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Adhesion, Autoaggregation and Hydrophobicity of Six *Lactobacillus* Strains

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The aim of this study was to assess probiotic attributes such as adhesion, auto aggregation, hydrophobicity and antibacterial activity of *Lactobacillus* strains from dairy products.
Methodology: In this study, the autoaggregation, coaggregation, hydrophobicity and adhering abilities and antimicrobial activities of six *Lactobacillus* strains belonging to different species were assessed. Hydrophobicity was determined by bacterial adherence to hydrocarbons, xylene, n-hexadecane and chloroform.
Results: The percentage of hydrophobicity of the strains range from 29.5% to 77.4% as measured by the described test. The autoaggregation among *Lactobacillus* strains range from 15.8% to 63.1%, while coaggregation range from 18.6% to 55.1%. Adhesion of the tested strains to buccal epithelial cells range from 8.0% to 50%. The tested *Lactobacillus* strains demonstrated variable inhibitory activity against pathogenic bacteria.
Conclusion: Our findings indicated that one *Lactobacillus* strain expressed broad antibacterial activities against a group of bacterial pathogens and along 2 other strains exhibited ability to adhere to epithelial cells as shown by aggregation, coaggregation and hydrophobicity, indicating that such isolates can be good candidates for probiotic use.

Keywords: Lactobacillus; autoaggregation; coaggregation; hydrophobicity; antimicrobial activity.

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1. INTRODUCTION

The genus *Lactobacillus* consists of a genetically and physiologically diverse group of Gram positive, rod shaped, catalase negative, non-spore forming bacteria [1]. Some members of this genus are considered among the most important lactic acid bacteria due to their role in various food and feed fermentations, production of many important metabolites. Their role in the prevention of food spoilage by acting as antagonists against other pathogens through the production of antimicrobials and bacteriocin are being increasingly studied as probiotics due to their health- promoting effects [2,3].

One of the frequently exploited activities used to screen probiotic candidates is adhesion to the host gut, which is presumed to be prerequisite for sufficient host – interaction to confer health benefits [4]. The essential characteristics for lactic acid bacteria (LAB) to be used as probiotics include the following: safety, viability during processing and storage, antagonistic effect against pathogens, capable of surviving in the intestinal ecosystem, and adherence to the intestinal epithelium of the host [5]. Adhesion to intestinal epithelial cells is an important prerequisite for colonization of probiotic strains in the gastrointestinal tract [6]. The hydrophobic properties of bacterial surfaces are a major determinant in the adhesion of bacteria and in the formation of biofilms by bacteria on animate and inanimate surfaces [7]. Hydrophobicity is likely due to a complex interplay between negatively-charged, positively-charged, hydrophobic and hydrophilic components on the surface of the bacteria. Relative hydrophobicity of bacterial cells has been determined by several methods, such as microbial adherence to hydrocarbons (MATH), hydrophobic interaction chromatography (HIC), aggregation in the presence of different salt solutions and adhesion to nitrocellulose filters [8].

The aim of this study was to assess some properties related to the probiotic nature of *Lactobacillus* strains such as adhesion, autoaggregation, hydrophobicity and antimicrobial activity.

2. MATERIALS AND METHODS

2.1 Bacterial Strains and Growth Conditions

Lactobacillus paracasei (lac 1), *Lactobacillus acidophilus* (lac 2), *Lactobacillus acidophilus* (lac 3), *Lactobacillus acidophilus* (lac 4), *Lactobacillus fermentum* (lac 5), and *Lactobacillus plantarum* (lac 6) were laboratory isolates recovered from raw milk (Lac1), Yoghurt (Lac 2 ,3 and 4) cream (Lac 5) and cheese (Lac 6). All Lactobacilli were grown in MRS broth (Himedia, India) at 37ºC for 24 hours, and characterized as previously described [9].

