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Evaluation of the probiotic potential of *Pediococcus* strains from fermented dairy product kefir

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Abstract: Fermented dairy products mostly harbour some microbiota, also known as probiotics. Over the last years, there has been a significant increase in interest in probiotics. Among them, *Pediococcus* strains also exist in fermented dairy products, including kefir. However, the probiotic potential of *Pediococcus* strains has not been explored extensively. This study was performed to evaluate the probiotic potential of *Pediococcus* strains from traditionally produced kefir samples. To do this, a total of 32 kefir samples from Marmara and Central Anatolia regions in Turkey was collected. The samples were exposed to conventional microbiological analysis to culture lactic acid bacteria (LAB), followed by identification using VITEK® mass spectrometer (MS), and molecular characterisation of *Pediococcus* strains by polymerase chain reaction (PCR). After that, the probiotic potential of each *Pediococcus* strain was tested for resistance to gastric acidity and bile salt and property of hydrophobicity. Overall, 22 strains (34.9%) were identified as *Pediococcus* spp. out of 63 LAB isolates. Among them, only one isolate, *Pediococcus pentosaceus* K6, was found to be resistant to gastric acidity, bile salt and to have hydrophobic properties. In conclusion, our study suggests that a limited number of strains could reveal their potential probiotic action on the host organism. Thus, *Pediococcus* strains of diverse natural origins can provide more insights into further probiotic supplement designs in human nutrition without genomic intervention.

Keywords: fermented foods; lactic acid bacteria; probiotics; *Pediococcus* spp.

A healthy lifestyle is provided by a well-balanced diet, including a broad range of nutrients from different food sources. Among them, fermented foods such as yoghurt, cheeses, fermented sausage, sourdough bread, soy sauce, fish sauce, kefir, kombucha, beer, wine, miso, kimchi, and sauerkraut constitute 5–40% of the human diet, partly due to their health-promoting potential (González et al. 2019). Particularly, fermented foods mostly harbour some microbiota, also known as probiotics. Therefore, probiotic-rich foods are considered important supplements that support the health status. The health benefits of probiotics vary depending on the strains of bacteria. Their colonisation in the intestinal tract improves the intestinal microbiota balance, strengthens the defence against pathogens, and improves oxidative stress resistance. In addition, other beneficial effects are lowering of blood cholesterol levels, synthesis of some vitamins, anticarcinogenic and antimicrobial metabolites (Gamba et al. 2020).

The studies indicate that the microbiota of fermented food origin can survive the gastric transit and reach the colon. Indeed, the majority of them are composed of lactic acid bacteria (LAB) with peculiar intrinsic characteristics, enabling them to promote their abil-

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ity to survive the gastric transit (for instance, acid and bile tolerance with a pH range of 1.5–4.5). For a microorganism to exhibit probiotic properties, it must reach the intestines alive. For this reason, they should primarily resist the gastric acid (pH 1.5–3.0) and maintain their viability. Probiotic bacteria that exceed the acidity of the stomach then encounter bile in the small intestine, and therefore they must be resistant to the bile to maintain their viability. Probiotic bacteria, which pass into the colon while maintaining their viability in the small intestine, must be able to attach to the epithelial and mucosal surfaces in order to proliferate in the intestinal surfaces to form a colony. In addition, fermented food-associated LAB make up 0.1% and 1% of the bacteria in the large intestine and a comparable proportion in the small intestine (Dogan and Ozpinar 2017).

There are over 3 500 fermented foods in the world, and most of them are fermented dairy products (ISAPP 2021). Among them, kefir is one of the best-known fermented dairy products which are consumed in European countries, in the USA, Japan, and Turkey. This slightly acidic, alcoholic, and carbonated dairy product is manufactured by inoculating milk with kefir grains, which is an exopolysaccharide and protein complex. Kefir grains have a unique microbiota (*Lactobacillus brevis, L. kefiri, L. acidophilus, L. casei, L. bulgaricus, Leuconostoc dextranicum, Acetobacter aceti, Streptococcus lactis, Str. durans, Str. cremoris, Str. citrovorum,* and *Str. diacetylactis)*, and its LAB content varies depending on the origin of kefir grains (Gamba et al. 2020).

