

CHARACTERIZATION OF *LACTOBACILLUS* SPECIES PROPOSED AS PROBIOTICS

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ABSTRACT

An isolated *Lactobacillus* from several various sources were identified depending on morphological, microscopical, and biochemical tests in vitro analysis of probiotic properties that included: an ability to tolerate in different concentrations of bile salt, survival in acidic conditions, their antimicrobial activity, and S-layer characterizations were carried out. It was noticed that isolates of *Lactobacillus rhamnosus* and *L. delbrueckii* have a broad activity of antimicrobial and found the isolate *L. rhamnosus* represented with a survival percentage of 6.9% at pH 4.5 and 5.1% at pH 2.0) also *L. rhamnosus* (5.7% at pH 4.5 and 4.9% at pH 2.0) tolerated acidic media, *Lactobacillus* spp. has antimicrobial activity against all gram-positive and negative tested isolates. 70 kDa of S-layer protein bands were detected with whole-cell SDS-PAGE analysis, and it's predominant in cells of isolates grown in MRS broth anaerobically. It was noticed that the collected *Lactobacillus* isolates could be used as pprobiotics

Keywords: *Lactobacillus* spp.; acidic tolerance; bile tolerance; S-Layer.

INTRODUCTION

All types of bacteria in the gastrointestinal tract (GIT) are influenced by the most important conditions; physiologic, e.g. (acidity, enzymes, and bile salt), and the existence of normal inhabitant bacteria of GIT. Since gastric juice is composed of acids, salts, and enzymes, it provides a chemical environment in the stomach that devastates the microorganisms found naturally there (Falk et al., 1998; Sghir et al., 2000). Lactic acid bacteria were found in a minor proportion with a hundred species of bacteria that were included in the complex microbial community of GIT. *Lactobacillus* species are non-pathogenic bacteria, positively react to Gram stain, and represent necessary microbiota in the lumen of the intestinal tract. The occurrences of *Lactobacillus* have been widely studied concerning their health-promoting effects and their role in maintain normally GIT habitat microbial balance and protects the intestine from infection. Moreover, the role of lactobacilli in reliefs of lactose intolerance improves food nutritional value, supports nonspecific immunity, and reduces serum cholesterol levels (Hooper et al., 1999; Perdigon et al., 2000; Suvarna and Boby, 2005; Kim et al., 2008). The benefits of *Lactobacillus* bacteria that are classified as probiotic in the Generally Recognized as Safe list (GRAS) to the host body have been extensively discussed in the literature. Their probiotic's beneficial properties have been proposed including their ability to produce the antimicrobial substance, their antagonism activity, their role to improve non-specific immunity, and their influence on gut metabolic activities (Marteau and

Rambaud, 2002). The majority of *Lactobacillus* spp. have been characterized as probiotics ability which has been isolated from humans and animals. These isolated species are considered to be a starter culture for fermented foods, particularly milk or milk-derived products (Caplice and Fitzgerald, 1999). Recently, different kinds of plants such as vegetables, fruits, medicinal plants, grains, and cereals have been produced and marketed in the form of lactic acid fermented plant beverages (FPBs) or foods for health-promoting in Thailand (Duangjitcharoen et al., 2009). Currently, *Lactobacillus* strains are predominantly administered as probiotic remedies in pharmaceutical forms of capsules or powders as well sold out as enriched yogurt or milk in the dairy and food markets (Sanders and Klaenhammer, 2001; De Roos, 2000). Bacterial strains of probiotic colonized GIT are dependent on their ability to adhere to intestinal epithelial cells through adherent factors. The process is mediated either non-specifically via physicochemical factors such as hydrophobicity or specifically by adhesive bacterial surface molecules and epithelial receptor molecules such as S-layer, fibronectin, and mucin binding proteins, which is essential for adherent and colonization (Altermann et al., 2004; Holzappel et al., 1998). S-layers monomolecular crystalline arrays and recognized in many different species of Bacteria and Archaea as an outermost structure of the cell envelope (Sára and Sleytr, 2000). A few pathogenic bacteria including *Clostridium difficile*, and *Bacillus anthracis* possess S-layer protein consisting of two types of S-layer subunits (Takeoka et al., 1991; Mesnage et al., 1997). Whereas among the lactic bacteria spp., S-

layers have been found in most species of the genus *Lactobacillus* (Masuda, 1992; Yasui, Yoda and Kamiya, 1995). The S-layer represents a typical surface structure in a lot of Lactobacilli spp. and has been demonstrated probiotic properties for human and animal consumers (Åvall-Jääskeläinen and Palva, 2005; Bernardeau et al., 2000). Thus, S-layers will be targeted in the future for their great potential roles in applications of Nanobio technologies.

