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ORIGINAL PAPER

Identification of *Lactobacillus plantarum* TW29-1 isolated from Iranian fermented cereal-dairy product (Yellow Zabol Kashk): probiotic characteristics, antimicrobial activity and safety evaluation

Mahboobe Saboktakin-Rizi[1] · Behrooz Alizadeh Behbahani[1] · Mohammad Hojjati[1] · Mohammad Noshad[1]

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Abstract
In this study, the probiotic characteristics of *Lactobacillus plantarum* isolated from an Iranian traditional fermented product, Yellow Zabol Kashk (YZK), were evaluated. The isolated strain was identified as *Lactobacillus plantarum* TW29-1 by 16S rRNA gene sequencing. *L. plantarum* TW29-1 had a remarkable tolerance to acidic pHs, bile salts, and simulated gastrointestinal juices. The strain also showed a 51.44% cell surface hydrophobicity, 40.49% auto-aggregation, and 28.63% co-aggregation. *L. plantarum* TW29-1 was found to have 12.5% adhesion, and it was also able to compete (50.19%) and inhibit (48.87%) *Salmonella typhimurium* adherence to Caco-2 cells. The growth of pathogenic bacteria (i.e., *Pseudomonas aeruginosa*, *Listeria innocua*, *Staphylococcus aureus*, *Escherichia coli*, and *S. typhimurium*) was strongly inhibited by the isolate and *S. aureus* was the sensitive strain. *L. plantarum* TW29-1 did not induce *DNase* or haemolytic activity, confirming its safety aspects. Based on the results, *L. plantarum* TW29-1 could be introduced as a novel probiotic strain with therapeutic and preservation properties for food and health-promoting purposes.

Keywords Adhesion · Anti-infection ability · Caco-2 cells · Cell surface hydrophobicity · Haemolytic activity

Introduction

Traditionally, fermentation has been employed to conserve and increase the sensory properties, functional features, and shelf-life of food. It is generally agreed that fermented foods contain beneficial microorganisms and their consumption is therefore considered as a supreme dietary strategy for health-promoting purposes [1]. Indeed, the interactions between human host and microbiota are physiologically important in health and diseases [2].

Fermented foods are predominantly rich in lactic acid bacteria (LAB), such as *Lactobacillus*, *Streptococcus*, and *Leuconostoc* species, which their beneficial roles on the immune system have been extensively reported in the literature [1]. LAB have the potential to improve the nutritional value of food and lactose digestion and control the intestinal infections, cancers, and serum cholesterol levels [3]. It is noteworthy that LABs with health-enhancing effects and high stability to harsh conditions are known as probiotics. The probiotic bacteria confer positive functions after post-colonization in the human gut, such as pathogen growth-suppression effect, reducing the formation of toxic compounds, deconjugation of bile salts, decreasing the serum cholesterol levels, inhibiting intestinal infections, preventing colon cancers, and immune system regulation [4].

Lactobacillus species are known as the most widespread group of the LAB family with some characteristics include non-spore formation, Gram-positive, rod-shaped, acid-resistant, facultative anaerobic, and catalase-negative with optimum growth temperature of 30–40 °C [5]. These species have been frequently isolated from different kinds of Iranian traditional fermented foods. YZK (Sistani) is a traditional cereal-dairy fermented product, which is highly consumed in Sistan-Baluchistan province. It is composed of some ingredients such as sour buttermilk, wheat flour, cumin, dill seeds, coriander, black pepper, garlic, turmeric, and onion. The mixture is placed in a cotton bag for 5–7 days which is then removed, kneaded, and powdered. The final product is

✉ Behrooz Alizadeh Behbahani
B.alizadeh@asnrukh.ac.ir

[1] Department of Food Science and Technology, Faculty of Animal Science and Food Technology, Agricultural Sciences and Natural Resources University of Khuzestan, Mollasani, Iran

cooked as a nutritious food in a short period of time, and it is rich in LABs such as *Lactobacillus* species [6].

Among the Lactobacillus species, *Lactobacillus plantarum* is the most ubiquitous one with useful characteristics which is normally found in a variety of fermented foodstuffs [7, 8]. Furthermore, *Lactobacillus plantarum* is widely applied in the industrial processing and fermentation of raw food materials. It is "generally recognized as safe" (GRAS) and has qualified presumption of safety (QPS) status [8, 9]. *Lactobacillus plantarum* strains must have a large capability of surviving in the gastrointestinal tract and adhere to its epithelial cells. Moreover, it should be a safe strain (Food and Agriculture Organization and World Health Organization) of animals and human [8, 10].

This study was therefore aimed to investigate the probiotic properties of *Lactobacillus plantarum* TW29-1 isolated from YZK, through assessing its resistance to the simulated gastric and small intestinal conditions, antibacterial and anti-adhesion effects against some food-borne pathogenic bacteria, haemolytic activity, and adhesion to intestinal cells.

Materials and methods

Materials

Chemicals and reagents used in this study were Genomic DNA Isolation VI kit (Asian Dena-Zist Co., Iran), PCR kit (Parstous Biotech, Mashhad, Iran), peptone water, de Man-Rogosa-Sharpe (MRS) agar and broth, Muller Hinton agar (Merck, Darmstadt, Germany), pepsin, trypsin, bile salt, Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin (10,000 U/mL), trypsin–EDTA, Triton X-100, gentamicin, erythromycin, tetracycline, cefixime, ampicillin, vancomycin, chloramphenicol, and kanamycin (Sigma-Aldrich, USA) and *DNase* medium (HiMedia, Mumbai, India).

