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Original Article

Correlation of Biofilm Formation and Caco-2 Cell Attachment Properties in Colonization Ability of Acid-Bile Resistant Fecal *Lactobacillus plantarum* Isolates

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Introduction: The strain-specific capabilities of lactobacilli are critical for gut colonization. In this study, we evaluated various colonization determinants of 42 fecal *Lactobacillus plantarum* isolates from the healthy human fecal samples. **Methods:** We investigated the attachment to the Caco-2 cell line, biofilm formation ability and cell surface activity of the isolates. Such properties were comparatively studied, and the regression between these features was statistically analyzed. **Results:** Among our results 18 (42.9%) were non-adhesive, 11 (26.2%) moderate adhesive and 13 (31%) strongly adhesive. The results showed that some isolates were significantly capable of biofilm formation. The highest rate of auto-aggregation was recorded for *L. plantarum* isolate RPR 240 (58%). **Conclusion:** Our results revealed a high degree of variability among colonization characteristics of the *L. plantarum* isolates indicating strain-specificity. Also, the colonization properties of the potential probiotic isolates had no significant correlation with each other. Our results confirmed the necessity of further *in vivo* colonization assays for selection of probiotic candidates. *J Med Microbiol Infec Dis, 2018, 6 (1): 13-19*.

Keywords: Probiotics, Strain specificity, Colonization ability, Caco-2 cell, Cell surface activity.

INTRODUCTION

The microbiome of the human gastrointestinal tract is an entity affecting the functional features of our body including immunity and metabolism. This dynamic and complex microbial community is linked with our nutrition and physiology [1]. The gut microbiome comprises a vast diversity of microorganisms, and probiotics as members of the microbiome have received much attention from biomedicine specialist [2, 3]. The probiotic microorganisms are among the most exciting groups of the gut microbiome. They are beneficial microbes that confer health benefits upon the host when consumed appropriately [1]. In the last few years, various branches of health sciences have focused on identification of potential probiotics by screening for unique and main probiotic properties of the microorganism [4].

Lactobacillus, one of the two main probiotic genera, include over 200 species with hundreds of strains introduced as probiotics [5-7]. Due to the economic significance of these microbes, various biological features of them have been exhaustively studied [1]. Lactobacilli are actually among the most extensively studied probiotics due to: a) potential anti-inflammatory and anti-allergic effects, b) secretion of antimicrobial compounds, c) growing in aerobic and anaerobic conditions, and e) comprising many species identified as Generally Recognized As Safe (GRAS) by the Food and Drug Administration (FDA). Considering the niche-specific genome evolution of these lactic acid bacteria to specific environments like the gastrointestinal tract of various animals, the diverse array of the physiological traits, which are known as the probiotic properties, have become species and even strain specific [8].

The intestinal epithelium and the mucosa lining the GIT form a defensive physical barrier against potential exogenous invasions. Probiotics are expected to be able to survive transit through the harsh stomach environment and resist digestive bile salts [9, 10]. The microbes that resist the acid and bile salts must also possess a variety of characteristics which are critical for a potential probiotic strain to become stabilized in GIT. These features include adhesion to the intestinal epithelial cells, colonization in the intestinal tract, production of antimicrobial factors, and inhibition of enteropathogens [11-14]. The resident gut microbiome including members of the probiotic genera, mainly bifidobacteria and lactobacilli, are involved in improving the biological functions of their hosts by conferring them a health benefit. This phenomenon occurs via various ways including preserving the niche balance of the gut microbial community, preventing pathogens from colonization and reducing the probability of their invasive effects, promoting the mucosal and epithelial integrity and

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improving the immune system function [15-18]. It was postulated that the adhesion is a prerequisite for the colonization of the GIT by probiotics. Like other probiotic characters, adhesion and attachment capabilities are strain-specific, and thus, the strains with the highest adhesion ability have the most significant probiotic performance [19, 20]. Therefore, adhesion property could be a valid criterion for screening putative probiotic isolates.