Scientific hypothesis

The hypothesis of this work is to select qualified *Lactobacillus* spp. bacteria to propose as probiotic, and spotlight on particular properties such as S-layer and other properties that make *Lactobacillus* suitable candidates for probiotics.

MATERIAL AND METHODOLOGY

The study was done in laboratories of the Food Contamination Research Center/Iraq Ministry of Science and Technology. Fifty food samples (Dairy milk and cheese) were collected from local markets in Baghdad. The process was sampling and it lasted from February 2019 to September 2019.

Lactobacillus isolation

Lactobacillus isolation from food sources was performed following (Perelmuter et al., 2008); one g of food sample was mixed in 10mL of sterile normal saline then, stirred vigorously and let the suspension settle for 10 min at room temperature. One hundred μ L of the suspension was spread on previously prepared and sterile MRS medium (Oxoid LTD, limited, Hamshire, UK), all inoculated plates were incubated at 37 °C under aerobic conditions for 24 – 48 h. The growth colonies were purified and picked up for further analysis.

Lactobacillus Biochemical characterization

Lactobacillus isolates were tested to be based on utilizing various carbohydrates according to (Kandler and Weiss, 1986). The biochemical identification of the obtained isolates at the genus/species level was performed according to established phenotypic criteria, and for acid and gas production from glucose, arginine hydrolysis, and fermentation of L-arabinose, lactose, mannitol, melibiose, raffinose, ribose, sucrose, sorbitol, xylose, and salicin (Merk) as described by (Samells et al., 1995).

Bile salt and acidic tolerance

The minimal inhibitory concentration (MIC) of bile salt was evaluated for each of the *Lactobacillus* isolates, Then 20 μ L of bacterial growth, 1×10^8 (cfu.mL⁻¹), of each collected isolates were transferred to 980 μ L of newly prepared sterile MRS broth containing 3% bile salts and incubated at 37 °C for 2 h.

For bacteria survival rate examination under the acidic condition at 37 °C, all isolates were tested following (Perdigon et al., 2002); about 20 μ L of 1×10^8 (cfu.mL⁻¹) of each fresh isolates were moved to 980 μ L of an acidic buffer consisting of 3.5 g of D-glucose, 2.05 g from NaCl and 0.6 g of KH₂PO₄, 0.11 g of CaCl₂ and 0.37 g of KCl per liter, with adjusted pH to 2.0 and 4.5.

Then, isolates were examined for tolerance to bile salt and acid when samples were taken at 0 min and 2 h and plated on freshly prepared sterile MRS agar plates after making of serial dilution, and plates were incubated at 37 °C for 48 h, the plates were enumerated by choosing a couple of plates (30 – 300 colony) for each dilution.

Antimicrobial Activity

Lactobacillus antimicrobial activity was determined through the agar spot method, following (Casey et al., 2004; Baccigalupi et al., 2005), with minor modifications; briefly, about 5 μ L of (1×10^8 cfu.mL⁻¹) of freshly prepared growth to each obtained isolate was plated into agar spots made previously on the MRS agar plate. All inoculated plates were incubated at 37 °C for the development of spots. Each of the indicator bacteria (bacteria spp. used is mentioned below) was inoculated on Luria broth and incubated overnight at 37 °C with shaking. Then, the bacteria were mixed with 15 mL of Luria soft agar (0.7% agar, w/v) and dispensed onto MRS agar plates that were full of grown colonies of *Lactobacillus*. All plates were incubated overnight at 37 °C and the inhibition zones were subjected to measure and record (Bauer et al., 1996). The indicators were *Shigella dysentery*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Escherichia coli*.