Methods

Isolation and identification

The YZK samples were randomly collected from a local market (Zabol, Iran) and transferred to the laboratory under refrigeration conditions. The samples (5 g) were added to peptone water (0.1%; 45 mL) and homogenized (Seaward, Germany), followed by serial dilution preparation (10^{-1} to10^{-6}) and culture on MRS agar. The strain was isolated from the culture medium and it was subsequently subjected to Gram-staining and catalase assays. After that, the genomic DNA was extracted from the strain by Genomic DNA Isolation VI kit and cultured overnight in the MRS broth. The universal primers used for 16S rRNA gene amplification were 27FYM (5′-AGAGTTTGATYMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). DNA amplification was performed in 25.15 µL reaction volumes (16.5 µL water, 2.5 µL 10×buffer, 2 µL dNTP, 1.2 µL MgCl2, 1.25 µL primers, 0.2 µL Taq Polymerase, and 1.5 µL DNA template) using master PCR kit, and the PCR conditions were initial denaturation at 95 °C for 5 min (1 cycle), 35 cycles (94 °C for 30 s, 54 °C for 30 s, and 72 °C for 2 min), and final elongation at 72 °C for 10 min (1 cycle). The PCR product was then analysed for segment's amplification by agarose gel electrophoresis at 95 V for 45 min. The amplified gene was sequenced and the obtained nucleotide sequences were analysed for homology. The results revealed that the isolate, with catalase-negative and Gram-positive properties, is belonged to *L. plantarum* TW29-1 at a similarity value of 99% [6]. PCR product shown in Fig. 1.

pH stability

The stability of the strain to acidic pHs was evaluated according to the method described by Hojjati et al. (2020) with some changes. To do this, the strain was firstly

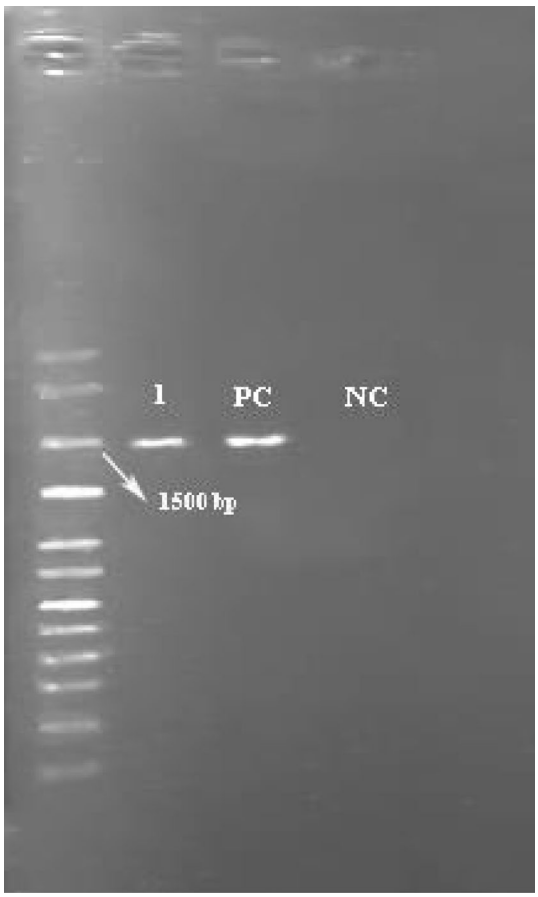

Fig. 1 PCR product amplified using primers 27FYM and 1492R. 1, subjected strain; *PC* positive control, *NC* negative control

inoculated into MRS broth and incubated at 37 °C for 24 h under anaerobic conditions. The microbial suspension (20 mL) was centrifuged at 9000×*g* for 5 min at 4 °C, and the precipitate (bacterial cells) was collected, washed with sterile phosphate buffer, and re-centrifuged. Subsequently, the precipitate was dissolved in sterile phosphate buffer to reach 0.6 absorbance at 600 nm. The microbial suspension (50 µL) was then inoculated into acidic phosphate buffer (450 µL; pHs 3, 4.5, and 6) and incubated at 37 °C for 2.5 h under anaerobic conditions. Afterward, serial dilutions (up to 10^{-10} in sterile phosphate buffer) were prepared and cultured on the MRS agar, which was followed by incubation at 37 °C under anaerobic conditions for 24 h. The colonies were finally counted and the survivability (%) of the strain was calculated [11].

Bile stability

The microbial strain was initially activated through inoculation in MRS broth and subsequent incubation at 37 °C for 24 h. The suspension was then centrifuged (9000×*g*, 5 min, 4 °C), and the precipitated cells were washed with sterile phosphate buffer and re-centrifuged based on the above conditions. The microbial suspension (100 µL) was cultured on MRS agar containing different bile salt concentrations (0.2, 0.5, 0.8, and 1.1% w/v). The culture medium was then incubated under anaerobic conditions (37 °C for 24 h) and the results were observed visually [12].

Stability to gastrointestinal digestion

The cell viability of the strain under gastrointestinal digestion was evaluated based on a procedure reported by Vasiee et al. (2020). Briefly, the bacterium was firstly inoculated in simulated gastric juice (containing 3 g L^{-1} pepsin in phosphate buffer; pH 3) for 1 h at 37 °C and the digested sample (1 mL) was then mixed with 9 mL simulated intestinal juice (containing 1 g L^{-1} trypsin in phosphate buffer; pH 8) and incubated for 2 h at 37 °C. The viable bacterial cells were cultured on MRS media at time intervals of 0, 1, 2, and 3 h [4].

Hydrophobicity

To determine the surface hydrophobicity of the strain, the probiotic *L. plantarum* TW29-1 was firstly harvested by centrifugation (5000×*g*, 15 min) of the microbial suspension, followed by washing the pellet with phosphate buffer and re-suspending in the same buffer to reach approximately 1.0 absorbance at 600 nm (H_1). The resulting bacterial suspension (3 mL) was then added to *n*-hexadecane (0.6 mL) and the mixture was vortexed for 2 min. After incubation (25 °C, 1 h), the absorbance of the aqueous phase (i.e., lower phase) was recorded at 600 nm (H_2), and the microbial cell surface hydrophobicity of the *L. plantarum* TW29-1 cells was calculated as below [11]:

$$\text{Surface hydrophobicity } (\%) = \left(\frac{H1 - H2}{H1}\right) \times 100.$$

