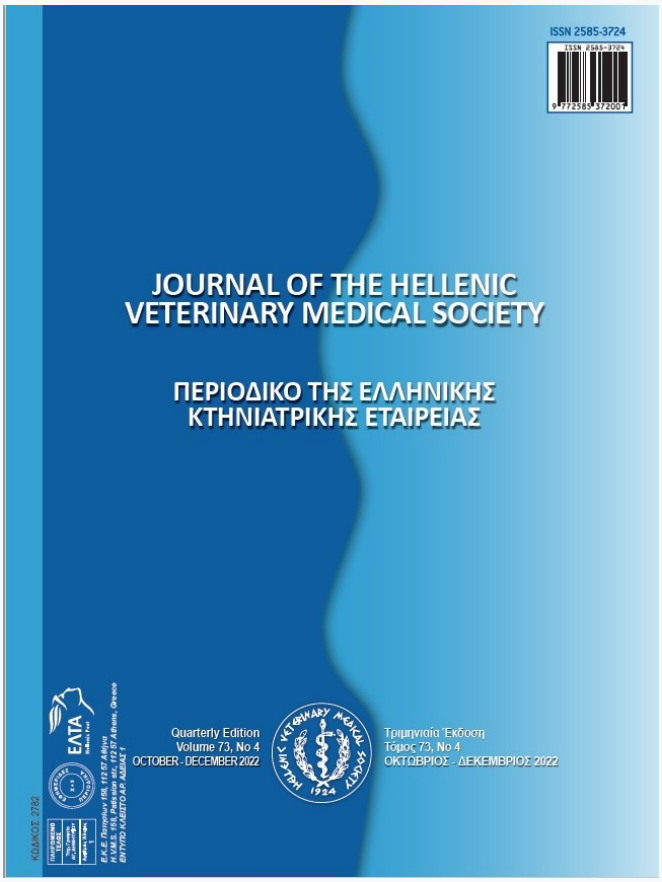
ePublishing

ΕΘΝΙΚΟ ΚΕΝΤΡΟ ΤΕΚΜΗΡΙΩΣΗΣ
EKT
NATIONAL DOCUMENTATION CENTRE

Journal of the Hellenic Veterinary Medical Society

Vol 73, No 4 (2022)



Probiotic potential and anti-quorum sensing activity of Enterococcus faecalis and Lactobacillus kunkeei isolates from Apis mellifera

ME Kiymaci, D Simsek, KC Tok, D Dirican, M Gumustas

doi: 10.12681/jhvms.26743

Copyright © 2023, Merve Eylul Kiymaci, Duygu Simsek, Kenan Can Tok, Dilay Dirican, Mehmet Gumustas

This work is licensed under a Creative Commons Attribution-NonCommercial 4.0.

To cite this article:

Kiymaci, M., Simsek, D., Tok, K., Dirican, D., & Gumustas, M. (2023). Probiotic potential and anti-quorum sensing activity of Enterococcus faecalis and Lactobacillus kunkeei isolates from Apis mellifera. *Journal of the Hellenic Veterinary Medical Society*, *73*(4), 4717–4728. https://doi.org/10.12681/jhvms.26743 (Original work published January 20, 2023)

https://epublishing.ekt.gr | **e-Publisher:** EKT | **Downloaded at:** 01/11/2024 03:22:24

J HELLENIC VET MED SOC 2022, 73 (4): 4717-4728
ΠΕΚΕ 2022, 73 (4): 4717-4728

Research article
Ερευνητικό άρθρο

Probiotic potential and anti-quorum sensing activity of Enterococcus faecalis and Lactobacillus kunkeei isolates from Apis mellifera

M.E. Kiymaci[1], D. Simsek[2], K.C. Tok[3], D. Dirican[4], M. Gumustas[3]

[1]University of Health Sciences Turkey, Gülhane Faculty of Pharmacy, Department of Pharmaceutical Microbiology, Ankara, Turkey

[2]Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology, Ankara, Turkey

[3]Ankara University, Institute of Forensic Sciences, Department of Forensic Toxicology, Ankara, Turkey

[4]Republic of Turkey Ministry of Health, Bilkent City Hospital Central Laboratory of Microbiology, Ankara, Turkey

ABSTRACT: This study aimed to investigate the antimicrobial, anti-quorum sensing activity and potential probiotic properties of *Lactobacillus kunkeei* and *Enterococcus faecalis* 1 and 2 isolates from *Apis mellifera* gut. The antimicrobial and anti-QS activity of isolates were determined by the broth microdilution method and the *Chromobacterium violaceum* biosensor strain, respectively. The probiotic potential was evaluated according to EFSA standards. The organic acid content of culture supernatants of isolates was determined quantitatively by a HPLC method. Results showed that isolates were found to be resistant to gentamicin, streptomycin, erythromycin, and clindamycin. Also, isolates were found gamma haemolytic and resistant to acidic and enzymatic environmental conditions. *E. faecalis* isolates showed antimicrobial activity against *Escherichia coli* ATCC 25922, *E. faecalis* ATCC 29212, and *Pseudomonas aeruginosa* ATCC 27853. Anti-quorum sensing activity of the culture supernatants of all isolates was found as a violacein pigment inhibition against *Chromobacterium violaceum* biosensor strain. The concentrations of lactic acid, acetic acid and butyric acid produced by isolates were found as 1.7 - 7.5 g/L, 0.94 - 2.20 g/L and 0.22 - 0.38 g/L, respectively.

Keywords: *Lactobacillus kunkeei*, *Enterococcus faecalis*, probiotic, anti-quorum sensing, bee gut

Corresponding Author:
Merve Eylul Kiymaci, University of Health Sciences Turkey, Gülhane Faculty of Pharmacy, Department of Pharmaceutical Microbiology, 06018, Kecioren-Ankara/ Turkey
E-mail address: merveeylul.kiymaci@sbu.edu.tr

Date of initial submission: 09-04-2021
Date of acceptance: 31-05-2022

INTRODUCTION

Apis mellifera is a honey bee that pollinates flowering plants along with the other pollinator insects. Pollination is a vital condition for the sustainability of ecosystems and human societies. Bees need the nectar of flowers as food to survive, while plants need pollen to spread their pollen and thus reproduce. In pollination, this task mostly falls on the bees (Aslan et al. 2016; Lika et al. 2021; Puvača 2018). It has been shown that the diversity of microorganisms composing the gastrointestinal system (GIS) of the honey bee is different from other living organisms. Hence, the resistance of bees against changing climatic and environmental conditions, stress factors, pollutants, and contaminants varies depending on this microbiota (Endo and Salminen 2013). Although the honey bee's gut microbiota and its functions remain largely unexplored, these microorganisms mostly include lactic acid bacteria (LAB) (Janashia et al. 2016).

Lactic acid bacteria are Gram (+) and catalase (-) microorganisms in cocci/bacillus morphology, in particular resistance to low pH values (Hayek 2013). LAB have significant roles in the GIS of animals and humans as well as multiple functions in various foods, such as prebiotics, synbiotics etc. Many members of LAB have quite industrial importance. These bacteria are used in food fermentation, especially for improving taste and texture, as well as regulating organisms' health (e.g. probiotics) (Tomičić et al. 2019). LAB produce some metabolites, as a result of their metabolic activities, that have economic value for humans. In addition, LAB can be found naturally in many foods such as meat, milk, cereal products, alcohol-containing products, and pickles, or they can be used in the maturation of foods by adding them as a starter culture (Bintsis 2018).

