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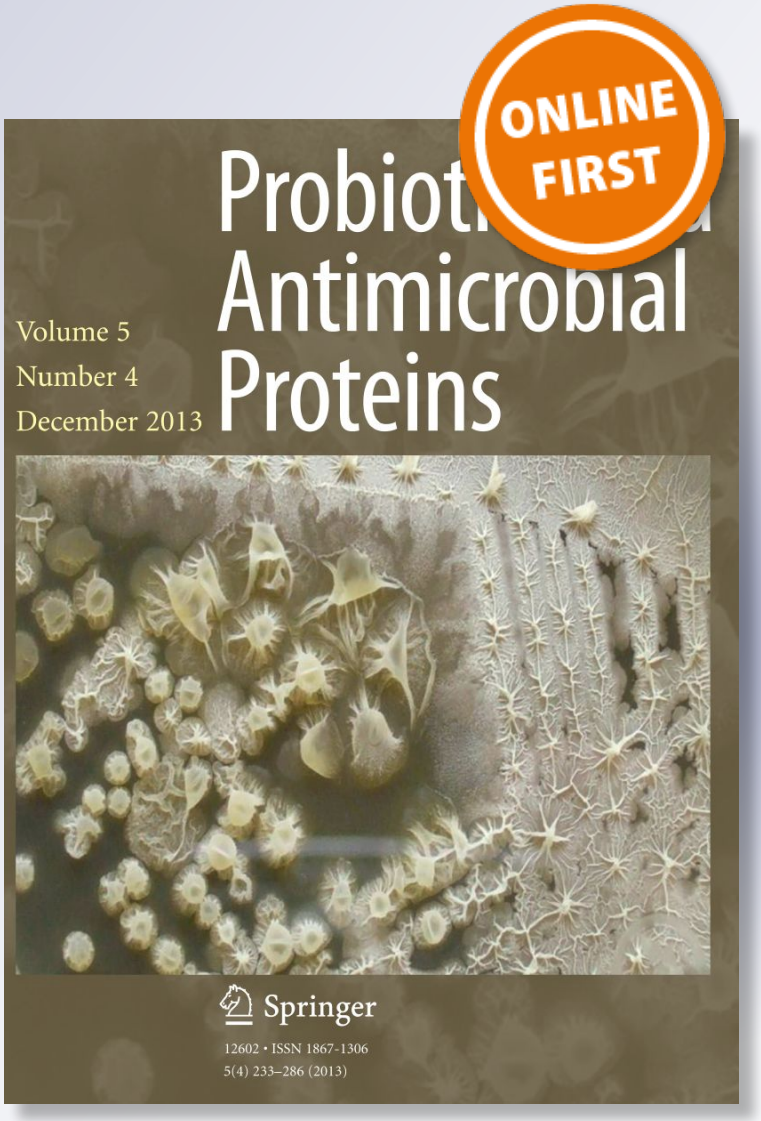

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Safety Evaluation and Colonisation Abilities of Four Lactic Acid Bacteria as Future Probiotics

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Abstract

The study evaluated the safety and colonisation properties of four lactic acid bacteria (LAB), by determining their cell hydrophobicity and aggregation abilities. In addition, the presence of virulence and resistance genes was assayed in these probiotic candidates. *Lactobacillus reuteri* ZJ625, *Lactobacillus reuteri* VB4, *Lactobacillus salivarius* ZJ614 and *Streptococcus salivarius* NBRC13956 were tested for cell surface hydrophobicity abilities against xylene, chloroform and ethyl acetate. The isolates were also tested for auto-aggregation and co-aggregation abilities; the optical densities of cell growth were measured after 1, 2, 3 and 4 h of experimental set-up. DNA was extracted from all the four isolates and amplified using PCR with specific primers to detect virulence genes of adhesion collagen protein (*ace*) and aggregation substances (*agg* and *asa*); also, resistance genes of Vancomycin *vanA*, Vancomycin *vanC1* and Vancomycin *vanC2/3* were assayed in the four isolates. The isolates showed high hydrophobicity to all solvents: xylene (78–84%), chloroform (68–75%) and ethyl acetate (52–60%). High auto- and co-aggregations ranging from 60 to 70% and from 45 to 56% respectively were observed in the isolates after 4 h of incubation at 37 °C. Some of the tested isolates showed the presence of virulence and resistance genes; however, this does not indicate that these genes are unsafe because their transmission and expression abilities are unknown. Therefore, in this study, the isolates studied are considered safe for use as future probiotics, as revealed from results presented, which generally represents the scanned safety evaluations of the isolates as promising probiotics.