Auto-aggregation capacity

The method of Fadda et al. (2017) with some modification was applied for evaluation of the auto-aggregation capacity of *L. plantarum* TW29-1. To do this, the overnight culture was centrifuged (10 min at 6000 rpm) and the precipitate was washed bcold phosphate buffer and dissolved in the same buffer to reach 0.6 absorbance at 600 nm (A_0). After that, the bacterial suspension was incubated (25 °C, 30 min) and its optical density was then recorded at 600 nm (A_1). In the final step, the auto-aggregation of the strain was calculated according to the following equation [13]:

$$\text{Auto-aggregation } (\%) = \left(\frac{A0 - A1}{A0}\right) \times 100.$$

Co-aggregation

To measure the co-aggregation, the absorbance of *L. plantarum* TW29-1 (A_L) and *Salmonella typhimurium* ATCC 14028 (A_S) suspensions were individually recorded at 600 nm. Then, the suspensions were mixed together for at least 30 s and after incubating t obtained mixture (30 min at 25 °C), its absorbance was determined at 600 nm (A_M) and co-aggregation was subsequently measured according to the following equation [14]:

$$\text{Co-aggregation } (\%) = \left(1 - \frac{AM}{\frac{AL+AS}{2}}\right) \times 100.$$

Adhesion capacity

Caco-2 cell lines were applied to determine the adhesion capacity of the strain. The cells were cultured in DMEM (containing 10% heat inactivated FBS and 1% penicillin–streptomycin) at 37 °C under humidified atmosphere of 5% CO_2. After 80% confluency, the trypsinization process by 1% trypsin–EDTA was performed to detach Caco-2 cells from the medium and the cells were then transferred to 6-well tissue plates at 30,000 cells/cm^2 density. The cells were incubated and the medium was regularly changed until a differentiated cell monolayer was obtained. To discard the old media, particularly penicillin–streptomycin, the plates were then emptied and washed with sterile phosphate buffer. In the next step,

the overnight culture of the strain was centrifuged (6000 rpm, 10 min) and the precipitate was washed by cold phosphate buffer (2-time), followed by suspending the bacterial cells in the DMEM at 10^8 bacteria/mL concentration and applying on confluent Caco-2 monolayer cells. The plates were then incubated (37 °C, 1 h, under 95% air-5% CO_2 atmosphere), and the unbounded bacteria were removed by sequential emptying and washing steps of the wells with phosphate buffer. The Caco-2 cells were then lysed by Triton X-100 (0.1% v/v) upon incubation for 10 min and the bacterial count was carried out on MRS agar. Finally, the adhesion capacity of the strain was measured as below [4]:

$$\text{Adhesion capacity } (\%) = \left(\frac{\text{A}}{\text{B}}\right) \times 100,$$

where, A is the number of adhered bacteria and B is the total number of bacteria incorporated into the wells.

Anti-adhesion activity

The anti-adhesion potential of *L. plantarum* TW29-1 against *Salmonella typhimurium* ATCC 14028 was investigated through competition and inhibition tests [11]. The equal number of the both bacteria was transferred to the wells and incubated under 5% CO_2 atmosphere (37 °C, 1 h). The free (unbounded) bacteria were washed by sterile phosphate buffer, and the adhered bacteria (the probiotic and pathogenic species) and Caco-2 cells were detached by 0.05% Triton X-100. A selective culture medium was used to count the pathogen, and the adherence competition between the species to bind to Caco-2 cells was measured according to the below equation:

$$\text{Competition } (\%) = \left(\frac{\text{Adhered } \textit{S.typhimurium} \text{ combination with } \textit{L.plantarum}}{\text{Bounded } \textit{S.typhimurium} \text{ in the absence of } \textit{L.plantarum}}\right) \times 100.$$

The inhibitory effect of *L. plantarum* on pathogen adhesion to the intestinal cells was evaluated by the inhibition assay. To do this, *L. plantarum* was firstly transferred to a Caco-2 cell-loaded well and incubated (1 h, 37 °C, under CO_2 pressure). Free bacteria were discarded through washing the well by phosphate buffer and the well was subsequently loaded with *S. typhimurium*. After incubating for 1 h and removing the unbounded *S. typhimurium* by phosphate buffer, the Caco-2 cells and bacterial species were detached by Triton X-100 and the bacteria count was performed. Finally, the inhibitory effect was calculated as below:

$$\text{Inhibition } (\%) = \left(1 - \frac{\textit{S.typhimurium} \text{ adhesion in the presence of } \textit{L.plantarum}}{\textit{S.typhimurium} \text{ adhesion in the absence of } \textit{L.plantarum}}\right) \times 100.$$

Antibacterial effect

Well and disc diffusion methods Antibacterial effect of *L. plantarum* TW29-1 was evaluated against some food-borne pathogenic bacteria (*Pseudomonas aeruginosa* PTCC 1707, *Escherichia coli* ATCC 2922, *Staphylococcus aureus* ATCC 25923, *Salmonella typhimurium* ATCC 14028, and *Listeria innocua* ATCC 33090), according to a procedure described by Georgieva et al. (2015), with some modifications. The strain was cultured in MRS broth (28 h, 37 °C) and the medium was then centrifuged (5000 rpm, 4 °C, 20 min). Part of the cell-free supernatant (CFS) was employed without pH changing; whilst, the other part of the CFS was adjusted to pH 5.5 to neutralize the effect of acidic conditions caused by organic acids. The mixtures were filtered (0.22 µm) and the neutralized and acid CFSs (nCFSs and aCFSs) fractions were freeze-dried. Sterile distilled water (2 mL) was used to hydrate the samples and 100 µL of the CFS fractions were then incorporated into the wells (6.8 mm in diameter) on the Muller-Hinton-Agar and loaded with pathogenic bacteria (0.5 McFarland concentration). The disc diffusion agar method was also used to investigate the antimicrobial potential of the isolate. The discs (6.2 mm in diameter) were firstly impregnated with the bacterial suspension and then placed on the Muller–Hinton–Agar medium. The culture medium was incubated for 48 h at 37 °C and inhibition zone around the wells/discs was measured [15]

Modified double layer method The antagonistic activity of the isolate was also evaluated by the spot-on-lawn or double layer method based on the procedure of Momenzadeh et al. (2021) with some changes [16]. The probiotic strain was cultured in MRS broth (12 h, 37 °C) and it was then spotted onto the MRS agar followed by incubation (24 h, 37 °C) and overloading with melted Muller Hinton agar. After solidification of the medium, it was inoculated with the pathogenic bacteria (100 µL) and then re-incubated (24 h, 37 °C). In the final step, the clear zones around spots were measured and reported as the sensitivity of the food-borne pathogens to the isolate.

