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Farklı Bitkilerden ve Arıların Sindirim Sisteminden Fruktofilik Laktik Asit Bakterilerinin İzolasyonu ve *İn-Vitro* Probiyotik Karakterizasyonu

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Öne Çıkanlar:
- Fruktofilik laktik asit bakterileri
- *Enterococcus faecalis*
- Antimikrobiyal

Anahtar Kelimeler:
- Fruktofilik laktik asit bakterileri
- Probiyotik
- Mikrobiyal tiplendirme
- 16S rRNA

ÖZET:

Bu çalışmanın amacı, genellikle fruktoz bakımından zengin nişlerden (arı sindirim sistemi, yer elması meyvesi, fındık yaprağı, üzüm, domates, beyaz dut, armut, kivi) izole edilen fruktofilik laktik asit bakterilerinin probiyotik olarak kullanılabilmelerine yönelik niteliklerinin araştırılmasıdır. Söz konusu kaynaklardan izole edilen çok sayıda izolat içerisinden ilk etapta katalaz negatif ve Gram-pozitif reaksiyon veren 10 adet bakteriyel suş seçilmiştir. Seçilen suşların ilk aşamada hücre morfolojileri, genotipik özellikleri (RAPD-PZR, 16S rRNA dizileme) belirlenmiştir. Çalışma kapsamına dahil edilmiş fruktofilik özellikteki izolatlar *Enterococcus faecalis* olarak tanımlanmıştır. İlgili izolatların filogenetik analizleri gerçekleştirilmiş ve arı gastrointestinal sisteminden izole edilenler ile farklı bitkisel kaynaklardan izole edilen izolatlar birbirinden ayrı kladlar içerisinde toplanmıştır. Fenotipik ve genotipik karakteristikleri tanımlanan izolatlar daha sonrasında probiyotik özellikleri itibariyle test edilmiştir. Bu bağlamda izolatların, asit, pepsin ve pankreatin direnç profilleri çıkartılmıştır. Ayrıca A6, 7, 9 ve 10 kodlu izolatların potansiyel birer bakteriyosin üreticisi oldukları kanıtlanmıştır. İlgili bu çalışmada farklı bitkisel kaynaklardan da fruktofilik özellikli laktik asit bakterilerinin izole edilebileceği ve probiyotik potansiyel içerebilecekleri gösterilmiştir.

Isolation and *In-Vitro* Probiotic Characterization of Fructophilic Lactic Acid Bacteria from Different Plants and The Digestive System of Bees

Highlights:
- Fructophilic lactic acid bacteria
- *Enterococcus faecalis*
- Antimicrobial

Keywords:
- Fructophilic lactic acid bacteria
- Probiotic
- Microbial typing
- 16S rRNA

ABSTRACT:

The aim of this study was to investigate the characteristics of fructophilic lactic acid bacteria isolated from fructose-rich niches (bee digestive tract, yam fruit, hazelnut leaf, grape, tomato, white mulberry, pear, kiwi) for their use as probiotics. Among a large number of isolates from these sources, 10 catalase-negative and Gram-positive bacterial strains were initially selected. Cell morphology and genotypic characteristics (RAPD-PZR, 16S rRNA sequencing) of the selected strains were determined in the first stage. The fructophilic isolates included in the study were identified as *Enterococcus faecalis*. Phylogenetic analyses of the related isolates were performed and isolates isolated from the bee gastrointestinal tract and isolates isolated from different plant sources were grouped into separate clades. Isolates whose phenotypic and genotypic characteristics were defined were then tested for their potential probiotic properties. In this context, acid, pepsin and pancreatin resistance profiles of the isolates were determined. In addition, isolates coded A6, 7, 9 and 10 were proven to be potential bacteriocin producers. In this study, it was shown that fructophilic lactic acid bacteria can be isolated from different plant sources and may have probiotic potential.

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INTRODUCTION

Fructophilic Lactic Acid Bacteria (FLAB) constitute a subgroup within Lactic Acid Bacteria (LAB). The growth and proliferation of FLABs, which preferably use fructose as substrate and cannot fully ferment glucose, increases significantly with pyruvate, oxygen and fructose in the environment. Members of this group are mostly found in foods such as flowers, fruits, wine and cocoa beans and in the gastrointestinal tract of insects (Endo et al., 2011). The classification of FLAB group bacteria includes the genera *Fructobacillus* spp., and *Lactobacillus* spp. (Endo et al., 2009). *Fructobacillus* consists of five species: *F. fructosus*, *F. pseudoficulneus*, *F. ficulneus*, *F. durionis* and *F. tropaeoli* (Endo et al., 2015). The most frequently detected species in natural sources are *F. fructosus* and *F. pseudoficulneus* (Endo et al., 2013). FLAB members of the genus *Lactobacillus* are *L. kunkeei*, *L. apinorum* and *L. florum*. In a study, it has been found that *L. kunkeei* ingested with food and inactivated by heat increases intestinal peristalsis and has beneficial effects on immunity such as immunoglobulin A production (Neveling et al., 2012). Therefore, as with *Bacteroides xylanisolvens*, *L. kunkeei* can be used in heat-treated form in the food industry.

Recently, in terms of FLAB, honeybees and the products obtained from these bees (propolis, royal jelly) and especially the products obtained as a result of pollination are of ecological importance. Given the importance of bees in pollinating plants, large-scale declines in bee abundance and species richness over the last decade are of concern, according to FAO data. To better understand and prevent this decline, many studies addressing symbiotic and pathogenic interactions have been examined. According to data, it has been stated that the gastrointestinal tract of honeybees contains a core microbiota that is unlike any other animal, including humans (Vásquez et al., 2009; Kwong and Moran, 2016). Although FLAB and honey bees are thought to have a strong relationship with the gastrointestinal system; It has been concluded that intestinal function increases as a result of probiotic bacteria and foreign antigens or pathogens living a commensal life and limiting the migration of these bacteria (Endo et al., 2013). Fructophilic Lactic Acid bacteria tend to grow in Fructose-Yeast Extract-Polypeptone medium using fructose as a carbon source. FLAB, which develops very well in the presence of fructose, actually develops in the presence of glucose, although not very well. This occurs in the presence of electron acceptors (e.g. pyruvate, oxygen) (Maeno t al., 2016). Janashia *et al*. (2016) showed that fructophilic species of the *Enterococcus* genus could be isolated from the intestinal microbiota of the *Apis mellifera* species (Janashia et al., 2016).