Multiple criteria should be evaluated to introduce a microbe as a functionally probiotic microorganism [1, 12]. Among the properties the candidate probiotics are screened for, adhesion to a model gut epithelial cells is a critical one. However, regarding the difficulty of the *in vivo* models, *in vitro* assays with intestinal cell lines were developed for assessment of interactions between bacteria and host epithelia [21, 22]. Nevertheless, there is still considerable variability in the protocols used for studying the attachment of candidate probiotics to the mammalian cell [23-25].

Regarding the importance of the gut-colonization ability in functional probiotic isolates, we screened fecal *Lactobacillus plantarum* isolates for biofilm formation and Caco-2 cell attachment properties. The correlation between these two features was also studied, and finally, the surface activity of the selected isolates was measured by *in vitro* assays.

MATERIAL AND METHODS

Isolation of lactobacilli. We collected fecal samples from 59 individuals aged 1-36 years old from 2013 to 2014. The volunteers resided either in Tehran city, (n=21) or Bojnourd city and its surrounding villages (n=38), Iran. The criteria for selection of volunteers included lack of any antibiotic therapy or consuming any commercial probiotic products over the past six months and having no significant gastrointestinal (GI) disorders. [12]. The fecal samples were transferred to the laboratory in 24 h, (in the cases that transfer was not possible in this period, the samples were frozen in -20°C) and were serially diluted in PBS buffer (pH 7.4). Amounts of 100 µL from the fifth and sixth dilutions of the samples were plated on Man, Rogosa and Sharpe (MRS) agar (Merck, Germany) and incubated at 37°C for 48 h in anaerobic condition. From each sample, ten colonies were selected randomly and preserved at -80°C in MRS broth containing 20% glycerol.

Screening for acidic pH and bile resistant isolates. All isolates were initially tested for their ability to resist against acidic pH and bile salts as described previously [12]. In brief, all 470 isolates were grown overnight in MRS broth. The cultures were then centrifuged at 6000 rpm, and the pellets were washed with PBS buffer (pH 7.4). An initial count (C0) of the bacterial suspension was performed after a serial dilution (10^{-2} to 10^{-10}) and plating on MRS agar followed by incubating plates at 37°C for 48 h in anaerobic condition. Then bacterial pellets resuspended in 5 ml PBS buffer and the pH was adjusted to 3.0 with HCl 0.5 M (Merck, Germany) and incubated at 37°C for 3 h.

Screening for the bile resistance isolates was performed by resuspension of the pellets of the cultures in MRS broth containing 0.4% bile salts (Merck, Germany) followed by incubation for 6 h at the abovementioned conditions. The harvested bacterial biomass from the PBS at pH 3.0 was washed in PBS and the bacteria cells enumerated as described above. The viable cells were grouped as strongly resistant, resistant, intermediate and susceptible based on 2, 2-4, 4-6 and >6 log reduction in comparison with the initial suspension after 3 h and 6 h of incubation in acid and bile respectively [12].

Molecular identification of *L. plantarum*. Total DNA was extracted using peqGOLD Bacterial DNA Kit (peQlab, Germany) according to the manufacturer's instruction. Identification of *Lactobacillus* genus was carried out by PCR amplification using the previously designed *Lactobacillus* 16s rRNA specific primers, for-lac and Rev-lac [12]. The PCR amplifications began with an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec, and a final extension step at 72°C for 7 min. For identification of the species, *L. acidophilus*, *L. casei*-group, *L. delbrueckii*, *L. gasseri*, *L. plantarum*, *L. rhamnosus* and *L. reuteri*, a multiplex PCR amplification performed using species-specific primers as described elsewhere [26]. The PCR amplification program included one cycle at 94°C for 5 min, 40 cycles of 94°C for 30 sec, 51°C for 40 sec and 72°C for 30 sec, and a final extension was carried out at 72°C for 7 min [9].