Safety evaluation

Antibiotic susceptibility The antibiotic susceptibility of *L. plantarum* TW29-1 to common antibiotics gentamicin (10 µg), erythromycin (15 µg), tetracycline (30 µg), cefixime (5 µg), ampicillin (10 µg), vancomycin (30 µg), chloramphenicol (30 µg), and kanamycin (30 µg) was evaluated following the method of Zhou et al. (2005) with modifications. The strain (0.5 McFarland) was initially cultured in MRS agar and the antibiotic discs were then placed on the medium. The plates were subjected to fixation at room tem-

perature (10 min) and incubation at 37 °C for 48 h. The inhibition zone around the antibiotic discs was finally measured [17].

DNase activity The probiotic *L. plantarum* TW29-1 was tested for *DNase* activity using *DNase* medium for 48 h incubation at 37 °C. *DNase* production was confirmed if a clear and pinkish zone was formed around the colonies [18].

Haemolytic activity The strain was cultured in 7% v/v sheep blood agar and then incubated (48 h, 37 °C). The presence of blood lyses zones around the colonies was considered as haemolytic strain (β-haemolysis). If the strain created green-hued zones around the colonies or did not cause any change on the blood plate, it was considered as α-haemolysis or γ-haemolysis, respectively [19].

Statistical analysis

The results were analysed by SPSS software (version 22) through one-way analysis of variance (ANOVA). The differences between the means were determined by the Duncan test at confidence level of 95% ($p < 0.05$). All experiments were carried out at three replications.

Results and discussion

Acid and bile tolerance

Resistance to some lethal conditions such as bile salts and acidic pHs is an essential factor in predicting the survivability and growth statues of bacterial species in the digestive tract, and therefore is a prerequisite for selecting isolates with probiotic properties [19]. The isolate had a pH-dependent survivability; the cell viability decreased significantly from 99.20 to 56.25% as the pH value of the medium decreased from 6 to 3 (Fig. 2). The relatively high resistance of the isolate to acidic pHs could be ascribed to the constant gradient between intracellular and extracellular pHs. Indeed, it has been claimed that F_1F_0-ATPase is one of the main mechanisms for protecting Gram-positive microorganisms against acidic conditions. It is induced by acidic conditions and leads to the intracellular pH increment, thereby alleviating acidic-based lethal effects [20]. The composition of the cytoplasmic membrane has also a great effect on the acid tolerance of LABs, which it is largely affected by the bacterium type, growth medium composition, and the incubation conditions [21]. It should be also underlined that although acidic conditions have significant pathogen growth-suppression effects, they can also affect the growth of beneficial microorganisms; however, the food matrix and some compounds (e.g., prebiotics) could protect probiotics from lethal effects of acidic conditions [22].

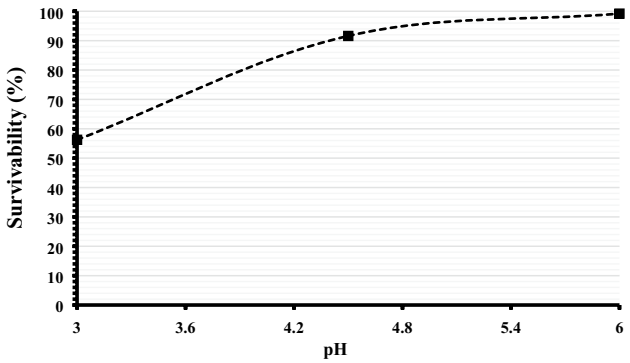

Fig. 2 Survivability (%) of *L. plantarum* TW29-1 under acidic conditions (pHs 3, 4.5, and 6)

Table 1 Survivability of *L. plantarum* TW29-1 under different bile salt concentrations

	Bile salt concentration (%)			
	0.2	0.5	0.8	1.1
Survivability	+++++	++++	+++	++

++ Moderate survivability
+++ Relatively high survivability
++++ High survivability
+++++ Very high survivability

Bile helps the enzymes of human body to breakdown fats to free fatty acids and facilitates fat molecules digestion in the small intestine through emulsion formation. All bacteria contain remarkable content of lipids in their cell walls which could be therefore degraded by bile salts, leading to the bacterial content leakage and cell death. In this context, bile tolerance is an essential property of a probiotic strain which enables it to survive, grow, and to impart its function in the small intestine [23]. Cell viability of the isolate was decreased as a function of bile salt concentration. The higher the bile concentration, the lower was the cell viability (Table 1). It is necessary to note that the bile salt levels in the body do not usually exceed 0.3% [4]. The cell viability of the strain in the present study was high in the bile concentration range of 0.2–0.5%. In line with our results, the viability of LABs under high bile salt stresses (e.g., 0.3% concentration) has been reported in some studies [4, 5, 23]. The bile tolerance of probiotic strains could be attributed to their hydrolysing activity to de-conjugate bile salts to amino acids and cholesterol [4, 24].

Tolerance to gastric and small intestinal juices

The simulated gastric conditions resulted in an approximately 1.24-fold reduction in the number of viable cells of the isolate (Fig. 3). This is mainly due to the acidic conditions of the gastric juice secreted in the stomach and the antimicrobial activity of pepsin. Indeed, pepsin facilitates bacterial death in the stomach and its antimicrobial effect is increased under acidic circumstances [25]. The simulated intestinal conditions had also a remarkable effect on the survivability of the isolate; incubation for 2 h led to an about 1.32-fold decrease and further incubation time caused an approximately 1.37-fold reduction in the number of *L. plantarum* TW29-1. The results of the present research are in accordance with other findings indicating resistance of *Lactobacillus* species and LABs to the lethal conditions of the gastrointestinal tract. By studying the survivability of *L. plantarum* strain L15 under simulated gastric conditions, Alizadeh Behbahani et al. (2019) stated that the number of bacteria decreased about 1.38-fold after 1 h incubation time under *in-vitro* gastric digestion [26]. In another study, it was shown that *Lactobacillus brevis* gp104 underwent an approximately 1.21-fold reduction in cell number after sequential gastrointestinal digestion (3 h) [11]. These studies revealed that LABs, specially *L. plantarum* TW29-1 are able to resist the detrimental effects of gastrointestinal tract and reach the colon to exert their beneficial functions.

