *Lb. plantarum* DM5 exhibited *in vitro* probiotic properties such as high resistance to gastric juice and bile salt, adherence to human adenocarcinoma (HT-29) cells.
- It showed bile salt hydrolase and cholesterol assimilation activity, thus able to reduce hypercholesterolemia.
- It showed broad bacteriocin activity against several food borne pathogens.
- Cytotoxicity analysis of purified bacteriocin (~15.2 kDa) on human cell lines revealed its nontoxic biocompatible nature rendering its use as biopreservant.

Characterization of a noncytotoxic bacteriocin from probiotic *Lactobacillus plantarum* DM5 with potential as food preservative

Deeplina Das and Arun Goyal*

Department of Biotechnology,
Indian Institute of Technology Guwahati,
Guwahati - 781 039, India

Corresponding author and address for correspondence:

Dr. Arun Goyal
Professor
Department of Biotechnology,
Indian Institute of Technology Guwahati
Guwahati 781 039, Assam
Email: arungoyl@iitg.ernet.in
Tel. 361-258 2208
Fax: 361-258 2249

ABSTRACT:

The aim of this work was to purify and characterize the bacteriocin produced by probiotic *Lactobacillus plantarum* DM5 in order to evaluate its potential as nutraceuticals. *Lb. plantarum* DM5 exhibited *in vitro* probiotic properties such as high resistance to gastric juice and bile salt, adherence to human adenocarcinoma (HT-29) cells, Bile salt hydrolase and cholesterol assimilation activity. Additionally, *Lb. plantarum* DM5 showed bacteriocin activity against several major food borne pathogens. Zymogram analysis of purified bacteriocin (plantaricin DM5) displayed the molecular size of ~15.2 kDa. Plantaricn DM5 was sensitive to proteolytic enzymes but stable in the pH range of 2.0-10.0. Plantaricin DM5 was heat resistant (121°C for 15 min) and remained active upon treatment with surfactants and detergents. Cytotoxicity analysis of plantaricin DM5 on human embryonic kidney 293 (HEK 293) and human cervical cancer (HeLa) cell lines revealed its nontoxic and biocompatible nature. This is to our knowledge, the first study on isolated strain expressing probiotic properties and broad antimicrobial activity without any cytotoxic effect on mammalian cells from indigenous fermented beverage Marcha from India, and thus contributes towards a growing concern to food industry as novel bio-preservant.

Keywords: *Lactobacillus plantarum*, Probiotic, bacteriocin, Marcha

1.0 Introduction

Probiotics are live microbial feed supplement which beneficially affect the host animal by improving its intestinal balance[1]. In order to provide beneficial health effect to the host, probiotic bacteria must survive through the barriers in the gastrointestinal tract in sufficient numbers, tolerating acid, bile and gastrointestinal enzymes, and then adhere and colonize in the intestinal epithelium [2,3]. Species of the genus Lactobacillus are widespread commensals in the gastrointestinal tract of humans and are potentially probiotic organisms that confer a health benefit of the host[1,4]. Production of bacteriocin is one of the desirable traits among probiotic bacteria from the perspective of controlling microbial populations in the gastrointestinal tract of host. In addition, bacteriocins offer potential applications in food preservation by reducing the use of chemical preservatives[5]. Several unique properties, such as activity over a wide pH range and stability at high temperature, make them suitable as biological preservatives to extend the shelf-life of refrigerated semi-preserved foods and canned foods[5,6]. In recent years, the main concern of the food industry has been used such a preservative that can selectively inhibit certain high risk bacteria such as *Listeria monocytogenes, Staphylococcus aureus, Camphylobacter jejuni* in food products without compromising the quality and taste of the product[5,7]. The potential application of probiotic and bacteriocin in the food industry have attracted tremendous interest, which has led to the search for a novel strains with bacteriocin producing probiotic characteristics.

The strain *Lactobacillus plantarum* has gained great attention in food industry due to its application as a starter culture for fermentation of vegetables[8,9] and as probiotic[10,11]; however; it is also well known for production of bacteriocin. Several bacteriocins producing *Lactobacillus plantarum* strains have been isolated earlier from fermented food sources such as, plantaricin C11 from cucumber[12]; plantaricin UG1 from sausage[13], plantaricin C19 from meat[14], plantaricin TF711 from Tenerife cheese[15], plantaricin ST16Pa from *Carica papaya*[16]

and plantaricin ST71KS from goat feta cheese[17]. Although these strains did not exhibit *in vitro* probiotic properties such as resistance to gastric juice and bile salts or *in vitro* adhesion ability. Till date only two commercial probiotic strains, *Lactobacillus casei* Shirota and *Lactobacillus johnsonii* La1 produce bacteriocins[18]. Therefore, bacteriocin producing probiotic *Lb. plantarum* strain remains to be explored, which will replace the chemical preservatives in food industry. This has prompted the screening of potential probiotic lactic acid bacteria with strong antimicrobial activity capable of inhibiting food borne pathogens.

In this regard, *Lactobacillus plantarum* DM5 was isolated from fermented beverage Marcha of Sikkim[19] and was explored for bioprotective potential. In the present study, probiotic properties of *Lb. plantarum* DM5 were evaluated by *in vitro* analysis. The production of bacteriocin by *Lb. plantarum* DM5 was also studied. The bacteriocin named as plantaricin DM5 was purified by ammonium sulphate precipitation, cation exchange chromatography and gel filtration. The purified plantaricin DM5 was also evaluated for cytotoxicity tests on human embryonic kidney (HEK 293) and human cervical cancer (HeLa) cell lines in order to develop it as a novel biopreservant with superior probiotic properties.

2.0 Results and Discussion

2.1 In vitro probiotic properties of *Lb. plantarum* DM5

2.1.1 Sensitivity of Lb. plantarum DM5 to gastric juice and bile salt

Resistance to gastric acidity and bile salts are most widely used *in vitro* test for the assessment of probiotic potential of a bacterial strain[2]. Probiotic microorganisms should not only be capable of surviving passage through the digestive tract but also have the capability to proliferate in the gut; as a result they must be resistant to gastric juices and able to grow in the presence of bile salt in the intestines. The sensitivity of *Lb. plantarum* DM5 towards the gastric juice (pH 2-4) and bile salts (0.3-1%) was determined and shown in Table 1. Exposure of *Lb. plantarum* DM5 to a simulated gastric juice containing pepsin at pH 2.0 resulted in a rapid loss of cell viability and 48% cell survival was observed after 5 h of incubation.

Compared to this, it showed 91% survival at pH 3 as the residual count decreased from 10^7 CFU mL^{-1} to 10^6 CFU mL^{-1} after 5 h of incubation; however; at pH 4 almost no change in cell viability was recorded after 5 h of incubation. This was significantly higher than *Lb. plantarum* 17 isolated from cooked meat product which could resist pH 3.0 for only 1 h and did not revive even after 4 h[20]. The isolate *Lb. plantarum* DM5 showed 15% higher survival rate as compared with novel isolate *Lb. plantarum* ST-III (~76% survival at artificial gastric juice after 5 h of incubation) which possessed probiotic as well as α-glucosidase inhibitory activity[21]. The percent survival of *Lb. plantarum* DM5 was also very high as compared to probiotic *Lb. plantarum* IMAU30055[22], *Lb. acidophilus* BFE 6056[3], *Lb. plantarum* Lp9[23] and *Lb. plantarum* B23[24] which showed 76%, 80%, 82% and 86% survival at pH 2 after 2 h of incubation. The elevated survival rate of *Lb. plantarum* DM5 at pH 2 indicated the good tolerance of *Lb. plantarum* DM5 against acidic conditions prevalent in the stomach.

Lactobacillus plantarum DM5 demonstrated excellent tolerance in presence of 0.3% and 0.5% bile salt as the viable cell count increased by 20% and 18%, respectively, after 30 h of incubation (Table 1). Although in presence of 1% bile salt marginal decrease of 3% observed in viable cell population. *Lactobacillus plantarum* DM5 confirmed high bile salt tolerance as it could survive 2.5 fold higher concentration (1%) than the usual bile salt concentration present in human stomach (0.3%)[25]. The results suggested that *Lb. plantarum* DM5 could survive passage through the high bile salt concentration of small intestine and may be used as potent probiotic.

