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Characterization of *Lactobacillus plantarum* as a Potential Probiotic in vitro and Use of a Dairy Product (Yogurt) as Food Carrier

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Abstract

Background and Objective: The current study was undertaken to check in vitro different characteristics of *Lactobacillus plantarum* as potential probiotic. These characteristics include viability of probiotic and pH during cold storage, tolerance to acid and bile, and antibiotic resistance.

Material and Methods: Samples of yogurt were stored at 4°C and analyzed in time 0, 1, 3, 7, 10 and 14 days of storage. In these periods, probiotic and starter cultures were enumerated and the pH parameter was analyzed.

Results and Conclusion: A gradual decline in pH was noticed throughout the storage. Counting of starter cultures decreased by 0.42 log cycle, and the probiotic's viability decreased by 0.68 log cycle at the end of storage, whereas the probiotic's viability in the samples subjected to re-pasteurization decreased by 0.30, 0.22 log cycles in the selective and reference media, respectively. The average viable cell counts of *Lactobacillus plantarum* decreased by 0.76, and 0.28 log cycles after incubation period (3 h) at 37ºC in the simulated gastric juice (pH 2.0 and 3.0), respectively. Generally, probiotic can maintain its viability by 76.672% in (1.0% w v $^{-1}$) bile. *Lactobacillus plantarum* was resistant to gentamicin, streptomycin, and vancomycin but susceptible to chloramphenicol, and tetracycline. Depended on these characteristics, *Lactobacillus plantarum* showed probiotic potential.

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1. Introduction

The awareness of using microorganisms to support an appropriate health and to prevent disease is not new. Primarily, several microorganisms have been used unintended in food industries such as dairy products and fermented vegetables [1]. The genus of Lactobacillus has an extended history of safe use, (particularly in dairy production), and plays a main role in the making of fermented milk products [2]. However, other species of lactobacilli as non-starter lactic acid bacteria species that are used in commercial probiotic products have been stated to provide some favorable effects through growth and action in the digestive tract such as save of normal gut microflora, improvement of the immune system, decrease of lactose - intolerance, and decrease of serum cholesterol amounts; this collection of bacteria and others are now often mentioned to as probiotics [3]. Varieties of microorganisms, typically food category lactic acid bacteria, have been assessed for their probiotic potential. Within the genus Lactobacillus, *Lactobacillus* (*L.*) *plantarum* is a member of the facultative hetero-fermentative group of lactobacilli [4]. *L. plantarum*, a mesophilic lactic acid bacteria, non-pathogenic is frequently isolated from food products and is widely used in the industrial production of fermented food. *L. plantarum* is one of the most studied lactic acid bacteria species, particularly because some strains are considered probiotic due to distinctive properties and because there is increasing consumer demand for nondairy-based probiotic products [5].

Today, there is a strong desire in the consumption of probiotic bacteria by using food products, especially probiotic dairy products; therefore, the preparation of dairy product involving probiotic bacteria is an important function [6]. Fermented milk products are the most common means of

carrying probiotic bacteria in food [7]. Probiotic food products are considered as an important collection of 'functional foods' [8]. Viability of probiotic bacteria (the amount of viable and active cells per g or mL of probiotic food products at the period of consumption) is the most serious value for these products because it decides their beneficial effectiveness. Consequently, it is essential to ensure high survival amount of the probiotic bacteria during production as well as during the storage time. To complete health benefits, probiotic bacteria should be viable and available in sufficient numbers of at least 1×10^6 CFU ml^{-1}or g^{-1} of product at the time of consumption [9]. Such high amounts could have been recommended to compensate for the potential decrease in the quantities of the probiotic organisms in passage through the gastrointestinal tract [10]. Standards for choosing a good probiotic strain have been registered by several researchers and comprise being of human origin, and a history of safe prolonged intake by a particularly sensitive population (infants), and they are adapted to reside in the human digestive tract and to interact with us in symbiosis. Main criteria for selection of probiotic include non-pathogenicity, survivability in stomach, maintaining their viability and metabolic activity in the intestine, bile salt tolerance, competition with pathogenic bacteria, and resistance to antibiotic [11,12]. Moreover, several technological characteristics have to be taken in a count when selecting of a probiotic bacteria-comprising viability during processing and stability in manufacturing and throughout storage [13].