Surface hydrophobicity

The cell surface hydrophobicity of a probiotic strain determines its ability to adhere to the epithelial cells surface. The surface hydrophobicity of the isolate was found to be 51.44% (Fig. 4). Similar results have been reported in the literature. *L. plantarum* strain L15 isolated from Horreh showed a hydrophobicity of approximately 53% [26]. A cell hydrophobicity range of 6.58 to 73.3% was also observed for *L. plantarum* strains isolated from Siahmazgi traditional cheese [27]. In another study, several species of LABs origin were isolated from kimchi, and *L. brevis* KU15006 had the highest surface hydrophobicity (48.07%) amongst other [28]. The cell surface hydrophobicity of 53.3% has been also reported for *L. brevis* ku200019 [29]. This characteristic is supposed to be an another important factor in determining the ability of probiotic bacteria to adhere to the intestinal cells and their subsequent proliferation.

Auto and co-aggregation

The adherence ability of a probiotic bacterium to the epithelial cells and mucosal surfaces is mediated by its auto-aggregation property; whilst, the co-aggregation helps probiotic bacteria to bind to pathogenic ones. In this way, the adherence of pathogens to the intestinal cells and their subsequent biofilm formation are greatly suppressed [4] The auto and co-aggregation capacity of the isolate were found to be 40.49% and 28.63%, respectively (Fig. 4). These results are in accordance with those of Gandomi et al. (2019), who worked on the probiotic properties of *L. plantarum* strains isolated from a traditional cheese, and showed that the auto-aggregation and co-aggregation of the isolates were ranged from 58.21–73.99 to 1.46–49%, respectively [27]. Moreover, it was shown that *L. plantarum* strains possess the potential to auto-aggregate and co-aggregate with different food-borne pathogens (i.e., enterohaemorrhagic *E. coli*, *Listeria monocytogenes*, and *Salmonella enterica* serotype Typhimurium). These properties are important in the selection of probiotic strains [30]. High auto-aggregation and hydrophobicity levels could lead to the attachment and colonization of probiotic bacteria on the intestinal epithelial cells. And low co-aggregation capacity could inhibit the biofilm formation ability of pathogens and therefore facilitate their removal from the gastrointestinal tract [31].

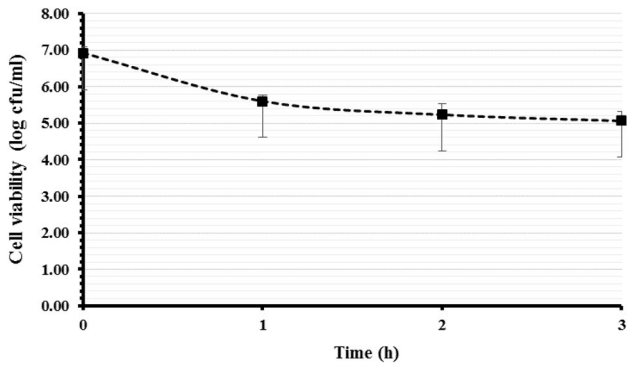

Fig. 3 Tolerance of *L. plantarum* TW29-1 to gastric and intestinal juices

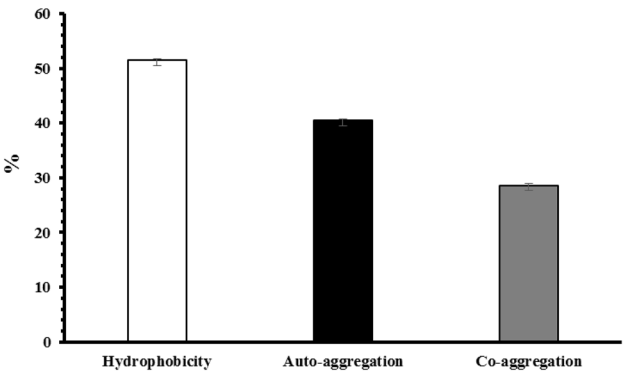

Fig. 4 Hydrophobicity, auto-aggregation, and co-aggregation of *L. plantarum* TW29-1

Adhesion capacity

A criterion to select probiotic bacteria is their intestinal colonization potential, at least temporarily, through adhering to the epithelium cells of intestine [13]. The adhesion potential of the isolate was investigated by a largely accepted in-vitro model, Caco-2 cells. The isolate had an adhesion capacity of 12.5% to the cells. In line with the results of the present research, it has been reported that *L. plantarum* Lp91 showed an adhesive degree of 10.2% to Caco-2 cells [32]. Similarly, it was shown that *L. plantarum* L15 had a potential to adhere to Caco-2 cells by a 12.2% adhesion degree [26]. The adhesive activities of 8.5% and 6.7% have been also reported for *L. plantarum* 122E and *L. plantarum* ATCC 8014, respectively [33, 34]. It has been claimed that a direct relationship is observed between in-vitro adhesion and in-vivo stability/colonization. And S-layer, lectin-like protein, lipoteichoic acid, and glyceraldehyde-3-phosphate dehydrogenase of probiotic bacteria facilitate their potential to adhere to the intestinal receptor cells and colonize the corresponding cells [11]. Probiotic strains could improve the immune system and heal the damaged intestinal tissues [5].

Anti-adhesion capacity

Pathogenic bacteria initially adhere to intestinal cells of the host to colonize, grow, and release enzymes/toxins to cause infections. In this context, probiotic strains are able to decrease the adhesion of pathogenic bacteria via producing antimicrobial compounds and blocking receptor molecules [35] The adhesion degree of *S. typhimurium* to the intestinal cells was decreased by 50.19 ± 0.29% when the probiotic isolate and *S. typhimurium* were simultaneously added to the culture medium containing Caco-2 cells. This anti-adhesion effect could be attributed to the competition between the strains for receptors and nutrients or the formation of antimicrobial substances by the isolate, such as bacteriocins, polysaccharides, organic acids, and hydrogen peroxide [36]. The inhibitory effect of the isolate on the adherence of *S. typhimurium* to the intestinal cells was observed to be 48.87 ± 0.49%. The anti-adhesion capacity of the isolate is similar to the reported values of 45–56% for *L. plantarum* L15 [26], 47.7% for *L. plantarum* CS24.2 [36], and 47–52% for *L. brevis* gp104 [11]. The adhesion properties of probiotic strains and pathogens could be influenced by in-vitro (such as growth medium, buffer composition, incubation time, and concentration of bacterium) and in-vivo (such as food matrix digestion and normal intestinal microbiota) conditions [37]. Accordingly, it has been reported that the higher is probiotic concentration and affinity to intestinal receptors, the lower is pathogens adhesion [38].