2.1.2 Bile salt hydrolase (BSH) activity and in vitro cholesterol assimilation of Lb. plantarum DM5

Bile salt hydrolase (BSH) activity is an important colonisation factor for probiotic bacteria as it provides resistance to the toxicity of conjugated bile salts in the duodenum[25,26]. The most abundant bile salts in humans are cholate, chenodeoxycholate and deoxycholate, which are normally conjugated with either glycine (75%) or taurine (25%)[23]. Therefore, the

BSH activity *Lb. plantarum* DM5 was measured for both glycine and taurine conjugated bile salt and is shown in Table 1. The isolate *Lb. plantarum* DM5 exhibited BSH activity of 0.63±0.02 U mg^{-1} towards TDCA, however, when bile salt mixture containing glycocholic, glycochenodeoxycholic, taurocholic, taurochenodeoxycholic and taurodeoxycholic acid was used it exhibited higher BSH activity of 1.18±0.03 U mg^{-1}. Similar results were also observed in case of *Lb. acidophilus* ATCC 4357 (0.64 U mg^{-1}) and *Lb. plantarum* Lp91 (0.12 U mg^{-1}) which showed lower activity towards TDCA[27,28], although; the strain *Lb. acidophilus* ATCC 4357 displayed higher BSH activity of 1.20 U mg^{-1} towards conjugated bile salt mixture. It has been reported that the strain *Lb. plantarum* Lp91 showed higher affinity towards bile salt GDCA as compared to TDCA[28]; however; *Lb. plantarum* BBE7 had a relatively high BSH activity toward both bile salts with a higher cholesterol-removing activity of 72.8%[29].

It also has been reported that the bile salt hydrolysing probiotic strains are able to control hypercholesterolaemia through interaction with host bile salt metabolism[26,27]. A high concentration of cholesterol in the blood streams of humans has been recognized as a risk factor for coronary heart disease[3]. The isolate *Lb. plantarum* DM5 was also tested for its ability to reduce cholesterol in the presence of TDCA and conjugated bile salt mixture (Table 1). The isolate *Lb. plantarum* DM5 was able to assimilate the *in vitro* cholesterol level in the range of 68-73%. However; higher cholesterol reduction was observed when the conjugated bile salt mixture was used, as compared with TDCA. This was in agreement with bile salt hydrolase activity of *Lb. plantarum* DM5 as cholesterol assimilation ability was dependent on bile salt hydroalse activity[30]. The aforementioned results indicate that *Lb. plantarum* DM5 could potentially decrease the concentration of bloodstream cholesterol in human beings.

2.1.3 In vitro cell adherence of Lb. plantarum DM5

A probiotic bacterium should be able to adhere and colonize in the human gastrointestinal tract for sustaining health promoting effect. The adherence ability of putative

probiotic is the most crucial criteria for its extended residence time in the host[20]. *Lb. plantarum* DM5 showed a good adhesion percentage of 8.63±3.03% with HT-29 cell line. The adhesion percentage of *Lb. plantarum* strains with HT-29 cell line were reported in between 5-13% [31,32]. A probiotic strain *Lb. plantarum* Lp9 and *Lb. plantarum* STIII showed adhesion percentage of 7.4±1.3% and 13±0.2% respectively with Caco-2 cell line[21]. The adhesion rate of probiotic *Lb. plantarum* S3 (0.9±0.3%) isolated from pickle was very low as compared to *Lb. plantarum* DM5suggesting the superior probiotic property of *Lb. plantarum* DM5[21]. The adhesion ability of *Lb. plantarum* DM5 with HT-29 cell line was also investigated by direct microscopic observation after Giemsa staining and is shown in Fig. 1. The microscopic image also confirmed the good adhesion ability of isolate *Lb. plantarum* DM5 with HT-29 cells. In our earlier study, we reported that *Lb. plantarum* DM5 possessed the good cell surface hydrophobicity of 53% and autoagrregasion of 43%, which is a required criterion for cell adhesion[19]. In the present study it is confirmed by adhesion assay of *Lb. plantarum* DM5 to HT-29 cell line. This result ensures the capability of *Lb. plantarum* DM5 to adhere in the gastrointestinal tract and resist their immediate elimination by peristalsis. The functional properties of bacterial strain such as gastrointestinal tolerance and adhesion to intestinal epithelium are critical factors in maintaining probiotic efficacy, therefore the strain *Lb. plantarum* DM5 can potentially used as commercial probiotic. Although future research is needed to determine the complex cell adhesion process between the bacterial cell membrane and interacting cell surfaces of mammalian cell line.

2.2 Antimicrobial spectrum of bacteriocin produced by *Lb. plantarum* DM5

The use of bacteriocins in the food industry can help to reduce the addition of chemical preservatives as well as the intensity of heat treatments, resulting in foods which are more naturally preserved and richer in organoleptic and nutritional properties[5]. In addition to functional probiotic properties, the strain *Lb. plantarum* DM5 showed broad antimicrobial

spectrum against major food borne pathogens such as *Listeria monocytogenes, Bacillus cereus, Alcaligenes feacalis, Pseudomonas aerogenosa, Staphylococcus epidermis, Salmonella enterica* as shown in Table 2. The antimicrobial activity of the cell free supernatant of *Lb. plantarum* DM5 was obtained as 6400 AU mL^{-1} against gram positive indicator strain *S. aureus* 737 and gram negative indicator strain *E. coli* DH5α as measured by agar well diffusion method[33]. In our earlier study, we have also reported that the bacteriocin from *Lb. plantarum* DM5 showed bactericidal action by repressing the growth of *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes* as analyzed by flow cytometry[18]; therefore it can be potentially used as bio-preservant.

2.3 Purification of bacteriocin from *Lb. plantarum* DM5

The cell free supernatant of *Lb. plantarum* DM5 containing bacteriocin (6400 AU mL^{-1}) was purified by ammonium sulphate precipitation. The precipitate obtained within 40-80% ammonium sulphate saturation showed specific activity of 2196 AU mg^{-1} with 5.4 fold increase in specific activity (Table 3) and was loaded on CM Sepharose column pre-equilibrated with 25 mM ammonium acetate buffer (pH 5.4). The sample was eluted in form of single-symmetrical peak at about 0.24 M to 0.27 M sodium chloride (Fig.2A). The bacteriocin activity was confined between 23^{th} to 27^{th} fractions which also showed maximum protein content (A_{280}). All the five fractions were pooled, which gave specific activity of 3137 AU mg^{-1} with 7.7 fold purification (Table 3). The pooled fraction was dialyzed and subjected to next step of purification by gel filtration. The sample eluted in form of two consecutive peaks as observed by A_{280} measurements, however, the bacteriocin activity was found only in the first peak of A_{280}, within 13^{th} to 18^{th} fractions. Among these fractions, the fraction 14 and 15 were pooled as they showed maximum bacteriocin activity and higher protein content (Fig. 2B). The pooled fractions showed specific activity of 10667 AU mg^{-1}

with 26 fold purification (Table 3). The pooled bacteriocin sample was lyophilised and subjected to SDS-PAGE for analyzing its purity and molecular mass.

2.4 Molecular mass of purified bacteriocin

The molecular size of purified bacteriocin by gel filtration was found to be approximately, 15.2 kDa as determined by SDS-PAGE (Fig. 3A). The result was also confirmed from the graph of relative mobility (R_f) versus log molecular weight (Log Mw) of the standard protein molecular mass markers (Fig. 3C). A prominent inhibition zone (Fig. 3B) was observed corresponding to the protein band of molecular weight 15.2 kDa, in the activity gel against the indicator strain *S. aureus* MTCC 737. The bacteriocin from *Lb. plantarum* DM5 was larger than bacteriocin produced by *Lb. plantarum* ST16PA (Mol. mass 6.5 kDa)[15], *Lb. plantarum* ST194BZ (Mol. mass 14 kDa)[33] but smaller than bacteriocin produced by *Pediococcus pentosaceus* ACCEL (Mol. mass 17.5 kDa)[34]. To the best of our knowledge, the present study is the first to report the molecular mass of bacteriocin from *Lactobacillus plantarum* sp. to be 15.2 kDa.