Probiotics are living microorganisms that benefit the host's health by creating a microbial balance in the body when they are ingested in adequate concentration. They play an important role in supporting the immune system by protecting the host with the help of antimicrobial metabolites. It has been recommended to use only the LAB isolated from the human GIS as probiotics by the Food&Agriculture Organization (FAO) and the World Health Organization (WHO). Due to these developments, in recent years, studies regarding human GIS increased and probiotic properties of some LAB obtained from the flora of the human GIS have been determined. On the other hand, several studies underlined that some strains derived from animal origin products and also non-dairy fermented products exhibit probiotic properties (Zielińska 2018).

Some bacteria belonging to the genus *Bifidobacterium* and *Lactobacillus* are more prominent among microorganisms used as probiotics and particularly used as the food supplements, due to their generally recognized as safe (GRAS) status. However, several other LAB such as *Aerococcus*, *Enterococcus,* or different microorganisms such as *Bacillus,* are also being studied for their probiotic potential, different strain advantages, and health-promoting properties. *Enterococcus* genus is another great part of LAB after *Lactobacillus* and *Streptococcus* (Franz et al. 2011). Some members of this genus could be found in environments such as water, soil (Ben Braiek et al. 2017), vegetables, and also dairy products such as raw milk, cheese, etc. (Ben Braiek and Smaoui 2019), In addition to these, human and animal body could be a host for the *Enterococcus* species as a commensal microorganism (Ben Braiek et al. 2017). This genus may also include strains known to be opportunistic pathogens that can cause different infections, especially in humans (O'Driscoll and Crank 2015). Some *Enterococcus* strains have been found to exhibit multi-drug resistance. Besides, it has been determined that antibiotic resistance genes and virulence factors can be transferable (Franz et al. 2001). Based on these and similar findings, concern has arisen that the pathogenicity and safety tests of enterococci should be properly investigated in the use of these bacteria as probiotics (Ben Braiek and Smaoui 2019).

Quorum sensing is a mechanism based on the regulation of gene expression of cells due to the increase in population density. The bacteria that detect population density, produce chemical signal molecules called autoinducers and release them into the environment. After these signal molecules reach a certain threshold concentration, they cause changes in the gene expression of many features, including virulence factors (Miller and Bassler 2001). Therefore, the inhibition of this system is investigated as an alternative treatment method to the use of chemotherapeutics in the fight against microorganisms.

Based on these, the present study aimed to determine the probiotic potential, antimicrobial and anti-quorum sensing activities of some LAB isolated from the *Apis mellifera* honey bee gut.

MATERIALS AND METHODS

Bacterial isolates

The tested bacteria were isolated from the intestinal flora of *Apis mellifera* honey bees. A total of 10 honey bees were collected from a non-migratory apiary located in Sivas/Turkey (39°43'41.4"N - 37°02'01.6"E) in 2017. The intestinal contents of honey bees were removed by dissection and the mid and hind intestine regions were separately transferred to test tubes under aseptic conditions. In particular, culture was performed on an MRS medium for the isolation of LAB. Intestines were crushed with a glass shaker, then added to 5 ml of MRS medium, vortexed, and incubated at 35 ± 2°C for 24 hours at 150 rpm in a shaking incubator. After incubation, 100 µl of the sample was taken and plated onto an MRS agar medium and incubated at 35 ± 2°C for 48 hours. Different morphological colonies were selected and Gram staining characteristics and catalase activities were determined at the end of incubation. Isolates suspected to be LAB were selected, and stored in distilled water containing 20% glycerol as a stock culture at -80 °C until used. The bacterial identification was performed with a VITEK® MS MALDI TOF mass spectrometer according to the manufacturer's instructions. Pure bacteria colonies were taken from blood agar with a 1-micron loop and rubbed into a well of the device's slide. One µl of alpha-cyano-4-hydroxy-cinnamic acid (CHCA) matrix was pipetted. Each run was calibrated with *Escherichia coli* (*E.coli*) ATCC 8739 strain on 3 wells of the slide to be run 48 samples. After applying the *E. coli* ATCC 8739 strain to the middle well, 1 µl of CHCA matrix was pipetted on it. Samples were saved in the program and the slide was loaded into the device. Identification was completed by the mass spectrometer method.

16S rRNA analysis

Identification of three isolates was also carried out with 16S rRNA analysis at BM Laboratories. EurX GeneMATRIX Bacterial & Yeast DNA isolation kit (Poland) used for DNA isolation. DNA amount and purity were checked by spectrophotometric measurement on Thermo Scientific Nanodrop 2000 (USA). The primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), were used for the amplification of the targeted gene regions. The reaction mixture contained 3 µl sample DNA, 1x PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTP mix, 0.3 µM F. primer, 0.3 µM R. primer, 2U Taq DNA polymerase. The PCR reaction conditions were: initial denaturation for 5 min at 95 °C; 45 sec; denaturation at 95 °C, 45 sec; annealing at 57 °C, 60 sec; extension at 72 °C as 40 cycles. The final extension was performed at 72 °C for 5 min. The PCR reaction was performed with Solis Biodyne (Estonia) FIREPol® DNA Polymerase Taq polymerase enzyme. Amplicons were put in a 1.5% agarose gel prepared with 1X TAE buffer at 100 volts for 90 minutes by electrophoresis and imaged under UV light. MAGBIO "HighPrep ™ PCR Clean-up System" (AC-60005) purification kit was used for the collection of the bands. Sanger sequencing was performed by the Macrogen Netherlands laboratory using the ABI 3730XL Sanger sequencer (Applied Biosystems, Foster City, CA) and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). While performing the readings, the CAP contig assembly algorithm was used in BioEdit software.

Tolerance to low pH values

Isolates with probiotic properties are expected to show resistance to varying pH values of the digestive system. On this basis, the isolates' resistance against pH 1.0, pH 2.0, and pH 3.0 was determined for 3 hours. Overnight cultures were centrifuged at 12000 rpm, at 4°C for 5 minutes. Supernatants were removed and pellets were washed twice with phosphate-buffered saline (PBS, pH 7.4). The pellets were then suspended in pH 1.0-PBS, pH 2.0-PBS, and pH 3.0-PBS that were prepared with 5M HCl. Neutral pH was used as a control. Viable bacterial colonies in samples at 0 to 3 hours were plated on MRS agar after 48 hours of incubation at 35 ± 2°C and calculated as log CFU/ml (Maragkoudakis et al. 2006).

Bile tolerance

To determine the tolerance to bile salts, MRS broth containing different proportions of bile salts was used. Overnight the bacterial cultures were centrifuged for 5 minutes at 12000 rpm, 4°C. Supernatants were removed and pellets were washed twice with PBS. The pellets were then suspended in MRS broth with bile salts (0.3%, 0.5%, and 1%), and incubated at 35 ± 2°C for 4 hours. Samples were taken at the 0th hour and the end of the 4th hour of incubation and then inoculated on MRS agar. Plates were incubated at 35 ± 2°C for 48 hours, and bacterial colonies were counted and calculated as log CFU/ml (Maragkoudakis et al. 2006).

Pepsin and pancreatin resistance

Charteris et al. (1998)'s method was used to evaluate the pepsin and pancreatin resistance of isolates. Fresh bacterial cultures were centrifuged for 5 minutes at 12000 rpm, 4°C. Supernatants were removed and pellets were washed twice with PBS. The pellets were then suspended in pepsin (3 mg/ml) - pH 2.0 PBS, pepsin (3 mg/ml) - pH 3.0 PBS and pancreatin (1 mg/ml) - pH 8.0 PBS and incubated at 35 ± 2°C for 0 to 3 h for pepsin PBSs and 0, 4 h for pancreatin PBSs. Pellets with neutral pH were used as a control. At the end of the incubation period, samples were taken, spread on MRS agar plates, and incubated at 35 ± 2°C for 48 hours. Viable colony counts were calculated as log CFU/ml.

