Archives of Microbiology (2022) 204:432
https://doi.org/10.1007/s00203-022-03000-x

ORIGINAL PAPER

Investigation of the probiotic and metabolic potential of *Fructobacillus tropaeoli* and *Apilactobacillus kunkeei* from apiaries

Duygu Simsek[1,2] · Merve Eylul Kiymaci[3] · Kenan Can Tok[4] · Mehmet Gumustas[4] · Nurten Altanlar[1]

Received: 24 January 2022 / Revised: 13 May 2022 / Accepted: 15 May 2022 / Published online: 27 June 2022

Abstract

Honeybee products have been among important consumer products throughout history. Microbiota has attracted attention in recent years due to both their probiotic value and industrial potential. Fructophilic lactic acid bacteria (FLAB), whose field of study has been expanding rapidly in the last 20 years, are among the groups that can be isolated from the bee gut. This study aimed to isolate FLAB from the honeybees of two different geographic regions in Turkey and investigate their probiotic, metabolic and anti-quorum sensing (anti-QS) potential. Metabolic properties were investigated based on fructose toleration and acid and diacetyl production while the probiotic properties of the isolates were determined by examining pH, pepsin, pancreatin resistance, antimicrobial susceptibility, and antimicrobial activity. Anti-QS activities were also evaluated with the *Chromobacterium violaceum* biosensor strain. Two FLAB members were isolated and identified by the 16S rRNA analysis as *Fructobacillus tropaeoli* and *Apilactobacillus kunkeei*, which were found to be tolerant to high fructose, low pH, pepsin, pancreatin, and bile salt environments. Both isolates showed anti-QS activity against the *C. violaceum* biosensor strain and no diacetyl production. The daily supernatants of the isolates inhibited the growth of *Enterococcus faecalis* ATCC 29212 among the selected pathogens. The isolates were found resistant to kanamycin, streptomycin, erythromycin, and clindamycin. In the evaluation of the probiotic potential of these species, the negative effect of antibiotics and other chemicals to which honeybees are directly or indirectly exposed draws attention within the scope of the "One Health" approach.

Communicated by Erko Stackebrandt.

Duygu Simsek and Merve Eylul Kiymaci contributed to the study equally.

✉ Duygu Simsek
dsimsek@ankara.edu.tr

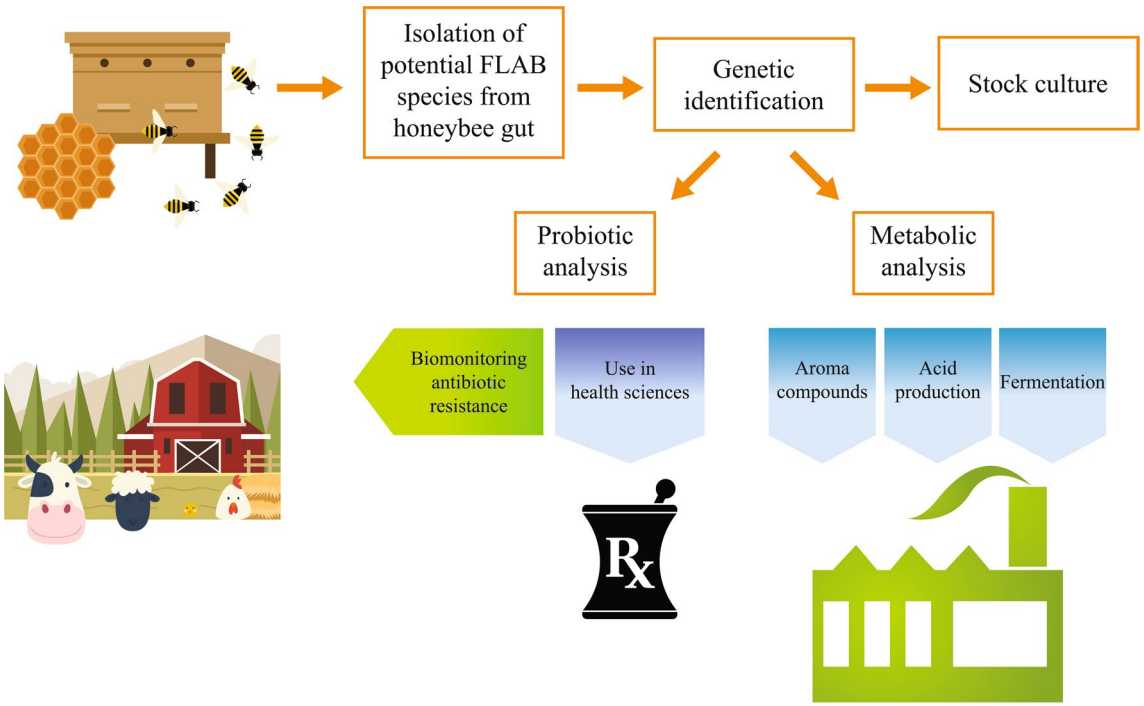
[1] Faculty of Pharmacy, Department of Pharmaceutical Microbiology, Ankara University, Ankara, Turkey

[2] Turkish Medicines and Medical Devices Agency, Ankara, Turkey

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

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

Graphical abstract



Keywords Fructophilic bacteria · Anti-quorum sensing · Antimicrobial resistance · One Health

Introduction

Conscious efforts are being made across the world to yield honeybee products (honey, royal jelly, pollen, propolis, venom, etc.), which have been among important resources throughout history (Basa et al. 2016). In addition to the usual honeybee-sourced products, the honeybee microbiota has also attracted attention worldwide in recent years (Bonilla-Rosso and Engel, 2018). These microbiota members are used both to develop an alternative solution to the challenges in combating microbial honeybee diseases and to obtain microorganism-derived bio-products on an industrial scale (Filannino et al. 2019).