2.5 Sensitivity of bacteriocin to heat, pH, proteolytic enzymes and detergents

The sensitivity of bacteriocin was tested at different temperatures (40, 60, 80, 100 and 120°C) and under pH conditions from pH 4 to 10, at which it displayed remarkable thermal and pH stability (Table 4), however; no activity was observed at pH 12. The activity of bacteriocin decreased after incubation at 120ºC for 30 min and completely lost thereafter (data not shown). Furthermore, the antimicrobial activity of bacteriocin remained unaltered when treated with catalase, α-amylase and lysozyme, suggesting that the antimicrobial activity is not dependent on hydrogen peroxide, carbohydrate or any lipid molecule present in cell free supernatant (Table 4). However, it was inactivated by proteolitic enzymes such as pepsin, trypsin and protinase K confirming its proteinaceous nature. Similar observations were also reported for plantaricin C19[14], plantaricin 163[6], plantaricin ST16Pa[16] and

plantaricin L4/14[35]. The sensitivity of antimicrobial compound from *Lb. plantarum* DM5 to heat and proteolytic enzymes demonstrated its proteinaceous nature and confirmed the antimicrobial compound produced by *Lb. plantarum* DM5 is bacteriocin. The physicochemical properties of bacteriocin identified in the present study are in agreement with the characteristic features of bacteriocin from other *Lactobacillus plantarum* sp and named as plantaricin DM5. The high thermostability of planatricin DM5 suggests that it can be easily exploited in food industry as bio-perseverant and will not get affected during the food processing and can satisfy the increasing consumer's demands for safe, fresh-tasting, and minimally-processed foods. In our previous study, we have shown the cell morphology of plantaricin DM5 treated *E. coli*, *S. aureus* and *L. monocytogenes* was completely deformed and vesiculated[19]. The aforementioned results suggest that the plantaricin DM5 is heat stable and acts by a pore formation mechanism, corroborating with the hypothesis that this is a class IIa bacteriocin[36]. Similar observation was also reported in *Lactobacillus sakei* MBSa1, isolated from salami produced Class II bacteriocin with anti-Listeria activity (6400 AU mL^{-1})[7]. The activity of plantaricin DM5 was unaffected after treatment with ethanol, methanol, acetone, ethyl acetate, n-butanol, isopropanol, Tween 20, Tween 80, Triton X-100 and SDS (Table 4) like other reported plantaricin 163[6], plantaricin ST16Pa[16] and plantaricin ST71KS[17].

2.6 In vitro cyotoxicity assay of plantaricin DM5

The plantaricin DM5 was subjected to cytotoxicity assay using HEK-293 and HeLa cells in order to elucidate if it is biocompatible and if it can be commercially exploited as food additive and bio-preservative. The HEK-293 and HeLa cells were exposed to various concentrations of purified plantaricin DM5 (0, 20, 50, 100, 250, 500 and 1000 μg mL^{-1}) dissolved in serum free DMEM medium for 3, 6, 12, 24 and 48 h. It was observed that the viability of both HEK-293 and HeLa cells remained constant at all concentrations of

bacteriocin revealing its non-toxic nature (Fig. 4). In the presence of 1000 µg mL^{-1} of plantaricin DM5, almost 85-90% of HEK-293 and HeLa cells remained viable over an incubation period of 48 h, however plantaricin DM5 did not influence the cell proliferation. It was observed that the viability of treated HEK cells (Fig. 4A) and HeLa cells (Fig. 4B) was alike as compared with the respective untreated cells in serum free medium with no significant toxic effect at all concentrations. All these results suggested that the bacteriocin from *Lb. plantarum* DM5 possessed the ability to eliminate or retard the growth of potent food borne pathogens *viz. S. aureus, E. coli* and *L. monocytogens* without any cytotoxic effect on mammalian cells and can be exploited for bio-preservation of various food, feed and beverages.

3.0 Experimental section

3.1 Bacterial strains, mammalian cell lines and growth conditions

Lactobacillus plantarum DM5 was isolated from an ethnic fermented beverage Marcha of north eastern Himalayas[19] and was maintained in MRS medium[37] at 37ºC. The indicator strains (as listed in Table 2) were procured from Agricultural Research Service Culture Collection (Peoria, USA) and Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The human embryonic kidney (HEK-293), human colon adenocarcinoma (HT-29) and the human cervical cancer (HeLa) cell lines were purchased from National Centre for Cell Science (NCCS), Pune, India. The mammalian cell lines were maintained in DMEM containing 4.0 mmol L^{-1} L-glutamine and 110 mg L^{-1} sodium pyruvate (Sigma Aldrich, USA) supplemented with 10% (v/v) heat-inactivated FBS (Sigma Aldrich, USA), 50 µg mL^{-1} streptomycin and 50 IU mL^{-1} penicillin (Hi-Media Pvt. Ltd., India) at 37°C in 5% carbon dioxide atmosphere.

3.2 In vitro probiotic properties of *Lb. plantarum* DM5

3.2.1 Resistance to gastric juice and bile salt

The tolerance of *Lb. plantarum* DM5 to artificial gastric juice and bile salt was evaluated. The artificial gastric juice was prepared by supplementing MRS medium with pepsin (Sigma Aldrich, USA) solution of 1000 UmL^{-1} and the pH of the medium was adjusted to 2, 3 or 4 with 2N HCl[38]. The isolate *Lb. plantarum* DM5 was inoculated in each 5 ml artificial gastric juice at a conc. of 10^7 CFU mL^{-1} and incubated at 37ºC for 5 h under static condition. The total number of viable cells was counted after exposure to acidic conditions for 5 h by plate count method. The resistance of *Lb. plantarum* DM5 to bile salt was determined according to the method of Zheng et al.[24]. *Lactobacillus plantarum* DM5 (initial conc. ~10^7 CFU mL^{-1}) was grown separately in sterile 50 mL MRS medium supplemented with 0.3, 0.5 and 1% (w/v) of bile salt (Sigma Aldrich, USA) and incubated at 37°C for 30 h under static condition. Bacterial growth was analyzed by determination of CFU mL^{-1} at 30 h.

3.2.2Bile salt hydrolase activity

Bile salt hydrolase activity of *Lb. plantarum* DM5 was quantitatively measured by determining the amount of amino acids liberated from conjugated bile salts[27]. The strain *Lb. plantarum* DM5 was grown in MRS medium at 37ºC for 20 h under static condition and the cell pellet was obtained by centrifugation at 10,000*g* at 4ºC for 10 min. The cell pellet was washed twice with deionised water and re-suspended into 10 mL of 0.1M sodium phosphate buffer (pH 7.0). The cell concentration was adjusted to an absorbance (A_{600}) of 1 unit at 600 nm and 5 mL of the cell suspension was subjected to sonication (Sonics, Vibra cell) for three, 1 min intervals, by keeping cells in an ice bath. The cell debris was removed by centrifugation at 10,000*g* at 4ºC for 10 min and the cell free extract obtained of 100 µL was mixed with 1.8 mL of 0.1 M sodium phosphate buffer (pH 7.0) and 0.1 mL of 6 mmol L^{-1} conjugated bile

salts. The conjugated bile salts used in the experiment were 6 mmol L^{-1} TDCA or 6 mmol L^{-1} conjugated bile salt mixture. The mixture was incubated at 37ºC for 30 min and the enzymatic reaction was terminated by adding 0.5 mL of trichloroacetic acid (15% w/v) to 0.5 mL of sample. The mixture was centrifuged (780*g* at 4ºC for 10 min) and 0.2 mL of supernatant obtained was added to 0.5 mL of distilled water and 0.5 mL of ninhydrin reagent (0.5mL of 1% ninhydrin in 0.5 M citrate buffer pH 5.5, 1.2 mL of 30% (v/v) glycerol, 0.2 mL of 0.5M citrate buffer pH 5.5). The preparation was mixed on a vortex and boiled for 15 min. After subsequent cooling, the absorbance (A_{570}) was measured at 570 nm. The glycine (in case of conjugated bile salt mixture) or taurine (in case of TDCA) was used as standards. One unit of BSH activity was defined as the amount of enzyme that liberated 1 µmole of amino acid (glycine or taurine) from substrate per min at 37ºC.

