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In vitro evaluation of probiotic and bacteriocinogenic potentiality of *Lactobacillus plantarum* and *Lactobacillus delbrueckii* isolated from vegetables in Chittagong region, Bangladesh

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ABSTRACT

Aims: Reducing indiscriminate and over use of antibiotics and chemical preservatives, finding better probiotics and new bacteriocins should get paramount importance which will eventually contribute to save lives of newborn to elderly. Some probiotic *Lactobacillus* produces bacteriocins or bacteriocin-like-substances (BLS) which may be considered as candidates for biopreservatives. The aims of this study was to find probiotic *Lactobacillus* and assessing their bacteriocinogenic activity.
Methodology and results: Five vegetables were processed and isolated 38 Lactic acid bacteria (LAB) by using De Man Rogosa Sharpe (MRS) medium. Among 38 LAB, only 8 (21%) showed potential antibacterial activity against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Salmonella* Typhii in agar well diffusion method. Finally, we selected two *Lactobacillus* species such as *Lactobacillus plantarum* MG1 and *Lactobacillus delbrueckii* MT4 for further *in vitro* testing. Both isolates showed growth capability at wide range of temperatures (27-45°C), pH (2-9), NaCl (1-7%), bile salt (0.5-2%) and could produce bacteriocin or BLS; which indicated they have potentiality to be probiotic. Bacteriocin or BLS produced by *L. plantarum* inhibited *E. coli* and *S.* Typhii whereas bacteriocin or BLS of *L. delbrueckii* inhibited *S. aureus*, *E. coli* and *S.* Typhi. These crude bacteriocin or BLS reduced initial bacterial load of vegetables up to 79% after 48 h while 5% of its mixed with vegetables in room temperature.
Conclusion, significance and impact of study: The study showed that our isolated *L. plantarum* and *L. delbrueckii* could be used as probiotic to improve public health and their bacteriocin or BLS could be used as biopreservatives.

Keywords: Probiotics, bacteriocin, biopreservatives, lactic acid bacteria, Antimicrobials

INTRODUCTION

Saving and improving the quality of life, we are continuously fighting against different types of fatal pathogens. Although a lot of antibiotics are currently available and playing significant roles against many pathogens, these are not enough because of emergence of new potential multiple drug resistance strains. Considering the facts, scientists are interested in using new antimicrobial agents in the treatment of infectious diseases including infections of the enteric system. There is evidence that some probiotics can inhibit gastrointestinal infections by blocking adherence of the pathogens to the intestinal epithelium cells. However, this effect of probiotics depends on both the specific probiotic strain and the pathogen (Abedi *et al*., 2013).

Excessive use of chemical preservatives in preserving food and food products is another concern because many of those chemical preservatives may have deteriorative effects on human health which demand exploring alternative to chemical preservatives such as biopreservatives. Bacteriocins are ribosomally synthesized antimicrobial peptides produced by various bacteria, including Lactic acid bacteria (LAB). Some of them have great potential in food preservation and can reduce or eliminate the need for addition of chemical preservatives (Perez *et al*., 2014).

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FAO and WHO defined probiotics as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" and probiotics should not be referred to as biotherapeutic agents (Mcfarland and Elamar, 1995). Probiotic LAB produces organic acids (lactic acid and acetic acid) which result in lowered pH. LAB also produce antimicrobial compounds including hydrogen peroxide, CO_2, diacetyl, acetaldehyde, D-isomers of amino acids, reuterin and bacteriocins (Cintas *et al*., 2001). There is increasing evidence that probiotics are beneficial in gastrointestinal disturbances, such as diarrhea, dysentery, typhoid etc. (Tambekar and Bhutada, 2010). In many cases, their effects are mainly prophylactic in nature, rather than therapeutic, *i.e.* preventive rather than curative (Suskovic *et al*., 2001). Majority of microorganisms used as probiotics belong to the LAB and bifidobacteria. The group of LAB, *Lactobacillus* species are most commonly utilized group of microorganisms for their potential beneficiary properties as probiotics. The antagonistic activity of such bacteria is known to inhibit a large number of enteric and urinary pathogenic bacteria (Hutt *et al*., 2006). They cause reduced lactose intolerance alleviation of some diarrheas, lowered blood cholesterol, increased immune response and prevention of cancer. The selection criteria for probiotic LAB include: safety, viability or activity in delivery vehicles, resistance to acid and bile, adherence to gut epithelial tissue ability to colonize the gastrointestinal tract, production of antimicrobial substances, ability to stimulate a host immune response and the ability to influence metabolic activities such as vitamin production, cholesterol assimilation and lactose activity (Pundir *et al*., 2013).

Bacteriocins are active against other bacteria, either of the same species or across genera. Nisin, produced by *Lactococcus lactis*, is the most thoroughly studied bacteriocin to date and has been applied as an additive to certain foods worldwide. Other bacteriocins such as pediocin, may also have potential applications in foods, though they are not currently approved as antimicrobial food additives (Yang *et al*., 2012). Bacteriocins can be used to confer a rudimentary form of innate immunity to foodstuffs (Cotter *et al*., 2005). Lot of studies have focused on the application of bacteriocin in preservation of vegetables, and appeared as a good alternative to chemical compounds and antibiotics (Nithya *et al*., 2012).