Antibiotics Susceptibility of Lactobacillus isolates

Anti-Bacterial sensitivity of *lactobacilli* in vitro was studied via the disc diffusion method according to the guidelines of the National Committee for Clinical Laboratory Standards (Wayne, 2015). It was studied against eight antibiotics: Ampicillin 25 μ g, Chloramphenicol 50 μ g, Gentamycin 10 μ g Carbenicillin 100 μ g, Nalidixic acid 30 μ g, Methenamine Mandelate 3 μ g, and Cortimoxazole 25 μ g.

Protein extraction and SDS-PAGE

For extraction of S-layer, *Lactobacillus* spp. was cultivated anaerobically in MRS broth at 37 °C. In general, 100 mL of pre-warmed MRS medium was inoculated 1: 100 (v/v) with an overnight culture and cultivated until the optical density at 600 nm reached 0.4 and 0.7. Cells were harvested by centrifugation at 12000 \times g for 15 min at 4 °C. The cells were washed twice with 100 mL of ice-cold water. The cell pellet was extracted with 0.1 volume of 4 M guanidine hydrochloride (pH 7) for one hour at 37 °C and centrifuged at 12000 \times g for 15 min. The supernatant, containing S-layer protein monomers, was dialyzed against water at 4 °C for 16 – 24 h (Boot et al., 1993). The dialyzed extracts were analyzed by SDS-PAGE (Smit et al., 2001). SDS-PAGE of protein samples was carried out using Precision Plus Protein Standard (low molecular weight marker (10 – 250 kDa) – Hi-media). The samples were run on 12% polyacrylamide gel at 100 V. Protein bands were visualized by Coomassie blue staining. Protein concentration was determined according to Bradford's method (Bradford, 1976). For normalization of the measured absorption values, BSA (Hi-media) was used. Finally, all samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following Laemmli method on a vertical Slab gel

(Laemmli, 1970); Acrylamide gel in form of resolving 12% and stacking gel 4% was prepared and the samples were run at 100 V. Protein bands were seen after gel stained with Coomassie Brilliant Blue stain (Merck) R-250 (Dunn, 1993).

Statistical Analysis

The percentage of survival rate and the mean cell forming units were implemented by using EXCEL 2010 software.

RESULTS AND DISCUSSION

Isolation and identification of *Lactobacillus*

Based on the isolation of *Lactobacillus spp.*, the bacteria were positive to grow on MRS selective media, and when examined under the light microscope, all isolates acquired blue-purple stain hence they react positively when stained with Gram. Also, based on biochemical tests displayed in Table 1, all isolates were negatively reacting with Catalase. Results of gas production from glucose to all inoculated test tubes after observation for 2 – 3 days showed that all isolates were positive for gas production from glucose fermentation. Findings of Carbohydrates fermentation analysis were listed in positive and negative reactions. Outcomes of

testing the ability of isolates to grow at different temperatures showed the ability of all isolates to grow at 45 °C whereas no growth was seen at 10 °C and 15 °C.

Bile and acid tolerance

From the different concentrations of bile salts taken, 3% bile salts in MRS broth was the minimal inhibitory concentration of bile from most *Lactobacillus* isolates as shown in Table 2 and Figure 1, hence the survival of *lactobacillus* post 2 h. Incubation in MRS containing 3% bile salts was analyzed and the results are shown in Table 2, isolate of *L. delbrueckii* showed an excellent survival of 89%, LGG showed the ratio of survival 91%. Also, *lactobacillus* survival rates in acidic buffer with pH 4.5 and 2.0 were examined by the difference in viable cell counts following 0 min 2 h of incubation as shown in Table 3 and Figure 2. The isolated *L. rhamnosus* 6.90% at pH 4.5, 5.12% at pH 2.0, showed the highest survival in acidic pH, immediately followed by *L. plantarum*, *L. delbrueckii*, and *L. fermentum* 5.70, 5.40 and 4.03% at pH 4.5, 4.90, 4.31 and 2.33% at pH 2.0 respectively.

Table 1 Biochemical test results of collected *Lactobacillus* isolates.