Some recent works have revealed that *Pediococcus* bacteria also exist in kefir grains (Mantzourani et al. 2019). *Pediococcus* strains are non-motile, coccus-shaped (0.36–1.43 μm in diameter), Gram-positive, catalase-negative, microaerophilic, and homofermentative bacteria within the family Lactobacillaceae, and do not form spores or capsules. The growth temperature range is between 25 °C and 40 °C (optimally around 35 °C). In addition, *Pediococcus* strains can be considered probiotics due to their low pH and bile salt resistance (Hecer et al. 2019). *Pediococcus acidilactici* and *P. pentosaceus* are the most important members of the genus *Pediococcus*, and are mainly isolated from dairy products (Vasiee et al. 2020). The effects of dietary inclusion of *Pediococcus* strains for humans have been under investigation. For instance, in humans, probiotic potential and anticancer properties (Jafari-Nasab et al. 2021), cholesterol-lowering effect (Jitpakdee et al. 2021), weight loss, triglyceride reduction (Zhao et al. 2012) and lowering induced allergic diarrhoea (Masuda et al. 2010) of *Pediococcus* spp. were studied.

Pediococcus strains of diverse natural origins can provide more insights into further probiotic supplement designs in human nutrition without genomic intervention. In this study, we aimed to evaluate the probiotic potential of *Pediococcus* strains from traditionally produced kefir samples.

MATERIAL AND METHODS

Sample collection

During the period of August 2020 to October 2020, a total of 32 kefir samples (22 in Marmara and 10 in Central Anatolia) was collected from traditional producers in different regions of Turkey. All samples were placed into sterile sampling bags (Interscience, France) and transported to the laboratory in a thermobox container at 4 °C (Thermobox 50; Avatherm, Turkey). The sample preparation and further examinations were quickly started after sampling.

Sample preparation, isolation and identification of LAB strains

Kefir samples (10 g in 90 mL of physiological saline solution) were homogenised in a sterile bag (Interscience, France) for 2 min by a stomacher (EasyMix; AES Chemunex, France), and 10^{-2} and 10^{-3} dilutions were prepared using the physiological saline solution. After that, 1 mL of the suspension was directly streaked onto De Man, Rogosa and Sharpe (MRS) agar (110660; Merck, Germany) or on M17 agar (115108; Merck, Germany). The inoculated plates were incubated at 37 °C for 24–48 h under aerobic conditions following the manufacturer's instructions (CO2 incubator; Binder, Germany). After the incubation, morphology and purity of each single colony were initially determined by a microscope (DM3000 + DFC295; Leica, Germany). Crystal violet (C.I. 42555; Sigma-Aldrich, USA) was used for cultivation and identification of LAB species, Safranine (109217; Merck, Germany) and Lugol dye (109261; Merck, Germany) were used for biochemical and morphological tests, physiological saline solution 8.5% (T20159; Mustela, Turkey) for dilution processes, and 20% glycerol (818709; Sigma-Aldrich, USA) for storing isolates. Subsequently, the presumptive colonies were inoculated into MRS (110661; Merck, Germany) and/or M17 broth (115029; Merck; Germany) at 37 °C for 24 h under aerobic and anaerobic conditions for verification of purity. Matte cream-coloured colonies were evaluated as presumptive LAB species. Then, staining methods were used to determine Gram (+) ones with examination under microscopy, followed by a catalase test to se-

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lect the catalase (–) isolates. Finally, each presumptive colony was enriched again in broth and then stored at –80 °C in MRS broth and/or M17 broth medium containing 20% glycerol (VWR 650; VWR, USA). All procedures were conducted according to the instructions by ISO 11133:2014. The presumptive LAB colonies were then sub-cultured into MRS and/or M17 broth and incubated at 37 °C for 24–48 h under aerobic and anaerobic conditions (CO2 incubator; Binder, Germany). The species identification of suspected LAB isolates was performed by VITEK® MS (bioMérieux; France). *Escherichia coli* ATCC® 25922 was used as a control strain (Dubois et al. 2012).

