Mehmet Burak YİĞİT

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DETERMINATION OF PROBIOTIC PROPERTIES OF LACTIC ACID BACTERIA ISOLATED FROM TRADITIONAL FOOD PRODUCTS

A THESIS
SUBMITTED TO THE DEPARTMENT OF BIOENGINEERING
AND THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF ABDULLAH GUL UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

By
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SCIENTIFIC ETHICS COMPLIANCE

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Name-Surname: Mehmet Burak YİĞİT

Signature :

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Prepared By	Advisor
Mehmet Burak YİĞİT	Asst. Prof. Dr. Aysun CEBECİ AYDIN

Head of the Bioengineering Program

Asst. Prof. Altan ERCAN

ACCEPTANCE AND APPROVAL

M.Sc. thesis titled "Determination of Probiotic Properties of Lactic Acid Bacteria Isolated from Traditional Food Products" and prepared by Mehmet Burak YİĞİT has been accepted by the jury in the Bioengineering Graduate Program at Abdullah Gül University, Graduate School of Engineering & Science.

13/09/2023

JURY:

Advisor : Asst. Prof. Dr. Aysun CEBECİ AYDIN ……..…….

Member : Asst. Prof. Dr. Emel Başak GENCER AKÇOK ……..…….

Member : Asst. Prof. Dr. Mehmet Sefa ULUTAŞ ……..…….

APPROVAL:

The acceptance of this M.Sc. thesis has been approved by the decision of the Abdullah Gül University, Graduate School of Engineering & Science, Executive Board dated ….. /….. / …….. and numbered ……..……. .

…….. /…….. / ……..

(Date)

Graduate School Dean
Prof. Dr. İrfan ALAN

ABSTRACT

DETERMINATION OF PROBIOTIC PROPERTIES OF LACTIC ACID BACTERIA ISOLATED FROM TRADITIONAL FOOD PRODUCTS

Mehmet Burak YİĞİT
MSc. in Bioengineering
Advisor: Asst. Prof. Dr. Aysun CEBECİ AYDIN

September 2023

Probiotics are microorganisms that live in our bodies and positively affect health when consumed regularly. One of the ways to have a healthy body is to have a healthy microbiota. Because of that, the importance given to the consumption of probiotic foods among the public is increasing. Since probiotics are especially abundant in fermented and traditional foods, consuming these foods is vital to have a healthy microflora. In this thesis, probiotic potentials of bacteria isolated from tarhana, einkorn sourdough, Turkish and Bulgarian-type boza and pickled beetroot foods were investigated, and obtained results were discussed. Based on acid and bile salt tolerance tests, MRS ES-2-3-7-11-12-17, MRS PT-2-14-16, MRS N-1, MRS EB-3, MRS T-1, M17 N-2 -3-4 showed higher viability in acidic environments (pH 2.0 and 3.0) than the control groups, M17 N-3-4 and M17 TB-1-2 strains showed higher viability at 0.3% and 0.5% bile salt conditions than other strains. For 10 strains which are selected for further tests, in the adhesion to Caco-2 cells, MRS ES-3, MRS N-1, MRS T-2, M17 BB-7, M17 N-2 and M17 N-3 showed over 35% adhesion, especially, MRS N-1 and M17 N-2 showed over 85% adhesion to Caco-2 cells. For the antimicrobial activity test, ES-3 strain showed limited effect on *S. aureus* ATCC 6538 and *K. pneumoniae* ATCC 4352 pathogens, while other strains showed no inhibitory effect on pathogens. Finally, according to the results of 16S rRNA sequencing, it was determined that MRS ES-3, ES-7, PT-14 strains belonged to *L. plantarum*, MRS ES-11 strain belonged to *L. brevis*, M17 BB-7 strain belonged to *E. faecium* and M17 TB-2 strain belonged to *E. durans* species.