Biochemical test	Collected <i>Lactobacilli</i> isolates			
	<i>L. delbrueckii</i>	<i>L. fermentum</i>	<i>L. plantarum</i>	<i>L. rhamnosus</i>
Catalase	-	-	-	-
Gas from glucose	+	+	+	+
Ammonia from Arginine	-	+	+	-
Growth at 10°C	-	-	-	-
Growth at 15 °C	-	-	-	-
Growth at 45 °C	+	+	+	+
Glucose	+	+	+	+
Xylose	+	D	+	+
Ribose	+	+	+	+
Melezitose	-	-	-	-
Arabinose	+	D	+	+
Mannitol	ND	-	-	-
Trehalose	d	d	d	d
Melibiose	+	+	+	+
Raffinose	+	+	+	+
Galactose	+	+	+	+
Salicin	-	-	-	+
Maltose	+	+	+	+
Sucrose	+	+	+	+
Mannose	D	W	-	+
Fructose	+	+	+	+
Lactose	+	+	+	+
Rhamnose	-	-	-	-
Sorbitol	ND	ND	-	-

Note: Symbols: w – weak positive reaction; ND – no data available; d – different.

Table 2 Concentration and survival rate of *Lactobacillus* isolates in the presence of 3% bile salts.

Isolates	Pre-incubation		Bile tolerance	
	Mean * 10 ⁸ cfu.mL ⁻¹	Survival rate %	Mean * 10 ⁸ cfu.mL ⁻¹	Survival rate %
LGG	6.57	100	6.30	91%
<i>L. delbrueckii</i>	6.43	100	6.11	89%
<i>L. fermentum</i>	5.93	100	4.85	82%
<i>L. plantrum</i>	6.18	100	5.1	82%
<i>L. rhamnosus</i>	6.19	100	4.98	80%

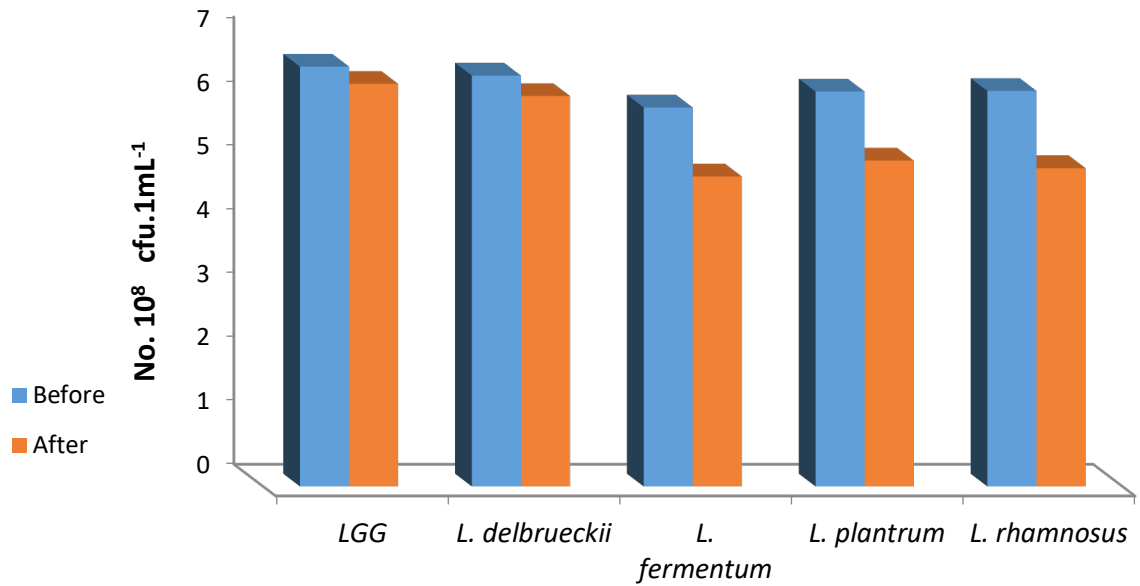


Figure 1 Concentration and survival rate of *Lactobacillus* isolates in the presence of 3% bile salts.