3.2.3 In vitro cholesterol assimilation activity

In vitro cholesterol assimilation of *Lb. plantarum* DM5 was determined by growing cells at 37ºC in 50 mL MRS broth supplemented with 0.5% w/v TDCA and 0.1 g of water-soluble cholesterol/L (Sigma Aldrich, USA). After the incubation, bacterial cells were harvested by centrifugation (16,000g at 4ºC for 5 min), and 100 µL of cell free supernatant was used for determining the cholesterol content[3]. An un-inoculated MRS medium supplemented with TDCA and cholesterol was used as reference.

***3.2.4 In vitro* cell adhesion assay**

The *in vitro* adhesion ability of isolate *Lb. plantarum* DM5 was assayed using HT-29 cell line. The HT-29 cells were seeded in each well of six-well tissue culture plates at a density of $4x10^4$ cell/cm^2 and incubated at 37ºC for 24 h. After 24 h, the DMEM medium was removed and the cells were washed with 1x PBS (pH-7.4). The *Lb. plantarum* DM5 was initially grown in MRS medium at 37ºC for 18 h under static condition and the cell pellet was obtained by centrifugation at 10,000*g* at 4ºC for 10 min. The cell pellet was washed twice

with 1x PBS (pH-7.4) and re-suspended in DMEM medium without serum and antibiotics to obtain $1x10^8$ CFU mL^{-1}. Adhesion assay was done by adding 1 mL of bacterial cell suspension to each well of the six-well tissue culture plate containing monolayer of HT-29 cells. The tissue culture plate was then incubated at 37ºC for 2 h in the presence of 5% CO_2/95% air atmosphere. After incubation, the monolayer was washed with sterile 1x PBS (pH-7.4) and 2 mL of methanol was added to each well for fixing and incubated at 37ºC for 10 min. After that the methanol was completely removed and the fixed cells were stained with 1 mL of Giemsa solution (1x) at 37ºC for 20 min[31]. The wells were washed with absolute ethanol to remove the excess stain and air dried. The plate was then monitored under inverted microscope (TS100-F, Nikon International Inc.). To determine the percent adhesion of isolate *Lb. plantarum* DM5 to HT-29 cells, bacterial cells were detached from monolayer by trypsinization using 500 µL of Trypsin-EDTA solution. The tissue culture plate was then incubated at 37ºC for 15 min and the cell suspension was platted on MRS agar plate by serial dilution for determining the adherent bacterial cells. The MRS agar plate was incubated at 37ºC for 24 h and colonies were counted. The results of the adhesion assay were expressed as adhesion percentage[23];

$$\textbf{Adhesion (\%)} = (B_1/B_0)x100;$$

where, B_1 and B_0= No of viable cell (CFU mL^{-1}) of *Lb. plantarum* DM5 before and after adhesion.

3.3 Production of antimicrobial compound from *Lb. plantarum* DM5 and spectrum of activity

The agar well diffusion method using indicator strains *E. coli* DH5α and *S. aureus* MTCC 737 was used for preliminary screening of antimicrobial activity of neutralized cell free supernatant (pH 6.0) of *Lb. plantarum* DM5[19]. The efficacy of antimicrobial compound produced by *Lb. plantarum* DM5 was further tested against several indicator strains listed in Table 2. The sensitivity of a strain was scored according to the diameter of the inhibition

zone around the well by the agar well diffusion method. Antimicrobial activity was expressed as arbitrary units (AU) per mL[33].

3.4 Purification of antimicrobial compound from *Lb. plantarum* DM5

The cell free supernatant containing extracellular bacteriocin from *Lb. plantarum* DM5 was partially purified by ammonium sulphate precipitation. Ammonium sulphate was added very slowly to the supernatant with stirring at 4°C to 80% saturation. This mixture was then centrifuged at 10,000g at 4°C for 30 min and the resulting pellet was re-suspended in 25 mM ammonium acetate buffer (pH 6.0) and extensively dialyzed using a dialysis membrane (Molecular mass cut off 3.5 kDa) against the same buffer with regular change of buffer for a time period of 24 h. The ensuing sample was further purified by cation exchange chromatography using CM sepharose as matrix. The column (1.5x5.0 cm) containing CM sepharose was pre-equilibrated with 25 mM ammonium acetate buffer (pH 5.4) and 20 mL partially purified bacteriocin obtained after dialysis was loaded to the column. After sample loading, the column was washed with 20 mL of 25 mM ammonium acetate buffer (pH 5.4) at a flow rate of 1 mL min^{-1}. The bound protein was eluted with a linear gradient of NaCl (0.0 to 0.5 M) in 25 mM ammonium acetate buffer (pH 5.4) with a flow rate of 1.0 mL min^{-1} in FPLC (Akta Prime, GE Healthcare) and 2 mL fractions were collected for estimation of protein content (A_{280}) and bacteriocin activity (AU mL^{-1}) by agar well diffusion method using *S. aureus* MTCC 737 as indicator strain. The fractions having bacteriocin activity were pooled and dialyzed against 25 mM ammonium acetate buffer (pH 6.0) at 4°C. The dialyzed sample was further subjected to the next step of purification by gel filtration using a column (1.5x50.0 cm) containing Sephacryl S-200HR matrix. The column was pre-equilibrated with 25 mM ammonium acetate buffer (pH 6.0) and 2 mL partially purified bacteriocin by cation exchange chromatography was loaded onto the column. The sample was eluted by 25 mM ammonium acetate buffer (pH 6.0) at a flow rate of 0.5 mL min^{-1} and 2 mL fractions were

collected. The purified fractions showing maximum bacteriocin activity against *S. aureus* MTCC 737 by agar well diffusion method were pooled and analysed for protein concentration and specific activity.

3.5 Estimation of molecular mass of bacteriocin from *Lb. plantarum* DM5

The homogeneity of the purified plantaricin DM5 by gel filtration and its molecular mass was determined by SDS-PAGE using 15% (w/v) 1.5 mm gel run on a vertical slab mini gel unit (Mini-PROTEAN®Tetra cell, BioRad, USA). The purified plantaricin DM5 was subjected to denaturing conditions for molecular mass determination and the samples were prepared in 0.0625 mol L^{-1} Tris–HCl buffer (pH 6.8) containing 2.8% (w/v) SDS, 10% (w/v) glycerol, 5%(w/v) β-mercaptoethanol and 0.05% (w/v) bromophenol blue and boiled at 100ºC for 4 min[39]. Electrophoresis was carried out using Tris-glycine buffer (pH 8.3) with a current of 2 mA per lane and after the electrophoresis, the gel containing protein bands were stained with Coomassie Brilliant Blue R250 staining solution. *In situ* bacteriocin activity was determined by agar gel overlay method[40]. After electrophoresis the gel was divided into two vertical parts and one half of the gel was stained with Coomassie Brilliant Blue R250 as described earlier and the other part was used for detection of antimicrobial activity. For determining the *in situ* antimicrobial activity, the gel was fixed in 20% isopropanol and 10% acetic acid and washed in sterile double distilled water for 6 h with frequent water exchange. The gel was placed above the Nutrient base agar (1.8%) and after that it was overlaid with Nutrient soft agar (0.7%) medium inoculated with the indicator strain *S. aureus* MTCC 737 (~10^6 CFU mL^{-1}) and incubated at 37ºC for 24 h.