Hemolytic activity

Hemolytic activity was tested with Columbia agar including 5% sheep blood. Fresh cultures of isolates were plated on agar plates and incubated at 35 ± 2°C for 24 h. No zones around the bacterial colonies were determined as gamma haemolysis (Maragkoudakis et al. 2006).

Antimicrobial susceptibility

Antimicrobial susceptibility tests were evaluated by micro broth dilution method according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards with antibiotics specified by the European Food Safety Authority (EFSA) in line with the concentrations (Rychen 2018). Ampicillin, clindamycin, chloramphenicol, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline, and vancomycin were the tested antibiotics. All antibiotics were obtained from Sigma Aldrich (Germany). Antibiotic concentrations were determined separately for each antibiotic, taking into account the MIC limit value specified by EFSA in the range of 0.125-256 µg/ml.

Antimicrobial activity

Antimicrobial activity of supernatants of isolates was determined against *E. coli* ATCC 25922, *Enterococcus faecalis* (*E. faecalis*) ATCC 29212, *Klebsiella pneumoniae* (*K. pneumoniae)* ATCC 13883, *Staphylococcus aureus* (*S. aureus*) ATCC 29213 and *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 27853 by Lima et al. (2007)'s method. The cell-free culture supernatants (CFS) of isolates were prepared by centrifugation at 12000 rpm for 20 minutes and then filter sterilized by a 0.22 µm filter. The inhibition zones around the CFSs were evaluated as antimicrobial activity.

The antimicrobial activity test was also evaluated as minimal inhibition concentration (MIC) by the micro broth dilution method according to EUCAST standards (EUCAST 2021) with CFS. Isolates were inoculated into MRS broth and left for 24 hours of incubation at 35 ± 2°C. At the end of the incubation period, the fresh bacterial culture was centrifuged at 12000 rpm at 4°C for 15 minutes to separate CFS. The obtained CFS was filtered through a 0.22 µm membrane filter and sterilized. These supernatants were evaluated in the MIC test.

Diacetyl Production

Overnight bacterial cultures were seeded (1%) in 5 ml MRS broth and incubated at 30 ± 2°C for 48 hours. Then, 1 ml of alpha-naphthol solution (4%) and 1 ml of KOH (30%) were added to the 2 ml culture tube and incubated at 30 ± 2°C for 30 minutes. After incubation, red ring formation at the top of the cultures was identified as diacetyl production (King 1948). *Lactobacillus rhamnosus* GG ATCC 53103 was used as a positive control.

Auto-aggregation test

The auto-aggregation experiment was performed according to Del Re et al. (2000) method. LAB isolates were cultured in MRS broth for 24 hours, then centrifuged for 15 minutes at 4°C, 5000 g. The cells were washed three times with PBS (pH 7.4) and suspended in sterile PBS to achieve a concentration of 10^8 CFU/ml. A five mL suspension was vortexed for 10 seconds and incubated at 25 ± 2°C for 24 hours. After a 24-hour interval, auto-aggregation was measured at 600 nm. The percentage of auto-aggregation was expressed as follows:

Auto-aggregation (%) = [(OD1 - OD2) / OD1] × 100

OD1 represents the optical density at the onset and OD2 represents the data after 24 hours. All experiments were performed in triplicate. *L. rhamnosus* GG ATCC 53103 was used as a control.

Anti-quorum sensing activity

Erdonmez et al. (2018)'s method was performed with slight modifications. The fresh culture of *Chromobacterium violaceum* (*C. violaceum*) CV026 at 30 °C for 18 hours was taken and adjusted to Mc Farland 0.5 density (10^8 CFU/ml). A hundred µl *C. violaceum*

CV026 and 50 µl N-hexanoyl-L-homoserine lactone were added to 10 ml soft Luria Bertani agar (0.9%) medium and poured into Petri plates after vortexing. CFSs of isolates were dropped on (15 µl) agar plates and incubated at 30 ± 2°C for 48 hours. Tests were carried out in duplicate.

Determination and quantification of organic acids by high performance liquid chromatography

Before the analysis, all strains were incubated in the medium at 35 ± 2°C for 18 h and the bacterial culture was centrifuged at 12000 rpm for 20 min. CFS was separated using a 0.22 µm cellulose acetate filter. Aliquoted CFS samples were spiked with suitable amounts of lactic acid, acetic acid, butyric acid, and propionic acid for the calibration curve (n=5). The calibration curve was constructed by using a modified version of the De Baere et al. (2013) extraction method. The International Council on Harmonization (ICH) guidelines were used for the validation of the modified method (ICH 2014). The chromatographic separation was performed at 10°C, on an XBridge C18 (4.6x250mm, 5 µm) analytical column and connected with a guard column (4 mm × 3 mm) for the protection of the analytical column. Fifteen mM phosphate buffer (pH 2.1, adjusted with NaOH (5M)) and acetonitrile (70:30, v/v) were used as mobile phase. The buffer solution was filtered using a 0.45 µm cellulose acetate filter under vacuum. Both buffer and solvents were degassed before the analysis was carried out. The analysis was performed by adjusting the instrument settings to a flow rate of 1 mL/min, detector wavelength of 210 nm and an injection volume of 5 µl. The present modified method was validated according to the selectivity, linearity, LOD, LOQ, precision, accuracy, etc. as specified in the ICH guideline. A known amount of standard acids was spiked to the blank CFS to investigate the effect of excipients and interferences. All results were calculated after three repeated analyses.

RESULTS

Identification of bacterial isolates

Three Gram-positive and catalase-negative bacterial isolates from *Apis mellifera* gut were selected on MRS agar plates and one of the three isolates was identified as *Lactobacillus acidophilus* and the other two isolates were determined as *E. faecalis* and named as 1 and 2 by VITEK® MS MALDI TOF mass spectrometer. The isolate, which was identified as *Lactobacillus acidophilus* by VITEK® MS MALDI TOF mass spectrometer analysis, was identified as *Lactobacillus kunkeei* according to the results of 16S rRNA genetic analysis. Based on this, throughout the entire manuscript, the results and discussion section were organized according to the definition obtained from genetic analysis.

According to 16S rRNA analysis results, one of the three samples was identified as *Lactobacillus kunkeei* (*L. kunkeei*) with a 99.9% sequence matching ratio, and the other two isolates were identified as *E. faecalis* with a 100% similarity rate. Gene sequences were presented in Table in supplementary data 1.

Acid pH and enzyme tolerance

Low pH values, gastric enzyme environments with different pH ranges, and varied concentrations of bile salts were used to simulate the GIS conditions. Isolates were tested for 3 and/or 4 hours. It was eval-

Table supplementary data 1. Gene sequences of isolates

	27F-1492R primer, 99,9 %similarity ratio
Lactobacillus kunkeei	ACACGTGGGTAACCTGCCCCGAAGCGGGGGATAACATTTGGAAACAAGTGCTAATACCGCATAATT AGTTGGAACCGCATGGTTCCAACTTGAAAGATGGCTCTGCTATCACTTTGGGATGGACCCGCGCCG TATTAGTTAGTTGGTGAGATAAAAGCCCACCAAGACGATGATACGTAGCCGACCTGAGAGGGTAATC GGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACA ATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGT TGTTAAAGAAGAACAAGTGTTAGAGTAACTGTTAACACTTTGACGGTATTTAACCAGAAAGCCACG GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTA AAGCGAGCGCAGGCGGTTTTGTAAGTCTGCTGTGAAAGCCCTCAGCTCAACTGAGGAAGTGCAGT GGAAACTACAAAACTTGAGTACAGAAGAGGAAAGTGGAACTCCATGTGTAGCGGTGAAATGCGTA GATATATGGAAGAACACCAGTGGCGAAGGCGGCTTTCTGGTCTGTTACTGACGCTGAGGCTCGAAA GCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAATACTAGGTGTTG GAGGGTTTCCGCCCTTCAGTGCCGCAGCTAACGCATTAAGTATTCCGCCTGGGGAGTACGACCGCA AGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGTGGTGGAGCATGTGGTTTAATTCGATG CTACGCGAAGAACCTTACCAGCTCTTGACATCTTCTGCCAACCCAAGAGATTGGGCGTTCCCTTCGG GGACAGAATGACAGGTGGTGCATGGTNGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG CAACGAGCGCAACCCTTATTATTAGTTGCCAGCATTTAGTTGGGCACTCTAG