L. plantarum exerts inhibitory activity against *Escherichia coli* (including *E. coli* 0157:H7), *Pseudomonas aeruginosa*, *Helicobacter pylori*, *Yersinia enterocolitica*, *Campylobacter jejuni*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Klebsiella*, *Salmonella*, *Shigella*, *Bacillus*, *Clostridium*, *Enterococcus*, *Lactobacillus spp.*, etc. The main antibacterial compounds of *L. plantarum* inhabiting the human digestive system improve the health and physiology of the host by interacting with epithelial cells and enhancing the host immune system. *L. plantarum* strains demonstrated the broadest spectrum of antimicrobial activity among the probiotic bacteria examined which makes it useful in veterinary, human medicine and food industry (Dinev *et al*., 2017). *L. plantarum* are ubiquitous in environments such as foods (dairy products, fermented meat, vegetables, fruits, and beverages); respiratory, gastrointestinal, and genital tracts of humans and animals, and in sewage and plant materials. The traditional recommendation that probiotic strains for humans should come from humans (species-specificity criterion) is becoming mitigated (Belicova *et al*., 2013).

Lactobacillus delbrueckii, a rare producer of bacteriocins, produce the bacteriocin named UO004, was proteinaceous, heat-stable, and had a bactericidal mode of action on a limited number of microorganisms, like most of the known bacteriocins produced by LAB (Boris *et al*., 2001).

Our study focused on isolation of probiotic *L. plantarum* and *L. delbrueckii* from five vegetables, whether they can produce bacteriocin or BLS and evaluation of the bacterion or BLS's food preserving capability.

MATERIALS AND METHODS

Sample collection, isolation and purification of LAB

Five vegetable samples were collected from the Chittagong area in Bangladesh. The samples were Tomato (*Solanum lycopersicum*), Ginger (*Zingiber officinale*), Cucumber (*Cucumis sativus*), Okra or Lady's finger (*Abelmoschus esculentus*) and Sweet potato (*Ipomoea batatas*). Multiple sets of each fresh sample were collected carefully. For isolation of LAB, serial dilution (10^{-1} to 10^{-6}) agar plate technique was used. LAB was purified by streak plate method on De Man Rogosa Sharpe (MRS) agar.

Test microorganisms

The target pathogenic organisms used in this study were *Staphylococcus aureus* ATCC25923*, Bacillus subtilis* IFSTIM22, *Pseudomonas aeruginosa* CRL (ICDDR,B)*, E. coli* ATCC25922, *Salmonella typhii* AE14296. The test microorganisms were standardized by using 0.5 McFarland standard. 0.5 McFarland gives approximate cell density of 1.5 x 10^8 CFU/mL, having absorbance of 0.132 at wavelength of 600nm (Andrews, 2001). We used this standardization technique for all the necessary steps of this study.

Screening of isolated LAB for antibacterial activity

MRS broth was used for antimicrobial metabolite production from lactic acid bacteria. 200 mL of MRS broth was autoclaved at 121 °C for 15 min and inoculated with the colonies of a LAB isolate and incubated at 37 °C for 2-3 days under stationary condition. Then it was centrifuged (Model 6930, Kubota, Japan) at 9000 rpm for 15 min at 4 °C. The supernatant was then filtered through Whatman No. 1 filter paper to remove residual cells. Petri-plates were prepared by pouring sterile molten Muellar Hinton medium and allowed it to solidify. A hundred microliters of

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each standardized test microorganisms were spread on agar plates. Two wells (each 7 mm in diameter) made into agar plates with sterile borer. The wells were loaded with 100 µL of filtered LAB culture supernatant and 100 µL sterile broth. Plates were incubated at 37 °C for 24 h. After incubation, diameter of zone of inhibition was observed and measured (Pundir *et al.,* 2013).

Identification of LAB isolates

Lactobacillus genus was identified by using real time PCR technology (QIAGEN) following manufactures guideline. We used forward primer (5´-TGGAAACAGRTGCTAATACCG-3´) and reverse primer (5´-GTCCATTGTGGAAGATTCCC-3´) of *Lactobacillus* produced by M. CHUN LEONG, Malaysia. After confirmation of genus the species was identified on the basis of their morphological characteristics including size and shape of the organism, arrangement of the cells, presence or absence of the spores, regular or irregular forms, acid fastness, gram reaction etc.; cultural and physiological characteristics including H_2S production, nitrate reduction, deep glucose agar test, fermentation of different carbohydrates etc. All these characteristics were then compared with the standard description of ''Bergey's Manual of Determinative Bacteriology'', 8th edition. (Buchanan and Gibbons, 1974).

Determination of probiotic efficiency of *L. plantarum* MG1 and *L. delbrueckii* MT4

pH tolerance and Temperature sensitivity

The *Lactobacillus* cultures were inoculated into sterile MRS broth tubes of varying pH, i.e., 2, 4, 7 and 9 and incubated at 37 °C for 24-48 h. Another set of inoculated MRS broth was grown at varying temperatures, *i.e.,* 27, 37, and 45 °C for 24-48 h. The absorbance of MRS broths were taken at 600 nm by a spectrophotometer (Model T60U, pg instruments, UK) to measure microbial load.

Bile salt and NaCl tolerance

The MRS broth media with varying concentrations of bile salt (0.5, 1.0 and 2.0%) and NaCl (1, 3 and 7%) were inoculated separately with each *Lactobacillus* culture and incubated at 37 °C for 48 h. Then the absorbance of MRS broths were taken at 600 nm by a spectrophotometer for measuring microbial load.

Lactose utilization

The acid production by *Lactobacillus* cultures was detected by observing the change in color of the medium. Sterilized fermentation medium (Peptone 10 g, NaCl 15 g, phenol red 0.018 g, lactose 5 g, for 1 L distilled water and final pH 7.0) was inoculated with *Lactobacillus* cultures and incubated at 37 °C for 24-48 h. Change in color from red to yellow indicates the production of acid (Ahmed and Kanwal, 2004).