Attachment to Caco-2 cells. The adherence of lactobacilli to Caco-2 cells was evaluated as described before [27]. First, the Caco-2 cells were cultured in RPMI (Gibco, Carlsbad, CA, USA) containing 20% (v/v) fetal calf serum (Gibco, Life Technology, USA), 100 U ml^{-1} penicillin, and 100 mg ml^{-1} streptomycin. The cultures incubated at 37ºC in a 5% CO_2 atmosphere. Then, 3 ml aliquots of Caco-2 cells, (1.5×10^5 cells/ml), were seeded on 6-well cell culture plates and after confluency, the cells were washed twice with 3 ml PBS. Two ml of RPMI (without antibiotics) was added to the wells and incubated at 37°C for 3 h. Amount of 1 mL RPMI1640 medium with no antibiotics containing 10^9 CFU/ml of each isolate was added to the wells. For releasing unbound bacteria, the plates were washed four times with PBS after incubation at 37°C with 5% CO_2 for 1 h. For fixation of the cells and attached bacteria, 1 ml methanol was added to the wells, and the plates were incubated at room temperature for 5-10 min. The cells were stained with 3 ml of Giemsa (1:20) (Sigma-Aldrich Co., Mo, USA) for 30 min at room temperature. Excess dye was washed with distilled water, and the plates were allowed to dry at 37°C overnight and then examined by oil immersion microscopy with a magnification of 100X. The adherent lactobacilli were counted by examining 20 random microscopic fields for each test. We assumed 0-40, 41-100, and >101 attached bacteria in 20 fields as non-adhesive, adhesive, and strongly adhesive, respectively.

***In vitro* biofilm formation assay.** All of acid and bile resistance *L. plantarum* isolates were subjected to biofilm formation assay as described previously [28] with some modifications. For biofilm formation, wells of a 96-well plate were filled with 200 µl of 3×10^7 CFU of testing

bacteria and incubated without shaking for 72 h at 37°C. The wells were washed with PBS, and the remaining attached bacteria were stained for 30 min with 200 µl 0.1% (wt/vol) crystal violet in an isopropanol-methanol-PBS solution (1:1:18 [vol/vol/vol]). Excess stain was washed with 200 µl water per well. Wells were air dried for 30 min, and the bounded dye was extracted with 200 µl ethanol-acetone (80:20). The optical density (OD) of 135 µl of each well was measured at 570 nm using a microplate reader. The sterile medium and *Pseudomonas aeruginosa* ATCC 27853 were included as negative and positive controls, respectively.

Surface activity properties. For determination of cell surface properties of the selected isolates, auto-aggregation and bacterial adhesion to solvent (BATS) assays were performed as described elsewhere [29]. For auto-aggregation determination, 1 ml from 18 h culture of the selected isolates in MRS broth was washed and resuspended in 5 ml PBS. The absorbance of the supernatant of each reaction was measured at 600 nm with a spectrophotometer (A0h) and incubated at 37°C for 24 h. The absorbance of each bacterial suspension was measured at A2h, A5h, and A24h, and auto-aggregation was calculated as Auto-aggregation (%) = (1- A2h, 5h, 24h/A0h) × 100.

The BATS assay was done for determination of hydrophobicity and hydrophilicity properties of outer proteins of selected isolates. For this, we used xylene as a polar solvent, chloroform as an electron donor and the ethyl acetate as an electron acceptor. One ml of 18 h cultured bacteria in MRS broth was centrifuged, washed and resuspended in 3 ml PBS. The absorbance of each bacterial suspension was measured at 600 nm with a spectrophotometer (Cecil, Germany) and 1 ml of each solvent was added to different assay tubes. Mixtures were vortexed for 1 min and allowed to separate into two phases. After 15 min, the absorbance of aquatic phase was measured, and surface properties of our selected isolates (hydrophobicity/hydrophilicity, electron donor/acceptor ability) were determined using the formula BATS (%) = (1-A15 min /A0 min) × 100.

Statistical Analyses. Statistical analysis was performed using SPSS 16.0 software. The correlation between the isolates biofilm formation, attachment, aggregation, and hydrophobicity were performed by Spearman's coefficient calculation.