2.2 Hydrophobicity Assay

Determination of cell surface hydrophobicity was evaluated based on the ability of the microorganisms to partition into hydrocarbon from phosphate buffer solution. This test was performed as described by Rosenberg et al. [10]. Bacterial strains were grown in MRS broth at 37ºC for 24 h, after centrifugation at 5000 x g for 15 minutes, the pellets were washed twice with phosphate buffer saline (pH 7.0) and optical densities of the bacteria were measured at 540_{nm} and adjusted to an optical density of A_{540}=1.0. One ml of bacterial suspension was added to 1 ml of each of the hydrocarbons (xylene, n-hexadecane and chloroform, sigma/USA) and vortexed vigorously for 30 sec. After phase separation (30 min),

the optical density of the aqueous phase was again measured and compared with the initial value. Hydrophobicity was calculated according to the equation:

$$(A_{540} \text{ initial} - A_{540} \text{ aqueous phase})/A_{540} \text{ initial}] \times 100 = \% \text{ hydrophobicity.}$$

2.3 Autoaggregation Assays

Autoaggregation assays were performed according to Del Re et al. [11], as modified by Kos et al. [12]. Bacteria were grown in MRS broth for 18 hours at 37ºC. After centrifugation at 5000 × g for 15 minutes, cells were washed twice and suspended in phosphate buffered saline (pH 7.0) to give viable counts of approximately 10^8 CFU/ml. Four ml of the cell suspension were mixed by vortexing for 10 s and autoaggregation was determined during 5 hours of incubation at room temperature. At hourly intervals, 100 µl of the upper suspension was transferred to another tube with 3.9 ml of PBS and the absorbance was measured at 600_{nm}. Autoaggregation was calculated according to the equation:

$$1 - (A_t / A_0) \times 100$$

Where A_t represents the absorbance at time t = 1,2,3,4 or 5 hours and A_0 is the absorbance at t= 0.

2.4 Coaggregation Assays

Coaggregation assays were performed according to Del Re et al. [11]. The cell suspensions were prepared as described above for autoaggregation assay. Equal volumes (2 ml) of broth cultures of tested *Lactobacillus* sp. strain and each pathogen strain were mixed by vortexing for 10 sec. The control tubes containing 4 ml of each bacterial suspension independently. The absorbance at 600_{nm} of the suspensions was measured after mixing and after 5 hours of incubation at room temperature. The percentage of coaggregation was calculated using the equation according to Handley et al. [13]:

$$\text{Coaggregation \%} = \{(A_X + A_Y)/2 - A(x+y)/(A_X + A_Y)/2\}] \times 100$$

Where x and y represent each of the two strains in the control tubes and (x + y) the mixture.

2.5 Adherence Assay

2.5.1 Preparation of buccal epithelial cells

Epithelial cells were obtained from mouth cavity of healthy people. The cells were washed three times in PBS. The pellets were then resuspended in PBS to give approximately 1×10^5 cells/ml by using hematocytometer.

2.5.2 Preparation of bacterial suspension

Bacterial cells were grown in MRS broth and incubated overnight at 37ºC. The culture adjusted to give an approximately 1.5×10^8 bacteria /ml (about 0.5 A_{650}). Then the bacteria were washed twice in PBS and centrifuged for 20 minutes at 2000 rpm and resuspended in PBS.

2.6 *In vitro* Adherence Test

The test was performed by taking equal volumes of buccal epithelial cells (10^5 cells / ml) and bacterial suspensions that were mixed and incubated under shaking (80 rpm) at 37ºC for 60 min. Unattached bacteria were removed from the suspension by centrifugation three times in PBS for 10 minutes at 2000 rpm. The resulting pellets were dried on glass slides in air and fixed with methanol then were stained with Giemsa. A control for epithelial cells was performed by fixing and staining the epithelial cells alone with Giemsa stain. Adherent and non-adherent strains could easily be differentiated [14]. Adherent strains attached to at least 40% of the epithelial cells, while non adhering strains attached to less than 10% of the examined epithelial cells [15].

2.7 Antimicrobial Activities against Indicator Organisms

Antimicrobial effects of *Lactobacillus* strains against *Serratia marcescens* (B 2), *Vibrio* vulnificus (B 5) *Enterococcus faecalius* (B 8), *Morganella morganii* (B 9), *Staphylococcus aureus* (B 4) and *Salmonella enterica* susp. *enterica* serovar Typhi strain (B3), were determined by the agar diffusion method. Overnight cultures of the indicator strains were used to inoculate agar growth media at 37ºC. Wells of 5 mm diameter were cut into BHI agar plates. To detect antibacterial activity of the strain, 10 ml of broth was inoculated with each *Lactobacillus* strain, and was incubated at 37ºC for 48 hours. Cell-free solution was obtained by centrifuging the culture at 6000 $\times$ g for 15 min, following by filtration of the supernatant through a 0.2 µm pore size filters. The pH of the filtered supernatants were adjusted to pH 6.5 with 1N NaOH, then 50 µl of supernatant fluid from each tested *Lactobacillus* strain was added to each well cut into the respective pathogen plate and incubated at 37ºC for 24 h before zones of growth inhibition were measured [16].