3.6 Characterization of bacteriocin from *Lb. plantarum* DM5

3.6.1 Effect of enzymes, pH, detergents and temperature on bacteriocin activity from Lb. plantarum DM5

The bacteriocin from *Lb. plantarum* DM5 was characterized with respect to thermal and pH stability and susceptibility towards different enzymes, organic solvents and

detergents. The effect of temperature on the bacteriocin was determined by heating the cell-free supernatants (pH 6.0) at different temperature ranging from 40, 60, 80, 100 and 120°C for 90 min and the residual antimicrobial activity was determined by agar well diffusion method using *S. aureus* MTCC 737 as indicator strain. For pH stability of plantaricin DM5, the cell-free supernatant of *Lb. plantarum* DM5 was adjusted to pH 2.0-12.0 with sterile 1 N HCl or 1 N NaOH and incubated for 2 h of at 30°C. After incubation, the pH of samples were readjusted to 6.5 with sterile 1 N HCl or 1 N NaOH and the antimicrobial activity was determined as described earlier. Bacteriocin was also tested for its susceptibility towards enzymes (1 mg mL^{-1} of pepsin, trypsin, α-amylase, catalase, lysozyme or proteinase K), organic solvents (1% , v/v of ethanol, methanol, acetone, ethyl acetate, n-butanol, isopropanol), surfactants (1% , v/v of Tween 20, Tween 80, Triton X-100) and detergent 1%, w/v of SDS following the method of Todorov and Dicks, (2005)[33]. The 2 ml cell free supernatant of *Lb. plantarum* DM5 was incubated at 37ºC for 1 h with above mentioned reagents and after incubation, residual antimicrobial activity was determined by agar well diffusion method using *S. aurues* MTCC 737 as indicator strain.

3.6.2 Assessment of cytotoxicity of bacteriocin from *Lb. plantarum* DM5

Lyophilized plantaricin DM5 with specific activity of 2196 AU mg^{-1} was used for cytotoxicity assay by colorimetric assay using MTT[41]. The human embryonic kidney cell line (HEK-293) and the human cervical cancer cell line (HeLa-293) were seeded separately in 250 mL, 75 cm^2 vent cap tissue culture flasks and after ~90% confluence, the cells were detached by 1x trypsin EDTA solution (Sigma Aldrich, USA) and counted by haemocytometer. For cytotoxicity test, the cells were re-suspended in FBS containing DMEM medium and 200 µL of this solution containing $2x10^4$ HEK 293 or HeLa cells per well were separately seeded in a 96 well plate. The plates were incubated at 37ºC for 12 h for cell adherence in CO_2 incubator (5%). After the incubation, the complete DMEM medium was completely removed and the

adhered cells were exposed to different concentrations of bacteriocin (ranging between 10 µg mL^{-1} and 1000 µg mL^{-1}) dissolved in FBS free DMEM medium. The MTT assay was done at regular time interval by removing the whole 200 µL medium and washing the each well containing the adherent cells in the bottom of the well with 200 µL of 1x PBS (pH 7.1) to remove any bacterial contamination. Finally, 100 µL MTT (500 µg mL^{-1}) was added to each well and the plates containing MTT solution was further incubated at 37ºC for 4 h. After the incubation, the 100 µL of MTT solution from each well was replaced with equal volume (100 µL) of DMSO. The absorbance at 570 nm, (A_{570}) was measured using a multi-mode micro-plate reader (Tecan, Infinite 200 Pro) and the viability (%) was calculated by an equation as mention below;

$$\textbf{Cell viability (\%)} = (N_t/N_c)\ \textbf{x100}$$

where, N_t is absorbance (A_{570}) of cells treated with glucan and N_c is the absorbance (A_{570}) of untreated cells.

4.0 Conclusions

Lactobacillus plantarum DM5 isolated from fermented beverage Marcha of Sikkim was analyzed for acid and bile tolerance to evaluate its potential probiotic properties. *Lb. plantarum* DM5 exhibited fairly high level of acid tolerance (pH 2-4) and bile tolerance (0.3-1%), suggesting that it could survive passage through the low acidic condition of stomach and high bile salt concentration of small intestine and may be used as potent probiotic. Apart from this, the isolate *Lb. plantarum* DM5 also expressed bile salt hydrolase activity and had ability to assimilate cholesterol *in vitro.* The strain *Lb. plantarum* DM5 was identified as bacteriocin producer and showed broad antimicrobial activity against Gram-positive and Gram-negative bacteria. The novel bacteriocin, plantaricin DM5, produced by Lb. plantarum 163 was purified by salt precipitation, cation exchange chromatography and gel filtration and showed molecular mass of 15.2 kDa. The plantaricin DM5 was further subjected to cytotoxicity assay

using HEK-293 and HeLa cells in order to commercially exploit it as food additives and bio-preservative; which revealed nontoxic biocompatible nature. Therefore, it can be used as can be used as alternative to chemical preservatives in food industry without any toxic effect to mammalian cells. With the aim of increasing food safety, the isolate *Lb. plantarum* DM5 has thus been considered as bacteriocin-producing probiotic strain and can be potentially used as "natural" food preservatives and as probiotic ingredient in foods products. Nevertheless, further investigations in an *in vivo* system are needed to validate the information.

Acknowledgement:

The authors thank Indian Institute of Technology Guwahati, Guwahati, India, for providing the experimental facilities and Council of Industrial and Scientific Research (CSIR), New Delhi, India, for providing fellowship to D.Das.

Conflict of Interest:

The authors have no conflict of interests.

Abbreviations

HT-29 cell: Human adenocarcinoma cell line, HeLa-293: Human cervical cancer cell line, Caco-2 cell: Human epithelial colorectal adenocarcinoma cell line, DMEM: Dulbecco's modified Eagle's medium, FBS: Fetal bovine serum, TDCA: Sodium taurocholate, CFU: Colony forming units, EDTA: Ethylenediaminetetraacetic acid, PAGE: Polyacrylamide gel electrophoresis, SDS: Sodium dodecyl sulphate, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, DMSO: Dimethyl sulfoxide, GDCA: Sodium glycholate.

References:

1. R. Fuller, Probiotics in man and animals. *J. Appl. Bacteriol*., 1989, **66**, 365-378.
2. M. Saarela, G. Morgensen, R. Forden, J. Matoto and T. Mattla-Sanholm, Probiotic bacteria: safety, functional and technological properties. *J. Bacteriol.*, 2000, **84**, 197-215.

3. J.M. Mathara, U. Schillinger, C. Guigas, C. Franz, P.M. Kutima, S.K. Mbugua, H.K Shin, and W.H. Holzapfel, Functional characteristics of Lactobacillus spp. from traditional Maasai fermented milk products in Kenya. *Int. J. Food Microbiol*., 2008, **126**, 57-64.
4. F. Lamari, K. Sadok, A. Bakhrouf and F.J Gatesoupe, Selection of lactic acid bacteria as candidate probiotics and *in vivo* test on *Artemia nauplii*. *Aquacult. Int*., 2014, **22**, 699-709.
5. A. Galvez, H. Abriouel, R.L. Lopez and N.B. Omar, Bacteriocin-based strategies for food biopreservation. *Int. J. Food Microbiol*., 2007, **120**, 51-70.
6. M. Hu, H. Zhao, C. Zhang, J. Yu and Z. Lu, Purification and characterization of Plantaricin 163, a novel bacteriocin produced by *Lactobacillus plantarum* 163 isolated from traditional Chinese fermented vegetables. *J. Agric. Food Chem*., 2013, **61**, 11676-11682.
7. M.S. Barbosa, S.D. Todorov, Y. Belguesmia, Y. Choiset, H. Rabesona, I.V. Ivanova, J.M. Chobert, T. Haertle and B.D.G.M. Franco, Purification and characterization of the bacteriocin produced by *Lactobacillus sakei* MBSa1 isolated from Brazilian salami, *J. Appl. Microbiol*., 2014, **116**, 1195-1208.
8. H.P. Fleming, R.F. McFeeters and M.A. Daeschel, The lactobacilli, pediococci and leuconostots: vegetable products. In: Gilliland SE. (Ed.), Bacterial starter cultures for foods. CRC Press Inc., Boca Raton, 1985, pp. 97- 118.
9. S.D Todorov and B.D.G.M. Franco, *Lactobacillus Plantarum*: Characterization of the species and application in food production. *Food Rev. Int*., 2010, **26**, 205-229.
10. L. Nissen, W. Chingwaru, B. Sgorbati, B. Biavati and A. Cencic, Gut health promoting activity of new putative probiotic/protective *Lactobacillus* spp. Strains: a functional study in the small intestinal cell model. *Int. J. Food Microbiol*., 2009, **135**, 288-294.