Antibiotic susceptibility test

The antibiotic susceptibility of *Lactobacillus* was assessed by using Kirby-Bauer discs diffusion method on MRS agar plates. The used antibiotics were penicillin g (10 IU), chloramphenicol (30 µg), erythromycin (15 µg), cefixime (5 µg), cephradine (30 µg), streptomycin (10 µg) and rifamycin (5 µg).

Determination of bacteriocin production capability of the *L. plantarum* MG1 and *L. delbrueckii* MT4

This experiment has been carried out according to the method described by Yang *et al*., 2012. 1 mL of frozen *Lactobacillus* isolate was cultured 24 h in 20 mL MRS broth. Then 1 mL culture was sub-cultured 24 h in 20 mL MRS broth. Cells were removed by centrifuging at 9000 rpm for 15 min. The supernatant was filtered through a sterile Whatman No. 1 filter paper and 100 µL of the pH unadjusted aliquot of cell free supernatant (CFS) was added to the first well. The remaining CFS was adjusted to pH 6.0 with 1M/IN NaOH in order to rule out possible inhibitory effects due to organic acids. 100 µL of the pH adjusted CFS was filtered and added to the second well. The neutralized CFS was then treated with 1mg/mL of catalase (Merck KGa A, Germany) at 25 °C for 30 min to eliminate the possible inhibitory action of H_2O_2 and filtered. Then 10 µL catalase treated CFS was placed in the third well. If inhibition zone were found in the third well, the isolates were considered to be able to produce bacteriocin or BLS). To confirm the production of a proteinaceous compound, CFS displaying antimicrobial production after acid neutralization and H_2O_2 elimination were treated with 1 mg/mL of proteolytic enzymes including papain and trypsin (Sigma-Aldrich Corporation, USA). 5 mL of bacteriocin was taken in test-tubes and treated with papain/trypsin (1 mg/mL) at pH 7. The test tubes with and without the enzyme (control) were incubated at 37 °C for 2 h and then heated at 100 °C for 3 min to denature the enzyme. Both the control and samples were assayed for antimicrobial activity by using agar well diffusion method.

Heat stability and Effect of pH on the crude Bacteriocin or BLS

Five millilitres of crude bacteriocin in different test-tubes was taken and then heated at 37, 45, 60, and 100 °C for 15 min respectively. In another set, 5 mL of crude bacteriocin or BLS was taken in test-tubes and the pH of the contents were adjusted to pH 2, 4, 7 and 9 separately, using either diluted NaOH or HCl and allowed to stand at room temperature for 2 h. The heat and pH treated crude bacteriocin or BLS samples were then assayed for antimicrobial activity. Agar well diffusion method was used and 100 µL of sample was added in each well (Nithya, *et al*., 2012).

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Bio-preservative efficiency of crude Bacterioicn or BLS

Vegetables (Tomato, Bean, Carrots, Cucumber and Ginger) were added with 5% of crude bacteriocin or BLS of *L. plantarum* MG1 and *L. delbrueckii* MT4 separately and kept at room temperature for 48 h. The controls were maintained without adding crude bacteriocin or BLS. After 24 h of incubation, the samples (both test and control) were serially diluted up to 10^{-6} and the plates were incubated at 37 °C for 24 h. The colony count was recorded and compared with the control (without crude bacteriocin or BLS) (Vinod *et al*., 2006).

RESULTS AND DISCUSSION

The present study aimed to isolate *Lactobacillus* spp. from vegetables, evaluation of their probiotic potentiality and assessment of their bacteriocin or bacteriocin-like-substance (BLS) production capability. The *Lactobacillus* isolates were primarily screened for their antibacterial activity as probiotic *Lactobacillus* should be good antimicrobial producer. *L. plantarum* MG1 showed antibacterial activity against *S. aureus* (15 mm), *E. coli* (13 mm), *S.* Typhii (14 mm), and *P. aeruginosa* (17 mm). *L. delbrueckii* MT4 also showed antibacterial activity against *S. aureus* (11 mm), *E. coli* (12 mm), *S.* Typhii (9 mm) and *P. aeruginosa* (16 mm). Both of them didn't show any antibacterial activity against *B. subtilis*.

Isolation, identification and screening of LAB on the basis of their antibacterial activity

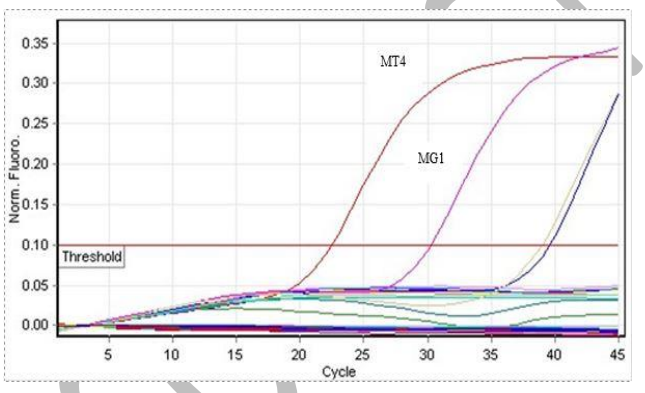

Figure 1. Fluorescence chart produced in real-time PCR. *L. plantarum* MG1 and *L. delbrueckii* MT4 crossed the threshold of Ct (Cycle threshold) value indicating closely relatedness to the genus *Lactobacillus.*