Yet, many probiotics comprising food products fail to preserve the recommended probiotic concentrations at the time of consumption due to instability of probiotics in food carriers [14].

Therefore, the aim of this study was to use of dairy product (yogurt) as carrier for probiotic (*L. plantarum*) into gastrointestinal tract, and to evaluate viability of probiotic bacteria in dairy product during storage period, the effect of gastric juice, bile salt, and antibiotics on probiotic bacteria. Owing to probiotic resistance to bile salt and antibiotics in selective/differential media, enumeration of *L. plantarum* from the lactic acid bacteria starters are used in fermented dairy products (yogurt).

2. Materials and Methods

2.1. Preparation of inocula

Probiotic bacterium *L. plantarum* PTCC 1745 (DSM 20174) was obtained from the Department of Food Science, Engineering and Technology, Faculty of Agricultural Engineering and Technology, University of Tehran. It was maintained at (-80°C) in 40% v v^{-1} glycerol, when required; then it was sub-cultured three times in de Man Rogosa Sharp (MRS) broth (Hi-Media, Mumbai, India) at 37°C overnight for routine analysis. The bacterium was categorized by cell morphology, and biochemical procedures. After (17-22 h of incubation) the cells were collected by centrifugation at 6000 rpm, 4°C for 10min. The pellet was washed twice in 0.85% sterile saline solution (pH 7.0) before suspension in UHT milk (1.5% fat). The purity of cultures was observed continually and at the start of each experiment by Gram staining [15]. The microbiological analysis was performed in time 0 at the end of fermentation, after 1, 3,7,10 and 14 days of cold storage.

2.2. Yogurt manufacture

Yogurt production took place at the plant in the Department of Food Science, Engineering and Technology, Faculty of Agricultural Engineering and Technology, University of Tehran. Yogurt production starts by the heating of raw milk to 85-90°C for 30 min.to destroy any undesirable bacteria that can create spoiled milk or are pathogenic. After pasteurization, milk is cooled to the preferred incubation temperature, commonly between 40°C and 43°C. Starter cultures for yogurt were added immediately, and consist of two organisms, *L. delbrueckii subsp. bulgaricus* and *Streptococcus* (*S.*) *thermophilus.* These two species are the only cultures required in products which so-called "Yogurt" according to the Code of Federal Regulations to be present [16]. The temperature of the mixture then preserved around 42°C for (3 h) until the pH arrives nearly 4.5.The probiotic culture (*L. plantarum*) was added immediately after completion of yogurt fermentation, just before transferring the samples to cold storage at a concentration of 5% v v^{-1} which was equivalent to more than 1×10^6 CFU g^{-1} at the time of inoculation. Probiotic was also added to another samples of yogurt were subjected to re-pasteurization by heating at 75°C for 15 min to kill all starter cultures at the end of fermentation just before transferring the samples to cold storage at a concentration of 5% (v v^{-1}).

2.3. Diluent of peptone and water

Bacteriological peptone-saline water and water diluent (0.15%, w v^{-1} peptone; 0.85%, w v^{-1} saline) were prepared by dissolving 1.5 g and 8.5 g of bacteriological peptone medium (Hi-Media, Mumbai, India), and pure Sodium chloride, respectively in 1L of distilled water. The pH was regulated to 7.0 ± 0.2 followed by sterilizing by autoclaving at 121°C for 15 min [17].

2.4. Selective media

Yogurt and fermented dairy products are examples of foods, which always involve mixtures of different lactic acid bacteria, therefore, the presence of many types of lactic acid bacteria that are closely related species in dairy products make the differential, enumeration, and isolation of probiotic and yogurt starter culture very difficult to perform due to similarity in growth requirements, morphology, and biochemical reaction [8].

2.5. Reference medium

MRS (de Man Rogosa Sharp) (Hi-Media, Mumbai, India) medium to be consider free selective chemical agents was depended as a reference medium because MRS medium supported optimum growth of "lactic acid bacteria " in general [18].

2.6. Counting of starter cultures

Counting of starter cultures *(L. delbrueckii subsp. Bulgaricus* and *S. thermophiles*) can be done by difference of colonies of *L. plantarum* on selective medium and the total counts of reference medium [19].