11. E. Lonnermark, V. Friman, G. Lappas, T. Sandberg, A. Berggren and I. Adlerberth, Intake of *Lactobacillus plantarum* reduces certain gastrointestinal symptoms during treatment with antibiotics. *J. Clin. Gastroenterol*., 2010, **44**, 106-112.
12. M.A. Daeschel, M.C. McKeney and L.C. McDonald, Bacteriocidal activity of *Lactobacillus plantarum* C-11. *Food Microbiol.,* 1990*,* **7,** 91-98.
13. G. Enan, A.A. Essawy, M. Uyttendaele and J. Debevere, Antibacterial activity of *Lactobacillus plantarum* UG1 isolated from dry sausage: Characterization, production and bactericidal action of plantaricin UG1. *Int. J. Microbiol*., 1996, **30**, 189-215.
14. A. Atrih, N. Rekhif, A.J.G. Moir, A. Lebrihi and G. Lefebvre, Mode of action, purification and amino acid sequence of plantaricin C19, an anti-Listeria bacteriocin produced by *Lactobacillus plantarum* C19. *Int. J. Food Microbiol*., 2001, **68**, 93-109.
15. D. Hernandez, E. Cardell and V. Zarate, Antimicrobial activity of lactic acid bacteria isolated from Tenerife cheese: Initial characterization of plantaricin TF711, a bacteriocin-like substance produced by *Lactobacillus plantarum* TF711. *J. Appl. Microbiol*., 2005, **99**, 77-84.
16. S.D. Todorov, H. Prevost, M. Lebois, X. Dousset, J.G. LeBlanc and B.D.G.M Franco, Bacteriocinogenic *Lactobacillus plantarum* ST16Pa isolated from papaya (*Carica papaya*)-From isolation to application: Characterization of a bacteriocin. *Food Res. Int*., 2011, **44,** 1351-1363.
17. R.C. Martinez, M. Wachsman, N.I. Torres, J.G. LeBlanc, S.D. Todorov and B.D. Franco, Biochemical, antimicrobial and molecular characterization of a noncytotoxic bacteriocin produced by *Lactobacillus plantarum* ST71KS. *Food Microbiol*., 2013, **34**, 376-381.
18. L. Avonts, E. Van Uytven and L. De Vuyst, Cell growth and bacteriocin production of probiotic *Lactobacillus* strains in different media. *Int. Dairy J*., 2004, **14,** 947-955.

19. D. Das and A. Goyal, Potential probiotic attributes and antagonistic activity of an indigenous isolate *Lactobacillus plantarum* DM5 from an ethnic fermented beverage Marcha of north eastern Himalayas. *Int. J. Food Sci. Nutr*., 2014, **65**, 335-344.
20. M.L. Ramirez-Chavarin, C. Wacher, C.A. Eslava-Campos and M.L. Perez-Chabela, Probiotic potential of thermo tolerant lactic acid bacteria strains isolated from cooked meat products. *Int. Food Res. J*., 2013, **20**, 991-1000.
21. P. Chen, Q. Zhang, H. Dang, X. Liu, F. Tian, J. Zhao, Y. Chen, H. Zhang and W. Chen, Screening for potential new probiotic based on probiotic properties and α-glucosidase inhibitory activity, *Food Control*, 2014, **35**, 65-72.
22. Y. Bao, Y. Zhang, H. Li, Y. Liu, S. Wang, X. Dong, F. Su, G. Yao, T. Sun and H. Zhang, *In vitro* screen of *Lactobacillus plantarum* as probiotic bacteria and their fermented characteristics in soymilk. *Ann. Microbiol*., 2012, **62**, 1311-1320.
23. J.K. Kaushik, A. Kumar, R.K. Duary, A.K. Mohanty, S. Grover and V.K. Batish, Functional and probiotic attributes of an indigenous isolate of *Lactobacillus plantarum*. *PloS One*, 2009, 4(12): Doi:10.1371/journal.pone.0008099.
24. Y. Zheng, Y. Lu, J. Wang, L. Yang, C. Pan and Y. Huang, Probiotic properties of *Lactobacillus* strains isolated from Tibetan kefir grains. *PloS One*, 2013, Doi:10.1371/journal.pone.0069868.
25. C.G. Vinderola and J.A. Reinheimer, Lactic acid starter and probiotic bacteria: a comparative in vitro study of probiotic characteristics and biological barrier resistance. *Food Res. Int*., 2003, **36**, 895-904.
26. I. De Smet, L. Van Hoorde, M. Vande Woestyne, H. Cristianes and W. Verstraete, Significance of bile salt hydrolytic activities of lactobacilli. *J. Appl. Bacteriol*., 1995, **79**, 292-301.

27. M.T. Liong and N.P. Shah, Acid and bile tolerance and cholesterol removal ability of lactobacilli strains. *J. Dairy Sci*., 2005, **88**, 55-66.

28. R. Kumar, S. Grover and V.K. Batish, (2012) Bile salt hydrolase (Bsh) activity screening of *Lactobacilli*: *in vitro* selection of indigenous *Lactobacillus* strains with potential bile salt hydrolysing and cholesterol-lowering ability. *Probiotics Antimicrobial. Prot*., 2012, **4**, 162-172.

29. Z. Dong, J. Zhang, B. Lee, H. Li, G. Du and J. Chen, A bile salt hydrolase gene of *Lactobacillus plantarum* BBE7 with high cholesterol-removing activity. *Eur. Food Res. Technol*., 2012, **235**, 419-427.

30. Y.T Ahn, B.H. Kim and H.U. Kim, Effect of the intake of acidophilus milk on the serum cholesterol level of Korean adult. *Korean J. Anim. Sci. Technol*., 2000, **42**, 223-234.

31. R.K. Duary, Y.S. Rajput, V.K. Batish and S. Grover, Assessing the adhesion of putative indigenous probiotic lactobacilli to human colonic epithelial cells. *Indian J. Med. Res*., 2011, **134**, 664-671.

32. R. Satish Kumar, P. Kanmani, N. Yuvaraj, K.A. Paari, V. Pattukumar and V. Arul, *Lactobacillus plantarum* AS1 binds to cultured human intestinal cell line HT-29 and inhibits cell attachment by enterovirulent bacterium *Vibrio parahaemolyticus*. *Lett. Appl. Microbiol*., 2011, **53**, 481-487.

33. S.D. Todorov and L.M.T. Dicks, Effect of growth medium on bacteriocin production by *Lactobacillus plantarum* ST194BZ, a Strain isolated from Boza. *Food Technol. Biotechnol*., 2005, **43**, 165-173.

34. C.W. Wu, L.J. Yin and S.T. Jiang, Purification and characterization of bacteriocin from *Pediococcus pentosaceus* ACCEL. *J. Agric. Food Chem*., 2004, **52**, 1146-1151.

589 35. S.K. Tiwari and S. Srivastava, Characterization of a bacteriocin from *Lactobacillus*
590 *plantarum* strain LR/14. *Food Biotechnol*., 2008, **22**, 247-261.
591 36. P.D. Cotter, C. Hill and R.P. Ross, Bacteriocins: developing innate immunity for food.
592 *Nat. Rev. Microbiol*., 2005, **3**, 778-788.
593 37. J.C. De Man, M. Ragosa and E. Sharpe, A medium for the cultivation of lactobacilli. *J.*
594 *Appl. Bact*., 1960, **23**,130-135.
595 38. S. Oh, S.H. Kim and R.W. Worobo, Characterization and purification of a bacteriocin
596 produced by a potential probiotic culture, *Lactobacillus acidophilus* 30SC. *J. Dairy Sci*.,
597 2000, **83**, 2747-2752.
598 39. U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of
599 bacteriophage T4. *Nature*, 1970, **227**, 680-685.
600 40. A.K. Bhunia, M.C. Johnson and B. Ray, Direct detection of an antiniicrobial peptide of
601 *Pediococcus acidilactici* in sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
602 *J. Ind. Microbiol*., 1987, **2**, 319-322.
603 41. J. van Meerloo, G.J. Kaspers and J. Cloos, Cell sensitivity assays: the MTT assay.
604 *Methods Mol Biol*., 2011, **731**, 237-245.
605
606
607

Table 1: Determination of probiotic characteristics of *Lactobacillus plantarum* DM5

Sensitivity of Lb. plantarum DM5 to gastric juice and bile slat		
Gastric juice[a]	**Log CFU mL^{-1} at 0 h**	**Log CFU mL^{-1} after 5 h**
pH 2	7.32±0.18	3.53±0.22
pH 3	7.32±0.18	6.61±0.16
pH 4	7.32±0.18	7.14±0.24
Bile salt conc. (w/v)	**Log CFU mL^{-1} at 0 h**	**Log CFU mL^{-1} after 30 h**
0.3%	7.11±0.17	8.85±0.26
0.5%	7.11±0.17	8.62±0.19
1%	7.11±0.17	6.90±0.17

Bile salt hydrolase (BSH) activity and Cholesterol assimilation of Lb. plantarum DM5			
Bile salt	**BSH Activity[b] (U mL^{-1})**	**Specific Activity (U mg^{-1})**	**% assimilation of cholesterol**
Sodium taurocholate (TDCA)	1.14±0.15	0.63±0.02	68.3±2.3
Conjugated bile mixture	2.11±0.36	1.18±0.03	72.7±1.9

All the experiments were carried out in triplicate (n =3) and the results are expressed as mean ± S.D.