Keywords Auto-aggregation · Co-aggregation · Hydrophobicity · Probiotics · Resistant genes · Virulent genes

Introduction

Lactic acid-producing bacteria are highly valued as beneficial microorganisms for their probiotic properties. Many probiotics, such *as Lactobacillus* strains, are currently in use extensively and produced on an industrial scale around the world [22]. However, the safety aspects of these isolates need to be considered, and possible undesirable effects from their consumptions and applications should be thoroughly and constantly been guided against [25]. In order to meet criteria for the selection as probiotic and the desirable beneficial health effects, probiotic bacteria have to be able to pass the gut survival challenges [16]. Criteria such as, abilities of isolates to aggregate and adhere to the intestinal epithelial cells of the host, are prerequisites for colonisation of probiotic strains in the gut. Aggregation, defined as the process of reversible accumulation of cells, causing them to naturally precipitate in the medium in which they are suspended, has two different types: auto-aggregation and co-aggregation. Auto-aggregation of probiotics is clumping of bacteria of the same strain and is necessary for adhesion of bacterial cells to intestinal epithelia walls. While co-aggregation is the result of cell-to-cell aggregation of two different bacterial strains [10], co-aggregation of probiotic bacteria with pathogens may prevent pathogens colonisation in the gut [14].

Adhesion of lactic acid bacteria (LAB) to host organism intestinal mucosa is essential for (a) colonisation in the intestinal tract, (b) antagonistic activity against enteric pathogens, (c) modulation of the immune system, and (d) increasing primary defence of the animal body, amidst many other functions [9]. Adherence of cells is normally associated with cell surface

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characteristics such as hydrophobicity, which may have effect on aggregation and adhesion of bacteria to different surfaces. Auto-aggregation ability test together with cell surface hydrophobicity and co-aggregation could be used as a preliminary screening identification of potential adherent bacteria with properties suitable for probiotics purposes [30].

Additionally, it is preferable that virulence genes should be absent from the genome of probiotics, the absence of transferrable genes providing resistance to clinically relevant antibiotics is also a desire [32]. Currently, the spread of antimicrobial resistance microorganisms is of serious concerns worldwide. Therefore, it is of utmost importance that potential probiotic strains be tested for antibiotic resistance genes [26], in order to prevent transmission of antibiotic resistance genes to the pathogens in the gut by the beneficial strains. The safety of selected probiotic strains should also be evaluated for potential virulence factors that might later initiate infection [17]. Since genes involved in these activities could be present, but not expressed, negative phenotypic results should be confirmed by proving the absence of virulence and resistance genes with molecular biology approach such as the use of polymerase chain reaction (PCR) techniques [31].

Nevertheless, antimicrobial resistance in potential probiotic strains is not considered a risk factor unless resistance is transferred to pathogens or it renders the probiotic untreatable in a very rare case of infections [34]. Therefore, individual evaluation of potential probiotic safety should be considered. Hence, this study investigated the presence of genes associated with virulence and resistance factors in the candidate isolates. Additionally, cell surface hydrophobicity, auto-aggregation and co-aggregation abilities of these isolates were evaluated. It is in our opinion that the study will give more understanding on the abilities of the chosen candidate strains to colonise the gastrointestinal tract with no aptitude to initiate infections and/or causes any harm to the host animal when consumed.

Materials and Methods

Origin of Bacterial Strains

The four bacterial isolates (*Lactobacillus reuteri* ZJ625, *Lactobacillus reuteri* VB4, *Lactobacillus salivarius* ZJ614 and *Streptococcus salivarius* NBRC 13956) used in this study were previously isolated and characterised from the gastrointestinal tract (GIT) of weaned piglets of indigenous South African Windsnyer pig breed. The donor piglets have the history of tolerance to diseases, high weaning rates and low incidences of post weaning diarrhoea (PWD). Combinations of both the molecular (16S ribosomal RNA) and analytical profile index (API® bioMerieux, SA) methods were used in identification of the isolates. These isolates were tested for probiotic properties, which include antimicrobial activities, bile salt tolerance, resistance to low pH and hydrolysis. The isolates were grown in De Man, Rogosa and Sharpe agar slants (MRS; Oxoid, England); the isolates were stored in ultra-low freezer (− 80 °C), until future use.