Molecular screening and confirmation of *Pediococcus* strains

The pure LAB were subjected to genomic deoxyribonucleic acid (DNA) extraction according to the manufacturer's protocol as described by a commercial DNA isolation kit (Hi-Media, India). Quantitative determination of isolated DNA samples was determined using NanoDrop 2000 Microvolume Spectrophotometer (Thermo Scientific™, USA). The absorbance was measured at 350 nm with a sample volume of 1 μL total DNA. The supernatant containing DNA was transferred to a new tube and stored at –20 °C for further molecular analyses (USD 3045 BK; Uğur, Turkey). The primer pairs for the specific target gene region IdhD as previously described by Mora et al. (1997) (Table 1), were used for screening of *Pediococcus* strains.

All primers were prepared by Ek-Im Biotechnology (Turkey). Amplification was performed in a singleplex PCR assay by a Thermal Cycler Prima Trio™ LA1006 (HiMedia, India). The PCR mixtures were prepared in a final volume of 25 μL. The thermocycler conditions were as follows: 40 s at 94 °C for denaturation, followed by annealing at 70 °C for 1 min, 35 cycles for 1 min at 70 °C, 50 s at 72 °C for extension, and finally 7 min at 72 °C, respectively. Gel electrophoresis of the PCR products was performed on a 1.5% agarose gel. The amplicons were photographed by GelDoc 2000 imaging system (BioRad, Turkey). Finally, the analysis was carried out using Quantity One 4.6.3 GelDoc XR Software (BioRad, Turkey).

Table 1. Primers, sequences and length

Primer	Sequence	Length (bp)
$ldhD_F$	GGACTTGATAACGTACCCGC	449
$ldhD_R$	GTTCCGTCTTGCATTTGACC	

Source: Mora et al. (1997)

Determination of probiotic potential of *Pedioccocus* strains

The probiotic potential of *Pediococcus* strains was evaluated according to their performance on resistance to gastric acidity and bile medium, and hydrophobicity for adhesion to the intestine (Gutiérrez-Cortés et al. 2018). All chemicals and reagents were selected according to the Instructions by ISO 11133:2014. All probiotic potential tests were repeated three times, and the results are given as mean ± standard deviation (SD).

Gastric acid resistance analysis. The gastric acid resistance was determined according to Klingberg et al. (2005) and Lee et al. (2016). To do this, 1 mol L^{-1} sterile hydrochloric acid (HCl) (320331; Sigma-Aldrich, USA) to create a medium similar to gastric acidity conditions, MRS and M17 broths with a pH value of 2.5 were used. Sterile HCl (1 mol L^{-1}) was added to MRS and M17 broths to adjust pH to 2.5, because the normal pH in the human stomach ranges from 1 to 3. The activated culture was centrifuged at 10 000 rpm for 10 min (3K-18; Sigma, Germany), and the supernatant was removed. The resulting pellet was initially suspended in 7 mL of physiological saline solution (8.5%) and then incubated for 3 h at 37 °C with 1% inoculation into 10 mL of broth with pH 2.5. Then the required serial dilutions were made again and incubated at 37 °C for 72 h (CO2 incubator; Binder, Germany). Finally, counting active cultures was performed.

Bile resistance analysis. For the bile salt resistance test, *Pediococcus* strains were inoculated into 7 mL of M17 and MRS broths containing 0.3% (w/v) 08175 Oxgall Bovine Bile (US Biological Life Sciences, USA). The activated cultures were centrifuged at 10 000 rpm for 10 min (3K-18; Sigma, Germany). The resulting pellets were suspended in 7 mL of physiological saline and then incubated at 37 °C for 3 h with 1% inoculation into 10 mL of broth containing Oxgall Bovine Bile. Finally, required serial dilutions were made and incubated again at 37 °C for 48–72 h (CO2 incubator; Binder, Germany), their viability was observed, and then active cultures were counted (Liong and Shah 2005).