3. RESULTS AND DISCUSSION

3.1 Hydrophobicity of Strains

The hydrophobicity percentages for the tested strains ranged between 77.4% and 29.5%. Strains with hydrophobicity more than 40% were considered hydrophobic [17]. The hydrophobicity of strain lac 1 showed greater affinity towards the solvents used for the study. The strain lac 1 showed 77.4% affinity towards xylene. All strains except lac 4 and lac 5 showed above 40% affinity towards xylene and n-hexadecane respectively. Results are shown in Fig. 1.

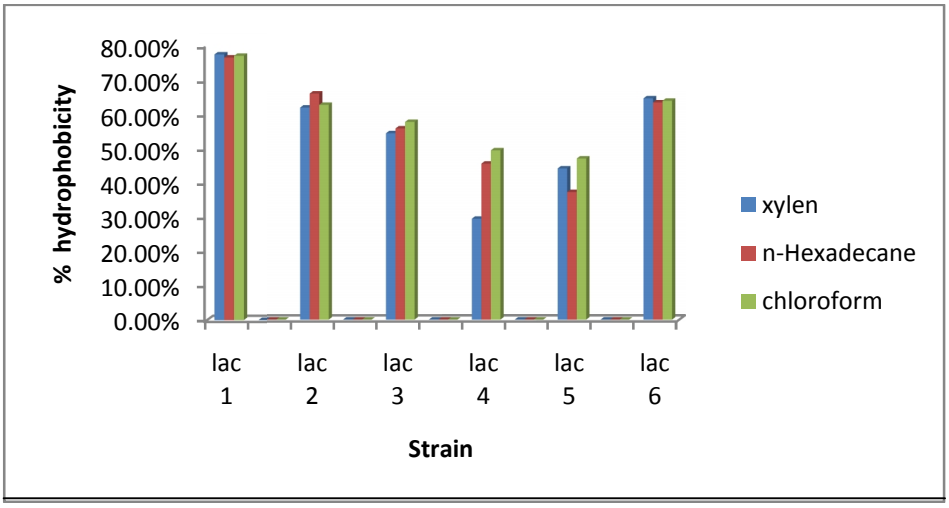

Fig. 1. Percentage hydrophobicity of *Lactobacillus* strains

3.2 Autoaggregation and Coaggregation

Autoaggregation assay of the tested strains was based on the test results of the three strains lac 1, lac 2 and lac 6 which had high hydrophobicity when grown in MRS broth. However, when the three strains were suspended in PBS, the percentages of auto aggregation were lower and ranged between 15.8% and 63.1%, with highest levels of autoaggregation obtained after 5 hours of incubation (Table 1 and Fig. 2).

Table 1. Autoaggregation of 3 *Lactobacillus* strains grown in MRS broth and phosphate buffered saline (PBS) measured at hourly bases

Strains	MRS broth - Percent autoaggregation on hourly bases					PBS - Percent autoaggregation on hourly bases				
	1 hr	2 hr	3 hr	4 hr	5 hr	1 hr	2 hr	3 hr	4 hr	5 hr
Lac 1	37.50	41.50	50.30	58.30	63.10	8.30	13.30	15.00	18.30	21.60
Lac 2	27.10	29.80	33.10	36.60	44.30	3.30	5.00	8.30	10.00	10.00
Lac 6	15.80	17.10	20.30	24.30	28	3.30	6.60	8.30	10.00	12