FLAB types are divided into two groups according to their chemical properties: obligate and facultative fructophilic LAB (Andrade-Velásquez et al., 2023). Obligate FLAB; while they grow well in fructose, their growth in the presence of glucose is only possible as long as they have external electron acceptors. These species convert glucose into almost equimolar amounts of lactic acid and trace amounts of ethanol. The low synthesis of ethanol is due to the presence of acetaldehyde dehydrogenase and weak alcohol dehydrogenase activity (Kouya et al., 2023). Facultative FLAB, on the other hand, grows well in a fructose environment and grows in a glucose environment, albeit with a delay, in the absence of electron acceptors. This group generally includes *Lactobacillus florum* and several *Lactobacillus* biotypes (Endo et al., 2013; Kim et al., 2013).

Fructophilic lactic acid bacteria, a newly discovered group, and their ecological potential are still in their early stages of research. Since these species are included in the LAB group, they have been included in the safe status after safety evaluations. After approval, they can be included in applications in many areas such as the food industry (Filannino et al., 2019).

Probiotics are defined as live microorganisms that benefit their host if administered to an acceptable extent. Lactic acid bacteria and bifidobacteria are the most common bacteria in the human gastrointestinal tract and represent a significant portion of commercial probiotics. Antimicrobial activity, inhibition of pathogens, strengthening of the intestinal epithelial barrier function, digestion of indigestible nutritional components and immunomodulation are the main benefits of probiotics used commercially for human health. Evaluations in the guidelines provided by FAO/WHO provide minimum criteria for a candidate microorganism to be considered a probiotic. Candidates with probiotic potential are frequently tested for their ability to survive when exposed to an environment simulating the upper gastrointestinal tract, to adhere to mucus or intestinal epithelial cells, and to modulate the immune system (Joint, 2002; Hotel and Cordoba, 2001; Resta-Lenert and Barrett, 2003; Smits et al., 2005).

This study was conducted to focus on the isolation and probiotic characterization of FLAB from different sources (bee digestive system and plant samples).

MATERIALS AND METHODS

Materials

In this study, 24 bacterial samples isolated from plant samples obtained from Kahramankazan district of Ankara province and honey bee gastrointestinal tract (GIT) were subjected to preliminary screening and characterisation procedures (Gram-staining and catalase test). Plant samples were weighed to 10 g and homogenized 90 mL sterile saline buffer (SF; 0.85% NaCl), bee gastrointestinal samples were weighed to 1 g and placed in 9 mL SF. Serial dilutions of these samples homogenized with SF were prepared up to 10^{-7} and 100 µL of these dilutions were taken and sown on Fructose Yeast Peptone (FYP: Fructose 10 g/L, Meat Extract 20 g /L, Peptone 5g /L, Sodium acetate 2 g/L, Yeast extract 10 g/L, Tween 80 0.5 g/L, $MgSO_4.7H_2O$ 0.2 g/L, $MnSO_44H_20$ 0.1 g/L, $FeSO_4$ 0.1 g/L, Soydum azide 0.5 g/L, Sodium chloride 0.1 g/L) agar medium by smear inoculation method and incubated aerobically without skaking 24 h 37°C (Endo and Sanae 2008). The isolated and purity-checked bacteria were enriched in FYP medium for 18 h 37°C and then transferred to microfuge tubes and centrifuged at 12000 x *g* for 90 seconds. The pellets were then washed twice with 1 mL sterile saline (0.85% NaCl), suspended in 1 mL sterile 50% glycerol solution and stored at -86°C (Todorov et al., 1999).

Genotypic Characterisation of The Isolates

For the DNA isolation method, 1.5 mL of active cultures grown at 37°C for 18 h were centrifuged at *12000 x* g for 1 minute and the supernatant was removed. The same procedure was repeated once more. Genomic DNA isolation from the obtained pellets was performed using PureLink™ Microbiome DNA Purification Kit (A29790). For amplification of the 16S rRNA region, primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-CTA CGG CTA CCT TGT TAC GA-3') were used. For this purpose, 13.875 µL PCR water, 5 µL standard Taq buffer (NEB, UK), 2.5 µL 25 mM $MgCl_2$ (NEB, UK), 0.5 µL 10 mM deoxynucleotide triphosphate (dNTP, Promega, USA), 0.5 µL each of 10 mM primers (a different primer was used for each reaction), 0.125 µL of standard Taq DNA polymerase enzyme (NEB, UK) and 2 µL of template DNA were used. PCR was performed in a Gradient PCR Thermocycler/Eppendorf' device. PCR tubes were placed in the device and followed a protocol consisting of a pre-denaturation at 94°C for 5 min followed by 35 cycles, one cycle of which consisted of denaturation at 94°C for 30 seconds, primer binding at 35°C for 1 min and elongation at 68°C for 1 min and 45 seconds. Purification and sequence analyses of PCR products were performed by BM Labosis (Ankara, Turkey). Sequence analysis results were compared with the database using the BLAST (Basic Local Alignment Search Tool) programme, and the microorganism to which the sequence

searched as a result of the scanning was determined by the percentage of similarity (Tang et al., 1998; Maragkoudakis et al., 2006).

Determination of Bacteriocin Production Properties of Strains

To determine the bacteriocin production properties of the strains, 1 mL of the cultures incubated at 37°C for 18 h was taken and centrifuged at 12,000 rpm for 10 min. The obtained supernatants were used to determine the antimicrobial activity by adjusting the pH values to 7, treating with catalase, passing them through 0.22µm diameter filters (Milex, Milipore, France) and boiling at 100°C for 5 min. Indicator bacteria and pathogenic strains (*Listeria monocytogenes* NCTC 5348, *Salmonella enterica Typhimirium* ATCC 14028, Shiga toxin-producing *Escherichia coli* O157:H7 ATCC 35150, *Escherichia coli* ATCC 25922 and *Pediococcus acidilactici* PedL) grown on appropriate media for 18 h were added to 5 mL of semi-solid medium at 0.1%. The semi-solid medium containing the indicators was spread-plated on the solid medium. For each sample to be tested for bacteriocin, 5 µL of the heated, pH neutralised and catalase-treated culture supernatant was spot inoculated. Petri dishes were incubated at 37°C for 18 h. After 18 h, it was checked whether there was an inhibition zone (Bhumia et al., 1988).