Hydrophobicity analysis. Finally, the hydrophobicity test was conducted based on the bacterial adherence to hydrocarbons (BATH) assay. For this, the pellets of active isolates were suspended in physiological saline to an optical density (OD) absorbance at 600 nm of 0.5 to 0.7. In tubes containing 3 mL of bacterial culture suspension, various volumes of test hydrocarbons (hexadecane, toluene, and xylene) were added. Aliquots of 0.1 M potassium nitrate (KNO_3) (319198; Sigma-Aldrich, USA) and 0.3 mL xylene (214736;

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Sigma-Aldrich, USA) were used for hydrophobicity testing. After incubation for 4 h at room conditions, the mixtures were vortexed for 90 s (VWR Vortex Mixers; VWR, USA), the tubes were let stand for 15 min to separate in two phases, and absorbance of the aqueous phase was measured at 550 nm and expressed as a percentage of the absorbance of the applied cell suspension (Gusils et al. 1999).

RESULTS AND DISCUSSION

Identification of LAB strains and *Pediococcus* strains. The microbiological and mass-spectrometry results showed that a total of 63 LAB strains were recovered, and among them, 22 (34.9%) were *Pediococcus* strains. The demographic distribution of the isolated LAB strains was found as 12 *P. pentosaceus*, 10 *P. acidilactici*, 15 *L. plantarum*, 9 *L. pentosus*, 6 *L. lactis*, 8 *L. casei*, and 3 *E. faecium* (Figure 1).

In addition, the identified *Pedioccus* strains (19.0% *P. pentosaceus* and 15.8% *P. acidilactici*) were also confirmed by molecular PCR screening and characterisation, as seen in Figure 2.

In this study, the probiotic potential of *Pediococcus* strains from 32 traditionally produced kefir samples manufactured in different regions of Turkey (Marmara and Central Anatolia) was evaluated. The results showed that only one *P. pentosaceus* K6 strain showed

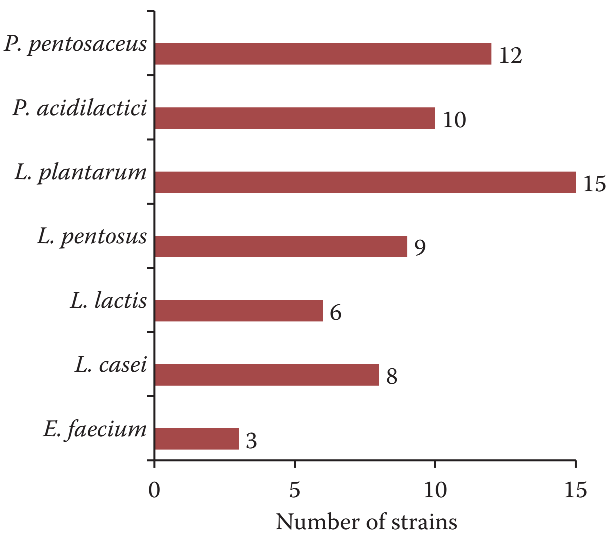

Figure 1. Demographic distribution of LAB strains

LAB – lactic acid bacteria; *P. pentosaceus* – *Pediococcus pentosaceus; P. acidilactici* – *Pediococcus acidilactici*; *L. plantarum* – *Lactobacillus plantarum*; *L. pentosus* – *Lactobacillus pentosus*; *L. lactis* – *Lactobacillus lactis*; *L. casei* – *Lactobacillus casei*; *E. faecium* – *Enterococcus faecium*

high performance in terms of probiotic properties and met the probiotic criteria.