2.7. Enumeration of *L. plantarum* in combination with other cultures by selective media

MRS-vancomycine agar was prepared according to the method described by Ong and Shah [19]. The medium was prepared by adding 2 ml of 0.5 mg ml^{-1} vancomycin solution to 1 L of fluid MRS agar just before pouring to get 1 mg l^{-1} of ending concentration. Lapsiri et al. demonstrated that all strains of *L. plantarum* have resistance to vancomycin in their study [20]. MRS-bile (0.2%) agar was prepared according to method described by Tharmaraj and Shah [17]. Then 2 g of pure bile salts (Sigma-Aidrich, USA) to 1 L was added to gain 0.2% MRS-bile agar. According to Sanders et al. Lactobacillus strains that might grow in typical physical bile concentration. Agar powder was added to each broth at the rate of 1.8% and the medium was autoclaved at 121 ˚C for 15 min [17,21].

2.8. Microbiological analysis and pH determination

One gram of probiotic yogurt sample was diluted aseptically with 9 ml of 0.15% peptone water (HiMedia, Mumbai, India) and mixed homogeneously by a vortex mixer. Then serial dilutions were organized and viable numbers were enumerated using pour plate technique. The plates were incubated at 37˚ C for 48-hr.The cell counts plates containing 25 to 250 colonies were counted and documented as colony forming units (CFU) per gram of yogurt. The viable cell counts were identified as $\log_{10}$ value. All tests and analyses were repeated at least twice.

The results offered are average of triplicates. Counts of *L. plantarum* were enumerated on MRS-vancomycine agar, MRS-bile (0.2%) agar. The pH value of the yogurt was determined by the pH meter (GLp22, CRISON. EEC.). By inserting the electrode directly into the yogurt sample.

2.9. Tolerance to low pH (tolerance to simulated gastric juice)

To determine the transit tolerance over the simulated gastric juice, the procedure of Vinderola and Reinheimer was used with slight modifications. Simulated gastric juice consisted of filter-sterilized pepsin (SIGMA-AIDRICH, Germany) (0.3% w v^{-1}) and NaCl (0.5% w v^{-1}) adjusted to pH 2.0 and 3.0. Overnight cultures (30 ml) were centrifuged (6000 ×g, 20 min, 5°C) and the pellets were washed twice with 0.85% sterile saline solution (pH 7.0) to eliminate the media [22]. Then they were re-suspended in 3 ml of the same buffer. One milliter of the washed cell suspension was collected by centrifugation (12.000 ×g, 20 min, 5°C) and suspended in 10 ml of gastric solution at pH 2.0 and 3.0. Total viable counts of *L. plantarum* were done on MRS agar, before and after an incubation period of 3 h at 37°C.

2.10. Bile resistance

The ability of *L. plantarum* to grow in the presence of bile was determined according to the method of Vinderola and Reinheimer [22]. Each culture was inoculated (2% v v^{-1}) into MRS broth with 0.3, 0.5 or 1% (w v^{-1}) of bile (sigma-aldrich, USA).The cultures were incubated at 37°C and, after 24 h, A560 nm (Spectrophotometer CECIL CE 2502 2000 SERIES, Bio-Quest, UK) was determined and compared to a control culture (without bile salts). The results were stated as the percentage of growth (A560 nm) in the presence of bile salts compared to the control.

2.11. Antibiotic resistance assay

The antibiotic sensitivity of probiotic bacteria was determined by the Bauer-Kirby method [23]. According to this method, the optical density at 600 nm of the overnight culture was adjusted to 0.08-0.1 (correspondent to 1-2 × 10^8 CFU ml^{-1}). The culture were spread evenly over the entire surface of the MRS agar plates; then the antibiotics paper discs were put on the plates. After incubation at 37°C for 24 hr., the inhibition zones were determined inclusive of the diameter of the discs. The results were stated as susceptible(S), intermediate (I) and resistant (R).

2. 12. Statistical analysis

The data were assessed using the analysis of variance (ANOVA) with a level of significance at ($p \leq 0.05$). All statistical analyses were performed using the SPSS software (version 22), and the means of treatments were compared using Duncan's test.