Total 38 LAB isolated from the vegetables samples by using MRS medium. Among the 38 isolates only 8 (21%) isolates showed potential antibacterial activity against the test pathogens. The isolates were identified by using combination of real time PCR (qPCR) using forward primer (5´-TGGAAACAGRTGCTAATACCG-3´) and reverse primer (5´-GTCCATTGTGGAAGATTCCC-3´), and conventional methods. Among them 3 isolates showed closely relatedness with the *Lactobacillus* genus in qPCR (Figure 1). The species was confirmed by conventional methods using cultural, morphological characteristics and biochemical reactions as described in ''Bergey's Manual of Determinative Bacteriology'', 8th edition. (Buchanan and Gibbons, 1974) (Table 1). Finally, we selected two *Lactobacillus* spp. such as *L. plantarum* MG1(Ginger) and *L. delbrueckii* MT4 (Tomato) for further experiments.

Evaluation of probiotic potentiality of *L. plantarum* MG1 and *L. delbrueckii* MT4

pH tolerance, Temperature sensitivity, Bile salt and NaCl tolerance

The growth of *L. plantarum* MG1 remain almost steady at pH 2-9 whereas *L. delbrueckii* MT4's optimum growth found at pH 7 and then growth rate decreased with increasing of pH (Figure 2a). The resistance to low pH is an important selection criterion for probiotic microorganisms, because gastric juice in the stomach destroys most microorganisms ingested. Many other studies have confirmed that the exposure of *Lactobacillus* strains to pH values of 2.5–4.0 does not influence their survival rate, but it dropped at lower pH values. The ability of lactobacilli to survive the passage through media with physiological pH of 2-3 (to mimic the stomach environment) was reported to be variable and strain dependent, but with a survival rate of approximately 85%, which is very significant for the probiotic (Belicova *et al*., 2013).

An efficient probiotic *Lactobacillus* must have the ability to survive at wide range of pH and temperature, tolerance to different concentrations of bile salt and NaCl, it can utilize lactose and resistant to several antibiotics. Both isolates were able to tolerate a wide range of temperatures (37–45 °C) but best growth found at 37 °C (Figure 2b). The reason for choosing this temperature range was to determine whether the *Lactobacillus* were able to grow within range of normal body temperature, survival within the human gut, an essential property of probiotics to show their effectiveness.

Tolerance to bile salts is a prerequisite for colonization and metabolic activity of bacteria in the small intestine of the host. This will help Lactobacilli to reach the small intestine and colon and contribute in balancing the intestinal microflora (Tambekar and Bhutada, 2010). Both isolates were able to tolerate or maintain their growth at 0.5-3% concentration of bile salt *in vitro* growth media (Figure 2c). *L. plantarum* MG1's optimum growth found with 1% bile salt whereas *L. delbrueckii* MT4's at 1-3%.

Both isolates were also able to grow well at 1-7% of NaCl concentrations and their best growth found with 7% NaCl (Figure 2d). NaCl is an inhibitory substance which may inhibit growth of certain types of bacteria. Hoque *et al*. (2010) observed that NaCl (1–9%) tolerance of their *Lactobacillus* sp. isolated from yoghurts. The present experimental results of our study were also similar to the observations made by Pundir *et al*., (2013).

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Antibiotic susceptibility

The small-scale antibiotic susceptibility testing revealed that *L. plantarum* MG1 is resistant to penicillin G, chloramphenicol, erythromycin, cefixime, chephradine, streptomycin and rifamycin whereas *L. delbrueckii* MT4 is resistant to cefixime and streptomycin and sensitive to penicillin G (28 mm), chloramphenicol (38 mm), erythromycin (32 mm), chephradine (24 mm), and rifamycin (28 mm) (Figure 3).

Table 1. Morphological, cultural and biochemical characteristics of *L. plantarum* MG1 and *L. delbrueckii* MT4.

Parameters	***L. plantarum* MG1**	***L. delbrueckii* MT4**
Colony characteristics		
Form	Circular	Irregular
Elevation	Raised	Umbonate
Margin	Entire	Curled
Surface	Smooth	Radiate
Color	Whitish	Cream
Slant Character (Growth)	Echinulate	Beaded
Broth Character (Growth)	Sediment	Turbid growth with sediment
Microscopic Observations (vegetative cell)	Short rod, single, double and cluster, 2.9-3.68 µm in length and 1.56-2.2 µm in width.	Short rod, mostly single, diplobacilli and slightly curved. 2.9–3.87 µm in length and 1.95–2.31 µm in width.
Gram staining	Gram positive	Gram positive
Spore staining	Non spore former	Non spore former
Acid fast staining	Non acid fast	Non acid fast
Motility test	Non motile	Non motile
Indole test	Negative	Negative
Methyl Red (M.R) test	Negative	Negative
Voges Proskauer (V.P) test	Negative	Negative
Deep glucose agar test	Strict aerobes	Strict aerobes
Glucose broth (Growth)	Sediment	Turbid growth with slightly sediment
Growth in synthetic media	Sediment	Turbid
Growth in inorganic salt	No growth	No growth
H_2S production test	Positive	Positive
Urease test	Negative	Positive
Nitrate reduction test	Negative	Positive
Citrate utilization test	Negative	Turbid with sediment
Catalase activity	Negative	Negative
Starch hydrolysis	Negative	Negative
Casein hydrolysis	Negative	Negative
Egg albumin test	Negative	Negative
Gelatin liquefication	Negative	Negative
Fermentation test		
Acid production with gas formation	Lactose	-
Acid production without gas formation	Glucose, Fructose, Galctose, Sucrose, Inulin.	Glucose, Fructose, Galactose, Sucrose, Inulin, Mannitol, Rhamnose, Raffinose and Starch.
Alkali production without gas formation	Mannitol, Rhamnose, Xylose and Raffinose.	-
No Change	Maltose and Starch.	Maltose, Lactose and Xylose