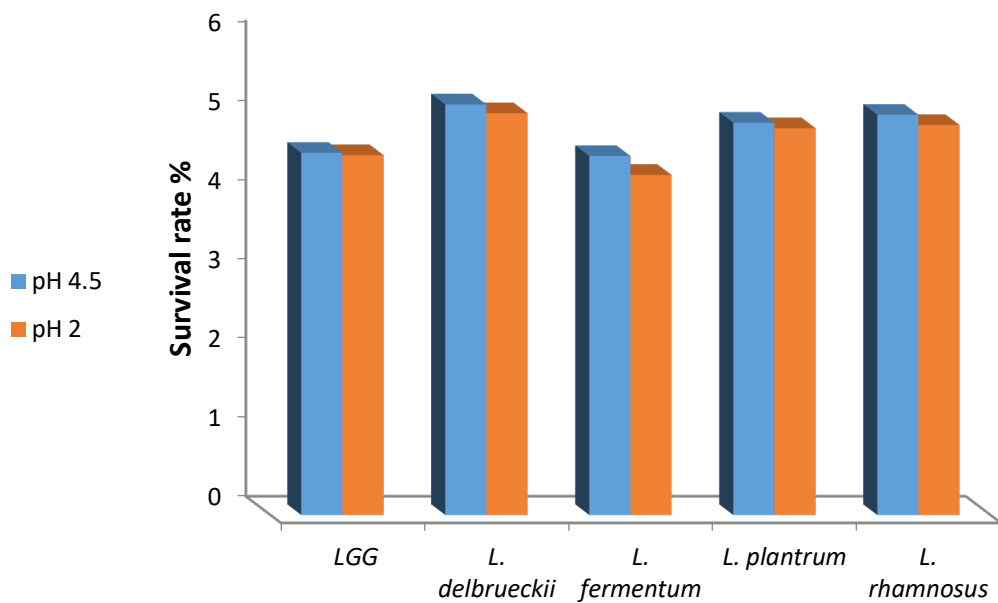


Figure 2 Concentration and survival rate of *Lactobacillus* isolates at the acidic condition.

Table 3 Concentration and survival rate of *Lactobacillus* isolates at the acidic condition.

Isolates	pH 4.5		pH 2.0	
	Mean * 10 ⁷ cfu.mL ⁻¹	Survival rate %	Mean * 10 ⁷ cfu.mL ⁻¹	Survival rate %
LGG	4.57	1.0	4.54	0.91
<i>L. delbrueckii</i>	5.18	5.40	5.07	4.31
<i>L. fermentum</i>	4.53	4.03	4.29	2.33
<i>L. plantarum</i>	4.95	5.70	4.88	4.90
<i>L. rhamnosus</i>	5.05	6.90	4.92	5.12

Tolerance to acid and alkaline conditions

Based on bile salt and acid tolerance: different concentrations of bile salt in MRS broth were prepared and tested on lactobacillus isolates to determine the minimal inhibitory concentration of bile salt tolerance as shown in Table 2 and Figure 1. Results showed that MIC was 3%. The vitality of *lactobacillus* post 2 h, incubation in MRS containing 3% bile salts that *L. delbrueckii* showed an excellent survival ratio of 89%, LGG showed a ratio of survival 91%. Also, lactobacilli survival rates in acidic buffer with pH 4.5 and 2.0 were examined by the difference in viable cell counts following 0 min 2 h, incubation as shown in Table 3 and Figure 2.

The isolated *L. rhamnosus* showed the highest survival in acidic pH; 6.90% at pH 4.5, 5.12% at pH 2.0, immediately followed by *L. plantarum*, *L. delbrueckii*, and *L. fermentum* showed; 5.70, 5.40 and 4.03 % at pH 4.5, 4.90, 4.31 and 2.33% at pH 2.0, respectively. *Lactobacillus* bacteria colonized the hindgut region in the human host and should be able to survive with the acid condition and bile salt before reaching the target tissue.

Therefore, tolerance of *Lactobacillus* to acid and bile salt equally is an important issue to select *Lactobacillus* bacteria as a probiotic.

Table 4 Antimicrobial substances activity exhibited by *Lactobacillus* isolates.

Strains	<i>E. coli</i>	<i>Salmonella typhi</i>	<i>Pseudo. areuginosa</i>	<i>Staph. aureus</i>	<i>Shigella dysenteriae</i>
LGG	++	+	+	+++	+++
<i>L. delbrueckii</i>	++	++	++	+++	+++
<i>L. fermentum</i>	+++	+	-/+	+++	+++
<i>L. plantarum</i>	+++	++	-/+	+++	+++
<i>L. rhamnosus</i>	+++	++	++	+++	+++

Table 5 The antibiotic susceptibility test of *Lactobacillus* isolates.