Antibacterial activity

Antagonistic activity against food-borne pathogens is an another important property of probiotic strains. As can be seen in Table 2, the aCFS fraction of *L. plantarum* TW29-1 was able to inhibit the growth of food-borne pathogens; however, the antimicrobial effect of the isolate was decreased upon pH neutralization and the nCFS fraction did not show any antibacterial effect against *E. coli* and *S. typhimurium* in well diffusion agar assay and against *E. coli*, *S. typhimurium*, and *P. aeruginosa* in disc diffusion agar method. Indeed, the growth of *S. aureus* was suppressed to a greater extent compared to the other pathogens. The results of antimicrobial activity of Lactobacillus plantarum TW29-1 by modified double layer method on pathogenic bacteria are shown in Table 2.

The antimicrobial activity of probiotics is mainly due to the production of bacteriocins, organic acids, and short-chain fatty acids. The growth of Gram-positive pathogens is usually inhibited by bacteriocins, whereas organic acids, hydroxyl fatty acids, and hydrogen peroxide are more active against Gram-negative pathogens [39–41]. In congruent with our study, it has been reported that *L. plantarum* AF1 and

Table 2 Antimicrobial effect of *L. plantarum* TW29-1 against pathogenic bacteria, through well diffusion agar, disc diffusion agar and modified double layer methods

Antimicrobial method	Pathogenic strains				
	E. coli	*P. aeruginosa*	*S. typhimurium*	*L. innocua*	*S. aureus*
Well diffusion agar (mm)					
aCFS	16.10 ± 0.42^{d}	17.70 ± 0.18^{c}	10.80 ± 0.50^{e}	18.80 ± 0.30^{b}	20.20 ± 0.38^{a}
nCFS	–	7.10 ± 0.46^{b}	–	11.90 ± 0.52^{a}	12.60 ± 0.56^{a}
Disc diffusion agar (mm)					
aCFS	15.00 ± 0.19^{c}	16.40 ± 0.63^{b}	9.00 ± 0.58^{d}	17.40 ± 0.48^{b}	19.70 ± 0.55^{a}
nCFS	–	–	–	8.60 ± 0.34^{b}	11.80 ± 0.50^{a}
Modified double layer method (mm)	20.10 ± 0.32^{d}	21.30 ± 0.58^{c}	12.20 ± 0.50^{e}	24.90 ± 0.46^{b}	29.80 ± 0.63^{a}

aCFS acidic cell-free supernatants, *nCFS* neutralize cell-free supernatants, – no growth inhibition zone different letters (a, b, c, d and e) in each row show significant difference at $P \leq 0.05$

L. plantarum NO1 isolated from kimchi showed strong antibacterial effect against *E. coli*, *S. aureus*, *Salmonella typhi*, and *L. monocytogenes*, with *S. aureus* being more sensitive than other pathogens [42]. And this beneficial effect was attributed to the release of inhibitory substances by the LAB strains, such as organic acids, hydrogen peroxide, carbon dioxide, bacteriocins, cyclo (Leu-Leu), and δ-dodecalactone. In another research, it was shown that *L. acidophilus* LAP5 and *L. fermentum* LF33 (isolated from wine and poultry) were able to strongly prevent the growth of *E. coli*, *S. typhimurium*, *S. aureus*, and *Bacillus cereus* [43]. The production of antimicrobial compounds, which prevent the growth of pathogens, is a beneficial property of probiotic strains.

Safety evaluation

The isolate *L. plantarum* TW29-1 did not show *DNase* activity or induce haemolysis on blood agar, in agreement with the findings of Ryu and Chang [42], demonstrating its safety for potential health-promoting purposes. Resistance to antibiotics is an another characteristics of probiotic bacteria which determines their safety for food consumption. The isolate was resistant to amoxicillin, erythromycin, and gentamycin, semi-sensitive to ampicillin and vancomycin, and fully sensitive to tetracycline and chloramphenicol (Fig. 5). Antibiotics show antimicrobial effect through different mechanisms include (i) inhibition of protein synthesis by erythromycin, streptomycin, tetracycline, and chloramphenicol, (ii) preventing mRNA synthesis by metronidazole and rifampicin, and (iii) destruction of bacterial cell wall by penicillin, vancomycin, and ampicillin. It is noteworthy that probiotic strains with native resistance to common antibiotics could be beneficial for survivability/colonization in the intestinal tract to restore gut microbiota after antibiotic therapy. Nonetheless, some probiotics carry resistant genes of therapeutic antibiotics and have the potential to transmit them to gastrointestinal pathogens and in turn cause serious safety issues [11, 16]. For safety reasons, it has been claimed that the commercially exploitable probiotics should be sensitive to commonly used antibiotics to be employed as starter cultures or co-cultures [18]. The present isolate was mainly sensitive or semi-sensitive to most of the antibiotics tested, and it can be therefore used as a potentially probiotic bacterium for food or health-promoting applications.