Preparation of Bacterial Isolates

Lactobacillus reuteri ZJ625, *Lactobacillus reuteri* VB4, *Lactobacillus salivarius* ZJ614 and *Streptococcus salivarius* NBRC 13956 were revived by inoculation in De Man, Rogosa and Sharpe broth (MRS; Oxoid, England) and grown in an anaerobic jar with CO_2-generating kits (Anaerogen; Thermofisher, UK) at 37 °C for 24 h.

Hydrophobicity Assay for Bacterial Isolates

Cell surface hydrophobicity, evaluated according to the method of Andrabi et al. [1], three different solvents, i.e. xylene, chloroform and ethyl acetate, were used for hydrophobicity analysis. Isolates were grown anaerobically in MRS broth for 24 h at 37 °C and the cells were harvested by centrifuging 5000×*g* for 15 min. The cell pellets were washed twice with Ringer's solution (6% NaCl, 0.0075% KCl, 0.01$CaCl_2$ and 0.01 $NaHCO_3$), suspended in the same solution, and the absorbance was measured at 580 nm (A_{580} initial). One millilitre of Ringer's solution was added to 3 mL of cell suspension and incubated for 10 min at room temperature; after incubation, the two-phase system was mixed by vortexing for 2 min, again incubated at room temperature for another 10 min to allow the phases to separate. Thereafter, the aqueous phase was carefully discarded and absorbance was measured (A_{580} solvent) using a Spekol® 1500 UV spectrophotometer. Hydrophobicity percentage was calculated using the formula:

$$\text{Hydrophobicity (H\%)} = [(A_{\text{initial}} - A_{\text{solvent}})/A_{\text{initial}}] \times 100$$

where A_{initial} and A_{solvent} represent the absorbance of aqueous phase before and after addition of absorbance, respectively.

Auto-aggregation Assay and Co-aggregation Ability of Bacterial Isolates

Four bacterial isolates were tested for auto-aggregation and co-aggregation abilities according to the procedure of Escamilla-Montes et al. [11]. Bacteria were grown anaerobically in MRS broth for 24 h at 37 °C; the cells were harvested by centrifuge at 5000×*g* for 15 min, washed twice, and suspended in phosphate-buffered saline (PBS) to obtain 10^8 cfu/mL. Bacterial cell suspensions (4 mL) were mixed by vortexing for 10 s and incubated at room temperature for 5 h. Every hour, 0.1 mL of the upper suspension was transferred to another 3.9 mL of PBS and absorbance measured at

600 nm using the Spekol® 1500 UV spectrophotometer; PBS was used as a blank solution to calibrate the UV spectrophotometer. The auto-aggregation percentage expressed as $1 - [A_t \quad A_0] \times 100$, where A_t represents the absorbance at time $t = 1, 2, 3, 4$ or 5 h and A_0 the absorbance at $t = 0$. The method for preparing the cell suspensions for co-aggregation was the same as that for auto-aggregation assay. *Enterococcus faecalis* ATCC 29212 represents pathogenic isolate and was grown in Trypticase soy broth. Cell suspension (2 mL) of *Enterococcus faecalis* ATCC 29212 was mixed with cell suspension (2 mL) of isolates by vortexing for 10 s. Centrifuge tubes containing 4 mL of each bacterial isolate or *Enterococcus faecalis* ATCC 29212 were used as control. The absorbance was measured as above and the percentage of co-aggregation was calculated using the equation of Balakrishna [5] as $[1 - 2A_{mix} \quad (A_{probio} + A_{indicat})] \times 100$ where A_{probio} and $A_{indicat}$ represented each bacterial isolate and *Enterococcus faecalis* ATCC 29212 (pathogenic bacterial isolate), respectively, while A_{mix} denotes the mixture of each bacterial isolate and pathogenic isolate.

Preparation of Bacterial Cell Cultures

Bacterial isolates, namely, *Lactobacillus reuteri* ZJ625, *Lactobacillus reuteri* VB4, *Lactobacillus salivarius* ZJ614 and *Streptococcus salivarius* NBRC13956, were cultured in MRS broth and incubated anaerobically with CO_2-generating kits (Anaerogen; Thermofisher, UK) at 37 °C for 24 h, following the procedure described by Casey et al. [8].