Keywords: Probiotics, lactic acid bacteria, traditional foods, characterization

ÖZET

GELENEKSEL GIDA ÜRÜNLERİNDEN İZOLE EDİLEN LAKTİK ASİT BAKTERİLERİNİN PROBİYOTİK ÖZELLİKLERİNİN BELİRLENMESİ

Mehmet Burak YİĞİT
Biyomühendislik Anabilim Dalı Yüksek Lisans
Tez Yöneticisi: Dr. Öğr. Üyesi Aysun CEBECİ AYDIN

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Probiyotikler vücudumuzda yaşayan ve düzenli tüketildiğinde sağlığa olumlu etkileri olan mikroorganizmalardır. Sağlıklı bir vücuda sahip olmanın yollarından biri de sağlıklı bir mikrobiyotaya sahip olmaktır ve bu nedenle halk arasında probiyotik gıdaların tüketimine verilen önem giderek artmaktadır. Probiyotikler özellikle fermente ve geleneksel gıdalarda bol miktarda bulunduğundan, sağlıklı bir mikrofloraya sahip olmak için bu tür gıdaları tüketmek önem arzetmektedir. Bu tez çalışmasında; tarhana, siyez ekşimaya, Türk ve Bulgar tipi boza ve pancar turşusu gıdalarından izole edilen bakterilerin probiyotik potansiyelleri incelenmiş ve elde edilen sonuçlar tartışılmıştır. Asit ve safra tuzu tolerans testlerine göre, MRS ES-2-3-7-11-12-17, MRS PT-2-14-16, MRS N-1, MRS EB-3, MRS T-1, M17 N -2 -3-4 asidik ortamlarda (pH 2.0 ve 3.0) kontrol gruplarına göre daha yüksek oranda canlılık göstermiştir, M17 N-3-4 ve M17 TB-1-2 suşları ise, %0.3 ve %0.5 safra tuzu koşullarında diğer suşlardan daha yüksek oranda canlılık göstermiştir. Sonraki deneyler için seçilen 10 suşun Caco-2 hücrelerine adezyon testinde MRS ES-3, MRS N-1, MRS T-2, M17 BB-7, M17 N-2 ve M17 N-3 suşları %35'in üzerinde, özellikle, MRS N-1 ve M17 N-2 suşları %85'in üzerinde oranla Caco-2 hücrelerine adezyon göstermiştir. Antimikrobiyal aktivite testinde ES-3 suşu *S. aureus* ATCC 6538 ve *K. pneumoniae* ATCC 4352 patojen suşları üzerinde sınırlı etki gösterirken, diğer suşlar patojenler üzerinde inhibitör etki gösterememiştir. Son olarak, 16S rRNA dizilemesi sonuçlarına göre MRS ES-3, ES-7, PT-14 suşlarının *L. plantarum*, MRS ES-11 suşunun *L. brevis*, M17 BB-7 suşunun *E. faecium* ve M17 TB-2 suşunun *E. durans* türlerine ait olduğu tespit edilmiştir.

Anahtar kelimeler: Probiyotik, laktik asit bakterileri, geleneksel gıdalar, karakterizasyon

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BB	Bulgarian-Type Boza
BSH	Bile Salt Hydrolase
CLSI	Clinical and Laboratory Standards Institute
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
EPS	Exopolysaccharide
ES	Einkorn Sourdough
EUCAST	European Committee on Antimicrobial Susceptibility Testing
IL	Interleukin
LAB	Lactic Acid Bacteria
LGG	*Lactobacillus rhamnosus* GG
LPS	Lipopolysaccharide
NF-κB	Nuclear Factor Kappa-B
PAMP	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PT	Pickled Beetroot
REGIIIγ	Regenerating Islet-Derived Protein 3 Gamma
TB	Turkish-Type Boza
TGF-β	Transforming Growth Factor-Beta
TLR	Toll-like Receptor
TNF-α	Tumor Necrosis Factor-Alpha

To My Family

Chapter 1

Introduction

1.1. Probiotics

Probiotics are living microorganisms existing in foods, when they are consumed in sufficient amounts regularly, they become beneficial to human health [1]. The word probiotic has both Latin and Greek origin and the meaning of this word is "for life". When they are consumed, they protect the host's mouth, stomach, intestinal health and support the immune system to cope with pathogens [2].