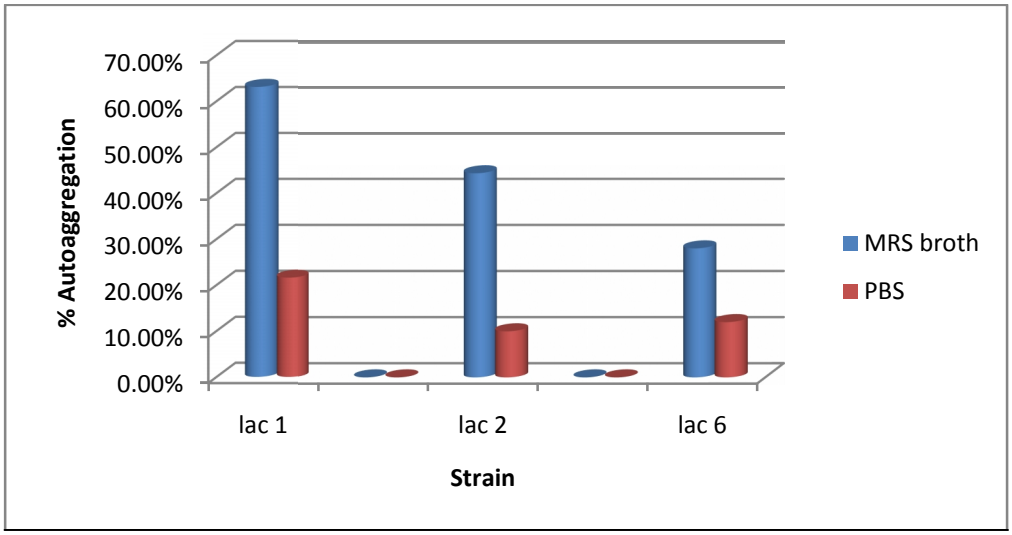

Fig. 2. Autoaggregation assay of *Lactobacillus paracasei* (lac 1), *Lactobacillus acidophilus* (lac 2) and *Lactobacillus plantarum* (lac 6) during incubation period of 5 hr in MRS & PBS buffer

Coaggregation with *Serratia marcescens, Morganella morganii, Staphylococcus aureus,* and *Salmonella typhi* was highest for the strain lac 1 (55.1%) with *Serratia marcescens* and lowest for the strains lac 6 (18.6%) with *Salmonella typhi* (Fig. 3).

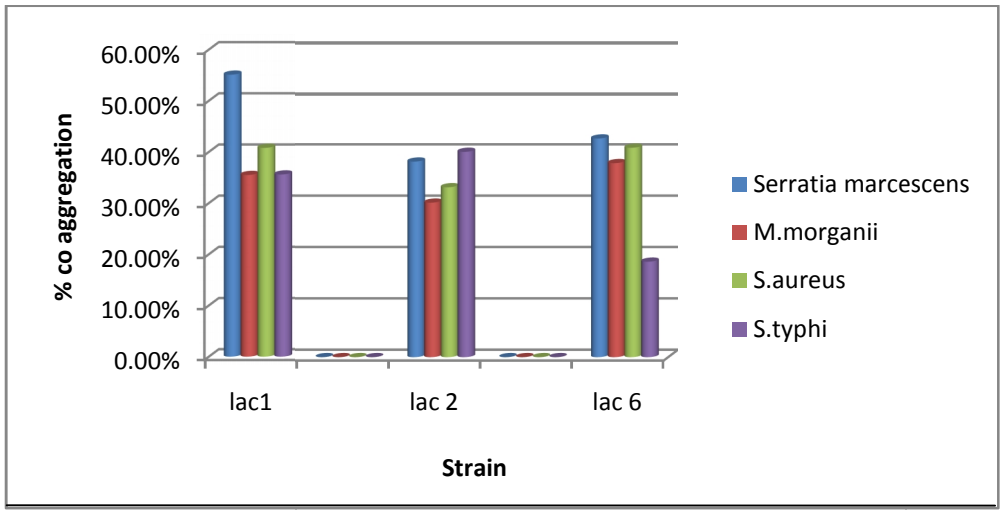

Fig. 3. Coaggregation assay of *Lactobacillus paracasei* (lac 1), *Lactobacillus acidophilus* (lac 2) and *Lactobacillus plantarum* (lac 6) after incubation of 5 hr

3.3 Adhesion Assay on Human Buccal Epithelial Cells

Adhesion property was assessed for the six *Lactobacillus* strains using described method [14,15]. Human buccal cavity cells from healthy people were used to measure the frequency

of adhesion of the tested bacteria to these cells .The adhesion average of lac 1 was 50 bacterial cells/ buccal epithelial cell whereas the adhesion average of lac 6 and lac 2 were 40 and 30% cell /buccal epithelial cell respectively. Adhesion average of lac 4, lac 3 and lac 5 were 10, 8 and 8% cell/epithelial cell, respectively. Therefore, lac 1, lac 2 and lac 6 were considered as strong adhesive strains, whereas lac 4, lac 3 and lac 5 were non adhering strains. Results are shown in Fig 4.