Conclusions

New species and more specific strains of bacteria are sought to develop novel probiotics. In this way, traditional fermented foods could be considered as safe sources of new microorganisms with probiotic features. Therefore, this study focused on the functionality and safety of

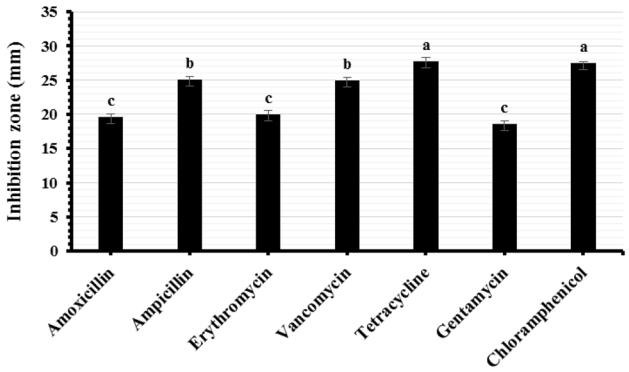

Fig. 5 Effect of common antibiotics on the growth of *L. plantarum* TW29-1

Lactobacillus plantarum TW29-1 strain isolated from YZK. By investigating its acid/bile tolerance, gastrointestinal fate, cell adherence, antagonistic activity against pathogens, and haemolytic and *DNase* activity, the isolate appears to meet the functional criteria needed to be a health-promoting probiotic strain. *L. plantarum* TW29-1 could be therefore considered as a novel probiotic candidate.

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Compliance with ethical standards

Conflict of interest There are no conflict of interest to declare.

References

1. S. Rezac, C.R. Kok, M. Heermann, R. Hutkins, Fermented foods as a dietary source of live organisms. Front. Microbiol. **9**, 1785 (2018)
2. A. Peters, P. Krumbholz, E. Jäger, A. Heintz-Buschart, M.V. Çakir, S. Rothemund, A. Gaudl, U. Ceglarek, T. Schöneberg, C. Stäubert, Metabolites of lactic acid bacteria present in fermented foods are highly potent agonists of human hydroxycarboxylic acid receptor 3. PLoS Genet. **15**(3), e1008145 (2019)
3. S.E. Gilliland, Health and nutritional benefits from lactic acid bacteria. FEMS Microbiol. Rev. **7**(1–2), 175–188 (1990)
4. A. Vasiee, F. Falah, B. Alizadeh Behbahani, F. Tabatabaee-Yazdi, Probiotic characterization of Pediococcus strains isolated from Iranian cereal-dairy fermented product: interaction with pathogenic bacteria and the enteric cell line Caco-2. J. Biosci. Bioeng. **130**(5), 471–479 (2020)
5. F. Falah, A. Vasiee, B. Alizadeh Behbahani, F.T. Yazdi, S. Moradi, S.A. Mortazavi, S. Roshanak, Evaluation of adherence and anti-infective properties of probiotic *Lactobacillus fermentum* strain 4–17 against *Escherichia coli* causing urinary tract infection in humans. Microb. Pathog. **131**, 246–253 (2019)