Tissier and Metchnikoff proposed that consuming *Bifidobacterium* and *Lactobacillus* rich dairy products helps to have a healthy gut and to cope with pathogens. More than a hundred years ago, their studies made the consumption of dairy products (especially yoghurt) worldwide popular. Metchnikoff put forward the idea that foods rich in beneficial bacteria such as yoghurt should be used to destroy pathogenic bacteria in the body. These beneficial bacteria would fight and replace pathogens for health. He was awarded the Nobel Prize (1908) for these studies [3].

For the first time, probiotics were mentioned by Kollath in literature (1953) and defined by Lilly and Stillwell (1965) as "growth-promoting factors produced by microorganisms" [4]. Since then, over 44000 articles about probiotics have been published in PubMed and the number of studies increases. In PubMed, 3141 studies in 2018, 3855 in 2019, 4646 in 2020, 5325 in 2021, 5916 in 2022 and 4145 studies on probiotics were published until September 2023 [5].

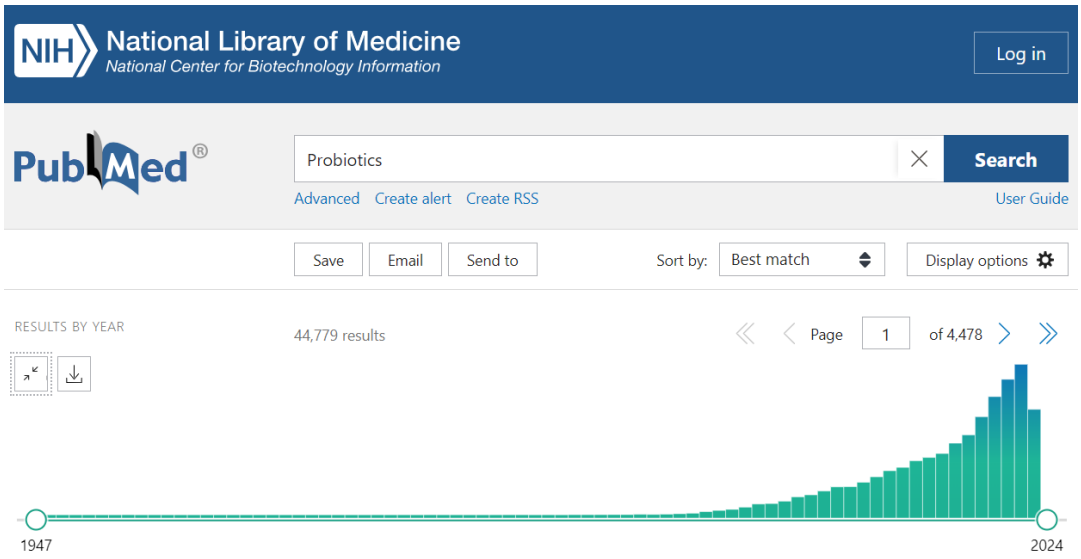

Figure 1. 1 Image showing the increasing number of studies on probiotics over the years on PubMed [5]**. (Accessed: Sep. 07, 2023)**

According to a survey conducted by the International Food Information Council (IFIC) in 2022 in the United States (n=1001), 24% of the participants believe gastrointestinal health is the most crucial factor to have overall body health. 67% of people are familiar with probiotics, 32% of respondents are trying to consume probiotics regularly and 60% of them are consuming probiotic foods daily basis and their primary source is yoghurt and kefir [6]. According to market research, in 2021, the global probiotics market size was valued at USD (United States Dollar) 58.17 billion, the estimated value in 2023 is USD 73.3 billion, and expected to extend this value to USD 105.5 billion in 2028. CAGR (compound annual growth rate) is expected to reach to 7.5% in 2028 [7,8].