In the kefir, a fermented dairy product with unique properties, there are up to fifty different types of bacteria and yeasts, mainly dominated by *Lactobacillus* species (Dertli and Çon 2017). Various factors such as geographical region of the grain and type of milk can affect the microbial composition of kefir, and knowing the composition is important for determining the potential impact of each strain on fermentation conditions, sensory characteristics, and health benefits (Purutoğlu et al. 2020). This study showed that the most dominant species in the analysed kefir samples were composed of 60% of the genus *Lactobacillus*, 5% *Enterococcus* and 35% *Pediococcus*. In a study conducted in Greece, 81.4% of the species isolated from kefir samples were of the genus *Lactobacillus* (*L. plantarum, L. casei,* and *L. paracasei*), whereas 18.6% were *P. pentosaceus* (Mantzourani et al. 2019). In another study carried out in Turkey, *Pediococcus* species (*P. dextrinicus, P. acidilactici,* and *P. pentosaceus)*, as well as other LAB species (*L. acidophilus, L. helveticus, L. casei,* and *L. lactis*) were recovered from the examined kefir samples (Sabir et al. 2010). Similarly to the previous works, Purutoğlu et al. (2020) dominantly characterised LAB species (*L. lactis, L. diolivorans, L. otakiensis, L. kefiri, L. kefiranofaciens*, and *L. paracasei*), as well as other species such as *Enterococcus durans, A. orientalis, A. okinawensis*, and *A. fabarum)* in the kefir samples, and Dertli and Çon (2017) isolated *L. kefiranofaciens, L. kefiri, L. apis, L. ultunensis, L. lactis, E. amnigenus, E. hormaechei, A. rhizosphaerae, A. calcoaceticus, Pseudomonas azotoformans, P. aeruginosa, P. otitidis*, and *Propionibacterium acnes* from kefir grains. Overall, our results regarding the types of species present in the analysed kefir samples were similar to the findings of national and international studies.

Probiotic potential of *Pediococcus* strains. The probiotic potential testing results of the *Pediococcus* strains indicated that 5 (41.6%) of *P. pentosaceus* strains and 4 (40.0%) of *P. acidilactici* strains were found to be resistant to gastric acid, 3 (25.0%) of *P. pentosaceus* strains, and 2 (20.0%) of *P. acidilactici* strains exhibited bile salt resistance, and only 1 (8.3%) *P. pentosaceus* strain K6 could pass the hydrophobicity test. Overall, one *P. pentosaceus* K6 isolate could show the highest performance and met the three criteria required for being considered as potentially probiotic culture (Table 2, Figure 3).

LAB vitality is the most important parameter for providing probiotic functions. Gastric acidity and bile salts affect the viability of probiotic bacteria

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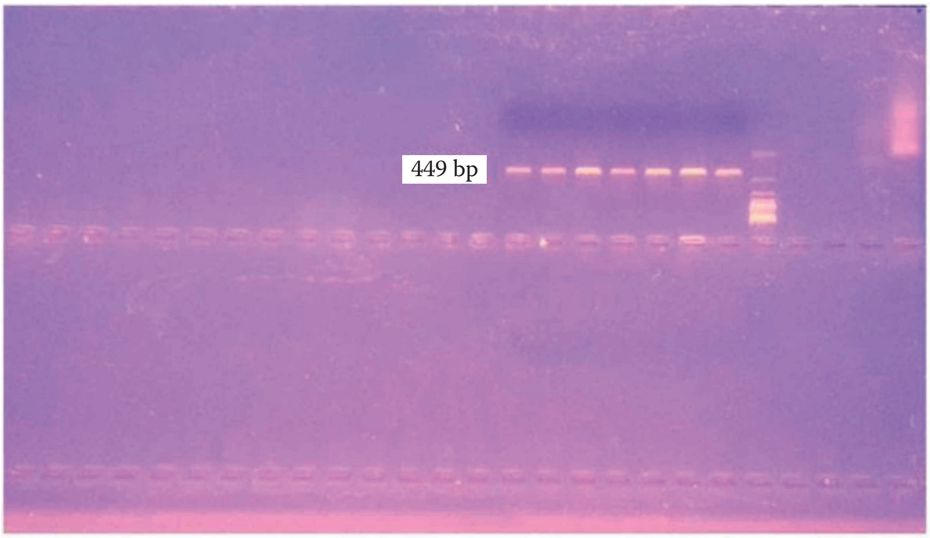

Figure 2. Agarose gel showing patterns of *Pediococcus* strains

Table 2. Experimental results of high-performance *Pediococcus* strains according to the potential of probiotic (n = 3; mean ± SD)