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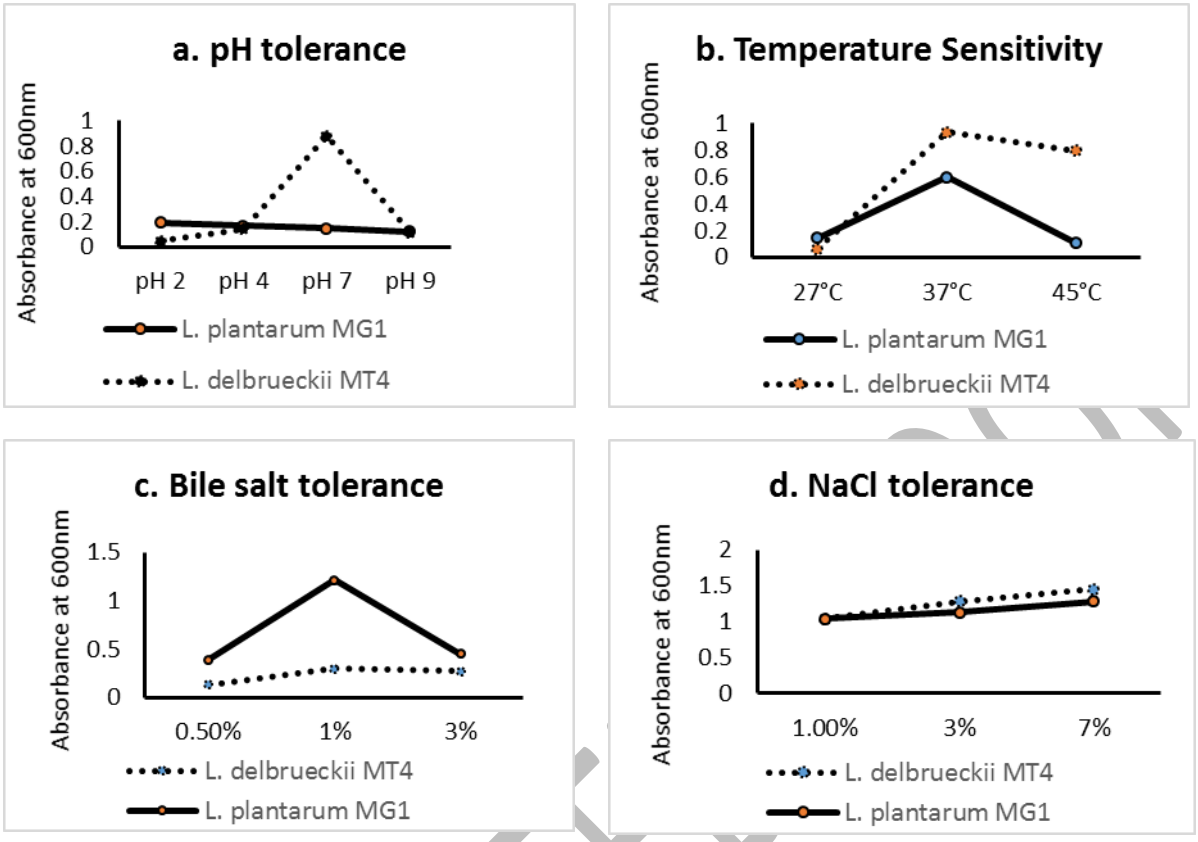

Figure 2. *Lactobacillus plantarum* MG1 and *L. delbrueckii* MT4 can tolerate a wide range of pH (2-4) (a), temperature (27-45°C) (b), bile salt (0.5-3%) (c) and NaCl (1-7%) (d).

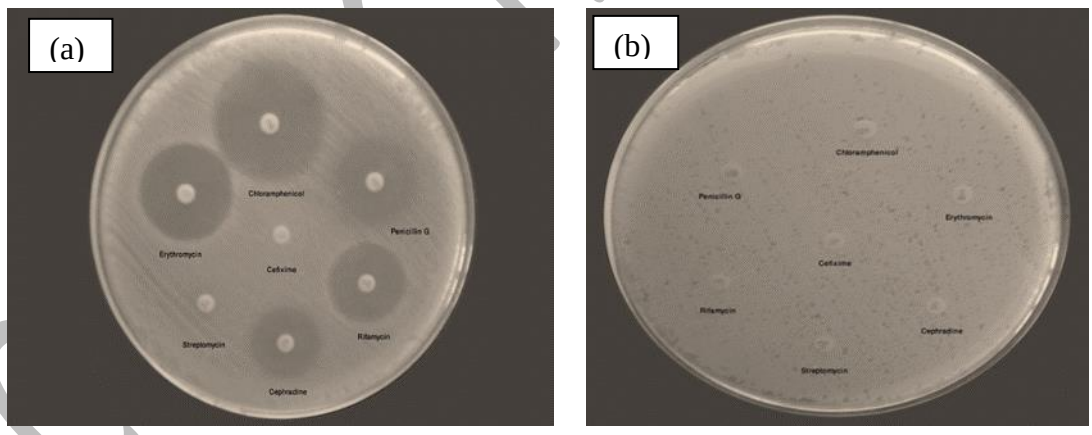

Figure 3: Antibiotic susceptibility pattern of: (a) *L. plantarum* MG1 and (b) *L. delbrueckii* MT4.