RESULTS

Isolation of lactobacilli and screening for pH and bile resistant isolates. Collectively, 470 lactobacilli were isolated from the 59 fecal samples. Screening for acid-bile resistance properties yielded 88 isolates (Table 1).

Molecular identification of *L. plantarum*. The multiplex PCR amplification and the resulting gel electrophoresis revealed a 428 bp band in 42 isolates (Fig. 1) indicative of the species *L. plantarum* according to others [26].

Table 1. *Lactobacillus plantarum* isolates detected in the feces samples

		L. plantarum
Age	Years (Mean+CD)	22.5±9.04
Sex	Male	31 (73%)
	Female	11 (27%)
Geography Residency (birth place)	Tehran (Tehran)	17 (40%)
	Tehran (Sanandaj)	1 (2.3%)
	Tehran (Kashan)	1 (2.3%)
	Tehran (Langrood)	2 (4.6 %)
	Tehran (Farsan)	1 (2.3%)
	Tehran (Tabriz)	3 (7.1%)
	Tehran (Chabahar)	1 (2.3%)
	Bojnourd/Peighoo (Peighoo)	13 (31%)
	Bojnourd/Chenaran (Chenaran)	3 (7.1%)
	Total	42 (100%)

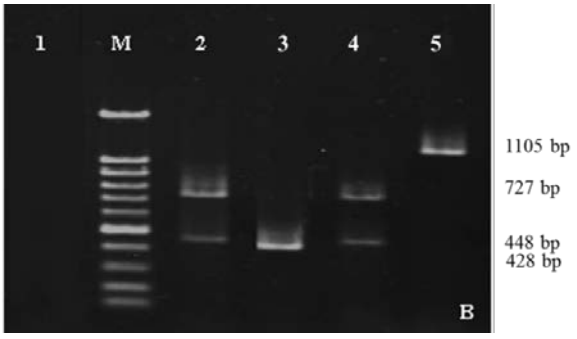

Fig. 1. The agarose gel electrophoresis of the multiplex PCR products of the fecal lactobacilli isolates obtained in this study. Lane 1, negative control; M, 100 bp ladder; lane 2, *L. rhamnosus*; lane 3, *L. plantarum*; lane 4, *L. rhamnosus*; lane 5, *L. reuteri*

Attachment to Caco-2 cells and *In vitro* biofilm formation assay. The biofilm formation and *in vitro* adhesion to Caco-2 cell line, an intestinal epithelium model cell was observed in 42 *L. plantarum* isolates (Fig. 2-3). A high variation in adhesion rate among 42 *L. plantarum* isolates was observed (Fig. 3), 18 (42.9%) were non-adhesive with fewer than 40 bacteria in 20 microscopic fields attached to Caco-2 cells, 11 (26.2%) moderate adhesive with 41-100 bacterial cells attached, and 13 (31%) strongly adhesive with >101 bacterial cell attached. The strongly adhesive *L. plantarum* isolates were much more interesting to us as they had one of the most critical criteria defined for a functionally probiotic isolate.