Determination of Antibiotic Susceptibility Profiles

The antibiotic resistance of the strains was determined by disc diffusion method using 9 different antibiotics (Rifampicin, Daptomycin, Trimethoprim-sulfamethoxazole, Ciprofloxacin, Penicillin, Amikacin, Erythromycin) (determined by European Food Safety Authority (EFSA). After the isolates were grown on MRS agar medium for 18 h, the colonies were collected with a sterile loop and suspended in physiological saline and their turbidity was adjusted based on the 0.5 McFarland standard. Standardised suspensions were inoculated onto MRS agar media with swabs. After placing the antibiotic discs on the petri dishes at appropriate distances, the petri dishes were incubated at 37°C for 18 h. After incubation, the diameter of the zones formed around the antibiotic discs was measured in mm (CLSI, 2015).

Determination of Resistance Properties of The Strains Against Low pH Values

To determine the ability of probiotics to reach the intestines alive, an attempt was made to create a pH environment similar to gastric fluid. Thus, the ability of the strains to pass through the acidic environment of the stomach and reach the intestines was observed. Active cultures were inoculated into MRS medium at a rate of 1% and incubated at 37°C for 18 h. At the end of incubation, 1 mL of active culture was taken and the supernatant was removed by centrifugation at 12000 rpm for 5 min at 4°C. The precipitate was washed 2 times with PBS (pH 7.4). The PBS buffer was then adjusted with 5 M HCl to pH 1.0, 2.0 and 3.0 and 1 mL each of the pH adjusted buffers was added to the pellets, vortexed and incubated at 37°C for 3 h. PBS with a pH value of 7.4 was used as control. Serial dilutions were performed by taking samples at 0, 1, and 3 h of incubation and samples taken were spread-plated on MRS agar in 3 parallel steps. At the end of 48 h incubation of the samples at 37°C, the colonies in the control and test groups were counted and their log-cfu/ml values were calculated (Dunne, 2001). The counting results were compared with the results obtained from the control groups and the logarithmic decrease was calculated. As a control group, samples treated in pH 7.4 buffer at the specified times were counted.

Determination of Resistance Properties of The Strains Against Pepsin

To determine the resistance properties of the strains against pepsin, PBS buffer containing 3 mg/mL pepsin (Merck, Germany) with pH 2.0 and pH 3.0 was used. 1 mL of active bacterial culture

was taken and centrifuged at 12000 rpm for 5 min at 4°C and the supernatant was removed. It was then washed twice with PBS (pH 7.4). The pellets were dissolved with pepsin-containing PBS (pH 2.0 and pH 3.0) buffers, and after 3 h of incubation at 37°C, the samples that were taken at the end of the 0^{th}, 1^{st} and 3^{rd} h were spread-plated on MRS agar in three parallel followed by serial dilutions. PBS with pH 7.4 was used as a control. At the end of the 48 h incubation of the samples at 37°C, the colonies in the control and test groups were counted and log/cfu values were calculated (Maragkoudakis et al., 2006).

Determination of Resistance Properties of The Strains Against Pancreatin

In order to determine the resistance properties of the strains against pancreatin, PBS buffers containing 1 mg/mL pancreatin (Merck, Germany) and pH value adjusted to 8.0 were used. The supernatant was removed by centrifuging 1 mL of the active bacterial culture at 12000 rpm for 5 min at 4°C and then washed twice with PBS (pH 7.4). It was then dissolved in PBS buffer containing pancreatin and pH adjusted to 8.0. After incubation at 37°C for 4 h, serial dilutions were made from the samples at the end of the 0^{th} and 4^{th} h and spread-plated on MRS agar in 3 parallel. PBS with pH 7.4 was used as control. After incubation of the samples at 37°C for 24 h, the colonies in the control and test groups were counted and the values were calculated as log/cfu (Maragkoudakis et al., 2006).

Determination of Resistance Properties of The Strains Against Bile Salt

To determine the strains bile salt resistance properties, MRS liquid media containing 0.3%, 0.5% and 1% bile salt (Merck, Germany) were used. MRS broth without bile salt was used as a control. 1 mL of active bacterial cultures was taken and centrifuged at 12000 rpm for 5 min at 4°C and the supernatant was removed. The pellets were then washed twice with PBS (pH 7.4). It was dissolved in MRS liquid media containing bile salt (0.3%, 0.5% and 1.0%), and after 4 h of incubation at 37°C, serial dilutions were made from the samples at the end of the 0^{th} and 4^{th} h and spread-plated on MRS agar in 3 parallel. At the end of the 48 h incubation of the samples at 37°C, colonies in the control and test groups were counted and the values were calculated as log/cfu (Maragkoudakis et al., 2006). Logarithmic percentage reduction was done as described previously

Determination of Haemolytic Activity of The Strains

The strains whose haemolytic activities were to be determined were cultured at 37°C for 18 h and then streak plated on Colombia agar (bioMerieux, Inc., France) containing 5% sheep blood. *Staphylococcus aureus* ATCC 6538 and *E. coli* ATCC 25295 strains were used as controls. After incubation at 37°C for 24 h, colonies forming a clear zone around were considered β-haemolytic, colonies forming a bright green zone were considered α-haemolytic and those not forming a zone were considered γ-haemolytic (Maragkoudakis et al., 2006).

Statistical Analyses

SPSS 15.0 (SPSS Inc., Chicago, IL, USA) statistical analysis software was used for statistical analyses. Data were evaluated using one-way analysis of variance ANOVA, Tukey posthoc test and independent T-test. In all statistical analyses, $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

As a starting point in the research, plants collected from the Kahramankazan district of Ankara province and isolates obtained from honey bees in the bee hives on the Ankara University, Faculty of Science campus were used. The codes given to the isolated bacteria during laboratory studies, isolation sources, Gram reaction results, catalase test results and cell morphologies are listed in (Table 1). After the first partial characterization processes, only 10 fructophilic lactic acid bacteria isolates were selected

among 24 isolates. These isolates are numbered as 1, 6, 7, 8, 9, 10, A1, A3, A4 and A6. These isolates are Gram-positive and catalase-negative, which are an important criteria for preselection of lactic acid bacteria. The cell morphology of all selected isolates is cocci-shaped. In order to evaluate the resistance potential of lactic acid bacteria to gastrointestinal system conditions, the tolerances of isolates isolated and identified from herbal samples and bee gastrointestinal tract to stomach acidity, pepsin, bile salt and pancreatin were tested within the scope of this work.

E. faecalis, a gram-positive, facultative anaerobic lactic acid bacterium, is a common member of the human commensal flora, but it should be noted that it may also pose a risk to human health due to its dualistic nature. Moreover, strains belonging to the genus *Enterococcus* are mostly associated with the intestinal tract of honey bees (Carina Audisio et al., 2011) and they are most commonly isolated from soil, surface waters, and raw plant and animal products (Johnston et al., 2004).