In this study both isolates could utilize lactose *in vitro* growth medium. Pundir *et al*., (2013) reported that lactose intolerant people cannot metabolize lactose due to the lack of essential enzyme β–galactosidase. When they consume milk or lactose containing products, symptoms including abdominal pain, cramping and diarrhea arise. If lactose passes through from the small intestine, it is converted to gas and acid in the large intestine by the colonic microflora. The studies provide that the addition of certain starter cultures to milk products, allows the lactose intolerant people to consume those products without the usual rise of breath hydrogen or associated symptoms (Pundir *et al*., 2013).

Lactobacillus delbrueckii MT4 was resistant to cefixime (5 µg) and streptomycin (10 µg) but *L. plantarum* MG1 was resistant to all the tested antibiotics (Figure 3).

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Such resistance to a wide spectrum of antibiotics indicated that if isolated probiotics induced in patients treated with antibiotic therapy may be helpful in faster recovery due to rapid establishment of desirable microbial flora. Resistance of the probiotic strains to some antibiotics could be used for both preventive and therapeutic purposes in controlling intestinal infections (EI–Naggar, 2004).

Lactobacillus plantarum is applied to preservative processes where they can contribute to the production of antimicrobial substances. Genome sequencing and comparative genomics have revealed a high genomic diversity and flexibility of *L. plantarum*, which can contribute to its success in diverse niches and applications. Although exact mechanisms of these effects are still not defined, some of these could contribute to immunomodulation of the host, competitive exclusion of pathogens, production of antimicrobial substances including bacteriocins and antioxidants in specific niches (Ratsep *et al*., 2014).

Considering above mentioned facts and empirical observations, it could be stated that our isolated *L. plantarum* MG1 and *L. delbrueckii* MT4 would be potential candidates for probiotic after successfully passing further safety and efficacy assessment and *in vivo* experiments.

Determination of bacteriocin or BLS production capability of the *L. plantarum* MG1 and *L. delbrueckii* MT4

To determine the presence of bacteriocin or BLS in the CFS of *L. plantarum* MG1 and *L. delbrueckii* MT4, the CFS has been neutralized to diminish the antimicrobial activity of organic acids and treated with catalase enzyme for reducing antimicrobial effect of H_2O_2 (Table 2; Figures 4-5).

If the CFS of *Lactobacillus* growing in MRS broth able to show antimicrobial activity after pH neutralization and catalase treatment, the isolates may be considered as bacteriocin or BLS producer (Yang *et al*., 2012). In this study, same test pathogens used to determine whether *Lactobacillu*s could produce bacteriocin or BLS. We found both isolates produce crude bacteriocin or BLS which has antibacterial activity against *E. coli* and *S.* Typhii (Table 2). The crude bacteriocin or BLS of *L. delbrueckii* MT4 also active against *S. aureus* (Figure 4). The crude bacteriocin or BLS of both isolates do not have any antibacterial activity against *P. aeruginosa*. The crude bacteriocin or BLS of both isolates lost its antibacterial activity after treatment with proteolytic enzymes (papain and trypsin) *i.e.*, they are proteinaceous in nature (Figure 5). Fircourt *et al*., (1994) demonstrated that LAB synthesizes bactericidal agents that vary in their spectra of activity and many of these agents are bacteriocins which are proteinaceous in nature. Our findings were also supported by the observations made by Nithya *et al*., (2012).

Effect of temperature and pH on the antibacterial activity of the crude bacteriocin or BLS of *L. plantarum* MG1 and *L. delbrueckii* MT4

The crude bacteriocin or BLS of both isolates retain their antibacterial activity at wide range of temperature (37 °C to 100 °C for 15 min) and pH (2–9 for 2 h) (Figure 6). But the maximum antibacterial activity observed after 37 °C heat and pH 2 treatments. The heat stability of the crude bacteriocin or BLS constitutes an advantage for potential use as biopreservatives in combination with thermal processing in order to preserve food products. Chen *et al*., (2013) investigated antibacterial properties of *L. plantarum* isolated from traditional fermented mustard. It was found to produce BLS against *Streptococcus mutans* BCRC10793. In addition, this BLS exhibited a strong antibacterial activity, heat stability (15 min at 121 °C) and pH stability (pH 2–4) against *S. mutans* BCRC10793, sensitive to proteolytic enzyme making it potential candidate for antibacterial agents. Jayachitra *et al*., (2012) also reported about bacteriocin producing *L. plantarum* where the effect of pH (2-12) on the crude protein extract was studied and the activity was not affected in pH 3-9. Boris *et al*., (2001) reported that the inhibitory activity of CFS of *L. delbrueckii* subsp. lactis UO004 was unaffected after 30 min at 100 °C, indicating that the bacteriocin is relatively resistant to heat, although inactivation was achieved after 60 min at 100 °C or after autoclaving at 121 °C for 5 min. On the other hand, the activity was remarkably stable between pH 3.0 and 10.0, as no difference was detected in the diameter of the inhibition halos at any pH (Boris *et al*., 2001).

Table 2: Determination of bacterion or BLS production by *L. plantarum* MG1 and *L. delbrueckii* MT4 by eliminating antibacterial effect of acids and H_2O_2.