6. Y.F. Tabatabaei, A. Vasiee, B.B. Alizadeh, S. Mortazavi, Diversity of lactic acid bacteria isolated from yellow zabol kashk using 16S rRNA gene sequence analysis. Iran. J. Food Sci. Technol. **59**(13), 25–36 (2017)
7. A. Guidone, T. Zotta, R.P. Ross, C. Stanton, M.C. Rea, E. Parente, A. Ricciardi, Functional properties of *Lactobacillus plantarum* strains: a multivariate screening study. LWT-Food Sci. Technol. **56**(1), 69–76 (2014)
8. S.S. Behera, R.C. Ray, N. Zdolec, *Lactobacillus plantarum* with functional properties: an approach to increase safety and shelf-life of fermented foods. BioMed. Res. Int. **2018**, 9361614 (2018)
9. E.P.O.B. Hazards, A. Ricci, A. Allende, D. Bolton, M. Chemaly, R. Davies, P.S.F. Escámez, Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 5: suitability of taxonomic units notified to EFSA until September 2016. EFSA J. **15**(3), e04663 (2017)
10. F.F. Jia, L.J. Zhang, X.H. Pang, X.X. Gu, A. Abdelazez, Y. Liang, S.R. Son, X.C. Meng, Complete genome sequence of bacteriocin-producing *Lactobacillus plantarum* KLDS1.0391, a probiotic strain with gastrointestinal tract resistance and adhesion to the intestinal epithelial cells. Genomics **109**(5–6), 432–437 (2017)
11. M. Hojjati, B. Alizadeh Behabahani, F. Falah, Aggregation, adherence, anti-adhesion and antagonistic activity properties relating to surface charge of probiotic *Lactobacillus brevis* gp104 against *Staphylococcus aureus*. Microb. Pathog. **147**, 104420 (2020)
12. A.M.O. Leite, M.A.L. Miguel, R.S. Peixoto, P. Ruas-Madiedo, V.M.F. Paschoalin, B. Mayo, S. Delgado, Probiotic potential of selected lactic acid bacteria strains isolated from Brazilian kefir grains. J. Dairy Sci. **98**(6), 3622–3632 (2015)
13. M.E. Fadda, V. Mosa, M. Deplano, M.B. Pisano, S. Cosentino, In vitro screening of Kluyveromyces strains isolated from Fiore Sardo cheese for potential use as probiotics. LWT **75**, 100–106 (2017)
14. E. Kiray, E. Kariptas, S.Y. Azarkan, Evaluation of vaginal lactobacilli with potential probiotic properties and biotherapeutic effects isolated from healthy Turkish women. PSM Microbiol. **4**(3), 56–70 (2019)
15. R. Georgieva, L. Yocheva, L. Tserovska, G. Zhelezova, N. Stefanova, A. Atanasova et al., Antimicrobial activity and antibiotic susceptibility of *Lactobacillus* and *Bifidobacterium* spp. intended for use as starter and probiotic cultures. Biotechnol. Biotech. Eq. **29**(1), 84–91 (2015)
16. S. Momenzadeh, H. Jooyandeh, B. Alizadeh Behbahani, H. Barzegar, Evaluation of probiotic and antibacterial properties of *Lactobacillus fermentum* SL163–4. Iran. J. Food Sci. Technol. Res. **17**(2), 233–242 (2021)
17. J.S. Zhou, C.J. Pillidge, P.K. Gopal, H.S. Gill, Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains. Int. J. Food Microbiol. **98**(2), 211–217 (2005)
18. H. Gupta, R.K. Malik, Incidence of virulence in bacteriocin-producing enterococcal isolates. Le Lait. **87**(6), 587–601 (2007)
19. S. Pieniz, R. Andreazza, T. Anghinoni, F. Camargo, A. Brandelli, Probiotic potential, antimicrobial and antioxidant activities of *Enterococcus durans* strain LAB18s. Food Control **37**, 251–256 (2014)
20. P.D. Cotter, C. Hill, Surviving the acid test: responses of Gram-positive bacteria to low pH. Microbiol. Mol. Boil. Rev. **67**(3), 429–453 (2003)
21. A.R. Madureira, C.I. Pereira, K. Truszkowska, A.M. Gomes, M.E. Pintado, F.X. Malcata, Survival of probiotic bacteria in a whey cheese vector submitted to environmental conditions prevailing in the gastrointestinal tract. Int. Dairy J. **15**(6–9), 921–927 (2005)
22. M. Nooshkam, A. Babazadeh, H. Jooyandeh, Lactulose: properties, techno-functional food applications, and food grade delivery system. Trends Food Sci. Technol. **80**, 23–34 (2018)
23. J. Šušković, B. Kos, S. Matošić, V. Besendorfer, The effect of bile salts on survival and morphology of a potential probiotic strain *Lactobacillus acidophilus* M92. World. J. Microbiol. Biotechnol. **16**(7), 673–678 (2000)
24. A. Bezkorovainy, Probiotics: determinants of survival and growth in the gut. Am. J. Clin. Nutr. **73**(3), 399s–405s (2001)
25. H. Zhu, C.A. Hart, D. Sales, N.B. Roberts, Bacterial killing in gastric juice–effect of pH and pepsin on *Escherichia coli* and *Helicobacter pylori*. J. Med. Microbiol. **55**(9), 1265–1270 (2006)
26. B. Alizadeh Behbahani, M. Noshad, F. Falah, Inhibition of *Escherichia coli* adhesion to human intestinal Caco-2 cells by probiotic candidate *Lactobacillus plantarum* strain L15. Microb. Pathog. **136**, 103677 (2019)
27. H. Gandomi, A. Farhangfar, A. Akhondzadeh Basti, A. Misaghi, N. Noori, Auto and co-aggregation, hydrophobicity and adhesion properties of *Lactobacillus plantarum* strains isolated from Siahmazgi traditional cheese. Food Health **2**(1), 1–5 (2019)
28. S.H. Son, H.L. Jeon, S.J. Yang, N.K. Lee, H.D. Paik, In vitro characterization of *Lactobacillus brevis* KU15006, an isolate from kimchi, reveals anti-adhesion activity against foodborne pathogens and antidiabetic properties. Microb. Pathog. **112**, 135–141 (2017)
29. K.M.G.M.M. Kariyawasam, S.J. Yang, N.K. Lee, H.D. Paik, Probiotic properties of *Lactobacillus brevis* KU200019 and synergistic activity with Fructooligosaccharides in antagonistic activity against foodborne pathogens. Food Sci. Anim. Resour. **40**(2), 297–310 (2020)
30. T. Janković, J. Frece, M. Abram, I. Gobin, Aggregation ability of potential probiotic *Lactobacillus plantarum* strains. Int. J. Sanit. Eng. Res **6**(1), 19–24 (2012)
31. D.N. Furtado, S.D. Todorov, M. Landgraf, M.T. Destro, B.D.G.M. Franco, Bacteriocinogenic *Lactococcus lactis* subsp: lactis DF04Mi isolated from goat milk: evaluation of the probiotic potential. Braz. J. Microbiol. **45**(3), 1047–1054 (2014)
32. R.K. Duary, Y.S. Rajput, V.K. Batish, S. Grover, Assessing the adhesion of putative indigenous probiotic lactobacilli to human colonic epithelial cells. Indian J. Med. Res. **134**(6), 664–671 (2011)
33. E.M. Tuomola, S.J. Salminen, Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. Int. J. Food Microbiol. **41**(1), 45–51 (1998)
34. M.A. Bianchi, D. Del Rio, N. Pellegrini, G. Sansebastiano, E. Neviani, F. Brighenti, A fluorescence-based method for the detection of adhesive properties of lactic acid bacteria to Caco-2 cells. Lett. Appl. Microbiol. **39**(3), 301–305 (2004)
35. A. Nowak, I. Motyl, In vitro anti-adherence effect of probiotic *Lactobacillus* strains on human enteropathogens. Biotechnol. Food Sci. **81**, 103–112 (2017)
36. A.S. Dhanani, S.B. Gaudana, T. Bagchi, The ability of Lactobacillus adhesin EF-Tu to interfere with pathogen adhesion. Eur. Food Res. Technol. **232**(5), 777–785 (2011)
37. A.C. Ouwehand, S. Salminen, In vitro adhesion assays for probiotics and their in vivo relevance: a review. Microb. Ecol. Health Dis. **15**(3), 175–184 (2003)
38. Y.K. Lee, C.Y. Lim, W.L. Teng, A.C. Ouwehand, E.M. Tuomola, S. Salminen, Quantitative approach in the study of adhesion of lactic acid bacteria to intestinal cells and their competition with enterobacteria. Appl. Environ. Microb. **66**(9), 3692–3697 (2000)
39. M.L. Chikindas, R. Weeks, D. Drider, V.A. Chistyakov, L.M. Dicks, Functions and emerging applications of bacteriocins. Curr. Opin. Biotechnol. **49**, 23–28 (2018)
40. C. Altieri, A. Bevilacqua, D. Cardillo, M. Sinigaglia, Effectiveness of fatty acids and their monoglycerides against Gram-negative pathogens. Int. J. Food Sci. Technol. **44**(2), 359–366 (2009)

41. L. Makras, L. De Vuyst, The in vitro inhibition of Gram-negative pathogenic bacteria by bifidobacteria is caused by the production of organic acids. Int. Dairy J. **16**(9), 1049–1057 (2006)
42. E.H. Ryu, H.C. Chang, In vitro study of potentially probiotic lactic acid bacteria strains isolated from kimchi. Ann. Microbiol. **63**(4), 1387–1395 (2013)
43. C.C. Tsai, H.Y. Hsih, H.H. Chiu, Y.Y. Lai, J.H. Liu, B. Yu, H.Y. Tsen, Antagonistic activity against Salmonella infection in vitro and in vivo for two Lactobacillus strains from swine and poultry. Int. J. Food Microbiol. **102**(2), 185–194 (2005)