Antibiotics [µg]	<i>L. delbrueckii</i>	<i>L. fermentum</i>	<i>L. plantarum</i>	<i>L. rhamnosus</i>
Ampicillin 5	-	-	-	-
Chloramphenicol 50	+	+	+	+
Nitrofurantion50	-	-	-	-
Gentamicin10	-	-	-	-
Carbenicillin 100	-	-	-	-
Nalidixic Acid 30	-	-	-	-
Cotrimoxazole 25	-	-	-	-
MethenamineMandelate 3 mg	-	-	-	-

Note: + Resistance, - Sensitive.

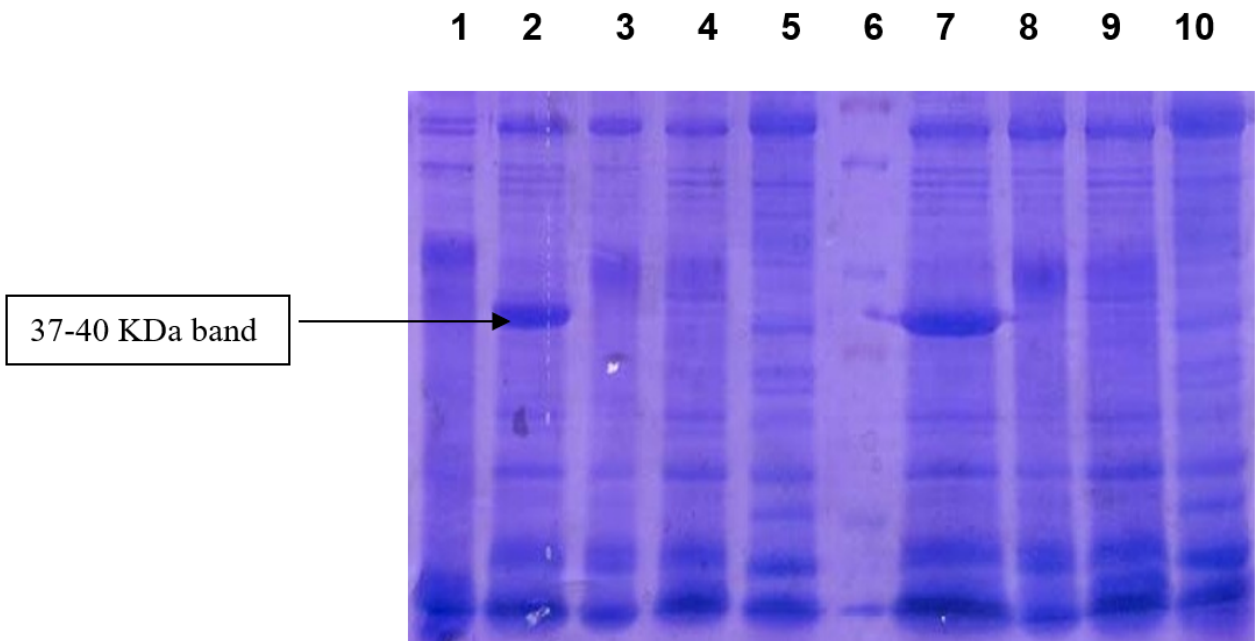


Figure 3 SDS-PAGE protein profiles of *Lactobacillus* isolates and the reference molecular protein marker. Note: lane 6: reference protein marker; lane 1: *L. rhamnosus*; lane 2: *L. fermentum*; lane 4: *L. delbrueckii*.

Our result of this study agreed with the outcomes of other researches, they reported a survival rate of *Lactobacillus* isolates in pH 2.5 (Kim et al., 2007; Perelmuter et al., 2008). Nevertheless, the survival rate of *Lactobacillus* has not been more than 0.2%.

Antimicrobial test

The Findings of antimicrobial activity are presented in Table 4; all collected *Lactobacillus* isolates showed antagonistic activity in various degrees when examined against gram-negative bacteria and gram-positive bacteria.