Table 1. Lactic acid bacteria isolated in the first stage of the thesis and isolation sources

Isolate codes	Source	Catalase	Morphology	Gram staining
1	Tomatoes	-	Coccus	Positive
2	Tomatoes	+	Bacil	Negative
3	Pear	+	Bacil	Negative
4	Pear	+	Coccus	Negative
5	Pear	+	Bacil	Negative
6	Mulberry	-	Coccus	Positive
7	Sweet potato	-	Coccus	Positive
8	Sweet potato	-	Coccus	Positive
9	Sweet potato	-	Coccus	Positive
10	Sweet potato	-	Coccus	Positive
11	Apple	+	Bacil	Negative
12	Apple	+	Bacil	Negative
13	Apple	+	Coccus	Negative
14	Kiwi	+	Coccus	Negative
15	Kiwi	+	Bacil	Negative
16	Kiwi	+	Coccus	Negative
17	Kiwi	+	Coccus	Negative
18	Grape	+	Coccus	Negative
19	Grape	+	Coccus	Negative
20	Grape	+	Coccus	Negative
A1	Bee	-	Coccus	Positive
A3	Bee	-	Coccus	Positive
A4	Bee	-	Coccus	Positive
A6	Bee	-	Coccus	Positive

Some strains of *E. faecalis* are opportunistic pathogens that exhibit multidrug resistance and can be frequently isolated in many nosocomial infections (Franz et al., 2003; Fisher and Phillips, 2009). However, in addition to these known alarming properties of some strains, the commercial use of some probiotic *E. faecalis* strains is quite common (Cebrián et al., 2012; Salek et al., 2023; Sonei et al., 2024; Daca and Jarzembowski, 2024). For example, some commercially available probiotic *E. faecalis* strains have strong properties such as the ability to colonize the intestine and stimulate the immune response in the host. Due to the two-way discussions mentioned above, candidate *E. faecalis* strains need to be handled more carefully than other lactic acid bacteria in order to be evaluated as starter cultures or probiotics (Nueno-Palop and Narbad, 2011; Al Atya et al., 2015; Kiymaci et al., 2023). However, unlike FAO/WHO, EFSA does not include these strains in the list of microorganisms for the Qualified Probability of Safety (QPS) due to the difficulties in distinguishing between *E. faecalis* strains that show virulence and those that do not (EFSA, 2010).

Enterococci with virulence properties can carry determinants associated with multidrug resistance and virulence factors such as adhesins, invasins and hemolysins. These characteristics of enterococci limit their use in foods and their use as probiotics. It has been reported that virulence factors can also be found in enterococci, members of the natural flora, isolated from some traditional fermented foods and vegetables. Virulence factors, which are generally the subject of debate, are more common in *E. faecalis* than in *E. faecium* (Willems et al., 2001; Leavis et al., 2004). *Enterococcus* probiotics are generally not used as starter cultures in foods, but can be used as food supplements. Strains with these qualities need to be consumed in high numbers to cure diseases such as irritable bowel syndrome, diarrhea or antibiotic-associated diarrhea, to reduce cholesterol levels and to have positive effects on immune regulation. In addition, it is known that probiotic enterococci taken as supplements interact synergistically with commensal enterococci found in the intestinal microflora (Fuller and Gibson, 1998; Im et al., 2023; Lou et al., 2024).

Genotypic Characterization of Isolates

In order to identify the lactic acid bacteria at the species level, 16S rRNA regions were first amplified with appropriate 27F and 1492R primers. Phylogenetic analysis of 16S rDNA sequences and BLAST analysis identified ten fructophilic lactic acid bacterial strains as *E. faecalis* (Table 2).

Table 2. Matching results of the 16S rRNA sequences of the isolates in the database

Isolate codes	Species	% Similarity
1	*Enterococcus faecalis*	99
6	*Enterococcus faecalis*	97.56
7	*Enterococcus faecalis*	98.06
8	*Enterococcus faecalis*	99
9	*Enterococcus faecalis*	99
10	*Enterococcus faecalis*	98.68
A1	*Enterococcus faecalis*	97.89
A3	*Enterococcus faecalis*	98.43
A4	*Enterococcus faecalis*	98.18
A6	*Enterococcus faecalis*	98.18

Bacteriocin Production Properties of Strains

Lactic acid bacteria produce a number of antimicrobial compounds that can be effective on closely related species and many pathogens such as hydrogen peroxide, bacteriocins, short-chain fatty acids and enzymes and these molecules have been important in their selection as probiotics (Sonei et al., 2024). Bacteriocins show antagonistic effects on many food pathogens and food spoilage microorganisms, especially Gram-positives (Knoll et al., 2008). Due to the increasing demand of consumers for natural products and the high cost of foodborne diseases, the increasing need for components that can be used as food preservatives is always up to date. Bacteriocins are the strongest candidates to replace chemical food preservatives (Casaus et al., 1997; Macwana and Muriana, 2012; Luenglusontigit et al., 2023; Öztürk et al., 2023, Al-S'adoon et al., 2023). The antimicrobial activity of the bee-derived isolate coded A6 on *Listeria monocytogenes* NCTC 5348 and the remarkable activity of isolates 7, 9 and 10 especially on *Salmonella enterica Typhimirium* ATCC 14028 are noteworthy outcomes of this study. The observation of antimicrobial activity on these two food pathogens in neutralised extracellular supernatants indicates that these isolates are potential bacteriocin producers. Detection of antimicrobial activity in the neutralized supernatant contents indirectly indicates the activity of a peptide-based component (bacteriocin or bacteriocin-like component) (Table 3). Al Atya et al. (2015) reported that lactic acid and bacteriocin production of strains named *E. faecalis* 28 and *E. faecalis* 93 isolated from meconium had an inhibitory effect on *S. aureus* ATCC 33862. Ali et all. (2023) investigate the

antibacterial activity of EPS-84B and their findings suggest that EPS-84B exerts bactericidal activity against the tested pathogens. Salek et al. (2023) invastegated of antimicrobial activity of potential probiotic *E. faecalis* KUMS-T48 and the CFS of *E. faecalis* KUMS-T48 showed significant anti-pathogenic activities against indicator microorganisms. This strain showed strong effects on *B. subtilis* and *L. monocytogenes* and a moderate effect on *Y. enterocolitica*, while indicating a weak effect on *K. pneumoniae* and *E. coli*. Overall, *E. faecalis* KUMS-T48 inhibited the growth of Gram-positive indicator bacteria more than Gram-negative indicator bacteria.