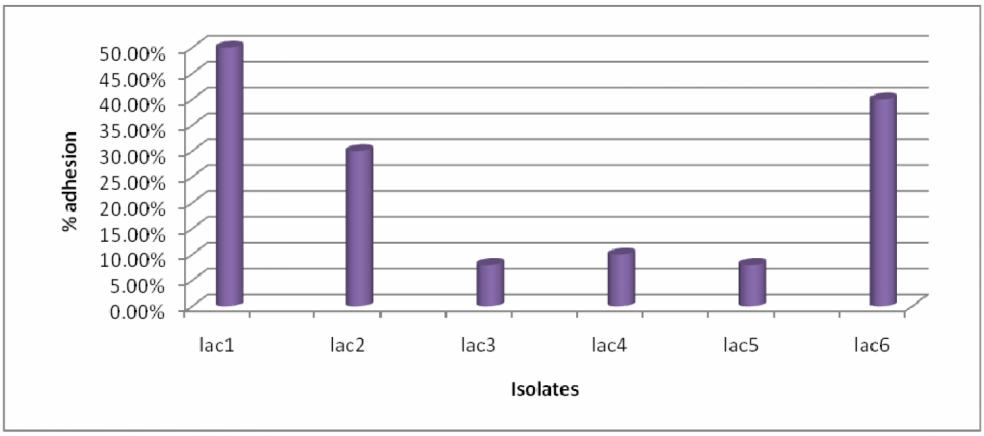

Fig. 4. Adhering ability of *Lactobacillus* strains

3.4 Antibacterial Activity

Local pure cultures of pathogenic strains *Serratia marcescens*. (B 2), *Vibrio* vulnificus(B 5)., *Enterococcus faecalius* (B 8), *Morganella morganii* (B 9), *Staphylococcus aureus* (B 4) and *Salmonella typhi* (B 3) were kindly provided by Hilla hospital microbiology laboratory at Babylon, Iraq and their taxonomic identity was confirmed according to the standard procedure [1]. Antibacterial activity of cell – free supernatant was evaluated on strains of *Serratia marcescens, Vibrio vulnificus, Enterococcus faecalius, Morganella morganii, Staphylococcus aureus* and *Salmonella typhi* using agar well diffusion method [16]. Results of the experiment showed that the *Serratia marcescens* is the most affected by filtered supernatant of six *Lactobacillus* strains among the pathological strains while the *Vibrio vulnificus* and *E. faecalius* are least affected, whereas the remaining strains showed varying response towards the filtered supernatant (Fig. 5). Based on results of this study, Lac 1 filtrate had the broadest spectrum of activity against the pathogenic microbes tested.

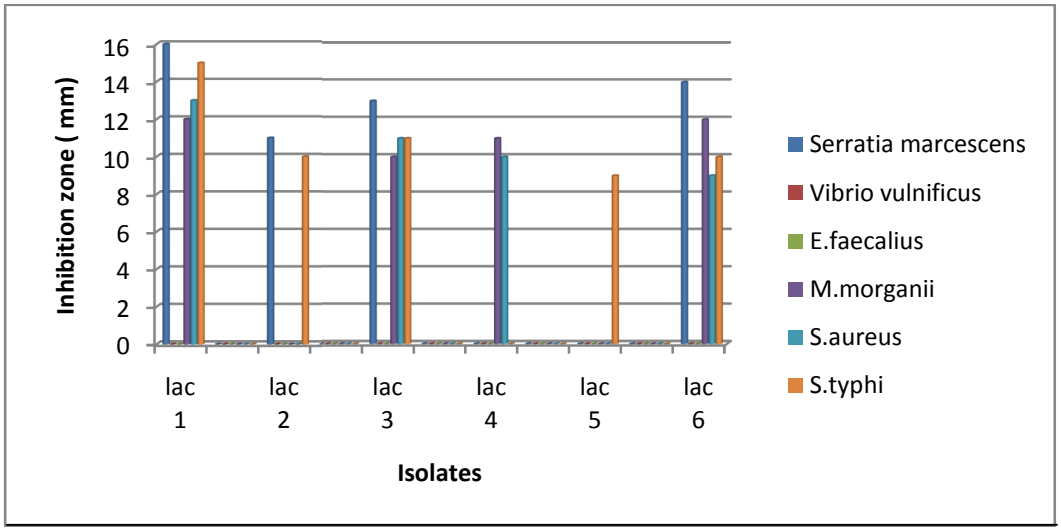

Fig. 5. Antimicrobial activity of *Lactobacillus* strains against indicator strains

The microbial adhesion to hydrocarbons has been widely used to measure the cell surface hydrophobicity of bacteria as adherence to the surface of host cells determines the colonization capability of bacteria, which is a crucial step in the establishment of probiotics in the intestine [18].