3. Results and Discussion

3.1. Changes in pH during the manufacture of yogurt and cold storage

Yogurts were prepared after the symbiotic growth of the two bacteria: *S. thermophiles* and *L. delbrueckii* ssp. *bulgaricus* [10]. Figure 1 shows the changes in pH for yogurt with probiotic and yogurt without probiotic as control. Observations were made after completion of the yogurt fermentation, during the 14-day storage of probiotic yogurt. Over 14 days of storage of all yogurt formulations at 4˚C, the mean pH values were significantly decreased. pH deceased significantly during storage period in samples containing probiotic from 4.10 at 0 h to 3.71 at 14 d. pH of samples

subjected to re-pasteurization before inoculating with probiotic changed from initial value of 4.11 at 0 h to 3.98 at 14 d. Significant differences ($p \leq 0.05$) in the mean of the pH values for control yogurt (without probiotic) and the initial pH (4.10 at 0 h) decreased to (3.74 at 14 d) (Figure 1).

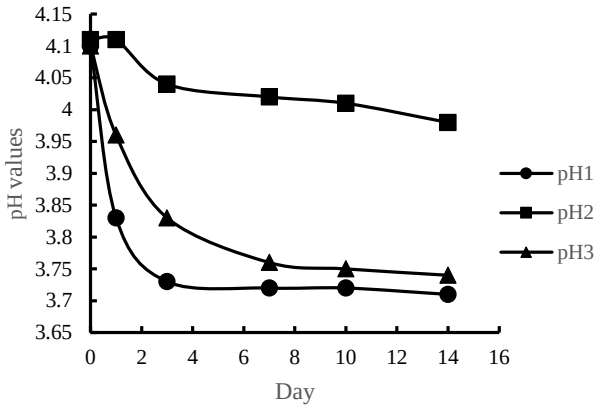

Figure 1. Changes in the pH during storage of probiotic yogurt (*L. plantarum).* pH 1 represents the mean of pH values for yogurt plus *L. plantarum*, pH 2 represents the mean of pH values for yogurt was subjected to re-pasteurization before inoculating with *L. plantarum*, and pH 3 represents the mean of pH values for yogurt without *L. plantarum* as control. 0-14 d: Observations were made after completion of the yogurt fermentation; during 14- day storage of probiotic yogurt respectively

A few change observed in the samples subjected to re-pasteurization before probiotic addition at the end of fermentation under refrigerated temperature where the growth of *L. delbrueckii* ssp. *bulgaricus* responsible of over-acidification was stopped by re-pasteurization. Another reason for the substantial gradual decrease in pH values at the start of fermentation is the buffering capacity of yogurt (Figure 1) [14]. According to Mani-Lopez et al., the reduction in the pH values of yogurts and fermented milks during storage is due to post acidification [24]. Shah reported that decline in pH values and acid production during refrigerated storage is due to the continued microorganisms' activity [10]. Beal et al. demonstrated that yogurt might undergo an occurrence called post-acidification that is the drop of pH during storage due to permanent metabolic activity of the starter culture added to the product (mainly *L. delbrueckii* subsp. *bulgaricus*). Our results are consistent with these findings, supporting the remaining acidification during storage [25].

3.2. Viability of probiotic and yogurt bacteria at the end of fermentation and storage time

Table 1 shows changes in the average values of *L. plantarum* during storage period in selective media, lactic acid starter of yogurt in reference medium (MRS) plus *L. plantarum*, and the average value of lactic acid starter of yogurt without *L. plantarum* (yogurt bacteria). The average viable cell counts of *L. plantarum* significantly decreased from 8.00 ± 0.03 log CFU g^{-1} on time 0 to 7.32 ± 0.05 log CFU g^{-1} on the day 14 while that of lactic acid starter plus *L. plantarum* significantly decreased from 8.25 ± 0.04 log CFU g^{-1} to 7.70 ± 0.05 log CFU g^{-1} during the same time. The average viable cell counts of lactic acid starter without *L. plantarum* (yogurt bacteria) significantly decreased from 7.89 ± 0.05 log CFU g^{-1} on time 0 to 7.47 ± 0.04 log CFU g^{-1} on the day 14. The average viable cell counts of yogurt bacteria (lactic acid starter) can be obtained by subtracting the average value of *L. plantarum* in selective media from the average value of lactic acid starter plus *L. plantarum* in reference medium (MRS) (Table 1).