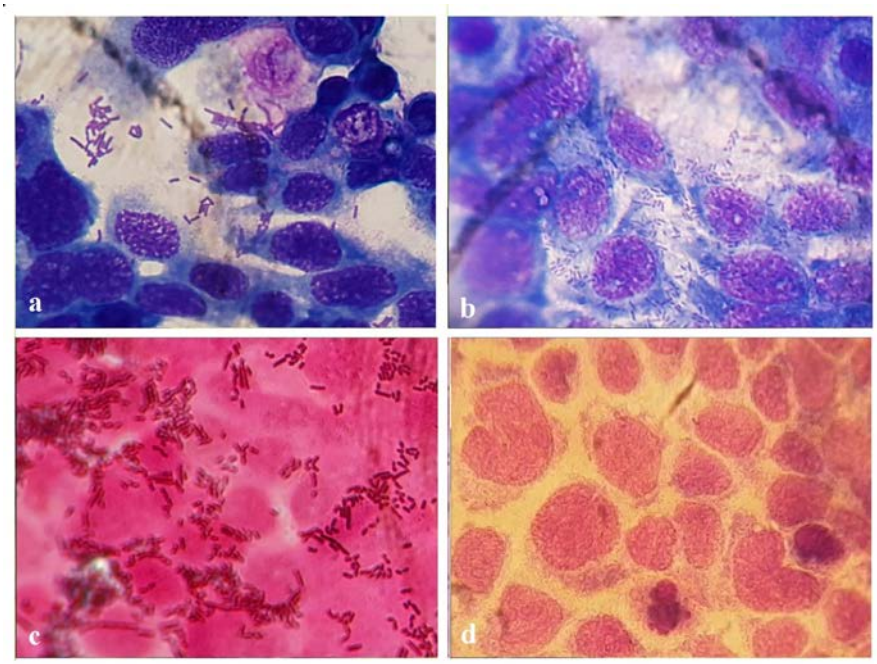

Fig. 2. Microphotographs (magnification of 1000x) showing the adhesion of *L. plantarum* RPR42 to the epithelial Caco-2 cell line. a, b, and c, positive; d, no attachment