Test Organisms	Raw CFS		CFS adjusted at pH 6		pH adjusted CFS after catalase treatment	
	L. plantarum MG1	*L. delbrueckii* MT4	*L. plantarum* MG1	*L. delbrueckii* MT4	*L. plantarum* MG1	*L. delbrueckii* MT4
E. coli	24	25	23	23	21	22
S. typhii	30	30	29	27	25	23
P. aeruginosa	21	16	14	11	0	0
S. aureus	15	18	12	17	0	16

*Mean value (diameter of zone of inhibition in mm) of triplet sets of tests.

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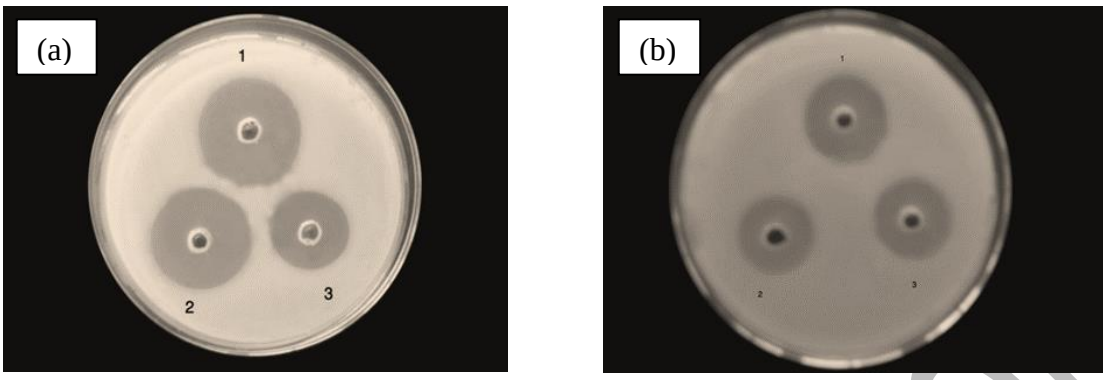

Figure 4: Production and antibacterial activity of bacteriocin or BLS (3) by (a) *L. plantarum* MG1 against *E. coli* and (b) *L. delbrueckii* MT4 against *S. aureus*.

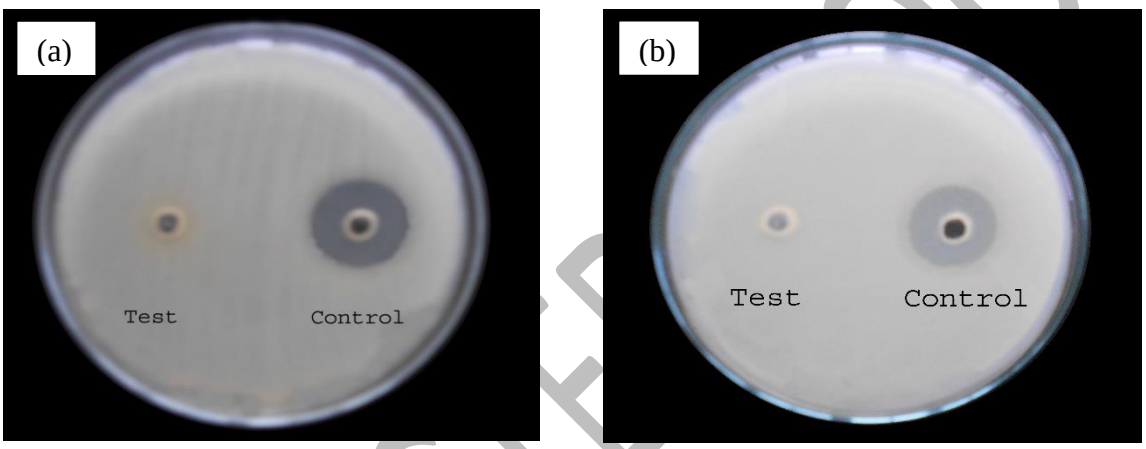

Figure 5: Proteolytic enzymes could eliminate antibacterial activity of the crude bacteriocin or BLS of: (a) *L. plantarum* MG1 (pappain) and *L. delbrueckii* MT4 (Trypsin). Crude bacteriocin or BLS without adding any proteolytic enzymes was used as control.

Bio-preservative efficiency of the crude bacteriocin or BLS

It was also observed that the 5% crude bacteriocin or BLS of both isolates could decrease microbial load significantly in the vegetable samples after 48 h of preservation at room temperature (Table 3). The 5% crude bacteriocin or BLS of *L. plantarum* MG1 decreased 68% initial microbial load of bean after treatment at room temperatures for 48 h whereas *L. delbrueckii* MT4's crude bacteriocin or BLS showed more efficiency which decreased 73%. In case of carrots, crude bacteriocin or BLS of *L. plantarum* MG1 decreased 79% of initial microbial load whereas *L. delbrueckii* MT4 decreased 77%. In case of tomato, crude bacteriocin or BLS of *L. plantarum* MG1 showed more efficiency in decreasing microbial load than *L. delbrueckii* MT4 where *L. plantarum* MG1 decreased 69% and *L. delbrueckii* MT4 decreased 54% of initial microbial load. In case of cucumber, crude bacteriocin or BLS of *L. plantarum* MG1 decreased 69% whereas *L. delbrueckii* MT4 decreased more than that (74%) of initial microbial load, and in case of ginger, crude bacteriocin or BLS of both isolates showed similar performance in decreasing initial microbial load 73% and 71% respectively. This very experimental data indicates that crude bacteriocin or BLS of both isolates could possibly be used in the preservation of vegetables if these crude bacteriocin or BLS are further purified and there is no toxicity, hypersensitivity or any other side effects to the human health. Nithya *et al*., (2012) used 5% crude bacteriocin of *L. fermentum* to the preservation of milk and mushroom and found satisfactory results. Udhayashree *et al*., (2012) successfully tested 5% crude bacteriocion to the preservation of apple juice and fish in similar way. Ensuring bacteriocin effectiveness when supplemented to the food it should be tested against specific target microorganisms in the type of food for which they are intended to be used. Most of the bacteriocins kill the susceptible bacteria by inducing permeabilization and pore formation on the cytoplasmic membrane or by interactions with essential enzymes. Because bacteriocins are degraded by the proteolytic enzymes of the gastrointestinal tract and seem to be non-toxic and non-antigenic to animals and humans they can be used to improve the safety and shelf-life of many food products (Dinev *et al*., 2017).