Table 3. Bacteriocin activities of lactic acid bacteria isolates

Isolate codes	Indicator strains				
	Listeria sp.	*Pediococcus acidilactici*	*Salmonella* sp.	*E. coli* ATCC 25922	*E. coli* O157:H7
1	-	-	-	-	-
6	-	-	-	-	-
7	-	-	+	-	-
8	-	-	-	-	-
9	-	-	+	-	-
10	-	+	+	-	-
A1	-	-	-	-	-
A3	-	-	-	-	-
A4	-	-	-	-	-
A6	+	-	-	-	-

"-"indicates the absence of any antimicrobial activity.

Antibiogram (disc diffusion) Test Results of Lactic Acid Bacteria

Table 4. Antibiogram (disc diffusion) test results of isolated lactic acid bacteria (S: Susceptible >20 mm, I: Intermediate 15–19 mm, R: Resistant ≤14 mm)

Isolate codes	Rifampicin	Daptomicin	Amikacin	Penicillin	Sulfaxomethaxazole	Erythromycin	Ciprofloxacin
	Zone diameter (mm)						
1	13 (R)	9 (R)	11 (R)	25 (S)	21 (S)	16 (I)	18 (I)
6	19 (I)	9 (R)	12 (R)	24 (S)	20 (S)	19 (I)	17 (I)
7	10 (R)	9 (R)	11 (R)	9 (R)	20 (S)	18 (I)	19 (I)
8	12 (R)	9 (R)	10 (R)	22 (S)	20 (S)	16 (I)	17 (I)
9	11 (R)	9 (R)	9 (R)	9 (R)	17 (I)	18 (I)	20 (S)
10	10 (R)	9 (R)	9 (R)	9 (R)	18 (I)	16 (I)	17 (I)
A1	18 (I)	9 (R)	10 (R)	9 (R)	18 (I)	17 (I)	12 (I)
A3	12 (R)	9 (R)	12 (R)	26 (S)	20 (S)	16 (I)	24 (S)
A4	12 (R)	9 (R)	15 (I)	21 (S)	18 (I)	15 (I)	19 (I)
A6	18 (I)	9 (R)	13 (R)	9 (R)	21 (S)	20 (S)	18 (I)

Antibiotics recommended by Clinical & Laboratory Standards Institute (CLSI) for the *Enterococcus* genus and susceptibility profiles determined based on reference values are given in Table 4. The only antibiotic to which all isolates were resistance was determined to be daptomicin. While all the isolate was resistenace to the antibiotic Amikacin except A6, while all the isolate was intermediate to the antibiotic Erythromycin except A6. The presence of daptomycin-resistant *Enterococcus* (DRE) strains has been previously reported (Tran et al., 2015). In a previous study tested 15 *Enterococcus* strains against tetracycline, vancomycin, erythromycin, penicillin, and ampicillin and found that most of the isolates were susceptible or moderately susceptible to all tested antibiotics such as tetracycline, vancomycin, erythromycin except penicillin and ampicillin (Sonei et al., 2024).

Resistance To Low pH Values (acidity)

Table 5. pH resistance results of lactic acid bacteria isolates

pH 1.0						
Isolate codes	**0 h**		**1 h**		**3 h**	
	Control (cfu/mL)	**Treated (cfu/mL)**	**Control (cfu/mL)**	**Treated (cfu/mL)**	**Control (cfu/mL)**	**Treated (cfu/mL)**
1	8.15±0.004	7.02±0.017	7.12±0.002	0	6.01±0.005	0
6	8.26±0.006	6.97±0.001	7.32±0.002	0	6.12±0.007	0
7	8.24±0.004	6.95±0.001	7.26±0.004	0	6.28±0.003	0
8	8.35±0.005	7.15±0.006	7.26±0.002	0	6.12±0.002	0
9	8.23±0.005	7.61±0.006	7.33±0.002	0	6.28±0.003	0
10	8.23±0.005	7.99±0.0	7.26±0.004	0	6.26±0.004	0
A1	8.37±0.002	6.76±0.001	7.23±0.004	0	6.12±0.005	0
A3	8.33±0.003	6.95±0.0	7.43±0.002	0	6.12±0.003	0
A4	8.28±0.005	7.04±0.006	7.26±0.001	0	6.23±0.003	0
A6	8.18±0.004	7.59±0.577	7.48±0.002	0	6.28±0.001	0
pH 2.0						
Isolate codes	**0 h**		**1 h**		**3 h**	
	Control (cfu/mL)	**Treated (cfu/mL)**	**Control (cfu/mL)**	**Treated (cfu/mL)**	**Control (cfu/mL)**	**Treated (cfu/mL)**
1	8.15±0.002	7.02±0.015	7.12±0.0	0	6.00±0.0	0
6	8.26±0.003	6.97±0.0	7.32±0.001	0	6.12±0.0	0
7	8.23±0.005	6.95±0.001	7.26±0.004	0	6.28±0.001	0
8	8.35±0.002	7.15±0.0	7.25±0.003	0	6.12±0.002	0
9	8.23±0.004	7.60±0.001	7.33±0.002	0	6.28±0.001	0
10	8.23±0.004	7.99±0.001	7.26±0.003	0	6.26±0.003	0
A1	8.36±0.0	6.76±0.001	7.23±0.003	0	6.12±0.005	0
A3	8.32±0.001	6.95±0.001	7.43±0.002	0	6.12±0.002	0
A4	8.28±0.001	7.01±0.005	7.26±0.001	0	6.24±0.001	0
A6	8.18±0.004	7.26±0.001	7.48±0.001	0	6.28±0.001	0
pH 3.0						
Isolate codes	**0 h**		**1 h**		**3 h**	
	Control (cfu/mL)	**Treated (cfu/mL)**	**Control (cfu/mL)**	**Treated (cfu/mL)**	**Control (cfu/mL)**	**Treated (cfu/mL)**
1	9.21±0.003	9.21±0.002	9.12±0.002	9.12±0.005	8.01±0.002	8.38±0.001
6	9.18±0.002	9.18±0.003	8.33±0.001	8.97±0.0	8.12±0.002	*7.26±0.001
7	9.18±0.002	9.18±0.002	9.28±0.001	9.28±0.001	8.26±0.002	7.21±0.002
8	9.01±0.002	9.01±0.002	8.26±0.003	8.48±0.001	8.26±0.002	8.23±0.001
9	8.98±0.001	8.98±0.0	8.33±0.001	0	8.28±0.001	0
10	9.12±0.002	9.12±0.004	8.30±0.001	8.99±0.0	8.26±0.003	8.93±0.0
A1	8.91±0.0	8.91±0.0	8.23±0.004	8.73±0.0	8.23±0.001	8.08±0.002
A3	9.12±0.002	9.12±0.002	8.43±0.002	8.28±0.002	8.12±0.004	*8.93±0.001
A4	9.23±0.001	9.23±0.003	8.26±0.001	0	9.26±0.001	0
A6	8.99±0.0	8.99±0.0	8.48±0.001	0	8.28±0.001	0

*Indicates a significant difference between the control and test groups at the indicated times ($p < 0.05$, Tukey "s Test).