Table supplementary data 1. Gene sequences of isolates - continued

	27F-1492R primer, 100% similarity ratio
***Enterococcus faecalis* 1**	GCTAATACCGCATAACAGTTTATGCCGCATGGCATAAGAGTGAAAGGCGCTTTCGGGTGTCGCTGAT GGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCCACGATGCATAGCC GACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA GTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTT CGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGACGTTAGTAACTGAACGTCCCCTGACGGTA TCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTG TCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTC AACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTG TAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACT GACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA CGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCAAACGCATTAAGCACTCCG CCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGA GCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAG AGATAGAGCTTTCCCTTCGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTG AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCCATCATTTAGTTGGGCAC TCTAGCGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTA TGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGTCGCTAGACCGCGAGGTCATGCAA ATCTCTTAAAGCTTCTCTCAGT
	27F-1492R primer, 100% similarity ratio
***Enterococcus faecalis* 2**	GGGGATAACACTTGGAAACAGGTGCTAATACCGCATAACAGTTTATGCCGCATGGCATAAGAGTGAA AGGCGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCT CACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGC CCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAAC GCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGACGTTAG TAACTGAACGTCCCCTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGG TAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAG TCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGA AGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGA AGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATA CCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGC AGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGAC GGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGT CTTGACATCCTTTGACCACTCTAGAGATAGAGCTTTCCCTTCGGGGACAAAGTGACAGGTGGTGCAT GGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTA GTTGCCATCATTTAGTTGGGCACTCTAGCGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATG ACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGTC GCTAGACCGCGAGGTCATGCAAATCTCTTAAAGCTTCTCTCAGTTCGGATTGCAGGCTGC

uated that *L. kunkeei* isolate lost its viability at pH 1.0 and pH 2.0 in two hours, and at pepsin pH 2.0 in three hours. *L. kunkeei* isolate maintained its vitality for 3 hours at pH 3.0 and pepsin pH 3.0 environments and 4 hours at pancreatin pH 8.0 and bile salts 0.3%, 0.5%, 1%. It was also evaluated that *E. faecalis* 1 and 2 displayed a loss of their viability at pH 1.0 in two hours and at pepsin pH 2.0 in three hours. Isolates maintained their vitality for 3 hours at pH 3.0, pepsin pH 3.0 and 4 hours at pancreatin pH 8.0 and bile salts 0.3%, 0.5%, 1%. But it was determined that their vitality decreased significantly after 3 hours at pH 2.0. The survival rates of isolates at 3h were given in Figure 1 and all survival results were tabulated in supplementary data 2.

Hemolytic activity

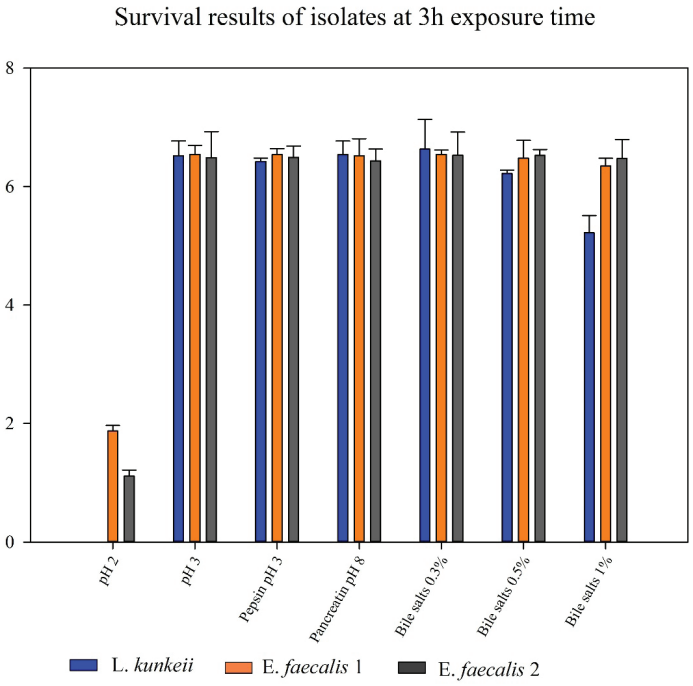
No hemolysis zones were determined around the *L. kunkeei*, *E. faecalis 1* and *E. faecalis 2* colonies on Columbia agar with 5% sheep blood, and isolates were evaluated as gamma hemolytic.

Antimicrobial susceptibility

Antimicrobial susceptibilities of isolates were tested by microdilution method with antibiotics specified by EFSA according to EUCAST standards (Rychen 2018). The results were shown in Table 1. *L. kunkeei* isolate was found resistant to gentamicin, kanamycin, streptomycin, erythromycin, tetracycline, and clindamycin. *E. faecalis* isolates 1 and 2 were found resistant to gentamicin, streptomycin, clindamycin, and erythromycin.

Antimicrobial activity

The antimicrobial activity of CFSs of isolates was



*Average results of three replicate experiments are indicated

Figure 1. Survival rates of isolates to gastric conditions as log CFU/ml.

Table supplementary data 2. Survival rates of isolates to gastric conditions as log CFU/ml

Parameters	Time	*Survival rates of isolates as log CFU/ml		
		L. kunkeii	*E. faecalis* 1	*E. faecalis* 2
pH 1.0	0. hour	6,505±0,001	6,540±0,17	6,494±0,02
	1. hour	1,079±0,014	1,602±0,09	1,851±0,08
	2. hour	0	0	0
	3. hour	0	0	0
pH 2.0	0. hour	6,591±0,11	6,492±0,1	6,551±0,15
	1. hour	1,672±0,13	6,487±0,29	6,506±0,06
	2. hour	0	6,276±0,07	5,812±0,02
	3. hour	0	1,875±0,09	1,113±0,1
pH 3.0	0. hour	6,499±0,04	6,515±0,09	6,491±0,11
	1. hour	6,527±0,18	6,525±0,09	6,562±0,38
	2. hour	6,506±0,27	6,517±0,14	6,477±0,16
	3. hour	6,522±0,25	6,546±0,15	6,491±0,44
Pepsin pH 2.0	0. hour	6,475±0,06	6,488±0,25	6,494±0,06
	1. hour	1,518±0,2	5,301±0,1	6,528±0,17
	2. hour	0,477±0,07	1,301±0,07	1,507±0,1
	3. hour	0	0	0
Pepsin pH 3.0	0. hour	6,551±0,17	6,492±0,18	6,552±0,22
	1. hour	6,537±0,08	6,521±0,17	6,546±0,15
	2. hour	6,457±0,15	6,513±0,22	6,478±0,18
	3. hour	6,424±0,06	6,542±0,1	6,494±0,19
Pancreatin pH 8.0	0. hour	6,495±0,16	6,474±0,17	6,565±0,08
	4. hour	6,540±0,23	6,522±0,29	6,436±0,2
Bile salts 0.3 %	0. hour	6,511±0,14	6,546±0,33	6,551±0,13
	4. hour	6,634±0,5	6,547±0,07	6,532±0,39
Bile salts 0.5 %	0. hour	6,502±0,17	6,457±0,17	6,463±0,06
	4. hour	6,222±0,06	6,481±0,3	6,526±0,1
Bile salts 1 %	0. hour	6,551±0,13	6,474±0,19	6,515±0,14
	4. hour	5,222±0,29	6,351±0,13	6,478±0,32

*Average results of three replicate experiments are indicated.

determined firstly as a zone of inhibition against *E. coli* ATCC 25922, *E. faecalis* ATCC 29212, *K. pneumoniae* ATCC 13883, *S. aureus* ATCC 29213, and *P. aeruginosa* ATCC 27853. The presence of the inhibition zone was shown as "+" and the absence as "-" in Table 2.