DNA Extraction from the Pure Cultures

The bacterial isolates were sub-cultured on MRS agar and incubated anaerobically at 37 °C for 24 h in order to obtain pure cultures. Subsequently, single colonies of bacterial cells were collected and the DNA was extracted using ZR Fungal/Bacterial DNA MiniPep™ (Zymo Research, USA) following the manufacturer's instructions. Bacterial samples were added directly to a ZR BashingBead™ Lysis Tube and efficiently lysed by bead beating without using organic denaturants or proteinases; the lysate was then washed on Zymo-Spin column, eluted, and the DNA was purified for PCR.

Detection of Virulence Genes and Resistance Genes by PCR

Isolates were screened for the presence of virulence genes *ace* (adhesion collagen protein), *agg* (aggregation) and *asa* (aggregation) and resistance genes *vanA* (Vancomycin), *vanC1* (Vancomycin) and *vanC2/3* (Vancomycin) by PCR following the method of Pieniz et al. [20]. The PCR was performed in total of 20 μL containing EconoTaq PLUS 2 × Master Mix (Lucigen), 7 μL of nuclease-free water, 1 μL of gDNA (10–30 ng/μL) and 1 μL each of forward and reverse primer (Table 1). The PCR conditions for virulence genes, *ace*, *asa* and *agg*, were as follows: initial denaturation at 95 °C for 5 min followed by 45 cycles of denaturation at 94 °C for 3 min, primers annealing at the 50 °C for each set of primers for 30 s, 68 °C extension for 2 min and final extension at 68 °C for 10 min. PCR conditions for resistance gene *van*A were as follows: initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 3 min, primers annealing at the 57 °C for each set of primers for 30 s, 68 °C extension for 1 min and final extension at 68 °C for 10 min. PCR conditions for resistance genes *van*C1 and *van*C2/3 were as follows: initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 3 min, primers annealing at the 54 °C for each set of primers for 30 s, 68 °C extension for 1 min and final extension at 68 °C for 10 min. The PCR products were analysed by electrophoresis on 1.5% agarose gel, stained by 5% (*w*/*v*) ethidium bromide solution, and the DNA bands were visualised under ultraviolet light.

Table 1 PCR primers and used to detect virulence and resistance genes on isolates

Primer	Sequence (5′-3′)	Product (bp)	Reference
ace f	AAAGTAGAATTAGATCACAC	320	[20]
ace r	TCTATCACATTCGGTTGCG		
agg f	AAGAAAAAGTAGACCAAC	1553	[20]
agg r	AACGGCAAGACAAGTAAATA		
asa f	GATACAAAGCCAATGTGGTTCCT	101	[20]
asa r	TAAAGAGTCGCCACGTTTCACA		
vanA f	GGGAAAACGACAATTGC	732	[20]
vanA r	GTACAATGCGGCCGTTA		
vanC1 f	GGTATCAAGGAAACCTC	822	[20]
vanC1 r	CTTCCGCCATCATAGCT		
vanC2/3 f	CGGGGAAGATGGCAGTAT	848	[20]
vanC2/3 r	CGCAGGGACGGTGATTTT		

Results

All bacterial isolates showed high hydrophobicity to all solvents used in the test. Hydrophobic cell surface was shown by high adherence to xylene (78–84%) followed by chloroform (68–75%) and the lowest was ethyl acetate (52–60%) (Fig. 1).

The auto-aggregation ability of isolates is presented on Fig. 2. All tested isolates showed high auto-aggregation ability at 4 h. *L .reuteri* VB4 showed highest auto-aggregation ability of 70% while *L. salivarius* ZJ614 showed low auto-aggregation ability of 60%.

All bacterial isolates exhibited a strong co-aggregation of 45 to 56% after 4 h of incubation at 37 °C as shown in Fig. 3. *L. reuteri* VB4 showed highest co-aggregation ability while *L. salivarius* ZJ614 showed the lowest co-aggregation ability. *E. faecalis* ATCC 29212 was used as positive control.

Discussion

Adhesion and cell surface properties such as hydrophobicity and auto-aggregation together with co-aggregation could be used as preliminary selection of isolates as probiotic bacteria candidate. These are very important attributes, which help in the attachment of various substrates that explain the probiotic nature of the microorganism [3].