Table 1. Changes in average values (log CFU g^{-1} ± SD, n=3) of *L. plantarum* in selective media, lactic acid starter of yogurt in reference medium (MRS) plus *L. plantarum*, and the average value of lactic acid starter of yogurt without *L. plantarum* (yogurt bacteria) as obtained by subtraction method during the storage of probiotic yogurt

Period	Mean values of *L. Plantarum* in selective media*.	Mean values of Lactic acid starter plus *L. plantarum* in MRS§	Mean values of Lactic acid starter without *L. Plantarum* (yogurt bacteria)
[0]Zero time	8.00 ± 0.03^{c}	8.25 ± 0.04^{c}	7.89 ± 0.05^{c}
1 d	8.63 ± 0.03^{a}	9.05 ± 0.02^{a}	8.84 ± 0.02^{a}
3 d	8.21 ± 0.03^{b}	8.51 ± 0.03^{b}	8.20 ± 0.03^{b}
7 d	7.49 ± 0.03^{d}	7.78 ± 0.04^{d}	7.47 ± 0.05^{d}
10 d	7.40 ± 0.06^{e}	7.72 ± 0.04^{de}	7.45 ± 0.03^{d}
14 d	7.32 ± 0.05^{e}	7.70 ± 0.05^{e}	7.47 ± 0.04^{d}

Different letters in columns represent significant differences ($p \leq 0.05$).
*MRS-vancomycine agar and MRS-bile (0.2%) agar.
§ reference medium

The viable counts of all these bacteria (probiotic and lactic acid starter) significantly increased up to the day 1, and then decreased during the storage. There were significant differences ($p \leq 0.05$) among the viable counts of the above-mentioned bacteria during the refrigerated storage of probiotic yogurt. The viability losses of all bacterial populations (in selective and reference media) at day 7 under refrigerated storage are presented in Table 1. The viability loss of all these bacteria were gradual and stable during the storage. The viable count of the probiotic (*L. plantarum*)

remained higher than the standard limit for probiotic foods until the end of storage above (6 log CFU g^{-1} or ml $^{-1}$).

Table 2 shows changes of *L. plantarum* in selective media, and in reference medium (MRS) in probiotic yogurt subjected to the re-pasteurization before inoculating with *L. plantarum.* The average viable cell counts of *L. plantarum* significantly decreased from 7.74 ± 0.04 log CFU g^{-1} on time 0 to 7.44 ± 0.03 log CFU g^{-1} on the day14 in the selective media while the average viable cell counts of *L. plantarum* significantly decreased from 7.68 ± 0.05 log CFU g^{-1} to 7.46 ± 0.03 log CFU g^{-1} in the reference media during this time. There were significant differences ($p \leq 0.05$) among the average values of *L. plantarum* in the selective and reference media during the refrigerated storage of probiotic yogurt. The probiotic bacteria in both the selective, and reference media exhibited good stability during the refrigerated storage (Table 2).

Table 2. Changes (log CFU g^{-1} ± SD, n=3) of *L. plantarum* in selective, and reference media (MRS) in yogurt samples subjected to re-pasteurization before *L. plantarum* inoculation.

Period	Mean values of *L. plantarum* in selective media*	Mean values of *L.plantarum* in MRS§
[0]Zero time	7.74 ± 0.04^{b}	7.68 ± 0.05^{b}
1 d	8.03 ± 0.02^{a}	8.04 ± 0.03^{a}
3 d	7.71 ± 0.04^{b}	7.71 ± 0.03^{b}
7 d	7.48 ± 0.05^{c}	7.51 ± 0.05^{c}
10 d	7.47 ± 0.05^{c}	7.48 ± 0.05^{c}
14 d	7.44 ± 0.03^{c}	7.46 ± 0.03^{c}

Different letters in columns represent significant differences ($p \leq 0.05$).
*MRS-vancomycine agar and MRS-bile (0.2%) agar
§ Reference medium.