And the antimicrobial activity was also tested by broth microdilution test as a minimal inhibition concentration against the same indicator microorganisms. It was determined that *L. kunkeei* CFS inhibited the growth of *E. faecalis* ATCC 29212. CFSs of *E. faecalis* 1 and 2 were found effective in the growth of *E. coli* ATCC 25922, *E. faecalis* ATCC 29212, and *P. aeruginosa* ATCC 27853. MIC values of CFSs were evaluated as 1/4 dilution of the culture supernatants in the first well of the microplates against indicator microorganisms. Considering that the antimicrobial activities of CFSs may be due to their inhibitor compound production such as bacteriocin, hydrogen peroxide, organic acid, etc. The organic acid content of the supernatants was determined by the HPLC method and these results are given in Table 3. Accordingly, it was detected that the isolates produced different amounts of lactic, acetic, and butyric acid.

Diacetyl Production

The red ring formation in test tubes containing bacterial cultures was identified as the production of diacetyl and the ring was defined as negative (-), weak (+), medium (++) or strong (+ + +), depending on the intensity of the color compared to reference *L. rhamnosus* GG ATCC 53103. According to the results, it was determined that *L. kunkeei* did not produce diacetyl, and *E. faecalis* isolates were strong diacetyl producers.

Auto-aggregation test

Autoaggregation values of three isolates ranged between 37 and 49. *E. faecalis* 1 and isolates exhibited higher aggregation (49±1.90, 43±0.83, respectively) than *L. kunkeei* isolate (37±0.10) and the control strain L. *rhamnosus* GG ATCC 53103 (65±1.46) had the highest score.

Anti-quorum sensing activity

CFSs of *L. kunkeei* and *E. faecalis* 1 and 2 had inhibitory activity on the N-hexanoyl-L-homoserine lactone signal molecule, as shown by the lower production of violacein pigment depending on the quorum sensing bacterial communication system (Figure supplementary data 3).

Determination and quantification of organic acids by high performance liquid chromatography

The concentration of organic acids produced by *L. kunkeei, E. faecalis* 1, and *E. faecalis* 2 were found between 1.7 - 7.5 g/L for lactic acid, 0.94 - 2.20 g/L for acetic acid (except *E. faecalis* 2), 0.22 - 0.38 g/L for butyric acid. All results are tabulated in Table 3.

Table 1. Minimum inhibitory concentration (MIC) results of isolates (Results were evaluated according to EFSA standards) (Rychen 2018)

Bacteria	MIC results for antibiotics (µg/ml)								
	AM	GM	K	S	E	CC	TE	C	VA
L. kunkeei	0,5	64	>256	128	>256	64	32	4	2
E. faecalis 1	<0,125	256	256	>256	>256	>256	0,5	4	4
E. faecalis 2	<0,125	256	256	>256	>256	>256	0,5	2	4

AM: Ampicillin, CC: Clindamycin, C: Chloramphenicol, E: Erythromycin, GM: Gentamicin, K: Kanamycin, S: Streptomycin, TE: Tetracycline, VA: Vancomycin

Table 2. Determination of the antimicrobial activity of tested isolates on indicator microorganisms

Isolates	Zone of inhibition (+/- and as mm)				
	E. coli ATCC 25922	*E. faecalis* ATCC 29212	*K. pneumoniae* ATCC 13883	*S. aureus* ATCC 29213	*P. aeruginosa* ATCC 27853
L. kunkeei CFS	-	+ (7 mm)	-	-	-
E. faecalis 1 CFS	+ (8 mm)	+ (10 mm)	-	-	+ (7 mm)
E. faecalis 2 CFS	+ (6 mm)	+ (9 mm)	-	-	+ (6 mm)

+: Zone of inhibition, -: Absence of inhibition zone

Table 3. Organic acid amounts of culture supernatants of isolates

	Lactic Acid	Acetic Acid	Butyric Acid
L. kunkeei	1,75 ± 0,03	2,21 ± 0,01	0,39 ± 0,01
E. faecalis 1	7,52 ± 0,23	0,95 ± 0,01	0,3 ± 0,02
E. faecalis 2	5,95 ± 0,02	<LOD	0,23 ± 002

LOD: Limit of detection

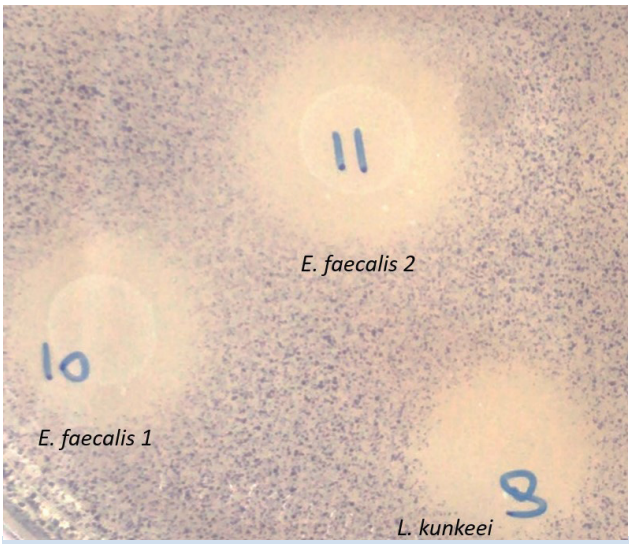


Figure supplementary data 3. Anti-QS activity of tested CFSs on *Chromobacterium violaceum* strain

DISCUSSION

Due to the increasing population, human habitats are expanding to different geographical regions, and accordingly, a life begins in close contact with the animals existing in these environments. As a result of the close contact, direct and/or indirect contact with animals provides more opportunities for microorganisms to pass between animals and humans (CDC 2022). This interaction affects the intestinal microbiota and the studies showed that microorganisms in the intestine plays a significant role for both the human and animal health along with the immune function (Guinane and Cotter 2013). The content of the gut microbiota varies depending on the dynamic changes that take place during the development, environment, nutritional state, physiological state, or the health of the host (Bromenshenk et al. 2010).