The standard strain LGG and the *Lactobacillus* isolates showed the highest inhibitory activity against *Staphylococcus aureus*, and *Shigella dysenteriae* whereas, only *L. fermentum*, *L. fermentum*, and *L. rhamnosus* displayed the highest inhibitory activity against *E. coli*. Strain LGG and the collected isolate *L. rhamnosus* belong to the same species, but they showed a difference in their antimicrobial activity since antimicrobial characteristics should be of a specific strain. Once they colonize the hindgut of the human intestine, they lose their antimicrobial activity in the acidic environment (Perelmuter et al., 2008; Enan et al., 2013). They are isolated, identified, and characterized seven small peptides by LGG that have a mass of about 1 kDa and that exert a bactericidal action against both Gram-positive and Gram-negative bacteria (Lu et al., 2009). While *Lactobacillus* spp. suppose to be getting their opportunity to display their antimicrobial activity due to production elements as; lactic acid, H₂O₂, and bacteriocins other antibacterial molecules (Eschenbach et al., 1989; Kučerová et al., 2007).

The result of this study agrees with that recorded by (Perelmuter et al., 2008; Casy et al., 2004), they had been observed a range of lactobacilli antimicrobial responses towards *E. coli* and bacteria besides, they approved the inhibitory activity of *Lactobacillus* spp. affected by the acidic environment. These results are following those reported by (Rodes et al., 2013).

Antibiotics Susceptibility Patterns:

Outcomes of antibacterial sensitivity of *Lactobacillus* isolates were revealed in Table 5: all collected isolates of food source were resistant to Chloramphenicol 50 µg whereas, all of them showed high sensitivity to; Ampicillin 5 µg, Nitrofurantoin 50 µg, Gentamicin 10 µg, Carbenicillin 100 µg, Nalidixic Acid 30 µg, Cotrimoxazole 25 µg and Methenamine Mandelate 3 mg. The safety of *Lactobacillus* isolates must be carefully evaluated. One safety aspect is the transfer or acquisition of antibiotic resistance. Because nonpathogenic enteric bacteria may also be a source for resistance genes that may spread to potential pathogens. Therefore, investigation activities to obtain *Lactobacillus* isolates for probiotic uses should include nonpathogenic as well as pathogenic bacteria (Katla et al., 2001). *Lactobacillus* isolates were collected in this research qualified to use as probiotics because they exhibited minor antibiotic resistance. Thus, it is most important for the selection of probiotic strains without any highly transferable antibiotic resistance or especially virulence mechanisms (Klein et al., 2000). Results obtained in this study agree with the findings reported by (Katla et al., 2001; Klein et al., 2000).

The S-layer protein profile:

The result of the detection S-layer protein profile through SDS-PAGE has demonstrated in Figure 3: SDS-PAGE protein of *Lactobacillus* isolates was quite similar except the *L. rhamnosus* produced patterns containing band about 37 – 40 kDa., according to the previous studies, one dominant band of 43 – 46 kDa, which is known as the S-protein (Boot et al., 1993; Smit et al., 2001). In our study, S-layer extraction was carried out according to Boot's method (Boot et al., 1993). The S-layer proteins characterized from the genus *Lactobacillus* range from 25 to 71 kDa in size (Sára and Sleyrtr, 2000). The Putative S-layer protein was detected by whole-cell SDS-PAGE analysis. S-layer analysis revealed the presence of a unique and single S-layer with an oblique structure. The whole-cell protein patterns of the *Lactobacillus* isolates are fairly homogeneous with some variability, primarily localized in the molecular mass region estimated 30 – 70 kDa.

CONCLUSION

This preliminary study was evaluated the presence of *Lactobacillus* spp. in foods and also it identified, and characterized some strains. Furthermore, it assessed collected isolates to determine their qualified characteristic to use as probiotics. The bacterial surface layer (S-layer) is an excellent candidate for *In vivo* and *In vitro* biotechnological applications. It had been shown to function as an adhesive, mediating binding of Lactobacilli to the host epithelial. The S-layer had been identified in several *Lactobacillus* spp. So, further investigations of *Lactobacillus* populations are necessary to obtain relevant data to be included in an important assessment that is related to food production.

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