Fructophilic lactic acid bacteria (FLAB) are a recently discovered group of lactic acid bacteria (LAB) and have received growing interest in the last few decades due to their unusual metabolic properties (Endo et al. 2018). The group taxonomy has recently been reclassified (Zheng et al. 2020), with *Apilactobacillus apinorum, Apilactobacillus kunkeei, Apilactobacillus micheneri, Apilactobacillus quenuiae, Apilactobacillus timberlakei, Fructobacillus durionis, Fructobacillus ficulneus, Fructobacillus fructosus, Fructobacillus pseudoficulneus*, *Fructobacillus tropaeoli*, *Fructilactobacillus florum*, *Lactiplantibacillus plantarum, and Levilactobacillus brevis* being members identified to date (Neveling et al. 2012; Pachla et al. 2018; Zheng et al. 2020; Maeno et al. 2021). The most important feature that distinguishes FLAB from the other bacteria in the LAB family is that FLAB prefer D-fructose, rather than D-glucose, because their glucose metabolism occurs under electron acceptor, their members metabolize very few carbohydrates and produce mannitol from fructose, and all FLAB are obligatory heterofermentative LAB but are distinguished from others by the production of acetate rather than ethanol (Endo et al. 2012). Flowers, fruits, and guts of fructose-feeding insects are common habitats for FLAB (Endo et al. 2018).

Fructobacillus tropaeoli was first isolated from a flower (*Tropaeolum majus*) in 2009 by Endo et al. and identified as a new species in the *Fructobacillus* genus. This new species is defined as Gram-positive, non-motile, catalase-negative, a facultative anaerobic bacterium with the dimensions of 0.8x1.5–6 µm, which ferments D-glucose, D-fructose, and mannitol. It has a lactic and acetic acid yield ratio of 1:1 and a relative trace amount of ethanol as a result of D-glucose consumption. It does not reduce nitrate or form dextran from sucrose, has a pH growth range of 4–8, and can survive at 10 °C but not at 45 °C (Endo et al. 2011). In the literature, FLAB species was identified by Snauwaert et al. (2013) from the strains that had been previously isolated through spontaneous fermenting cocoa pulp-beans in cocoa bean box and platforms. Rodríguez et al.

isolated *F. tropaeoli* from the fig, optimized the culture conditions for mannitol production (Ruiz Rodríguez et al. 2017), and reported the draft genome of the isolate with unique properties (Ruiz Rodríguez et al. 2020). The intracellular accumulation of nicotinamide mononucleotide and nicotinamide riboside in the presence of D-fructose has been previously shown (Sugiyama et al. 2021). In recent years, this species has attracted attention with their industrial potential; however, the number of available isolates is very limited.

Using previously isolated LAB showing sluggish alcoholic fermentation from a commercial grape wine, Edwards et al. (1998) were the first to identify *Apilactobacillus kunkeei* as a Gram-positive, catalase-positive (weak), facultative anaerobic rod shaped bacterium of 0.5x1–1.5 mm in dimensions. The characteristics of the species were revised and explained more comprehensively in the next two studies. The lactic and acetic acid yield ratio of this bacterium is 1:1 with a relative trace amount of ethanol. This species does not reduce nitrate or form dextran from sucrose. It ferments D-fructose, D-glucose, and sucrose. The fermentation of other various carbohydrates is strain-dependent. The growth range for pH has been determined as 4.5–6.8, and it can survive at 15 °C but not at 45 °C. This bacterium has been previously isolated from bees, flowers, fruits, and honey (Endo et al. 2012; Neveling et al. 2012). Due to the bacteriocin-like substances produced in the honeybee gut and some other properties, *A. kunkeei* is considered as a probiotic for honeybees (Endo and Salminen, 2013). The genome of a bee isolate indicates that *A. kunkeei* possesses fewer protein coding sequences than other small-genome lactobacilli and possesses a partial adhE gene (Maeno et al. 2016). The glucansucrase activity of some strains has been reported (Vasileva et al. 2017). It has also been investigated in terms of its probiotic potential in recent years (Vergalito et al. 2020).

There is an increasing interest in the search for new probiotics from "non-traditional sources" for human use (Sornplang and Piyadeatsoontorn, 2016). Among these, FLAB are recently characterized as a small group considered to be safe. The unique properties of the bee gut make it a suitable environment for the isolation of FLAB. This study aimed to isolate FLAB species from the honeybee gut and to investigate their antimicrobial susceptibility, probiotic and metabolic potential, and antimicrobial and anti-quorum sensing (anti-QS) activity. It is considered that the results will provide important data for the literature based on the 'One Health' approach.