Several studies have revealed the low viability of probiotics in yogurt [10,26,27]. Our findings showed that *L. plantarum* retained an acceptable level of viability during the refrigerated storage of probiotic yogurt. These are in consistent with finding by Dave and Shah, they reported that the lactic acid production by *L. delbrueckii* subsp [26]. *Bulgaricus* throughout the storage of yoghurt (so-called post-acidification) is one of the influences identified to affect the viability of probiotic bacteria in these products. Several factors can influence the viability of probiotic bacteria. Previous studies have described that the most important contributing reasons for loss of cell viability are pH decrease during storage (post-acidification) and accumulation of organic acids as a consequence of growth and fermentation. Lourens-Hattingh and Viljeon reported that the viability of probiotic bacteria in fermented dairy products depends on many factors such as the strains used, interaction between the species present, culture conditions, chemical composition of the fermentation medium, ultimate acidity, milk solids content, availability of nutrients, growth supporters and inhibitors, intensity of sugars, concentration of inoculation, incubation temperature, fermentation time and storage temperature [28]. The low pH of fermented foods is one of the most substantial factors creating evident viability loss of probiotics [10,14,27]. The inhibition growth at pH values lower than 4.5 is associated to a decrease in the intracellular pH of bacteria caused by un-dissociated lactic acid form, which is attributed to its lipolitic nature, its pass freely through the cell membrane and causing failure in the electrochemical gradient, supporting inhibition or killing properties [29].

In addition, because of the absence of *L. delbrueckii* ssp. *bulgaricus* that is responsible for "over- acidification" by re-pasteurization, probiotic was more stable throughout the refrigerated storage (Table 2). Over- acidification is found to cause loss of viability of probiotic bacteria. *L. delbrueckii* ssp. *bulgaricus* produces sufficient hydrogen peroxide to display inhibition of probiotic. Hydrogen peroxide can react with other components to form inhibitory components [10]. In the current work, the yogurt starters including *S. thermophiles*, and *L. delbrueckii* ssp .*bulgaricus*, decreased at the end of storage. This may be attributed to secretion of inhibitory metabolites (e.g., bacteriocins) created by probiotic or starter cultures that could affect species of the same genus. De Vuyst and Leory showed that a decline of pH cause a decrease in the adsorption of the bacteriocin molecules to the producer cells, and consequently, in an increase bioavailability These findings are consistent with the results of [30,31,32]. Generally, at the end of storage of probiotic yogurt, there was a decrease of all populations possibly caused by adverse conditions, such as deficiency of nutrient, low pH, and low temperature.

3.3. Viability of probiotic bacteria after exposure to simulated gastric juice

The effect of simulated gastric juices on the viability of *L. plantarum* is shown in Table 3. The average viable cell counts of *L. plantarum* decreased from 7.92 ± 0.02 log CFU ml^{-1} on time 0 (before incubation period) in pH 2.0 to 7.16 ± 0.04 log CFU ml^{-1} after incubation period (3 h) at 37 ºC in pH 2.0. Whereas the average viable cell counts of *L. plantarum* decreased from 8.76 ± 0.03 log CFU ml^{-1} on time 0 in pH 3.0 to 8.48 ± 0.03 log CFU ml^{-1} after incubation period (3 h) at 37ºC in pH 3.0. There were significant differences ($P \leq 0.05$) among the viable counts of *L. plantarum* during the incubation period (3 h) at 37ºC in pH 2.0 and 3.0 (Table 3). Based on these results (Table 3), the viability of *L. plantarum* has established to be successful to meet the minimum criterion of 1×10^{6} viable probiotic cells per ml at pH 2.0 and 3.0 after exposure to simulated gastric juice for 3 h. Zago et al. counted still more than 6 log CFU ml^{-1} of nine strains of *L. plantarum* when subjected to simulated gastric juice after 90 min at pH 2.2 [4].

Table 3. Resistance of probiotics bacteria to low pH during 3 h at 37°C (n=3)

Probiotic	pH	Mean values of *L. plantarum* (log CFU m^{-1} ± SD)

		Before incubation (time 0)	After incubation (3 h at 37°C)
L. plantarum	2.0	7.92 ± 0.02[a]	7.16 ± 0.04[b]
	3.0	8.76 ± 0.03[a]	8.48 ± 0.03[b]

Different letters in rows represent significant differences ($p \leq 0.05$).