The natural pH of an empty human stomach is on average 1.5. After the meal, this value can rise to pH 5-6 to prevent the entry and survival of unwanted bacteria in the intestinal tract. In order for probiotic bacteria to show their ideal contribution to their hosts, they must show an acceptable level of tolerance to all these challenging conditions. In this context, it is considered appropriate to test pH values between 1 and 5 in order to test the tolerance of a potential probiotic against stomach acidity (Huang and Adams, 2004). The ability of a probiotic candidate to tolerate pH 2.0 indicates that it is a good candidate due to this feature (Kiessling et al., 2002; Paramithiotis et al., 2006). In order for a strain to have probiotic properties, it must be resistant to the low pH of the stomach, which varies between 1-4, while passing through the digestive system. Since the residence time in the stomach was 3 h, a change

in viability of the strains was observed at low pH levels for 3 h (Dunne et al., 2001; Vinderola and Reinheimer, 2003). In this context, colony counts were performed by taking samples at 0, 1 and 3 h from the isolates treated in buffer solutions with pH 1.0, 2.0 and 3.0 (Table 5).

The results are presented as colony count results on a logarithmic scale. As can be seen from the colony-forming unit numbers in Table 5, acid tolerance was not observed at pH 1.0 and 2.0 in all lactic acid bacteria included in the study. However, at pH 3.0, acid tolerance was observed to continue at the end of the 3rd h in the remaining 7 isolates, except for the isolates coded 9, A4 and A6. In the said application, no significant difference was detected between the control groups and the test groups, except for isolates coded 6 and A3 ($p < 0.05$; Tukey's Test). In a previous study by Cebrian et al. (2012), *E. faecalis* UGRA10 was found to be highly resistant to acid and the reduction in viable count after 1 h exposure to pH 3 was less than 1 unit log CFU/ml.

Pepsin Resistance

The purpose of pepsin application at pH 2.0 and 3.0 is to determine *in vitro* the resistance levels of microorganisms reaching the digestive system to gastric conditions (Maragkoudakis et al.,2006). In order to better simulate stomach contents under *in vitro* conditions, unlike acid tolerance, LAB isolates were treated for different periods of time (1 and 3 h) in buffers modified with pepsin content and adjusted to pH 2.0 and 3.0 (Figures 1 and 2). The results are presented as colony count results on a logarithmic scale. In a previous study *Enterococcus* isolates were able to resist at pH 2.5 after 3 h as well as with in the presence of pepsin enzyme. The results obtained revealed that isolates had ≥90% tolerance to pH = 2.5 and The lowest resistance in these conditions belonged to C35 (*E. faecalis*) (Sonei et al., 2024).

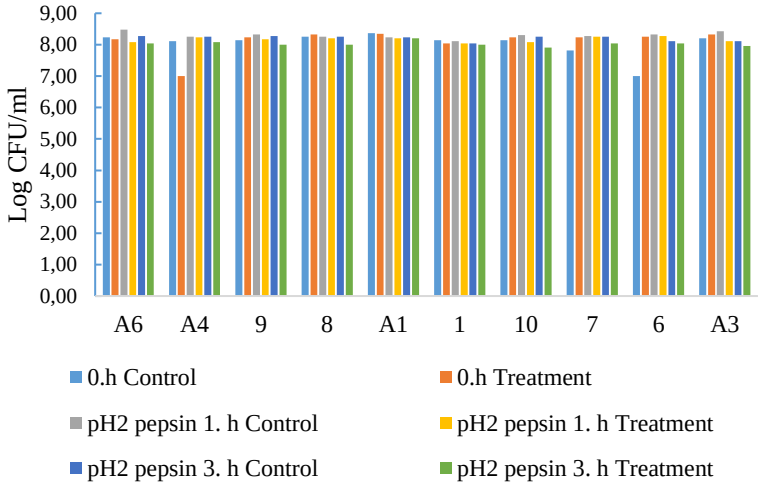

Figure 1. Pepsin (pH 2) resistance profiles (log cfu/ml) of the strains used in the study

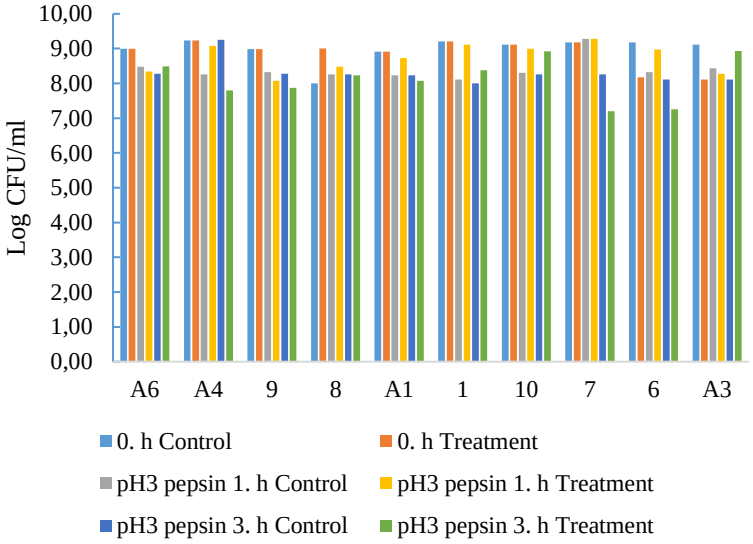

Figure 2. Pepsin (pH 3) resistance profiles (log cfu/ml) of the strains used in the study

Pancreatin Resistance

In order to determine the pancreatin resistance properties of the isolates, colony counting was performed by taking samples from the cultures treated for the 4th h in PBS buffers containing 1 mg/mL pancreatin and pH value adjusted to 8.0. The counting process was carried out in 3 parallels (A, B, C). No difference was detected between the colony count results obtained from the control groups and the count results obtained from the test groups exposed to pancreatin application. The results are given as colony count results on a logarithmic scale (Figure 3). Similar results were presented in the study by Özkan et al. (2021). They indicated a high survival rate of nine enterococcal isolates under bile-containing (0.25%) conditions in the range of 77–94%. These results are comparable to those of Zommiti et al. (2018), who found that the survival rates of five enterococci strains isolated from an artisanal Tunusian meat were 93.60–98.78% after 2 hours against a 1 bile salt concentration.