In the present study, the hydrophobicity of the strains was studied using chloroform, xylene and n-hexadecane and it was revealed that all the strains exhibited a different degree of hydrophobicity. Kos et al. [12] recorded maximum cell surface hydrophobicity in chloroform when the strains *L. acidophilus* M92, *Lactobacillus plantarum* L4 and *Enterococcus faecium* L3 were tested against xylene, chloroform and ethyl acetate. The microbial adhesion to n-hexadecane reflects cell surface hydrophobicity or hydrophilicity because electrostatic interactions are absent [19]. A high percentage of lac 1 cells adhered to xylene, a polar solvent, demonstrate hydrophobic cell surface of this strain. However, lac 3, lac 4 and lac 5 showed strong affinities to chloroform, which means they are strong electron donors. Surface hydrophobicity was determined in order to test for possible correlation between this physico-chemical property and the ability to adhere to the intestinal mucus as suggested by Wadstrom et al. [20]. Ngwai et al. [21] reported that physicochemical properties such as hydrophobicity play a major role in initial interaction with host tissue.

Hydrophobicity and surface charge of bacterial may differ between species strains and changes with variation in physiological state of cells and composition of suspension media or might involve expression of variable surface-associated proteins between strains [22]. Pelletier et al. [23] reported the physico-chemical properties of microbial cell surface, including the presence of (glycol-) proteinaceous material at the cell surface results in higher hydrophobicity, whereas hydrophilic surfaces are associated with the presence of polysaccharides.

Bacterial aggregation between cells of the same strain (autoaggregation) or between different species and strains (coaggregation) is of considerable importance in several ecological niches, especially in the human gut where probiotics are to be active [24]. The autoaggregation ability is one of the key factors that determine the ability of the probiotic

strain to adhere to the oral cavity, gastrointestinal tract and urogenital tract and coaggregation ability helps to from a barrier that prevents colonization by pathogens. Lactobacilli with aggregation ability and hydrophobic cell surface could have more chance for adhesion to intestinal cells [19]. In the present study, out of the six *Lactobacillus* strains checked, strain lac 1 showed maximum autoaggregation of 63.1% after 5 hours in MRS broth. The strains with the highest autoaggregation or coaggregation were selected for further tests in probiotic screening steps [25].

Kos et al. [12] reported that the cell surface proteins (S- layer proteins) influenced autoaggregation property and adhesiveness of *L. acidophilus M92*. Goh and Klaenhammer [26] reported that the aggregation promoting factors increases self-aggregation with incubation. Although we have not tested the strains for Apf proteins, our results are in general agreement with the results of Goh and Klaenhammer [26]. Del Re et al. [11] reported that aggregation ability is related to cell adherence properties. The method of coaggregation with gut pathogens may be useful for screening for potential probiotic strains [27]. In the current study, high autoaggregation was associated with lower coaggregation, mimicking the previous results [28] on a probiotic strain of *L. acidophilus M92* which showed a high score in autoaggregation but lower score in coaggregation with pathogens.

The ability to adhere to epithelial cells and mucosal surface has been suggested to be an important property of many bacterial strains used as probiotics. Cell adhesion is a multistep process involving contact of the bacterial cell membrane and interacting surfaces [12].

Lactic acid bacteria produce a variety of antimicrobial substances which made them a preferable choice for dairy starter cultures and also as food biopreservatives. The antibacterial activity of LAB has been attributed to the production of H_2O_2, organic acids (lactic acid, acetic acid and formic acid), proteinaceous compounds and cyclic dipeptides [29]. The characteristics of a successful probiotic consist of antimicrobial activity against intestinal pathogens, acid and bile tolerance and the ability to adhere to and colonies the intestinal tract [30].

4. CONCLUSION

Our findings indicate that one strain of *Lactobacillus* expressed broad antibacterial activities against bacterial pathogens and along 2 other strains exhibited ability to adhere to epithelial cells as shown by aggregation, coaggregation and hydrophobicity, indicating that such strains can be good candidates for probiotic use.

COMPETING INTERESTS

All authors declare that they have no competing interest related to this research work.

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