Materials and methods

Isolation and identification of bacteria

In August 2017, worker honeybee samples from two non-migratory apiaries located in Muğla (36°46'10.6" N–28°14'29.4"E) and Sivas (39°43'41.4" N–37°02'01.6"E), Turkey were taken using appropriate equipment, kept in jars, and transferred to the laboratory within 10 h. Before dissection, an antiseptic was applied to the surface of the bees to avoid any surface contamination. The intestines of the honeybees were dissected carefully, and the crop, midgut, and hindgut were separated aseptically and transferred to glass test tubes. For FLAB isolation, the formulated medium Fructose Yeast Peptone (FYP) broth (pH 6.8) was used following the protocols described by Endo et al. (2011) with a slight modification. After grounding the intestines with a glass rod, 5 mL of FYP was added, and the sample was vortexed for 30 s to obtain a homogeneous suspension. The suspension was incubated at 35 ± 2 °C and 150 rpm for 24 h. Then, each sample was re-inoculated into the FYP broth at a 100 µL volume, at 35 ± 2 °C for 24 h to reduce intestinal debris in the culture. Subsequently, 30% D-fructose-supplemented FYP was used in the FLAB selection. After overnight growth, the cultures were inoculated onto the FYP agar containing 0.5% calcium carbonate and incubated at 35 ± 2 °C for 24 h. Colonies were carefully selected by morphological observation, and then further inoculated onto both the FYP and Glucose Yeast Peptone (GYP) broth media (D-glucose rather than D-fructose) and statically incubated at 35 ± 2 °C for 24 h in anaerobic conditions. The isolates showing better growth with the FYP broth than the GYP broth were accepted as FLAB and further examined for Gram characteristics and catalase activity. The results of the carbohydrate fermentation test were also considered in the selection of isolates for the genetic analysis.

Finally, the 16S rRNA analysis of the two isolates selected from 20 isolates was conducted at BM Laboratories. The EurX GeneMATRIX Bacterial & Yeast DNA isolation kit (Poland) was used for DNA isolation. DNA amount and purity were checked by spectrophotometric measurement using Thermo Scientific Nanodrop 2000 (USA). With primers 27F (*5′-AGAGTTTGATCMTGGCTCAG-3′*) and 1492R (*5′-TACGGYTACCTTGTTACGACTT-3′*), the targeted gene regions for species identification were amplified. Amplification was performed in a reaction mixture (35 µL) containing 3 µL sample DNA, 1x PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.3 µM F. Primer, 0.3 µM R. Primer, and 2U Taq DNA Polymerase. The polymerase chain reaction was carried out with 40 cycles of the following steps: initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 45 s, annealing at 57 °C for 45 s, and extension at 72 °C for 60 s. The final extension was performed at 72 °C for 5 min. Lastly, the temperature was reduced to 4 °C, and the process was completed. The PCR procedure was performed with the Solis Biodyne (Estonia) FIREPol® DNA Polymerase Taq polymerase enzyme. The amplified genetic material obtained by PCR was subjected to electrophoresis in a 1.5% agarose gel prepared with 1X TAE buffer at 100 volts for 90 min

by electrophoresis and imaged under ultraviolet light. The MAGBIO "HighPrep™ PCR Clean-up System" (AC-60005) purification kit was used for the bands. Sanger sequencing was undertaken 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). The readings were performed with the CAP contig assembly algorithm included in BioEdit software. Using the NCBI GenBank records, evolutionary closeness detection was inferred with the maximum likelihood method and Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-6372.87) was determined. Evolutionary analyses were conducted using MEGA X (Kumar et al. 2018).

Metabolic analysis

Carbohydrate fermentation analysis

Analyses were performed using the API CHL 50 kit (BioMérieux, Marcy l'Etoile, France) as stated in the manufacturer's instructions. In brief, the inoculum of all the isolates prepared in API Suspension Medium ampoules (equivalent to McFarland 2 turbidity) was individually added to the wells, covered with mineral oil, incubated at 35 ± 2 °C for 24–96 h, and checked daily. The observation was conducted based on color changes in the wells, and the results were determined according to the manual provided with the kit. The experiments were performed in duplicate.

Organic acid production

The acetic, butyric, lactic and propionic acid production of the strains was investigated with high-performance liquid chromatography (HPLC). The overnight cultures of the strains were inoculated onto the 5 mL De Man-Rogosa and Sharpe (MRS) broth at McFarland 0.5 turbidity and incubated at 35 ± 2 °C for 18 h. From the cultures, 1 mL was centrifuged at 12,000 rpm at 4 °C for 20 min. The supernatants were filtered through a 22 µm sterile membrane filter. These cell-free supernatants (CFSs) were analyzed for organic acid production by HPLC. Agilent 1100 Series (Wilmington, DE, USA) was used for the analysis. Before preparing the calibration curve, blank CFS samples were spiked with a certain amount of organic acid standard mixture. The extraction method described by De Baere et al. (2013) was used to model calibration curves. The XBridge C18 column (0.46x25 cm, 5 µm) was utilized for separation at 10 °C. An acetonitrile mixture (70:30, v:v) and 5 mM phosphate buffer (pH 2.1) were used as the mobile phase. First, the buffer solution (0.45 µm filtered) and solvents were degassed. The following parameters were used: injection volume, 5 µL; flow rate, 1 mL min^{-1}; temperature, 10 °C; and detection wavelength, 210 nm. All the processes were validated according to the International Council for Harmonization (ICH) guidelines in terms of selectivity, linearity, limit of detection, limit of quantification, precision, and accuracy (ICH, 2014). The results were recorded as the averages of three measurements.