Cell Surface Hydrophobicity

Cell hydrophobicity is an important property for probiotic bacteria; it is a prerequisite for probiotics to adhere to the intestinal epithelium in order to colonise the gastrointestinal tract by exerting beneficial effect of probiotics, such as exclusion of enteropathogenic bacteria [24]. Hydrophobic cell surface was shown by high adherence to xylene (78–84%) followed by chloroform (68–75%) and the lowest was ethylacetate (52–60%) (Fig. 1). The cell surface hydrophobicity varied with the isolates tested, *L. reuteri* VB4 exhibited high hydrophobicity than *L. reuteri* ZJ625, *L. salivarius* ZJ614 and *S. salivarius* NBRC13959 in all the solvents used for the experiments. The results obtained in this study are in agreement with those of Haitham et al. [12], who reported that the probiotic tested in their study showed high degree of hydrophobicity against all solvents used in their study. On the contrary, Andriantsoanirina et al. [2] observed poor hydrophobicity on some LAB isolates tested against xylene.

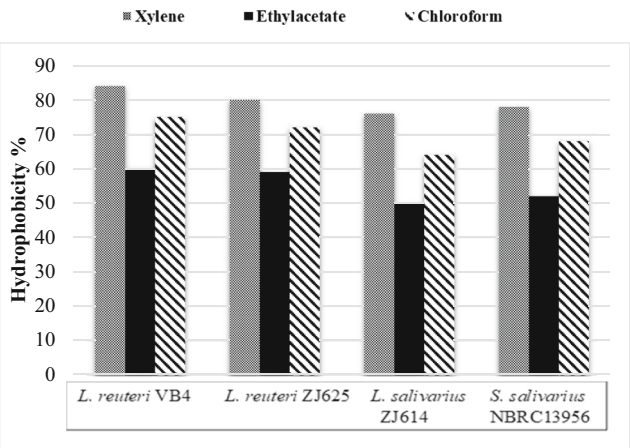

Fig. 1 Cell surface hydrophobicity of bacteria isolates against xylene, chloroform and ethyl acetate. ▩ Xylene, ■ ethylacetate and ◥ chloroform

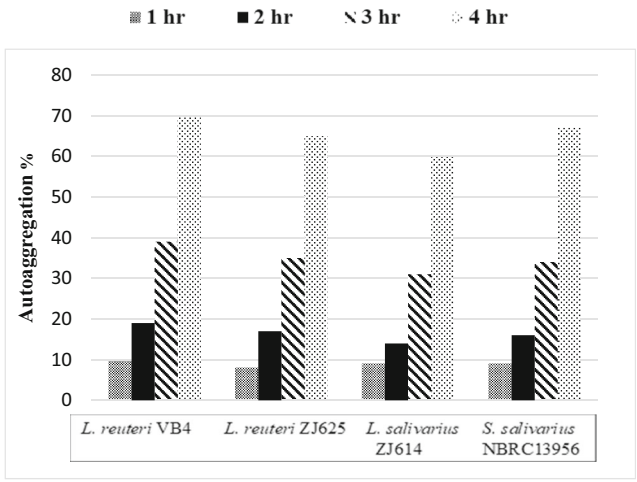

Fig. 2 Auto-aggregation ability of bacterial isolates at 1, 2, 3 and 4 h, serially. ▩ 1 h, ■ 2 h, ◥ 3 h and ⁘ 4 h

Auto-aggregation Ability

This phenomenon is very significant in the GI tract and it is mostly intended for action site for probiotics [21]. Auto-aggregation ability of cells plays a crucial role in adhesion to intestinal cells and prevention of pathogen colonisation. The auto-aggregation ability of bacterial isolates tested is presented in Fig. 2. All tested bacterial isolates showed high auto-

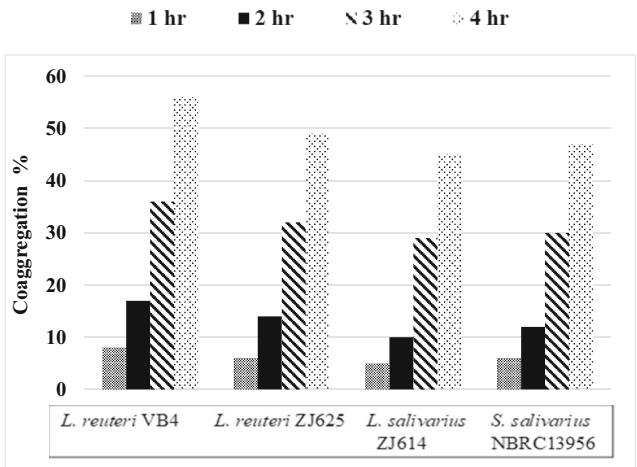

Fig. 3 Co-aggregation ability of bacteria isolates against pathogen *E. faecalis* ATCC 29212 at 1, 2, 3 and 4 h. ▩ 1 h, ■ 2 h, ◥ 3 h and ⁘ 4 h