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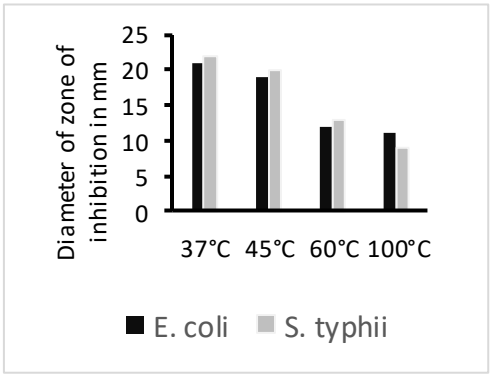
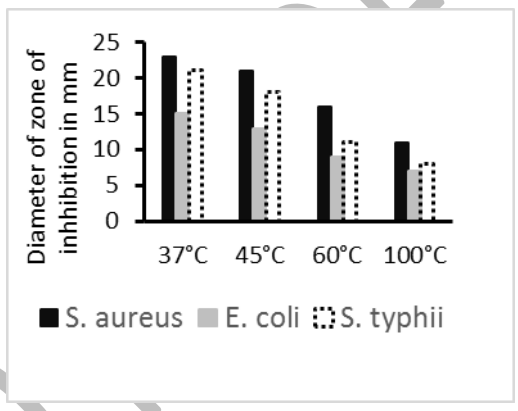
Table 3: Bio-preservative efficiency test of the crude bacteriocin or BLS on vegetables reveals that they can reduce microbial load in lab preservation of vegetables.

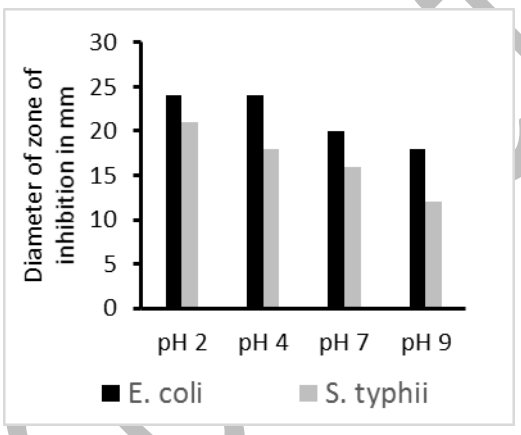
Vegetables	Control (CFU/mL)	*L. plantarum* MG1 (CFU/mL)	*L. delbrueckii* MT4 (CFU/mL)
Bean	13.4×10^6	4.2×10^6	3.6×10^6
Carrots	11×10^6	2.3×10^6	2.5×10^6
Tomato	10.5×10^6	3.3×10^6	4.8×10^6
Cucumber	11.1×10^6	3.4×10^6	2.9×10^6
Ginger	7.3×10^6	2×10^6	2.1×10^6



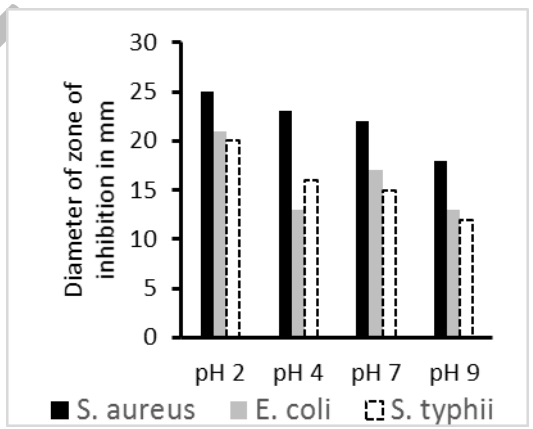
(a) Antibacterial activity of crude bacteriocin or BLS of *L. plantarum* MG1 after treatment at different temperatures.



(b) Antibacterial activity of crude bacteriocin or BLS of *L. delbrueckii* MT4 after treatment at different temperatures.



(c) Antibacterial activity of crude bacteriocin or BLS of *L. plantarum* MG1 after treatment at different pH.



(d) Antibacterial activity of crude bacteriocin or BLS of *L. delbrueckii* MT4 after treatment at different pH.

Figure 6: Crude bacteriocin or BLS of *L. plantarum* MG1 and *L. delbrueckii* MT4 can retain their antibacterial activity even after treatment at wide range of temperatures (37-100°C) and pH (2-9).

CONCLUSION

Lactobacillus plantarum MG1 and *L. delbrueckii* MT4 have showed poentiality to be probiotic and their crude extracts showed characteristics of bacteriocin or BLS. We hypothesized that if it will possible to purify these crude bacteriocin or BLSs, there is huge possibility to get new potential bio-preservatives.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

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