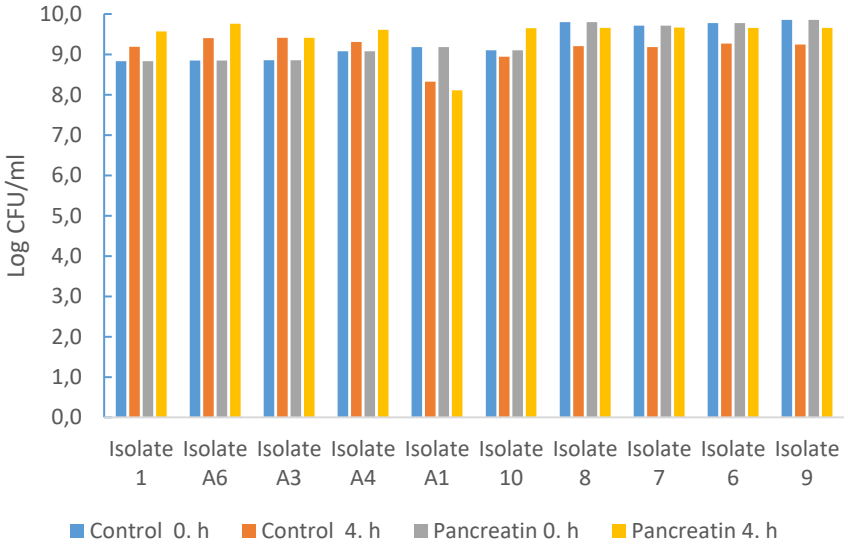

Figure 3. Pancreatin resistance results of lactic acid bacteria isolates (p>0,05)

Bile Salt Resistance

Resistance to bile salts is an extremely important characteristic of a candidate bacterial strain whose probiotic potential is being evaluated (Luo et al., 2012). Resistance to 0.15%-0.3% bile salts is

considered sufficient to identify a probiotic candidate for human use (Gilliland et al., 1984). In this study, lactic acid bacteria isolated at three different bile salt concentrations (0.3-0.5-1.0%) were tested. Even a 50% decrease in the viability of a potential probiotic bacterium tested for bile salt resistance even at 0.3% bile salt concentration shows that the tested bacterium is a good candidate within the mentioned criterion (Mathara et al., 2008). High levels of bile salt hydrolase enzyme activity are also observed in potential probiotics with high bile salt tolerance (Ahn et al., 2003). In this study, isolates 1, 7, 8 isolated from plant sources and A3, A6 isolated from bee gastrointestinal tract showed significant tolerance to bile salts. Due to these abilities, these isolates are again probiotic candidates.

Lactic acid bacteria within the scope of the study were exposed to three different (0.3%-0.5% and 1%) concentrations of bile salt content, 1th and 4th h). The resistance of bacteria to bile salt is given in Figure 5-7. Results are presented as reductions in bile satls groups colony counts with reference to count results in control groups. A significant decrease in bacterial viability is observed in all isolates with increasing bile salt concentrations and contact times. It is noteworthy that, except for isolates 1, 6 and 8, a significant level of viability could be maintained in all the remaining isolates even at the end of the 4th h and at a bile salt concentration of 1.0%. The resistance of the isolated lactic acid bacteria to bile salt at the specified contact times was also expressed as log cfu/ml in their viability as a result of comparison with the control groups. Çetin and Aktaş (2024) investegated of bile salt tolerance of *E. faecium* strains and all strains were tolerated bile salt containing 0.3, 0.5, and 1%. In the study by Sonei et all. (2024) All of the studied *Enterococcus* isolates were able to survive in 0.3% (w/v) of bile salts.

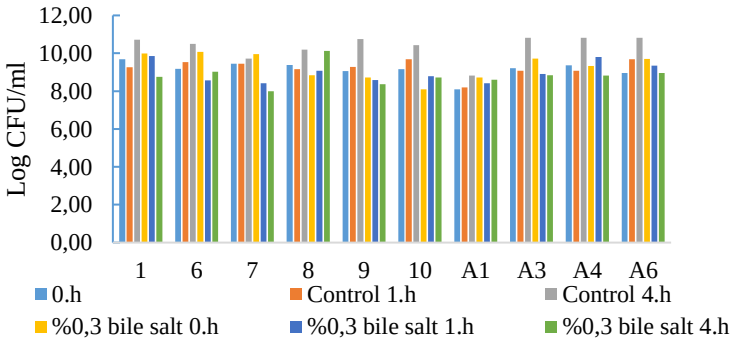

Figure 5. 0.3% bile salt resistance results of the strains used in the study (log cfu/ml) ($p < 0.05$)

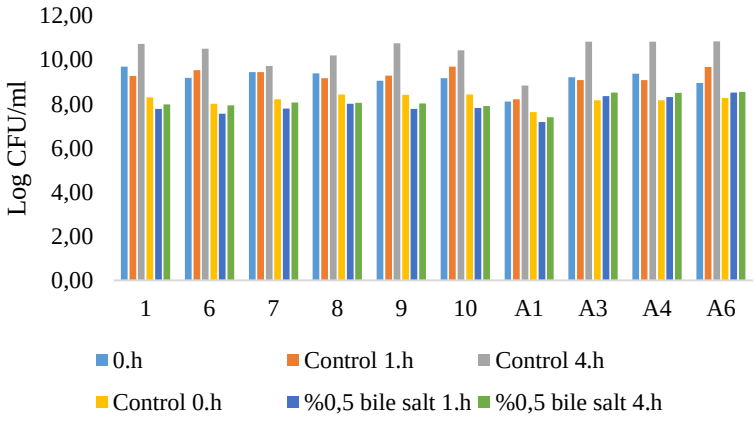

Figure 6. 0.5% bile salt resistance results of the strains used in the study (log cfu/ml) ($p < 0.05$)