[a]*Gastric juice was prepared by supplementing MRS medium with pepsin solution of 1000 UmL^{-1} and the pH of the medium was adjusted to 2, 3 or 4 with 2N HCl.*

[b]*BSH activity was measured according to Liong and Shah, 2005 and the conjugated bile salts used in the experiment was 6 mM sodium taurocholate or 6 mM conjugated bile salt mixture.*

Table 2: Antimicrobial spectrum of bacteriocin produced by *Lactobacillus plantarum* DM5

Test microorganism	Growth condition	[a]Activity
Alcaligenes feacalis MTCC 2952	Nutrient broth/30ºC	+
Bacillus cereus MTCC 8776	Nutrient broth/37ºC	+
Bacillus subtillis NRRL B-14596	Nutrient broth/37ºC	-
Enterobacter aerogenes MTCC 7016	Nutrient broth/30ºC	-
Escherichia coli DH5α	Nutrient broth/37ºC	+++
Listeria monocytogenes MTCC 1143	Nutrient broth/37ºC	++
Klebsiella oxytoca MTCC 3030	Nutrient broth/37ºC	-
Pseudomonas aeruginosa MTCC 7523	Nutrient broth/37ºC	+
Staphylococcous aureus MTCC 737	Nutrient broth/30ºC	+++
Staphylococcus epidermis MTCC 6810	Nutrient broth/30ºC	++
Salmonella enterica MTCC 1165	Nutrient broth/37ºC	+
Lactobacillus plantarum NRRL B-4496	MRS/ 30ºC	++
Lactobacillus acidophilus NRRL B-4495	MRS/30ºC	-
Pediococcus pentosaceus SPA	MRS/30ºC	++

[a]Activity: ***(+++):*** *zone>15 mm[b],* ***(++):*** *10 mm<zone< 15 mm[b],* ***(+):*** *5mm<zone<10mm[b],* ***(-):*** *no zone of inhibition.*
[b]Values in millimeter are the distance of zone of inhibition of growth of microorganism. All the individual experiments were carried out in triplicates and the results represent mean values of three data.

Table 3: Purification of bacteriocin from *Lb. plantarum* DM5

	Activity (AU/ml)	Total Units (AU)	Protein Conc. (mg/ml)	Total protein (mg)	Specific Activity (AU/mg)	Overall Activity Yield (%)	Fold Purification
Crude (CFS)	6400	640000	15.6	1560	410	100	–
Ammonium sulphate precipitation (80%)	12800	256000	5.83	116.6	2196	40	5.4
Ion exchange chromatography by CM sepharose	3200	32000	1.02	10.2	3137	5	7.7
Gel filtration by Sephacryl S-200HR	1600	6400	0.15	0.60	10667	1	26

Table 4: Effects of enzyme, temperature, pH, surfactant and detergents on the antimicrobial activity of plantaricin DM5

Treatment	Antimicrobial activity[a]
Control[b]	+++
• Enzyme	
Catalase	+++
α-amylase	+++
Lysozyme	+++
Pepsin	-
Trypsin	-
Protinase-K	-
• Temperature	
40	+++
60	+++
80	+++
100	++
120[c]	+
• pH	
2	++
4	++
6	+++
8	++
10	++
12	+
• Solvents	
Ethanol	+++
Methanol	+++
1-Butanol	+++
Iso-propanol	+++
Ethyl acetate	+++
Acetone	+++
• Surfactants/detergents	
SDS	+++
Tween 80	+++
Tween 20	+++
Triton X-100	+++

[a] *compared with the highest antimicrobial activity (6400 AU/mL) against indicator strain S. aureus by agar well diffusion method.*

[b] *The cell free supernatant (pH 6.0) of Lb. plantarum DM5 without any treatment.*

[c] *incubated at 120ºC for 30 min instead of 90 min.*

Figure Legends:

Fig. 1 Adhesion of *Lb. plantarum* DM5 on HT-29 cell line observed under inverted microscope (40x) after Giemsa staining. A) Control HT-29 cell line (B) and (C) treated HT-29 cells with *Lb. plantarum* DM5 for 2 h at 37ºC. The arrows indicate the attachment of bacterial cell to the mammalian HT-29 cells.

Fig. 2 Elution profile of (A) 80% ammonium sulphate precipitated plantaricin DM5 run on cation exchange chromatography using CM sepharose. The bound protein was eluted with linear salt gradient in range of 0.0-0.5M NaCl with a flow rate of 1 mL min^{-1} and 2 mL fractions were collected. (B) Plantaricin DM5 purified by cation exchange chromatography run on gel filtration column using Sephacryl S-200HR as matrix. The flow rate was 0.5 mL min^{-1} and 2 mL fractions were collected. In both the cases fractions were assayed for bacteriocin activity against *S. aureus* MTCC 737 (--●--) and protein concentration (--□--).

Fig. 3 SDS-PAGE analysis of purified plantaricin DM5 (A) Gel was stained with Coomassie Brilliant Blue R250. Lane M: Molecular mass marker (3.5-43 kDa), Lane 1: 80% ammonium sulphate purified fraction, Lane 2: plantaricin DM5 purified by cation exchange chromatography, Lane 3: plantaricin DM5 purified by gel filtration showing single band. (B) Clear zone of inhibition of indicator strain *S. aureus* MTCC 737 corresponding to the position of the protein band of 15.2 kDa. The gel was overlaid with indicator strain *S. aureus* MTCC 737 (~10^6) embedded in NB soft agar (0.7%) and incubated at 37ºC for 24 h. (C) Molecular mass determination of purified plantaricin Dm5 by R_f *vs* Log Mw plot.

Fig. 4 The *in vitro* cytotoxicity assay of plantaricin DM5 showing the cell viability (%) of (A) HEK-293 and (B) HeLa cells treated with various concentrations of plantaricin DM5 (10-1000 µg mL^{-1}) over a period of 3- 48 h of incubation.