No.	Type	Viability (log CFU g^{-1})		Hydrophobicity (%)
		pH 2.5	0.3% w/v Oxgall Bovine Bile	
1	*P. acidilactici* K1	4.32 ± 0.08	3.87 ± 0.13	–
2	*P. acidilactici* K2	–	–	–
3	*P. acidilactici* K3	–	–	–
4	*P. acidilactici* K4	–	–	–
5	*P. acidilactici* K5	5.01 ± 0.02	4.93 ± 0.11	–
6	*P. acidilactici* K6	5.71 ± 0.08	–	–
7	*P. acidilactici* K7	–	–	–
8	*P. acidilactici* K8	4.33 ± 0.04	–	–
9	*P. acidilactici* K9	–	–	–
10	*P. acidilactici* K10	–	–	–
11	*P. pentosaceus* K1	5.26 ± 0.07	–	–
12	*P. pentosaceus* K2	–	–	–
13	*P. pentosaceus* K3	4.57 ± 0.12	4.85 ± 0.03	–
14	*P. pentosaceus* K4	–	–	–
15	*P. pentosaceus* K5	–	–	–
16	*P. pentosaceus* K6	5.68 ± 0.09	4.41 ± 0.06	88.23 ± 1.7
17	*P. pentosaceus* K7	–	–	–
18	*P. pentosaceus* K8	5.21 ± 0.07	4.47 ± 0.08	–
19	*P. pentosaceus* K9	–	–	–
20	*P. pentosaceus* K10	4.86 ± 0.05	–	–
21	*P. pentosaceus* K11	–	–	–
22	*P. pentosaceus* K12	–	–	–

SD – standard deviation; CFU – colony-forming unit; *P. acidilactici* – *Pediococcus acidilactici*; *P. pentosaceus* – *Pediococcus pentosaceus*

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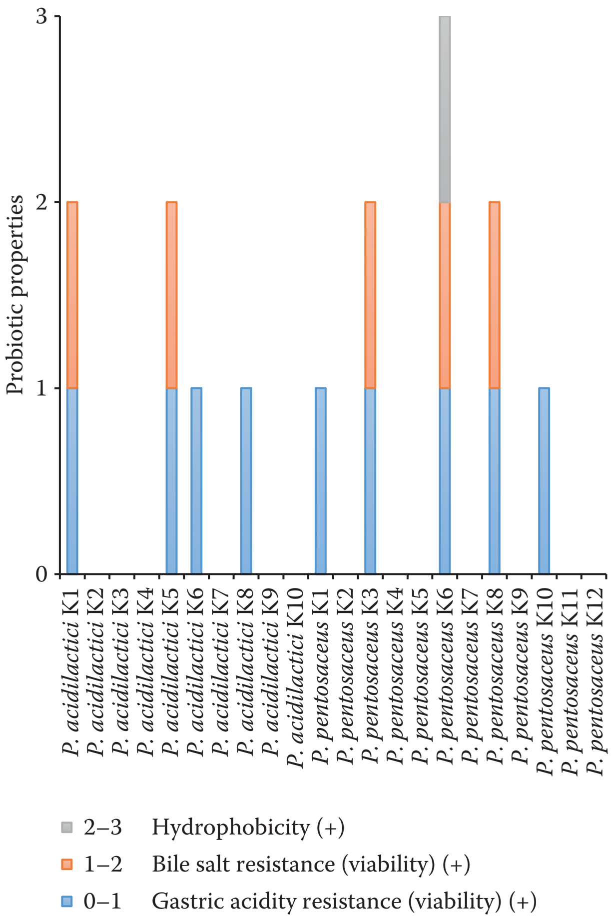