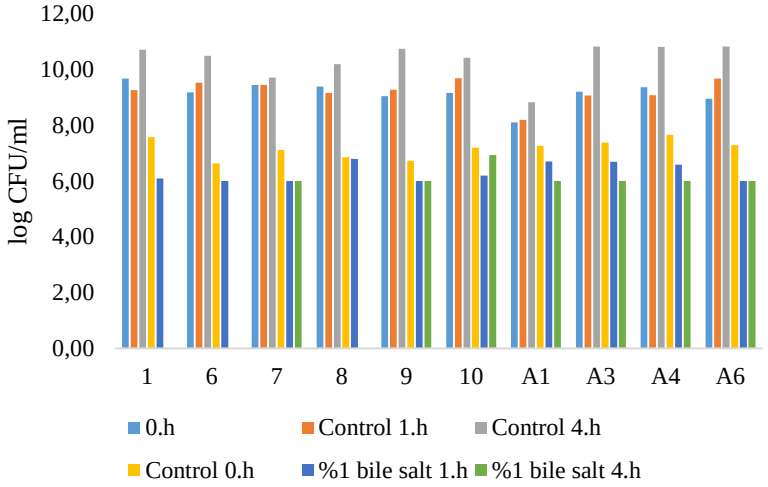

Figure 7. 1 % bile salt resistance results of the strains used in the study (log cfu/ml) ($p < 0.05$)

Hemolytic Activities of The Strains

One of the primary criteria in the evaluation of lactic acid bacteria with probiotic potential is the determination of haemolytic activity. In order for a bacterium with probiotic potential to be fully acceptable in this context, it is expected to show non-haemolytic properties. Many researchers agree that haemolytic activity should definitely not be observed at the point where the possibilities of safe use of probiotics are discussed (Bujnakova and Strakova, 2017; Nami et al., 2018; Mangia et al., 2019) since haemolytic activity is also a virulence feature.

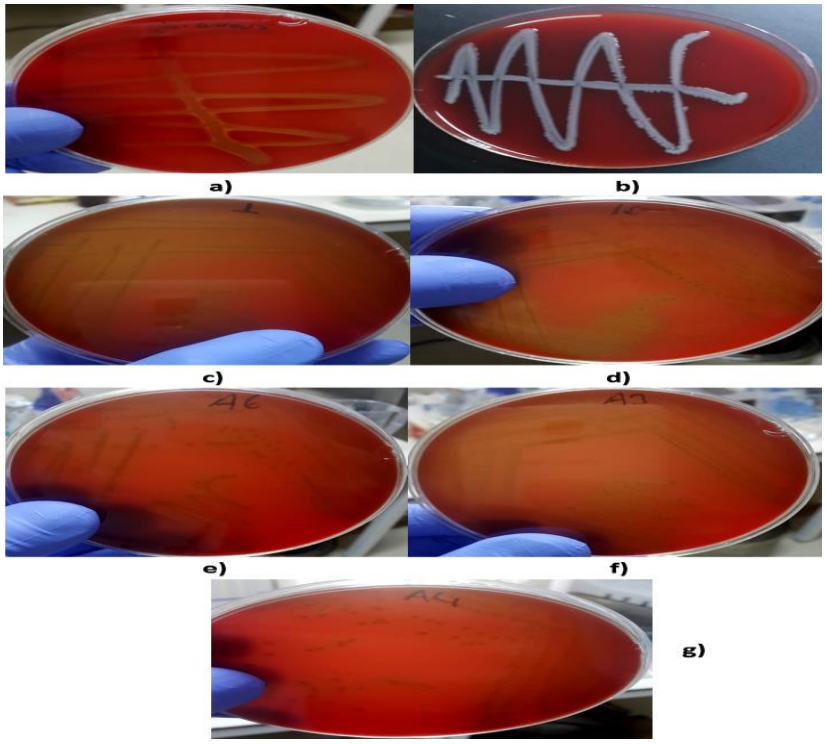

Figure 4. a) *Staphylococcus aureus* beta (β) hemolytic activity b) *Eschrichia coli* non-hemolytic activity c) isolate 1, d) isolate 10, e) isolate A6, f) isolate A3, g) isolate A4 (All lactic acid bacteria coded contain alpha (α) hemolytic activity

However, the virulence feature that is not accepted by other researchers in these evaluations is β-haemolytic activity. It is also seen that strains belonging to the genus *Enterococcus*, which are utilised in the processing of some fermented products, can be preferred in the processing of traditional products,

although they contain α-haemolytic activity (Nami et al., 2019). Lack of hemolytic activity (non-hemolytic activity and α-hemolytic activity) is one of the features that should be considered first when selecting probiotic strains. In fact, α-hemolytic activity can often be observed in *P. pentosaceus*, which has many probiotic strains (Colaninno et al., 2021). Similarly, α-hemolysis is also observed in some *Leuconostoc* and *Pediococcus* strains that have probiotic properties. In this study, the α-hemolytic feature of bee gastrointestinal tract lactic acid bacteria isolates and plant-origin fructophilic lactic acid bacteria isolates shows that they may have probiotic potential due to their non-hemolytic activity feature. All isolates within the scope of the study contain alpha (α) hemolytic activity on blood agar medium.

CONCLUSION

Fructophilic lactic acid bacteria (FLAB) are a specific group of lactic acid bacteria (LAB) that have recently been characterised and described. They prefer fructose as growth substrate and live only in fructose-rich niches. Honey bees are high fructose-consuming insects and important pollination tools in nature, but have unfortunately been reported to decline in the wild. There are very limited studies in the literature on fructophilic lactic acid bacteria, especially fructophilic *Enterococcus* species. Little is known about the presence of FLAB in the bee gut, let alone their probiotic properties. In this study, FLAB strains were isolated and identified from 8 different sources. After Gram-staining and catalase tests, the isolates were identified as *Enterococcus faecalis* by 16S rRNA gene sequencing. The isolates coded A1, A3, A4 and A6 were also identified as fructophilic lactic acid bacteria originating from bee microbiota. These findings clearly show that honey bees and their products are rich sources of FLAB and that FLAB are potential candidates for future bee probiotics. Another finding in this study, which is independent from the literature and contributes for the first time, is the demonstration that fructophilic lactic acid bacteria can be isolated from a wide variety of plant sources. Although the isolates exhibit potential probiotic properties, their pathogenicity properties in particular need to be investigated in detail in future studies.

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Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contributions

The authors declare that they have contributed equally to the article.

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