Anti-QS test

The biosensor strain *Chromobacterium violaceum* CV026 was incubated at 30 °C for 18 hours, and 100 µL of McFarland 0.5 turbidity suspension from that culture and 50 µL N-hexanoyl-L-homoserine lactone (C6-HSL) extract were added to 10 mL of the soft (0.9%) Luria Bertani agar, mixed quickly, and poured onto plates before the suspension solidified. CFSs of 15 µL (prepared as stated in organic acid production section) were dropped onto the agar plates. Inhibition zones were evaluated after incubation at 30 ± 2 °C for 48 h (Erdönmez et al. 2018). The experiments were performed in duplicate.

Diacetyl production

The method described by King (1948) was used with a slight modification. About 16-h cultures of bacteria were inoculated onto 5 mL FYP and GYP broths (1%, w/v) and incubated at 35 ± 2 °C for 48 h. One mL of α-naphthol (4%, w: v) and 1 mL of potassium hydroxide (30%, w: v) were transferred to 2 mL of the culture and incubated at 35 ± 2 °C for 30 min. The formation of a red or pink surface ring indicated diacetyl production. The results were qualitatively defined, depending on the intensity of the color. *Lactobacillus rhamnosus* ATCC 53103 was used as a positive control. The experiments were performed in duplicate.

Auto-aggregation test

FLAB were cultured in the MRS broth for 24 h. Cell pellets were obtained by centrifugation at 5,000 g and 4 °C for 15 min, and then washed three times with a phosphate buffer (PBS) at pH 7.0, adjusted to the McFarland 0.5 standard in density. A 5 mL suspension was vortexed for 10 s and incubated at 25 ± 2 °C for 24 h. At the end of this period, auto-aggregation was measured at 600 nm in terms of optic density (OD). The auto-aggregation percentage was expressed as $[(OD_{t0}–OD_{t24})/OD_{t0}] \times 100$. *Lactobacillus rhamnosus* GG ATCC 53103 was used as a control (Del Re et al. 2000). The experiments were performed in duplicate.

Probiotic potential

Antimicrobial susceptibility

Following the standards of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2021), the micro-broth dilution method was used for the antibiotics ampicillin, clindamycin, chloramphenicol, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline, and vancomycin at the concentrations specified by the European Food Safety Authority (EFSA) (Rychen et al. 2018). The experiments were performed in duplicate.

Antimicrobial activity

The method described by Lima et al. (2007) was used to determine the antimicrobial activity of the two FLAB isolates. The CFSs of the isolates were prepared and placed on agar surfaces which included the test ATCC strains separately. Antimicrobial activity was tested against *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, and *Staphylococcus aureus* ATCC 29213.

The micro-broth dilution method was applied with a slight modification for Lactobacilli stated in EUCAST (2021). The CFSs of the FLAB isolates were serially diluted in the MRS broth medium at a 100 µL volume and tested for the above-mentioned strains prepared in the MRS broth to yield a final concentration of approximately 10^5 cfu mL^{-1} in density and inoculated at a 100 µL volume. The microplates were left to incubate at 35 ± 2 °C for 24 h. The results were read by visual observation. The experiments were performed in duplicate.

Low pH tolerance

Bacterial cultures were incubated in the MRS broth overnight. The cell pellet was obtained as previously described and washed twice with PBS, pH 7.4. Then, 1 mL of each of the PBS buffers at pH 1.0, 2.0 and 3.0, adjusted with 5 M hydrochloric acid, was added to the pellets and vortexed. The suspensions were left to incubate at 35 ± 2 °C for 3 hours. As a control, a PBS buffer at pH 7.4 was used. At hours 0 and 3 following incubation, samples were taken and spread onto two parallel MRS agar plates. After 48 h of incubation at 35 ± 2 °C, colonies in the control and test groups were counted from the Petri dishes, and the values were recorded as log cfu mL^{-1} (Maragkoudakis et al. 2006).

Pepsin resistance

PBS buffers at pH 2.0 and 3.0 containing 3 mg mL^{-1} pepsin were used. The cell pellet was obtained as previously described and washed twice with a PBS buffer at a pH of 7.4. It was then dissolved with PBS buffers (pH 2.0 and 3.0) containing pepsin and incubated for at 35 ± 2 °C for 3 h. PBS with a pH of 7.4 was used as a control. At hours 0 and 3 following incubation, samples were taken and spread onto two parallel MRS agar plates. The results were obtained as log cfu mL^{-1} as previously described (Charteris et al. 1998).

Pancreatin resistance

PBS, containing 1 mg mL^{-1} pancreatin and adjusted to pH 8.0, was used. The cell pellet was obtained as previously described and washed twice with PBS, pH 7.4. Then, it was dissolved in PBS with a pH of 8.0 containing pancreatin and incubated at 35 ± 2 °C for 4 h. PBS at pH 8.0 was used as a control. At hours 0 and 4 following incubation, samples were taken and spread onto two parallel MRS agar plates. The results were calculated as log cfu mL^{-1} as previously described (Charteris et al. 1998).

Bile salt tolerance

MRS broths containing 0.3%, 0.5%, and 1% bile salt and a control MRS broth without bile salt were used. The cell pellet was obtained as previously described, and then dissolved in MRS broths containing different amounts of bile salt (0.3%, 0.5% and 1%) and incubated at 35 ± 2 °C for 4 h. At hours 0 and 4 following incubation, samples were taken and spread onto two parallel MRS agar plates. The results were obtained as log cfu mL^{-1} as previously described (Maragkoudakis et al. 2006).