Table 2 Detection of virulence and resistance genes from LAB isolates

Bacterial isolates	Virulence genes			Resistance genes		
	asa	*agg*	*ace*	*vanA*	*VanC2/3*	*VanC1*
L. reuteri ZJ625	–	–	–	–	–	–
L. salivarius ZJ414	–	–	–	–	–	–
S. salivarius NBRC13956	–	–	–	–	–	+
L. reuteri VB4	–	+	–	+	–	–

aggregation ability at 4 h. *L. reuteri* VB4 showed the highest auto-aggregation ability of 70% while *L. salivarius* ZJ614 showed low auto-aggregation ability of 60%. These results are in accordance with results of Sandrani et al. [23], who observed high ability auto-aggregation (72–88%) of *Lactobacillus* isolates. On contrary, Li et al. [15] observed different results. They reported that some of LAB isolates tested showed poor auto-aggregation ability.

Co-aggregation Ability

The co-aggregation ability percentages are shown in Fig. 3. All tested bacterial isolates showed some co-aggregation ability with *E. faecalis* ATCC 29212; however, this ability was also bacterial isolate-specific and the percentages gradually increased with time. The bacterial isolate *L. reuteri* VB4 (56%) showed the highest co-aggregation ability with *E. faecalis* ATCC 29212 while isolate *L. salivarius* ZJ14 (45%) showed lower co-aggregation ability. Co-aggregation is one of the desired properties for probiotics and it may play an important role in eliminating pathogens in the gastrointestinal tract by preventing adherence of pathogens to the host tissue [28]. Therefore, co-aggregation ability with potential pathogens may be used for preliminary screening of potentially probiotic bacteria suitable for administration to humans and animals [6]. These results correspond with those of Xu et al. [35], who reported all LAB strains tested presented co-aggregation with pathogens, in a strain-pathogen combination-dependent manner, while Taheri et al. [27] reported that their study showed no co-aggregation between the LAB isolates and pathogens tested. All isolates tested in this study showed high percentage of colonisation attributes (auto-aggregation, co-aggregation and cell hydrophobicity), with *L. reuteri* VB4 being the best of them all.

Safety status is one of the important aspects to consider when choosing a potential probiotic strain; hence, it is of importance to evaluate some safety aspects before probiotic cultures are put on the market, including screening of the presence of virulence genes and resistance genes in the potential probiotic strains [19].

The presence of virulence genes among tested strains is shown in Table 2. From all the tested strains, no presence of virulence gene *ace* and virulence gene *asa* was detected. Only virulence gene *agg* was detected on *L. reuteri* VB4 strains. The presence of virulence genes does not guarantee gene expression, and there is high possibility that these genes are not expressed or only weakly expressed [33]. These results are in accordance with those of Toğay et al. [29]; the authors observed the presence of virulence genes on some of the LAB strains tested, while Bennedsen et al. [7] found no virulence genes in all the tested LAB strains in their study.

The presence of resistance genes among all tested isolates is shown on Table 2. *L. reuteri* VB4 and *S. salivarius* NBRC13956 showed the presence of resistance gene *vanC* 2/3 and resistance gene *vanC1*, respectively. The presence of resistance gene *vanC 2/3* was not detected in all the isolates tested. In many cases, resistance genes are not transferrable; therefore, these isolates may not have safety concerns [4]. These results are similar to those of Hassan and Belal [13]; the authors found the presence of vancomycin-associated resistance genes on some of the tested enterococcus strains. On contrary, Nami et al. [18] did not find any presence of resistance genes on the tested LAB isolates. However, the presence of genes associated with virulence and resistance does not assure gene expression or transmissibility respectively. According to Wassenaar et al. [33], this does not make the LAB isolates virulence or resistance genes transmissible. Therefore, these isolates may be qualified as future probiotics.

Conclusion

The results from this study revealed high cell hydrophobicity, auto-aggregation and co-aggregation abilities for all bacterial isolates tested. In addition, these results indicate that the isolates tested might be good future probiotics to be used in animal feed, although some showed the presence of virulence and resistance genes. Research is ongoing in our labs on evaluating every other probiotic attributes, inherent in our chosen candidates, so that they may be released in the near future as commercial probiotics. From this study, it is recommended that more research be conducted regarding safety of the reported LAB isolates.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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