We carry 1.3 times more microorganisms in our body than our cells [9]. And the approximate weight of these organisms is one to two kilograms. Bacteria constitute the most significant part of these organisms, and these are mainly probiotics [10]. Most probiotics are bacteria, but some strains are *Saccharomyces* yeasts and also some fungal strains such as *Aspergillus niger* show probiotic properties [11,12]. Well-known and widely used probiotic genera are species of *Bifidobacterium* and *Lactobacilli* [13,14]. Most of the lactic acid bacteria strains and other widely used probiotic microorganisms are given in Table 1.1.

Table 1. 1 Identified probiotic microorganism species exist in human microbiota [14-21]**.**

Type	Species
Lactic Acid Bacteria	*Bifidobacterium bifidum, B. longum, B. thermophilum, B. adolescentis, B. animalis, B. breve, B. catenulatum, B. infantis*
	Lactobacillus rhamnosus, L. acidophilus, L. gasseri, L. casei, L. fermentum, L. plantarum, L. helveticus, L. salivarius, L. pentosus, L. delbrueckii, L. brevis, L. reuteri, L. curvatus, L. paracasei
	Lactococcus lactis
	Enterococcus faecium, E. faecalis
	Streptococcus thermophilus, S. salivarus K12
	Pediococcus acidilactici, P. pentosaceus
Yeast	*Saccharomyces cerevisiae, S. boulardii*
Other	*Bacillus clausii, B. subtilis, B. cereus*
	Escherichia coli Nissle 1917

1.1.1 Lactic Acid Bacteria

Lactic acid bacteria (LAB) comprise the largest group of probiotic microorganisms. Generally, they are Gram-positive and non-spore forming bacteria. They are mostly catalase and oxidase negative, anaerobe or facultative anaerobe, acid-tolerant, immobile, generally having cocci or rod shape, use only carbohydrates as carbon source and produce lactic acid as a major end product of carbohydrate fermentation. They contain *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, *Enterococcus*, *Streptococcus*, and *Pediococcus* genus. LABs are usually mesophilic microorganisms, most of them optimally grow at 37ºC [3,22,23]. They are widely used in the food and drug industry to produce and also bio-preservation of fermented food products (yoghurt, kefir, ayran, pickles, boza, tarhana, wine etc.) and probiotic supplements [14].

Lactic acid bacteria show antagonistic activity against pathogenic microorganisms. They are coping with pathogens via four main mechanisms. The first of them is producing hydrogen peroxide (H_2O_2) by *Lactobacillus spp*. which helps to inhibit the growth of pathogens non-specifically. The second mechanism is the production of lactic acid and acetic acid due to the fermentation of carbohydrates and lowering the pH of the environment. Many microorganisms in foods are sensitive to low pH and these organic acids, as a result, most pathogens cannot tolerate these conditions and their growth will be inhibited. The third mechanism is immunomodulation via stimulating the expression of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-10, IL-12, IFN-γ via *Lactobacilli* and *Bifidobacteria* induction. And the last mechanism is the production of antimicrobial bacteriocins to inhibit the growth of closely related pathogenic bacteria [24].

One of two major genera of LAB is *Lactobacillus*, they are rod-shaped, Gram-positive, non-spore forming, immobile, catalase and oxidase negative, optimally growing at 37ºC. They are extensively used in food industry as starter culture for extending shelf-life via expressing antimicrobial compounds and inhibiting unwanted bacterial growth [25]. In studies examining the probiotic potential of species belonging to the genus *Lactobacillus*, it has been shown that probiotic strains might induce the support mechanism of T cells and the development of dendritic cells, reverse the low antimicrobial response of the body, enhance the immune system, and improve the colon surface. It has been reported, a diet which contain probiotics with the strong colonization properties (such as *L. plantarum*) has an anti-obesity effect by inducing lipogenic gene expression in mice fed excessively fat [26-28].