Hemolytic activity

Fresh cultures of the isolates were inoculated onto the Columbia agar containing 5% sheep blood as a streak. After 24 h of incubation at 35 ± 2 °C, the formation of hemolysis around the colonies was evaluated (clear: β, bright green: α, no zone: ɣ) (Maragkoudakis et al. 2006).

The NCBI GenBank access codes of the bacteria used in the study were OK091171 and OK091172.

Results

Identification

According to the 16S rRNA analysis results carried out with the 27F-1492R primers, one of the two samples was

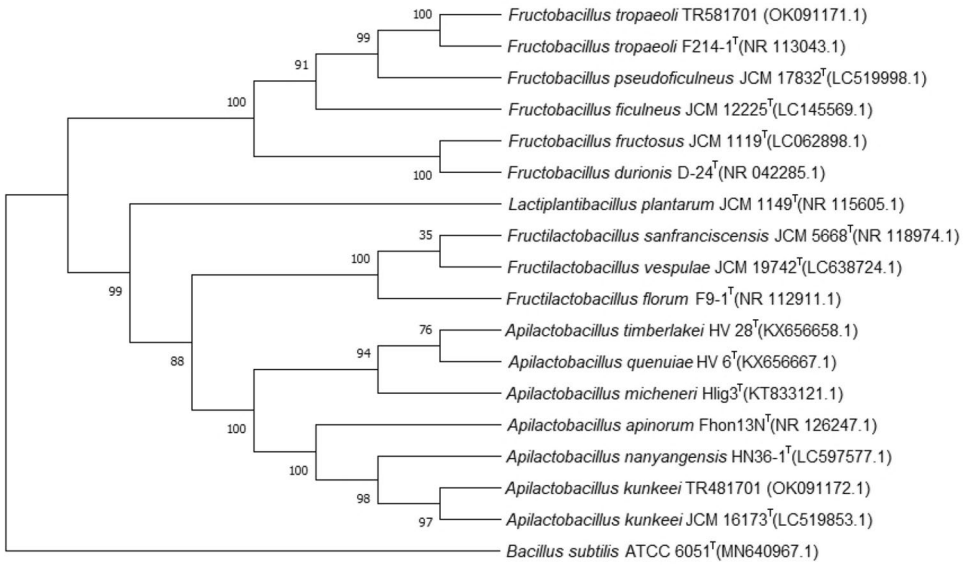


Fig. 1 Phylogenetic tree of *Fructobacillus tropaeoli* TR581701 and *Apilactobacillus kunkeei* TR481701. *Bacillus subtilis* ATCC 6051^T was used as an outgroup.

Table 1 List of acid production from the carbohydrates in the API CHL 50 kit

Bacteria	Fermented carbohydrates						
	Glucose	Fructose	Mannitol	Lactose	Saccharose	Trehalose	Potassium gluconate
Fructobacillus tropaeoli TR581701	Day 2	Day 1	Day 4^w	-	-	-	-
Apilactobacillus kunkeei TR481701	Day 2	Day 1	Day 3^w	Day 2	Day 2	Day 2	Day 4^w

Day refers to the day when positive results were seen, *w* weakly positive, – negative

identified as *F. tropaeoli* with a total of 1,402 bases, 100% sequence matching ratio, and 99.86% similarity ratio, and the other was identified as *A. kunkeei* with a total of 1423 bases, 100% sequence matching ratio, and 100% similarity ratio. Gene sequences are presented in supplementary data (SI). *F. tropaeoli* TR581701 was isolated from the midgut of the honeybee from Sivas region, and *A. kunkeei* TR481701 was isolated from the crop of the honeybee from Muğla region in Turkey. *F. tropaeoli* TR581701 and *A. kunkeei* TR481701 are included in the NCBI gene bank records and in the phylogenetic tree (presented in Fig. 1) with the OK091171.1 and OK091172.1 codes, respectively.

Metabolic analysis

Acid production from carbohydrates

F. tropaeoli TR581701 fermented only three and *A. kunkeei* TR481701 fermented five carbohydrates of the total 49. Fructose was well-fermented on the first day for both strains. The results are given in Table 1.

Table 2 Quantification of organic acids produced by the two FLAB isolates

	Lactic Acid	Acetic Acid	Butyric Acid
Fructobacillus tropaeoli TR581701	1.29 ± 0.01	2.26 ± 0.01	0.59 ± 0.02
Apilactobacillus kunkeei TR481701	2.86 ± 0.02	2.27 ± 0.01	0.67 ± 0.02

Organic acid production

Both bacteria produced lactic acid, acetic acid, and butyric acid. *F. tropaeoli* TR581701 produced lactic, acetic and butyric acids at a ratio of 2:4:1 and *A. kunkeei* TR481701 at a ratio of 4:3:1. The quantities are shown in Table 2. Propionic acid was not detected.

Anti-QS test

The anti-QS activity of the CFSs' of the isolates was examined based on their inhibition of the violacein pigment. The results revealed that *F. tropaeoli* TR581701 and *A. kunkeei* TR481701 inhibited the N-hexanoyl-L-homoserine lactone signal molecule via the quorum sensing bacterial communication system by inhibiting violacein production. The results are shown in Fig. 2.

Diacetyl production

There were no red/pink surface rings, and therefore, no diacetyl production was detected for either strain.