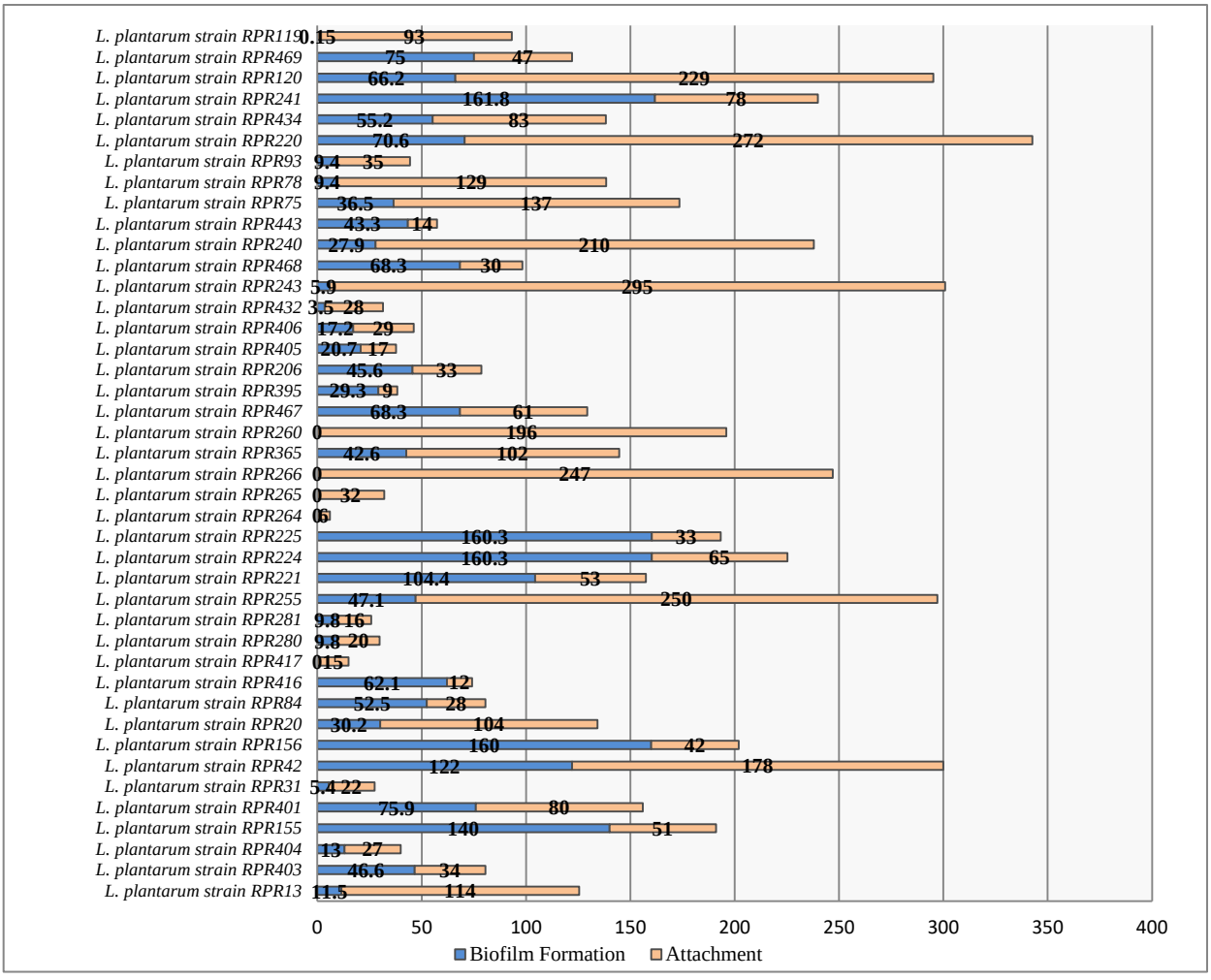

Fig. 3. The quality of the biofilm formation and attachment in the studied *L. plantarum* isolates. Blue horizontal bars show the percentage of biofilm formation and orange horizontal bars the number of attached lactobacilli in 20 microscopic fields to Caco-2 cell line. There was no correlation between biofilm formation and attachment ability.

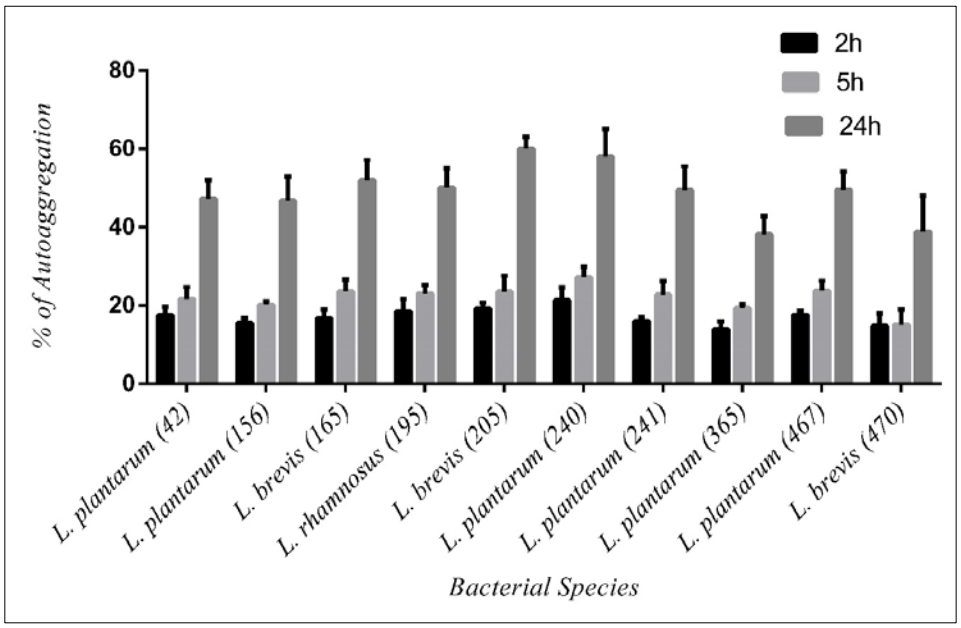

Fig. 4. The result of the 10 selected isolates from various species showing the highest auto-aggregation degree

Table 2. Comparison of different colonization determinants among 6 *L. plantarum* isolates with suitable potential probiotic characteristics that have been shown colonization ability with at least one of these three *in vitro* methods.

Isolate No.	Biofilm Formation (%)	Attachment	BATS (%)		
			X	C	E
RPR240	27.9	210	59.7	28.2	22.1
RPR42	122.0	178	48.2	42.7	20.5
RPR467	68.3	61	41.7	63.7	38.2
RPR365	42.6	102	0	38.8	39.2
RPR241	161.8	78	41.5	54.4	40.3
RPR156	160.0	42	16.6	15.3	22.7

BATS, Bacterial Adhesion To Solvent; X, Xylene; C, Chloroform; E, Ethyl acetate.