Acidophilus milk obtained by using *Lactobacillus acidophilus* is used as a probiotic culture as well as in the food industry [29]. *Lactobacillus casei*, a crucial bacterium in the food industry, is also used to ferment sourdough and pickled cheeses [30]. *Lactobacillus helveticus* is frequently used in cheese production and is often isolated from raw milk and raw milk products. These bacteria can also grow at high temperatures, such as 55°C and they are known as thermophiles [31]. *Lactobacillus rhamnosus* GG (LGG) has become one of the most used bacterial strains in probiotic food supplements because of its many benefits, such as immune system regulation, suppression of pathogens by producing antimicrobial substances and bio-preservation [32-34]. Studies on *Lactobacillus gasseri* strains found that obese adults lost more

than 8% of their belly fat after three months by adding these probiotic strains to their diets. In another study, it was observed that cholesterol levels in adults decreased as a result of consuming these bacteria [35,36]. *Lactococcus lactis* is a type of LAB found in raw milk and dairy products used in the food industry and can also be isolated from plant foods. It is used as a starter culture in foods and has an inhibitory effect against pathogens found in fermented milk products, and some *L. lactis* strains produce important bacteriocins such as nisin [37].

Second major group of LAB is *Bifidobacterium*, they are Gram-positive, mostly strict anaerobe, catalase-negative (except a few strains), non-spore forming, immobile, rod-shaped bacteria and they usually exist in the gastrointestinal tract of animals, humans and also gut flora of infants. They are optimally grown at 37ºC, and the ideal pH is around pH 6.5-7.0, but *B. animals* and *B. thermophilum* strains are metabolically active at pH 3.5-4.0. So far, many *Bifidobacterium* strains showed advantageous impacts on human well-being such as immunoregulation, helping the absorption of nutrients, repressing pathogens via expressing antimicrobial agents and promoting psychological health by producing neurotransmitters which are tryptophan, niacine (vitamin B3), and hypoxanthine [38,39].

B. bifidum strains can activate the host immune system and induce the expression of IL-6 (Interleukin-6) and IL-10 [40], help rebuild the intestine mucus layer and microflora balance of mice having ulcerative colitis (UC) by affecting NF-κB (nuclear factor kappa B) signaling pathway [41], and suppress the growth of colon cancer [42]. A mixture containing *B. lactis*, *B. bifidum*, *L. casei* and *L. acidophilus* can ameliorate IBS (irritable bowel syndrome) [43]. *B. bifidum*, *B. longum*, *B. infantis*, *L. rhamnosus* and *L. casei* strains can relieve symptoms of constipation [44]. *B. lactis* helps to decrease body fat and sugar intolerance in diabetic and obese mice by inhibiting the relocation of intestine microorganisms [45]. *B. longum* strains can reduce LDL-cholesterol (Low-density lipoprotein) and triglyceride levels and increase HDL-cholesterol (High-density lipoprotein) levels, hence they can prevent or relieve cardiovascular diseases [46]. Additionally, *B. longum* can hinder *Clostridium difficile* and *Klebsiella pneumoniae* infections by inducing ROS (reactive oxygen species) production [47,48].

1.1.2 Probiotic Rich Foods

Probiotic foods have been consumed for centuries in many parts of the world. Especially dairy products (such as yogurt, kefir, cheese), sourdough, vinegar, several types of pickles (sauerkraut, pickled beetroot, cornichon, pickled peppers and cucumber etc.), kimchi (Korea), kombucha (China), boza, turnip and tarhana (Türkiye), koumiss (kımız-Turkic Countries), lassi (India), tempeh (Indonesia), miso and natto (Japan) can be shown as probiotic foods consumed worldwide [49-52]. In this study, boza, pickles, sourdough and tarhana probiotic foods will be investigated in detail.

Boza is a traditional fermented non-alcoholic, viscous Turkish beverage produced by yeast and lactic acid fermentation by mixing wheat, millet, rice flour with sugar or saccharin. It has a pale-yellow color and has a sweet-sour taste. It is widely consumed in Türkiye, Bulgaria, Albania, Romania, and some Balkan Countries due to its taste and nutritional properties [53-55]. In the production of boza, lactic acid and ethyl alcohol fermentation occurs. While LAB increases acidity by producing lactic acid and provides a protective effect, metabolites produced from ethyl alcohol fermentation of yeasts create the taste and odor of boza [49,55]. Boza is comprised of 0.5-1.5% protein, 12-13% carbohydrate, 0.5% ethanol, 15-20% dry matter and 60-65% water. pH level of boza is between 3.15-4.0 [49,56]. In the boza preparation, raw materials are boiled together in water for 1-2 hours. Then, it is left to cool and filtered with a sieve. Sugar and yeast mixture will be added and left to ferment for 24 hours at 15-25ºC. After the incubation, extra water and sugar can be added to adjust taste and viscosity and then left to cool down below 15ºC. After this process, boza is ready to drink or bottle [56]. *L. plantarum, L. paracasei, L. pentosus, L. rhamnosus, L. brevis, L. acidophilus, L. fermentum, Lactococcus sp., Pediococcus sp., S. cerevisiae* strains are probiotics found to be present in boza [49,55-58].