In order for a bacterium to be identified as a probiotic, it should have beneficial effects on health and be scientifically recognized as safe. According to the FAO/WHO (2002) criteria, a strain with probiotic characteristics should be able to survive in the gastrointestinal system, and be resistant to acids and bile salts. In the present study, three LAB strains were isolated from the bee gut (*L. kunkeei*, *E. faecalis* 1 and 2) and investigated for their probiotic abilities, antimicrobial efficacy against some pathogen microorganisms, and anti-quorum sensing potential. Different pH values (pH 1, 2, 3, 4) and environments with pepsin and pancreatic enzymes, and bile salts (0.3%, 0.5%, 1%) were tested to determine what extent the strains could tolerate GIS conditions. The results showed that all strains had tolerance to pH 3, pepsin pH 3, pancreatin pH 8, and bile salt environments with better viability rates at the end of the determined periods. Wang et al. (2020) showed that *E. faecalis* isolates from infant faecal samples survived at pH 5.0 and 3% bile salt. Mohammad et al. (2020) evaluated *Leuconostoc mesenteroides*, *Lactobacillus musae*, *Lactobacillus crustorum*, *Lactobacillus mindensis*, *E. faecalis* isolates from bee bread which showed survival rates of more than 80% pH 3 for 3 h and more than 60% bile salts 0.3%. Baccouri et al. (2019) determined that *E. faecalis* strains isolated from traditional food products tolerated the GIS (acidity and bile salt) conditions. Hasali et al. (2018) detected that *Lactobacillus* strains from *Heterotrigona itama* honey were resistant to pH 2 and 0.3% bile salts concentration. Kim et al. (2017) reported that *E. faecalis* PSCT3-7 isolated from pig intestines showed tolerance to pH 3-8 and 0.3% bile salts, and showed antibacterial activity against *Salmonella* Typhimurium in a concentration-dependent manner. Khalkhali and Mojgani (2017) reported that *E. faecalis* TA102 isolated from human milk survived acidic and 1% bile salt conditions, simulated GIS conditions, and CFS of isolating inhibited the growth of *E. coli*, *Listeria monocytogenes*, *Salmonella typhi*, *S. aureus*, *Shigella dysenteriae,* and *Streptococcus agalactiae*. Shokryazdan et al. (2014) found that isolated *Lactobacillus* strains (*L. acidophilus*, *L. fermentum*, *L. buchneri*, *L. casei*) exhibited good acid tolerance at pH 3 for 3 h.

The present study had similar results to the published studies and determined that *L. kunkeei*, *E. faecalis* 1 and 2 isolates were found resistant to gentamicin, streptomycin, erythromycin, and clindamycin. Tetracycline and kanamycin resistance was also

shown in *L. kunkeei* isolate. Vergalito et al. (2020) evaluated that *Apilactobacillus kunkeei* isolates (K18, K34, DSM 12361) were resistant to ampicillin, chloramphenicol, and kanamycin. Wang et al. (2020) reported that *E. faecalis* isolates from healthy infant faeces were resistant to erythromycin, quinupristin/dalofopine, and clindamycin. Baccouri et al. (2019) report the occurrence of tetracycline resistance gene in *E. faecalis* OB14 by whole genome sequencing analysis. Joghataei et al. (2019) found that *Lactobacillus* strains isolated from Iranian traditional fermented food products were resistant to vancomycin and streptomycin. Kim et al. (2017) underlined that *E. faecalis* PSCT3-7 from pig intestines showed resistance to colistin, spectinomycin, streptomycin, chloramphenicol, florfenicol, norfloxacin, novobiocin, cephalexin, bacitracin, marbofloxacin, gentamicin. Khalkhali and Mojgani (2017) found vancomycin resistance in *E. faecalis* TA102 isolated from human milk. Al Atya et al. (2015) have examined that *E. faecalis* isolates isolated from meconium which was resistant to clindamycin and erythromycin. Hanchi et al. (2014) showed that *E. faecalis* 61B from Tunisian dairy products were resistant to erythromycin. This resistance profile was in agreement with the literature, Mathur and Singh (2005) stated the intrinsic resistance in enterococci to various antibiotics such as aminoglycosides and beta-lactams. Ribeiro et al. (2014) reported that antimicrobial agents may be used in the treatment and control of infectious diseases in bees as a cause of antibiotic resistance in microorganisms. This could also affect humankind indirectly in terms of antibiotic resistance in microorganisms considering the concept of one health (CDC 2022). s (Guinane and Cotter 2013).

Vergalito et al. (2020) found that *Apilactobacillus kunkeei* isolates (K18, K34, K45) had inhibitory activity against *E. faecalis* ATCC 29212 and *P. aeruginosa* ATCC 27853. Jomehzadeh et al. (2020) showed that *Lactobacillus* strains isolated from faeces of infants inhibited the growth of *Yersinia enterocolitica* ATCC 23715, *Shigella flexneri* ATCC 12022, *Salmonella enterica* ATCC 9270, and enteropathogenic *E. coli* (EPEC) ATCC 43887. Abhisingha et al. (2018) determined that *L. johnsonii* LJ202 from piglets was inhibited the growth of *Salmonella enterica* Enteritidis DMST7106 in 10 h. In literature, it was found that some LAB strains including *Lactobacillus* and *Enterococcus* strains from different environments had also antagonistic activity against *Pseudomonas spp*., *Proteus spp*. (Zugic Petrovic et al. 2020), *Listeria monocytogenes* (Valente et al. 2019), *Streptococcus mutans* (Fang et al. 2018), *S. aureus*, *Staphylococcus epidermidis* (Jabbari et al. 2017), *Pseudomonas putida*, *Pseudomonas fluorescens*, *Salmonella typhimurium*, *Shigella flexneri* and *Shigella sonnei* (Liu et al. 2016). In accordance with these studies, in the present study, it was determined that LAB isolated from bee gut had inhibitory activity on *E. faecalis* ATCC 29212, *E.coli* ATCC 25922, and *P. aeruginosa* ATCC 27853.

In the present study, similarly to the published studies, it was found that LAB isolates produced lactic acid between 1.7 - 7.5 g/L, acetic acid 0.94 - 2.20 g/L (except *E. faecalis* 2), butyric acid 0.22 - 0.38 g/L as previously stated (Table 3). Franco et al. (2020) evaluated the quantity of lactic and acetic acid produced by LAB from Quinoa sourdough fermentation and found that organic acid amounts changed according to quinoa flour type. The highest values of lactic and acetic acid were determined as 7.75 ± 0.15 mM/kg and 5.84 ± 0.11 mM/kg, respectively. Ouiddir et al. (2019) showed that the selected LAB strains for application in dairy and bakery products produced lactic acid between 1.04 ± 0.18 to 12.1 ± 1.61 and acetic acid 0.03 ± 0.006 to 0.64 ± 0.17. Al Atya et al. (2015) concluded that *E. faecalis* 28 and *E. faecalis* 93 isolates produced up to 7.06 g/l of lactic acid, after 24 h incubation.

The aggregation ability of the bacteria, which is an important criterion in terms of probiotics, allows them to attach to the cells and colonize dominantly. Due to this feature, LAB can form a biological barrier by adhering to the surface, one another, and colonizing the cells they occupied (Vlkova et al. 2008). Lactobacilli were reported to prevent the colonization of pathogenic bacteria by aggregation (Ferreira et al. 2011). In the present study, the highest autoaggregation score was registered for *E. faecalis* 1 with 49 ± 1.9 %. Results of a study on two *E. faecalis* isolated from Rigouta cheese were similar to our results with scores of 48,9 and 54.3 (Baccouri et al. 2019). Autoaggregation values of five Lactobacilli samples from dental caries of children ranged between 48-93% (Piwat et al. 2010; Sophatha et al. 2020). All these percentages are comparable with the human isolates. *E. faecalis* strains isolated from the meconium of human donors were determined to have an autoaggregation ratio between 35-49% (Al Atya et al. 2015). *Enterococcus spp.* isolated from a healthy human vagina and GIS was reported to have an aggregation ratio between 20-50% (Bhagwat et al. 2019).

Inhibition of quorum sensing-controlled pigment production of *C. violaceum* strain was achieved by the culture supernatants of the isolates in the present study. Onbas et al. (2019) observed that *Lactobacillus plantarum* F-10 inhibited the quorum sensing-controlled virulence factors of *P. aeruginosa* such as motility, protease, elastase, pyocyanin, and rhamnolipid production. Joshi et al. (2014) determined the anti-quorum sensing potential of *L. plantarum* NC8 against multidrug-resistant *P. aeruginosa* for infection control.

CONCLUSION

Bee gut was identified as a novel LAB source with significant probiotic characteristics. *E. faecalis* 1, 2, and *L. kunkeei* isolates were able to tolerate GIS conditions, showed antagonistic activity against some pathogen bacteria and were found to be gamma haemolytic. These isolates were found to be resistant to some of the antibiotics tested. Based on the results of new in vivo studies for genetic determination of safety assessment and virulence traits, and promising probiotic properties of isolates, it is thought that industrial use potentials will emerge.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare that are relevant to the content of this article.