Probiotic potential

Antimicrobial susceptibility

According to the antimicrobial susceptibility test results, neither strain was susceptible to any of the antibiotics tested, except ampicillin. The minimum inhibition concentration (MIC) values were remarkably high, especially for kanamycin, streptomycin, and erythromycin. The results are shown in Table 3.

Antimicrobial activity

The antimicrobial activity of the CFSs with the disc diffusion method showed that both strains generated zones only for *E. faecalis* ATCC 29212 among the tested bacteria. The MIC test results showed that the CFSs of the two FLAB had no antimicrobial activity against the tested microorganisms, except *E. faecalis.* It was found that the CFSs of *F. tropaeoli* TR581701 and *A. kunkeei* TR481701 inhibited the growth of *E. faecalis* ATCC 29212 in the first well of the microplates, in which the culture supernatants were diluted at a ratio of 1:4. Organic acids produced by isolates were identified and quantified by HPLC (Table 2), but further studies are needed to detect other possible inhibitor compounds in supernatants.

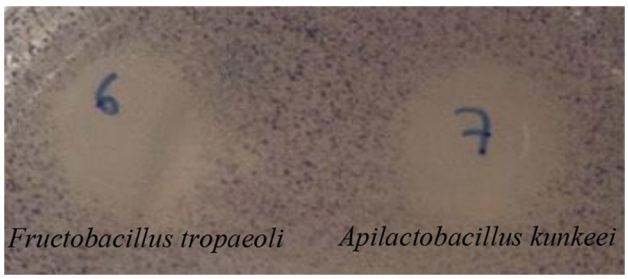


Fig. 2 Anti-QS activity of cell-free supernatants in violacein production from *Chromobacterium violaceum*

Low pH tolerance

The test results of pH tolerance revealed that *F. tropaeoli* TR581701 had no tolerance to pH 1.0 or 2.0, and the strain remained its vitality after 3 h at pH 3.0 despite a 3-log decrease. *A. kunkeei* TR481701 showed good growth after 3 h at pH 3.0 but had no tolerance to pH 2.0 and pH 1.0. The detailed data are shown in Table 4.

Pepsin resistance

A. kunkeei TR481701 was found to be resistant to pepsin (3 mg mL^{-1}) at pH 3.0 for 3 hours with only 1-log decrease. It was determined that *F. tropaeoli* TR581701 showed a 3-log decrease in growth at pH 3.0 at the end of the third hour. Neither species had any tolerance to pH 2.0 at the third hour. The detailed data are shown in Table 4.

Pancreatin resistance

The pancreatin resistance test results showed that both strains were resistant to pancreatin over the four-hour period. The data are shown in Table 4.

Bile salt tolerance

A. kunkeei TR481701 and *F. tropaeoli* TR581701 were found resistant to 0.3% bile salt at both time intervals. However, slight sensitivity was seen at 0.5% bile salt at four hours with 2-log and 3-log decreases for *F. tropaeoli* and *A. kunkeei*, respectively. The 1% bile salt ratio inhibited the growth

Table 3 Minimum inhibition concentration values (in µg mL^{-1}) of the two FLAB isolates and EFSA standards (Rychen et al., 2018)

Bacteria	Minimum inhibition concentrations (in µg mL^{-1})							
	AMP	GEN	KAN	STR	ERY	CLI	TET	CHL
Fructobacillus tropaeoli 581701	0.25	16	256	128	>256	64	8	4
Apilactobacillus kunkeei 481701	0.5	32	>256	128	>256	32	16	4
EFSA standards[a]	2	16	64	64	1	4	8	4

AMP Ampicillin, *GEN* gentamicin, *KAN* kanamycin, *STR* streptomycin, *ERY* erythromycin, *CLI* clindamycin, *TET* tetracycline, *CHL* chloramphenicol, [a]for obligate heterofermentative lactic acid bacteria

Table 4 Survival rates of the FLAB isolates in low pH, pepsin, pancreatin and bile salt environments (in log cfu mL^{-1})[1]

Bacteria	Low pH						Pepsin				Pancreatin		Bile salt					
	pH 1.0		pH 2.0		pH 3.0		Pepsin pH 2.0		Pepsin pH 3.0		Pancreatin pH 8.0		0.3%		0.5%		1%	
	Hour 0	Hour 3	Hour 0	Hour 3	Hour 0	Hour 3	Hour 0	Hour 3	Hour 0	Hour 3	Hour 0	Hour 4	Hour 0	Hour 4	Hour 0	Hour 4	Hour 0	Hour 4
Fructobacillus tropaeoli TR581701	7.55	0	7.47	0	7.49	4.55	7.53	0	7.61	4.23	7.58	7.08	7.56	7.02	7.51	5.6	7.48	0
Apilactobacillus kunkeei TR481701	7.64	0	7.49	0	7.63	7.04	7.5	0	7.57	6.54	7.61	7.35	7.47	7.39	7.56	3.95	7.61	0

[1] Average of the results of the experiments performed in duplicate

Table 5 Auto-aggregation percentages of the FLAB isolates

Bacteria	Auto-aggregation (%)
Fructobacillus tropaeoli TR581701	70.5 ± 0.35
Apilactobacillus kunkeei TR481701	32.9 ± 1.65
Lactobacillus rhamnosus GG ATCC 53103	65.7 ± 1.46

of both bacteria at four hours. The detailed data are shown in Table 4.