Around 2.5 L of gastric juice at a pH near 2.0 is excreted every day in the stomach, which result in the destruction of most of microorganisms consumed [22]. Therefore, resistance to human gastric transit is an essential selection criterion for probiotic; however, during the digestion process, the pH raises to almost 3.0 due to the existence of food [33]. Maragkoudakis et al. demonstrated that a good probiotic should survive at pH 3.0. They claimed that pH of stomach in individuals ranges from 1.0 during fasting, to 4.5 after meal, and food ingestion occurs during 3 h. Lourens-Hattingh and Viljeon [28] showed that the probiotics need to be viable and tolerant to the stressful conditions of the stomach and the anti-microbial activity of pepsin that act as effective barriers against the activity of bacteria in gut. In the current study, there was a reduction in the probiotics' counts, as they were exposed to pH 2.0 and pH 3.0 after 3 h of incubation at 37ºC. Our results are consistent with those obtained from prior similar studies where Lactobacillus strains were capable to retain their viability when exposed to pH around 3.0 but exhibited loss of viability at lower pH values [2,26]. Probiotic lactobacilli strains are exposed to extreme acid stress when they arrive at the gut where hydrochloric acid is existent. Some mechanisms control the homeostasis of internal pH. The proton-translocating ATPase is the greatest important for fermentative bacteria [34]. *L. plantarum* contains this enzyme in the cell membrane and its activity was discovered to be optimum at the pH range 5.0-5.5. The general proton permeability of the plasma membrane also participates to the adjustment of the internal pH. Least membrane permeability of *L. plantarum was* documented at pH 4.0, while that in the acid-sensitive organism was discovered at pH 6.0. It seems that proton- translocating ATPase's show main roles in moving protons out of the cells and in reducing their net permeability to protons [35].

3.4. Bile resistance

The concentration of bile in the gastrointestinal tract changes; thus, the concentrations of 0, 0.3, 0.5 and 1.0% (w v^{-1}) of bile were chosen for testing the resistance to bile. The results were stated as percentage against the values found in the medium without bile (control culture). Figure 2 and 3 show that the resistance of *L. plantarum* decreases after 24 h of incubation with increasing the bile concentration .There were significant differences ($p \leq 0.05$) in optical density were observed between the control and 0.5 -1% (v^{-1}) concentration of bile, but no Significant differences ($p>0.05$) were observed in optical density between the control and 0.3% (w v^{-1}) concentration of bile (Figure 2). After 24 h of incubation at 1.0% (w v^{-1}) bile, only 76.67% growth was noticed compared to the control (Figure 3). 0% bile operated as control for the research and it documented the highest growth. During exposure to bile, cellular homeostasis disorders causes the parting of lipid bilayer and integral protein of their plasma membranes, subsequent discharge of bacterial content, and eventually, cell death [36].

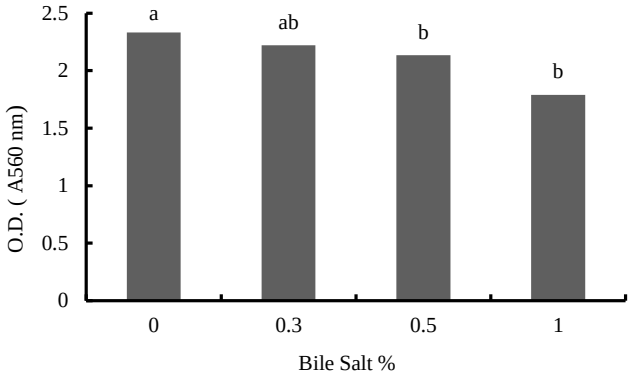

Figure 2. Effect of bile on the viability of *L. plantarum*. Different letters indicate significant differences ($P \leq 0.05$)