REFERENCES

Abhisingha M, Dumnil J, Pitaksutheepong C (2018) Selection of Potential Probiotic Lactobacillus with Inhibitory Activity Against Salmonella and Fecal Coliform Bacteria. Probiotics Antimicrob Proteins 10(2):218-227 doi:10.1007/s12602-017-9304-8

Al Atya AK, Drider-Hadiouche K, Ravallec R, Silvain A, Vachee A, Drider D (2015) Probiotic potential of Enterococcus faecalis strains isolated from meconium. Front Microbiol 6:227 doi:10.3389/fmicb.2015.00227

Aslan CE, Liang CT, Galindo B, Hill K, Topete W (2016) The Role of Honey Bees as Pollinators in Natural Areas. Nat Area J 36(4):478-488 doi:Doi 10.3375/043.036.0413

Baccouri O, Boukerb AM, Farhat LB, et al. (2019) Probiotic Potential and Safety Evaluation of Enterococcus faecalis OB14 and OB15, Isolated From Traditional Tunisian Testouri Cheese and Rigouta, Using Physiological and Genomic Analysis. Front Microbiol 10:881 doi:10.3389/fmicb.2019.00881

Ben Braiek O, Ghomrassi H, Cremonesi P, et al. (2017) Isolation and characterisation of an enterocin P-producing Enterococcus lactis strain from a fresh shrimp (Penaeus vannamei). Anton Leeuw Int J G 110(6):771-786 doi:10.1007/s10482-017-0847-1

Ben Braiek O, Smaoui S (2019) Enterococci: Between Emerging Pathogens and Potential Probiotics. Biomed Research International 2019 doi:Artn 5938210

10.1155/2019/5938210

Bhagwat A, Nandanwar YA, Warke R, Annapure US (2019) In vitro assessment of physiological properties of Enterococcus strains of human origin for possible probiotic use. Asian J Pharm Clin Res 12 doi:https://doi.org/10.22159/ajpcr.2019.v12i6.33294

Bintsis T (2018) Lactic acid bacteria: their applications in foods. Journal of Bacteriology & Mycology: Open Access 6(2):89-94

Bromenshenk JJ, Henderson CB, Wick CH, et al. (2010) Iridovirus and Microsporidian Linked to Honey Bee Colony Decline. Plos One 5(10) doi:ARTN e13181

10.1371/journal.pone.0013181

CDC (2018) One Health. Centers for Disease Control and Prevention , National Center for Emerging and Zoonotic Infectious Diseases. In. https://www.cdc.gov/onehealth/basics/index.html#:~:text=One%20Health%20is%20a%20collaborative,plants%2C%20and%20their%20shared%20environment

CDC (2022) One Health. Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases.

Charteris WP, Kelly PM, Morelli L, Collins JK (1998) Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic Lactobacillus and Bifidobacterium species in the upper human gastrointestinal tract. J Appl Microbiol 84(5):759-768

De Baere S, Eeckhaut V, Steppe M, et al. (2013) Development of a HPLC-UV method for the quantitative determination of four short-chain fatty acids and lactic acid produced by intestinal bacteria during in vitro fermentation. J Pharmaceut Biomed 80:107-115 doi:10.1016/j.jpba.2013.02.032

Del Re B, Sgorbati B, Miglioli M, Palenzona D (2000) Adhesion, autoaggregation and hydrophobicity of 13 strains of Bifidobacterium longum. Lett Appl Microbiol 31(6):438-42 doi:10.1046/j.1365-2672.2000.00845.x

Endo A, Salminen S (2013) Honeybees and beehives are rich sources for fructophilic lactic acid bacteria. Syst Appl Microbiol 36(6):444-448 doi:10.1016/j.syapm.2013.06.002

Erdonmez D, Rad AY, Aksoz N (2018) Anti-Quorum Sensing Potential of Antioxidant Quercetin and Resveratrol. Braz Arch Biol Techn 61

EUCAST (2021) Breakpoint tables for interpretation of MICs and zone diameters , . European Committee on Antimicrobial Susceptibility Testing Version 11.0(7.01.2021)

Fang F, Xu J, Li Q, Xia X, Du G (2018) Characterization of a Lactobacillus brevis strain with potential oral probiotic properties. BMC Microbiol 18(1):221 doi:10.1186/s12866-018-1369-3

FAO/WHO (2002) Guidelines for the Evaluation of Probiotics in Food. Report of a Joint FAO/WHO Working Group on

Drafting Guidelines for the Evaluation of Probiotics in Food.,

Ferreira CL, Grzeskowiak L, Collado MC, Salminen S (2011) In vitro evaluation of Lactobacillus gasseri strains of infant origin on adhesion and aggregation of specific pathogens. J Food Prot 74(9):1482-7 doi:10.4315/0362-028X.JFP-11-074

Franco W, Perez-Diaz IM, Connelly L, Diaz JT (2020) Isolation of Exopolysaccharide-Producing Yeast and Lactic Acid Bacteria from Quinoa (Chenopodium Quinoa) Sourdough Fermentation. Foods 9(3) doi:10.3390/foods9030337

Franz CMAP, Huch M, Abriouel H, Holzapfel W, Galvez A (2011) Enterococci as probiotics and their implications in food safety. Int J Food Microbiol 151(2):125-140 doi:10.1016/j.ijfoodmicro.2011.08.014

Franz CMAP, Muscholl-Silberhorn AB, Yousif NMK, Vancanneyt M, Swings J, Holzapfel WH (2001) Incidence of virulence factors and antibiotic resistance among enterococci isolated from food. Appl Environ Microb 67(9):4385-4389 doi:Doi 10.1128/Aem.67.9.4385-4389.2001

Guinane CM, Cotter PD (2013) Role of the gut microbiota in health and chronic gastrointestinal disease: understanding a hidden metabolic organ. Ther Adv Gastroenter 6(4):295-308 doi:10.1177/1756283x13482996

Hanchi H, Hammami R, Kourda R, Hamida JB, Fliss I (2014) Bacterioci-

nogenic properties and in vitro probiotic potential of enterococci from Tunisian dairy products. Arch Microbiol 196(5):331-44 doi:10.1007/s00203-014-0978-y

Hasali NH, Zamri AI, Lani MN, Mubarak A (2018) Antibiotic susceptibility, antibacterial activity and probiotic characterisation of isolated lactobacillus brevis strains from heterotrigona itama honey. Malaysian Applied Biology 47(6):105-112

Hayek SA, & Ibrahim, S. A. (2013) Current Limitations and Challenges with Lactic Acid Bacteria: A Review. Food and Nutrition Sciences

ICH (2014) CPMP/ICH/381/95 - ICH harmonised tripartite guideline - Validation of Analytical Procedures: Text and Methodology Q2(R1).