Fig. 1

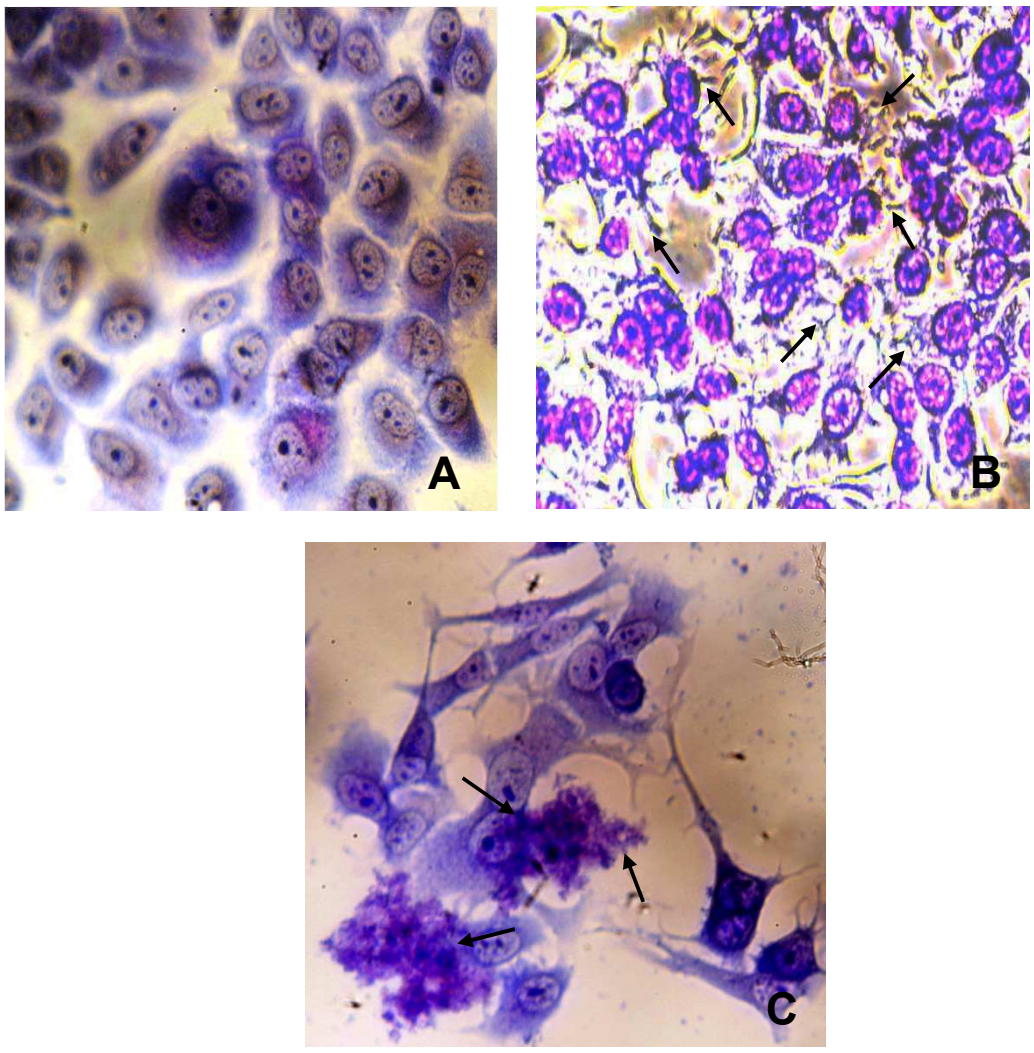


Fig. 2

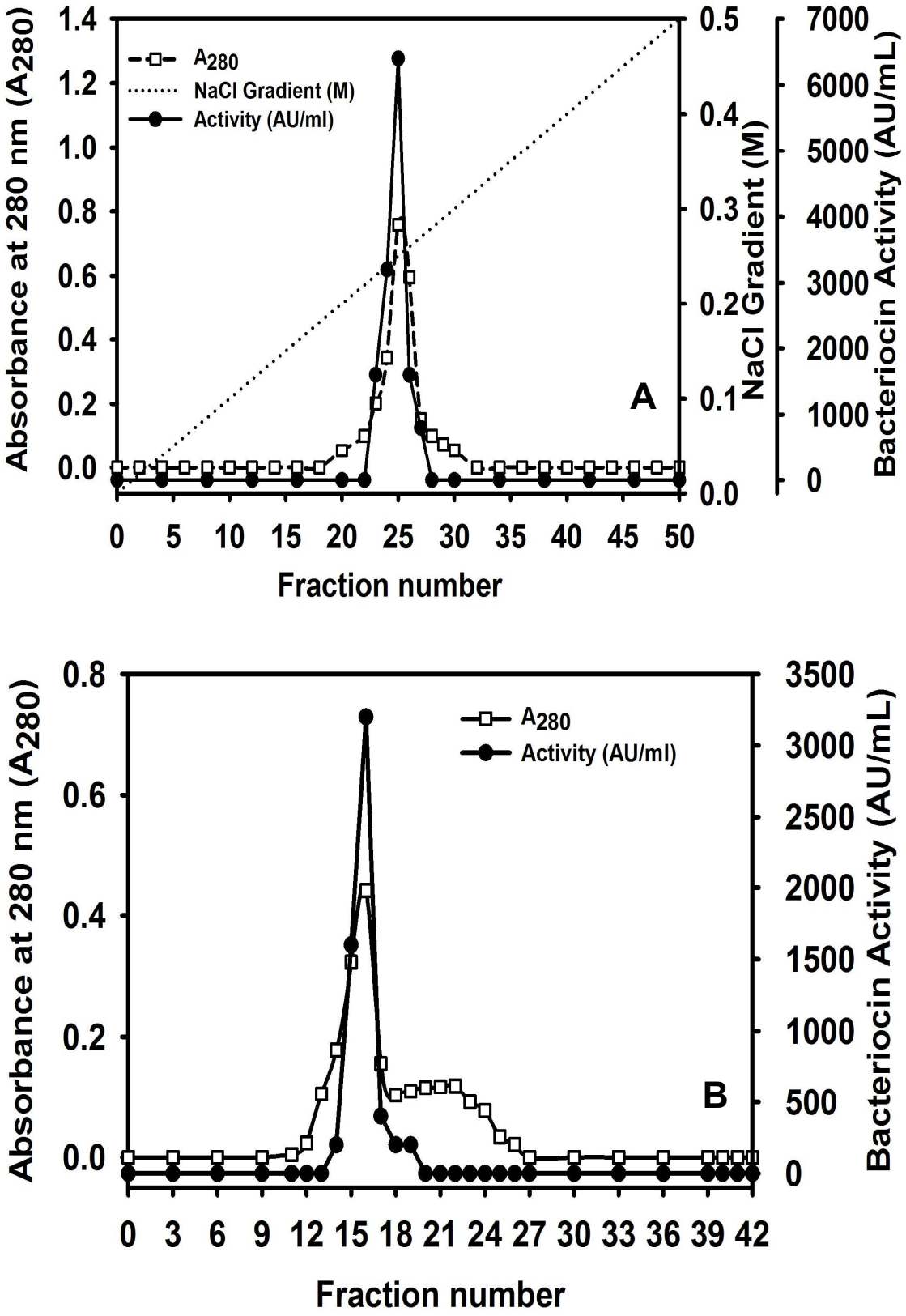


Fig. 3

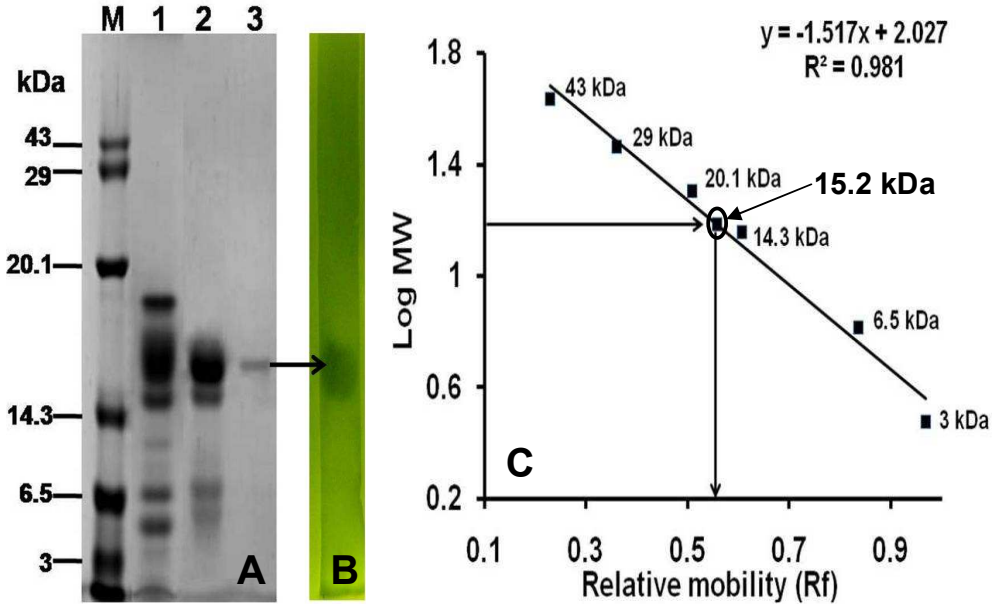


Fig.4