Figure 3. The potential of probiotic of *Pediococcus* strains

P. acidilactici – Pediococcus acidilactici; *P. pentosaceus – Pediococcus pentosaceus*

in the colon (Sabir et al. 2010). The human stomach is an acidic medium where the pH ranges between 1.0 and 4.5, and the ingested food may remain in the stomach for 3 h (Yang et al. 2020). Therefore, stomach pH is a critical performance factor for microorganisms. Probiotic strains are expected to resist an acidity of pH 3.0 or less. Similarly, the intestinal tract is a primary medium in which bile salts are present in concentrations ranging from 0.3% to 0.5% (Yerlikaya 2019). The residence time of food in the small intestine is approximately between 4 h and 6 h. For this reason, the resistance of probiotic bacteria against bile salts in the intestinal tract is a very important property (Mantzourani et al. 2019). In our study, a medium similar to pH 2.5 gastric acidity conditions was created to determine the gastric acidity resistance of 22 *Pediococcus* (10 *P. acidilactici* and 12 *P. pentosaceus*) strains isolated from kefir samples. The findings showed that 4 of 10 *P. acidilactici* strains (K1, K5, K6, and K8) and 5 of 12 *P. pentosaceus* strains (K1, K3, K6, K8, and K10) were found to be resistant to gastric acidity. Following that, the resistance of *Pediococcus* strains, which survived in the gastric acidity medium and could pass into the intestinal tract, against bile salts was determined in a medium with 0.3% bile salt concentration. Accordingly, 2 of 4 *P. acidilactici* (K1 and K5) strains and 3 of 5 *P. pentosaceus* (K3, K6, and K8) strains were detected to be resistant to bile salt conditions. The literature tells us that *P. pentosaceus* has relatively superior resistance to gastric acid and bile salts for *P. pentosaceus* strains from a grain-based drink in Korea, 'black gamju' (Yang et al. 2020). Mantzourani et al. (2019) also reported that *P. pentosaceus* from kefir grains was one of the best performing strains at extremely strong acidic conditions such as pH 2. Similarly, Cavicchioli et al. (2019) confirmed that *P. pentosaceus* strains isolated from cheeses could survive when exposed to the simulated stomach and/or intestinal conditions. Farag et al. (2020) found that *P. acidilactici* strain could survive at pH 1.0 and bile salt concentration of 0.5%, whereas *P. pentosaceus* had relatively low viability at pH 2.5 and lost its vitality at pH 1.0. Similarly, in the study conducted for the isolation of metabolically active bacteriocinogenic LAB population from local cheeses in Brazil, *P. pentosaceus* was found to be one of the LAB isolates showing sensitivity to high acidity (Gutiérrez-Cortés et al. 2018). Our results regarding the probiotic potential of *Pediococcus* isolates were very similar to national and international works. In this study, only one *P. pentosaceus* K6 isolate could meet the criteria against gastric acid and bile salt conditions and matched with the data given in the literature. Hence, it can be said that *P. pentosaceus* may survive through the gastrointestinal tract and play a pivotal probiotic role in the stomach and intestinal medium.

The probiotic effect is a requirement for probiotic bacteria to adhere and colonise the intestinal surface. Adhesion and colonisation can lead to the production of antimicrobial agents and the exclusion of pathogens by competition of binding sites in the epithelium (Mantzourani et al. 2019; Yerlikaya 2019). The probiotic bacteria that colonise in the gut form biofilms that can help bind effectively to the intestinal epithelium, thus preventing the pathogen adhesion. The ability to adhere to the intestinal surface is associated with hydrophobicity, and the hydrophobicity ability allows bacterial cells to adhere more strongly to the intestinal surface (Cavicchioli et al. 2019; Yang et al. 2020). In our study, the hydrophobicity ability of each strain was determined by calculating their attachment to the surface of some hydrocarbons, indicating a measure of the

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adhesion of microbiota to intestinal epithelial cells. Among 2 *P. acidilactici* and 3 *P. pentosaceus* strains, which were resistant to 0.3% (w/v) bile salt concentration, only 1 *P. pentosaceus* (K6) strain could exhibit hydrophobic characteristics. Yang et al. (2020) reported that *P. pentosaceus* had high adhesion and colonisation ability to human intestinal cells. Similarly, Mantzourani et al. (2019) and Cavicchioli et al. (2019) both reported that *P. pentosaceus* strains isolated from kefir and cheese were good in high adhesion ability.

CONCLUSION

In conclusion, our study suggests that a limited number of strains could reveal their potential probiotic action on host organisms and survival while passing through the upper part of the gastrointestinal tract. Thus, *Pediococcus* strains of diverse natural origins can provide more insights into further probiotic supplement designs in human nutrition without genomic intervention.

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