Jabbari V, Khiabani MS, Mokarram RR, et al. (2017) Lactobacillus plantarum as a Probiotic Potential from Kouzeh Cheese (Traditional Iranian Cheese) and Its Antimicrobial Activity. Probiotics Antimicrob Proteins 9(2):189-193 doi:10.1007/s12602-017-9255-0

Janashia I, Carminati D, Rossetti L, et al. (2016) Characterization of fructophilic lactic microbiota of Apis mellifera from the Caucasus Mountains. Ann Microbiol 66(4):1387-1395 doi:10.1007/s13213-016-1226-2

Joghataei M, Shahidi F, Pouladfar G, Mortazavi SA, Ghaderi A (2019) Probiotic potential comparison of Lactobacillus strains isolated from Iranian traditional food products and human feces with standard probiotic strains. J Sci Food Agric 99(15):6680-6688 doi:10.1002/jsfa.9945

Jomehzadeh N, Javaherizadeh H, Amin M, et al. (2020) Isolation and identification of potential probiotic Lactobacillus species from feces of infants in southwest Iran. Int J Infect Dis 96:524-530 doi:10.1016/j.ijid.2020.05.034

Joshi S, Kaur A, Sharma P, Harjai K, Capalash N (2014) Lactonase-expressing Lactobacillus plantarum NC8 attenuates the virulence factors of multiple drug resistant Pseudomonas aeruginosa in co-culturing environment. World J Microbiol Biotechnol 30(8):2241-9 doi:10.1007/s11274-014-1645-9

Khalkhali S, Mojgani N (2017) Bacteriocinogenic potential and virulence traits of Enterococcus faecium and E. faecalis isolated from human milk. Iran J Microbiol 9(4):224-233

Kim JY, Awji EG, Park NH, et al. (2017) Probiotic properties and adsorption of Enterococcus faecalis PSCT3-7 to vermiculite. J Vet Sci 18(1):95-99 doi:10.4142/jvs.2017.18.1.95

King N (1948) A modification of the Voges-Proskauer test for rapid colorimetric determination of acetylmethylcarbinol plus diacetyl in the butter cultures. Dairy Indust 13(9):860-861,878

Lika E, Kostić M, Vještica S, Milojević I, Puvača N (2021) Honeybee and plant products as natural antimicrobials in enhancement of poultry health and production. Sustainability 13(5):8467 doi:https://doi.org/10.3390/su13158467

Lima ET, Andreatti RL, Okamoto AS, Noujaim JC, Barros MR, Crocci AJ (2007) Evaluation in vitro of the antagonistic substances produced by Lactobacillus spp. isolated from chickens. Can J Vet Res 71(2):103-107

Liu H, Zhang L, Yi H, et al. (2016) A novel enterocin T1 with anti-Pseudomonas activity produced by Enterococcus faecium T1 from Chinese Tibet cheese. World J Microbiol Biotechnol 32(2):21 doi:10.1007/s11274-015-1973-4

Maragkoudakis PA, Zoumpopoulou G, Miaris C, Kalantzopoulos G, Pot B, Tsakalidou E (2006) Probiotic potential of Lactobacillus strains isolated from dairy products. Int Dairy J 16(3):189-199 doi:10.1016/j.idairyj.2005.02.009

Mathur S, Singh R (2005) Antibiotic resistance in food lactic acid bacteria--a review. Int J Food Microbiol 105(3):281-95 doi:10.1016/j.ijfoodmicro.2005.03.008

Miller MB, Bassler BL (2001) Quorum sensing in bacteria. Annu Rev Microbiol 55:165-199 doi:DOI 10.1146/annurev.micro.55.1.165

Mohammad SM, Mahmud-Ab-Rashid NK, Zawawi N (2020) Probiotic properties of bacteria isolated from bee bread of stingless bee Heterotrigona itama. Journal of Apicultural Research 60(1):172-187

O'Driscoll T, Crank CW (2015) Vancomycin-resistant enterococcal infections: epidemiology, clinical manifestations, and optimal management. Infect Drug Resist 8:217-30 doi:10.2147/IDR.S54125

Onbas T, Osmanagaoglu O, Kiran F (2019) Potential Properties of Lactobacillus plantarum F-10 as a Bio-control Strategy for Wound Infections. Probiotics Antimicrob Proteins 11(4):1110-1123 doi:10.1007/s12602-018-9486-8

Ouiddir M, Bettache G, Leyva Salas M, et al. (2019) Selection of Algerian lactic acid bacteria for use as antifungal bioprotective cultures and application in dairy and bakery products. Food Microbiol 82:160-170 doi:10.1016/j.fm.2019.01.020

Piwat S, Teanpaisan R, Thitasomakul S, Thearmontree A, Dahlen G (2010) Lactobacillus species and genotypes associated with dental caries in Thai preschool children. Mol Oral Microbiol 25(2):157-64 doi:10.1111/j.2041-1014.2009.00556.x

Puvača N (2018) Honeybee and medicinal plants products in poultry postantibiotic era production. Journal of Agronomy, Technology and Engineering Management 1(1):8-17

Ribeiro SC, Coelho MC, Todorov SD, Franco BD, Dapkevicius ML, Silva CC (2014) Technological properties of bacteriocin-producing lactic acid bacteria isolated from Pico cheese an artisanal cow's milk cheese. J Appl Microbiol 116(3):573-85 doi:10.1111/jam.12388

Rychen G, Aquilina, G., Azimonti, G., Bampidis, V., Bastos, M. de L., Bories, G., Chesson, A., Cocconcelli, P. S., Flachowsky, G., Gropp, J., Kolar, B., Kouba, M., López-Alonso, M., López Puente, S., Mantovani, A., Mayo, B., Ramos, F., Saarela, M., Villa, R. E., Wallace, R.J., Wester, P., Glandorf, B., Herman, L., Kärenlampi, S., Aguilera, J., Anguita, M., Brozzi, R., Galobart, J. (2018) Guidance on the characterisation of microorganisms used as feed additives or as production organisms EFSA Journal 16(3):1-24

Shokryazdan P, Sieo CC, Kalavathy R, et al. (2014) Probiotic Potential of Lactobacillus Strains with Antimicrobial Activity against Some Human Pathogenic Strains. Biomed Research International 2014 doi:Artn 927268
10.1155/2014/927268

Sophatha B, Piwat S, Teanpaisan R (2020) Adhesion, anti-adhesion and aggregation properties relating to surface charges of selected Lactobacillus strains: study in Caco-2 and H357 cells. Arch Microbiol 202(6):1349-1357 doi:10.1007/s00203-020-01846-7

Tomičić Z, Ivana Čabarkapa I, Čolović R, Đuragić O, Tomičić R (2019) SALMONELLA IN THE FEED INDUSTRY: PROBLEMS AND POTENTIAL SOLUTIONS. Journal of Agronomy, Technology and Engineering Management 2(1):130-137

Valente GLC, Acurcio LB, Freitas LPV, et al. (2019) Short communication: In vitro and in vivo probiotic potential of Lactobacillus plantarum B7 and Lactobacillus rhamnosus D1 isolated from Minas artisanal cheese. J Dairy Sci 102(7):5957-5961 doi:10.3168/jds.2018-15938

Vergalito F, Testa B, Cozzolino A, et al. (2020) Potential Application of Apilactobacillus kunkeei for Human Use: Evaluation of Probiotic and Functional Properties. Foods 9(11) doi:10.3390/foods9111535

Vlkova E, Rada V, Smehilova M, Killer J (2008) Auto-aggregation and co-aggregation ability in bifidobacteria and clostridia. Folia Microbiol (Praha) 53(3):263-9 doi:10.1007/s12223-008-0040-z

Wang J, Da R, Tuo X, et al. (2020) Probiotic and Safety Properties Screening of Enterococcus faecalis from Healthy Chinese Infants. Probiotics Antimicrob Proteins 12(3):1115-1125 doi:10.1007/s12602-019-09625-7

Zielińska D, Kolozyn-Krajewska, D., Laranjo, M. (2018) Food-Origin Lactic Acid Bacteria May Exhibit Probiotic Properties: Review. BioMed Research International:2018

Zugic Petrovic TD, Ilic PD, Grujovic MZ, Mladenovic KG, Kocic-Tanackov SD, Comic LR (2020) Assessment of safety aspect and probiotic potential of autochthonous Enterococcus faecium strains isolated from spontaneous fermented sausage. Biotechnol Lett 42(8):1513-1525 doi:10.1007/s10529-020-02874-5