Hemolytic activity

F. tropaeoli TR581701 and *A. kunkeii* TR481701 showed very weak alpha-hemolysis.

Auto-aggregation test

The auto-aggregation values of the isolates were 70.5 and 32.9 for *F. tropaeoli* TR581701 and *A. kunkeei* TR481701, respectively. *F. tropaeoli* TR581701 exhibited significantly higher aggregation than *A. kunkeei* TR481701 and comparable aggregation to the control strain *L. rhamnosus* GG ATCC 53103. The results are given in Table 5.

Discussion

If the world we live in is considered as a superorganism like a honeybee hive, many systems with their internal dynamics being also in interaction with each other makes it insufficient to observe and evaluate these systems separately. This resulted in the emergency of the One Health concept, which considers diseases, antimicrobial resistance, and environmental factors in human, animal and environmental tripods. The Food and Agriculture Organization of The United Nations (FAO), the World Organization for Animal Health (OIE), and the World Health Organization (WHO) collaborated to coordinate the One Health approach, and they have been preparing joint action plans to ensure the widespread adoption of this approach for more than 20 years (WHO.FAO.OIE, 2019). The observation of animals is a well-known method in monitoring the environment and public health (Reif, 2011). Honeybees are also among the microorganisms used as bio-indicators and biomonitoring agents and contribute to the continuity of plant species in nature through pollination (Quigley et al. 2019; Girotti et al. 2020; Piva et al. 2020). However, due to honeybees being non-targeted species for various ecological and anthropogenic suppressors, there is a decrease in their population, which causes a significant increase in the yield of agricultural products. A decrease in these pollinators may indirectly lead to dietary changes and micronutrient deficiency

in humans by reducing crop yield (Mahefarisoa et al. 2021). Biotic and abiotic threats affect the microbiota of honeybees (Daisley et al. 2020); therefore, so there is a need to monitor time- and condition-dependent changes in their microbiota. These changes can be observed through routine field analyses, allowing for the identification and stocking of the species for further studies. The specific characteristics of some members of the microbiota provide opportunities for industrial and/or probiotic use.

FLAB isolated from fermentation products or food, such as fruit and honey have been naturally consumed throughout history. Since these bacteria exist as symbionts of honeybees, most should be taken into the digestive system during the consumption of bee products (Vergalito et al. 2020). However, according to our review of the literature, they have not yet been detected in the human gastrointestinal system. *A. kunkeei* is one of the most dominant members of the honeybee gut (Endo and Salminen, 2013; Filannino et al. 2016). Some of the isolates of this species are under discussion in terms of their probiotic potential because the data obtained from limited studies on probiotic properties do not show complete parallelism. To our knowledge, Uğraş (2017) is the first to investigate the probiotic properties of *A. kunkeei* isolated from the honeybee gut, and reported that this isolate could tolerate pH (1.0, 2.0 and 3.0 for 3 h), pancreatin (1 mg mL^{-1} for 3 h), pepsin (3 mg mL^{-1} at pH 1.0, 2.0 and 3.0 for 3 h) environments and was susceptible to erythromycin and resistant to streptomycin. In addition, this strain was shown to be gamma-hemolytic and exhibit antibacterial activity against *K. pneumonia, E. coli, Listeria monocytogenes, Yersinia pseudotuberculosis, P. aeruginosa,* and *S. aureus*. Our strain was alpha-hemolytic, which prevents it from being evaluated as a probiotic for humans; however, it could still contribute to the health of bee colonies. These data suggest that information about species can be updated by isolation and analysis from different sources. In a very recent study, three *A. kunkeei* bee bread isolates were investigated for their survival capacity during simulated human gastrointestinal transit and their probiotic potential for human use (Vergalito et al. 2020). Unlike our results, only ampicillin, chloramphenicol, and kanamycin resistance was detected. During gastric juice tests, these isolates showed 90-min survival at pH 2.0 and pH 3.0, and therefore, they were considered to be highly resistant to these unfavorable conditions. This is also in agreement with our results indicating similar pepsin and pancreatin ratios at a similar time interval. It is also noteworthy that the isolate fermented a much higher number of carbohydrates than indicated in the general characteristics of the breed. Ebrahimi et al. (2021) investigated the probiotic properties of *A. kunkeei* isolated from honey and found it to be tolerant to low pH and biliary stress and high auto-aggregation (83.65 ± 4.07%). It was reported to have antibacterial effect against *Bacillus cereus* ATCC 14579, *E. coli* ATCC 11229, *Salmonella enterica* ATCC 35664, and *S. aureus* ATCC 23235. In addition, clindamycin, streptomycin, gentamicin, vancomycin and kanamycin resistance was detected in the antibiotic panel tested by the researchers. Our results are comparable to the previous study in terms of tolerance to stress factors. However, antibiotic susceptibility varies in different studies, indicating that local chemical/antibiotic use may be a factor. A recent study (Maeno et al. 2021) showed gene loss in FLAB similar to the host infection strategy in Gram-negative pathogens, the absence of two genes related to cadmium resistance, and tolerance in *E. coli*. The authors stated that cadmium was an industrial pollutant in fields, which is one of the pollination areas of honeybees, and there was an exposure risk that could affect their evolution. Exposure to heavy metals and various chemicals in nature as a result of human intervention may impede the existence of this group, with some members being colonized in the bee gut.