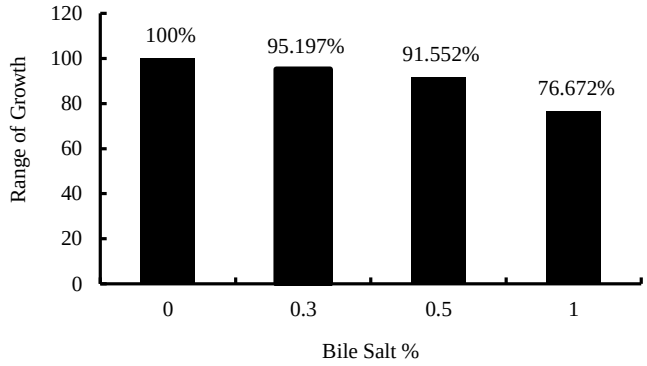

Figure 3. Growth (%) respect to a control in the presence of bile for *L. plantarum*

Lactobacilli have the capability to adapt in the environment of bile to reveal bile deconjugation activity as a defensive mechanism against the toxicity of conjugated bile salt. Additional essential element is the bile salt hydrolase (BSH) activity, which is related to the bile resistance. It is detected in several strains of Lactobacilli where BSH hydrolyse conjugated bile, and hence, decreases its bactericidal influence [20]. Liong and Shah showed that Lactobacillus ssp. from the stationary phase of static cultures apparently had higher BSH activities taking place at low pH [37]. It was assumed that high BSH deconjugation activity is correlated with the stationary phase of culture as a result of decreased pH levels in the medium. Lye et al. reported that deconjucation of bile salt and BSH activity is a mechanism by lactobacilli to eliminate or decrease the concentration of cholesterol in vitro [38].

3.5. Antibiotic resistance

Antibiotic disc diffusion susceptibility of *L. plantarum* is summarized in (Table 4). This bacterium is resistant to

gentamicin, streptomycin and vancomycin, whereas, it is sensitive to chloramphenicol and tetracycline (Figure 4). Our results of test bacteria displaying resistance to vancomycin are consistent with [20,39]. Holliman and Bonereported that vancomycin resistance in clinical isolates was limited to hetero-fermentative lactobacilli [40]. Ammor et al. reported that lactobacilli was commonly susceptible to antibiotics that inhibit protein synthesis such as chloramphenicol and tetracycline, but more resistant toward aminoglycosides such as streptomycin and gentamicin [29].

Table 4. Resistance of *L. plantarum* to antibiotics

Antibiotic	Disk symbol	Disk content (µg)	*L. plantarum* Effect
Chloramphenicol	C	30	Susceptible
Gentamicin	GM	10	Resistance
Streptomycin	S	10	Resistance
Tetracycline	T(TE)	30	Susceptible
Vancomycin	VA	30	Resistance

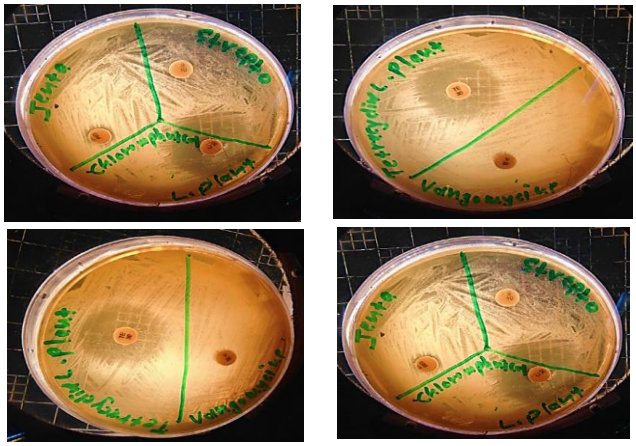

Figure 4. Resistance of *L. plantarum* to antibiotics.

4. Conclusions

This study showed how in vitro methods could be used for studying *L. plantarum* as potential probiotic. Probiotic bacteria have a long history of relationship with dairy products. Dairy products (e.g. yogurt) can provide an appropriate "food carrier" for probiotic into the gastrointestinal tract. Culture of probiotic remained viable at levels above the recommended 10^6 CFU g^{-1} after 14 d in the refrigerated storage; therefore, the concentration of initial inoculums is regarded as a critical factor. *L. plantarum* exhibited promising results for both acid and bile tolerance. Low pH and bile salt are two conditions encountered in passage through the gut. So it is not surprising that the bacteria developed controlled response to these two stresses.

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6. Conflict of interests

The authors declare no conflict of interest.

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