***In vitro* biofilm formation.** We calculated the ratio of biofilm formation by measuring the observed optical density (OD) of the isolates in comparison to *P. aeruginosa* as a positive control. The results showed that some isolates were significantly stronger than positive control in the biofilm formation (Fig. 2).

Surface properties of isolated isolates. The highest rate of auto-aggregation was recorded for *L. plantarum* isolate 240 (58%) (Fig. 4). In BATS assay *L. plantarum* isolates RPR42, RPR156, RPR240 and RPR241 showed a high ratio of surface activity (Fig. 4).

DISCUSSION

The essential characteristics of the probiotics are their ability to colonize and survive in the gastrointestinal tract, and the colonization capacity is a critical criterion for choosing the novel probiotic isolates. Primarily in different guidelines for novel probiotic selection, colonization and adhesion abilities were demonstrated by using Caco-2 cells model and plate biofilm formation assay [28]. Adhesion and colonization of the probiotic bacteria in the intestinal tract can stimulate the immune system which leads to the production of cytokines and activation of cellular response [30] with probable antagonistic effects against enteropathogens [31]. In our study, 21 isolates from 42 *L. plantarum* were identified as adhesive or strongly adhesive while demonstrating different ratios of biofilm formation ability. In the present study, we identified 9 *L. plantarum* isolates as strong, 4 as intermediate, and one as weak biofilm producers. The remaining isolates failed to produce biofilm using the *in vitro* model. Our results were in agreement with the previous works, showing an extensive variation in biofilm formation among the isolates [32, 27] and supported the notion of no correlation between *in vitro* attachment and biofilm formation abilities (r=0.11, *P*=0.51). However, the high ratios of these two abilities were observed only in one isolate, i.e., *L. plantarum* strain 42. Similarly, in our previous work on *Pediococcus* spp. isolates from broiler chickens, only one strain demonstrated high ratios for both these two abilities [33]. The results of this study and our previous work on *Pediococcus* spp., in agreement with other studies, emphasize that colonization ability for novel probiotic candidates is a critical feature and thoroughly a strain-specific characteristic [34] that should be evaluated individually for all probiotic candidates.

For confirmation of colonization ability of our reported novel potentially probiotic lactobacilli and their probable protection of GIT against foodborne pathogens colonization [12], cell surface properties of our final selected isolates were determined. The bacterial cell surface proteins and

polysaccharides provide hydrophobic and hydrophilic properties of bacterial isolates which are highly correlated with *in vivo* colonization ability of the microorganisms [35] and could affect the competition between the probiotic bacteria and enteropathogenic microorganisms [36]. Our results demonstrated a completely different surface activity among the isolates. However, no correlation between *in vitro* biofilm formation and cell attachment ability via surface activity was observed. Among our 42 *L. plantarum* isolates, 4 showed a high affinity to xylene (hydrophobicity). There was a correlation between aggregation after 2 h and 24 h of incubation and hydrophobicity (p=0.006 and p=0.017 respectively), but aggregation after 5 h incubation was not correlated with hydrophobicity. The results showed that aggregation (p=0.1), hydrophobicity (p=0.764), electron donor (p=0.356) and electron acceptor (p=0.509) abilities were not correlated with *in vitro* attachment. In accordance with our results, Nejati and colleagues (2016) in their study on *Lactococcus* spp. isolates from Iran showed that this ability was not directly correlated with colonization in the gut [37]. Among our *L. plantarum* isolates, 4 isolates showed a high affinity to xylene (hydrophobicity), suggesting that these potentially probiotic isolates could produce health benefits on their host due to their colonization and competition abilities (Table 2).

In this study, there was no correlation between three different phenotypic and *in vitro* assays commonly used for determination of colonization ability of probiotic candidates. In accordance with previous works [38], our results emphasize the importance of *in vitro* models for preliminary evaluation of the colonization abilities of probiotic candidates. However, confirmation of these abilities in probiotic candidates requires *in vivo* assays.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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