A. kunkeei has been investigated in terms of antimicrobial and anti-virulence activities against human pathogens, as well as healing properties. In addition to their probiotic properties, it has been suggested that this species may improve fructose-mediated irritable bowel syndrome (IBS) (Filannino et al. 2019; Sakandar et al. 2019). Our findings support that idea that butyric acid is involved in treating IBS (Borycka-Kiciak et al. 2017). There are patents for the use of this acid in food and pharmaceutical products (Matsuura et al. 2014; Olofsson and Vasquez, 2016; Haga et al. 2021).

F. tropaeoli has been investigated for its possible industrial use since being identified a decade ago. The carbohydrate consumption and organic acid production capacity, and mannitol production efficiency of this species have been reported in several publications. Endo et al. (2011) found that only D-fructose, D-glucose, and D-mannitol were metabolized in the API CHL 50 kit, and the ratio of lactic acid:acetic acid produced in the FYP broth medium was 1:1. Ruiz Rodríguez et al. (2017) found that fructose and glucose in the medium were consumed during the 24-h incubation period in the FYP medium, and the produced lactic acid:acetic acid ratio was 2:1. Our results from the API CHL 50 kit were the same as those reported by Endo et al. However, the results on organic acid production slightly differ between the three studies. The previous two studies were carried out using the FYP broth medium, and the differences in results may be strain-dependent. In the organic acid production test we conducted using the MRS broth, we found the lactic acid:acetic acid:butyric acid ratio to be 2:4:1 and observed no propionic acid production. To our knowledge, this is the first report showing the production of butyric acid. Differences in lactic and acetic acid ratios may be due to the media used or related to the strain. Further studies are needed to clarify this.

There is much new research pointing to the nutritional use of *F. tropaeoli.* It has been shown to produce intracellular nicotinamide mononucleotide, a high-priced industrial product and has anti-ageing activity via nicotinamide phosphoribosyl transferase during D-fructose consumption (Sugiyama et al. 2021). Selenium, an essential trace element in the human diet found in the most toxic form of selenite in nature, is transformed by *F. tropaeoli* to selenium nanoparticles and amino acids (Martínez et al. 2020). Our research is one of the primary studies investigating the potential use as probiotic. Our results indicate that it has no tolerance to pH 1.0 and 2.0 values, slight tolerance to pepsin (survived only in pH 3.0), moderate tolerance to bile salt (0.3 and 0.5% bile salt tolerated), and good tolerance to pancreatin (pH 8.0). The auto-aggregation ratio was found to be high (70.5%) when compared to the reference strain. Despite all these features, weak alpha-hemolysis and resistance to antibiotics specified in the EFSA standards were observed. Although it is not possible to classify these isolates as a potent probiotic for human use due to these features that are not compatible with the EFSA standards, they may be considered as growth promoters for honeybee colonies. The generation of antibiotic-resistant organisms and resistance genes are among the negative effects of intensive antibiotic use in non-therapeutic situations, such as prophylaxis in animal applications in the livestock sector (Zhang et al. 2011). We consider that antibiotic resistance is strain-dependent and reduces the possibility of use as a probiotic. The interactions between nearby apiaries via pollination areas may lead to the resistance gene pool in the environment being able to be shaped similarly. In addition, the reason why it was not isolated from human microbiota may be due to its intolerance to gastric pH. This indicates that the strains that are found suitable for other characteristics in supporting the treatment (e.g., IBS) must be prepared in a suitable pharmaceutical formulation in order to reach the intestines.

In this study, we detected the inhibitory activity of the culture supernatants of the isolates in the quorum sensing mechanism of the *Chromobacterium violaceum* biosensor strain. Although there are studies in the literature investigating the effects of lactic acid bacteria on the virulence of pathogenic microorganisms (Berríos et al. 2018), we found no study evaluating the anti-quorum sensing activity of *A. kunkeei* and *F. tropaeoli*.

The reason why *A. kunkeei* and *F. tropaeoli*, which are consumed with food and show varying rates of pancreatin, pepsin, pH and bile resistance, are not isolated from the human microbiota should be investigated in more detail to clarify their probiotic potential. All risk factors should be established before their direct external or internal use. Due to their metabolic properties, we consider them to be among the promising species for industrial product yield in the pharmaceutical field.

Conclusion

Due to the unique extreme properties of the honeybee gut, we believe that the screening and stocking of different FLAB species adapted to the honeybee digestive system should be carried out for use in both bee health and industry studies. In addition, the investigation of their responses to stress factors will help predict their adaptation to changes in the microflora caused by the climate change and pollutants. Since honeybees collect pollen from plants in areas a few kilometers from their hives, the obtained bacteria will also provide an idea of the fructophilic bacteria profile found in plants in these regions. The negative effect of antibiotics on FLAB due to the direct or indirect exposure of honeybees can be monitored within the scope of the 'One Health' approach. Only with a consortium of experts in the fields of agriculture, veterinary medicine, human health, and environmental health, the susceptible presence of FLAB in nature can be determined comprehensively, and their effective use in the field of health can be achieved.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00203-022-03000-x.

Author contributions The idea was conceptualized by DS and Material preparation, data collection, and data analysis were performed by DS, KCT, MG, and MEK. The first draft of the manuscript was prepared by DS, KCT, MG, and MEK. The manuscript was reviewed and edited by DS, KCT, MG, MEK, and NA. Supervision was provided by NA.

Funding The authors declare that no funds, grants, or other financial support were received for this study.

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors have no relevant financial or non-financial interest to disclose.

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