Pickle is a traditional food obtained by fermenting vegetables and sometimes fruits by lactic acid bacteria in certain concentrations of salt water or their juices. Pickles are a highly acidic and durable food. For centuries, it has been consumed by humans for its taste, nutritiousness, health benefits and long-term preservation. In many countries, pickles of different fruits and vegetables are made. While making pickles, the jar or bottle to be used is filled to the top with the vegetables and fruits to be pickled, and garlic will be added in between. Afterward, pickle juice is prepared in

another container. It is obtained by mixing vinegar, lemon salt, brine salt and water. This pickle juice is added to the container where the vegetables and fruits to be pickled are placed and filled to the top. The jar is tightly closed and kept in a cool place out of direct sunlight for 2-3 weeks for fermentation. Then the pickle is ready to eat [59]. Pickles have a rich microflora because of the microorganisms such as yeast, Gram-positive and Gram-negative bacteria, and mold on the vegetables and fruits. As LAB increases the acidity of the environment during the fermentation process, most harmful microorganisms lose their activity, while the activity of beneficial LABs increases, and they become dominant organisms in the container. Many pickles contain known probiotic strains such as *L. plantarum, L. brevis, L. fermentum, L. pentosus, P. pentosaceus, Leuconostoc mesenteroides* [59,60].

Sourdough is a product that has been used by humans to make bread for nearly four thousand years. It has been used throughout history in almost all parts of the world due to its taste, nutritiousness and continuous utilization by people [61]. It is known that sourdough contains many vitamins, minerals and beneficial substances such as vitamin E1, B1, B6, B12, magnesium, iron, zinc, potassium, calcium, thiamine, niacin, riboflavin and folate [62]. It has been shown in many studies that several types of probiotic bacteria exist in sourdough such as *L. brevis, L. plantarum, L. casei, L. paracasei, L. alimentarius, L. acidophilus, L. farciminis, Leu. lactis, L. lactis, L. delbrueckii, L. fermentum, L. reuteri* and *P. pentosaceus* [61-64].

Pre-prepared sourdough yeast is dissolved in drinking water at room temperature for the preparation of bread from yeast. Einkorn flour and rock salt will be added to the blend and mixed thoroughly until it becomes smooth. The dough is placed in the container to be cooked in and covered with a lid. After waiting for 1 hour at room temperature, it is placed into the refrigerator for 24 to 48 hours to ferment. At the end of fermentation, the bread dough will be placed at room temperature and kept for about 1 hour until its size is twice its initial state. To turn the dough into bread, it will be baked in a 220ºC oven for 35-50 minutes and then rest for 2-4 hours at room temperature to be ready to consume [62].

Tarhana is one of the traditional and local foods generally consumed in Türkiye. It is produced by the fermentation of wheat flour, yogurt, sourdough, herbs, vegetables and several spices [50]. Vegetables are chopped as small as possible and

boiled by adding spices and herbs to the mixture. When mixture has cooled, yoghurt and wheat flour are added, and dough is obtained. The dough is fermented at room temperature for up to a week. At the end, fermented dough is thinned and left to dry. Completely dried tarhana under the sun or in the dryer, is now ready to be consumed [65]. Tarhana has high nutrient value and long shelf-life, it can be consumed as a snack or soup, and the ingredients may vary according to the geographical region where it is produced. Metabolites (lactic acid, ethyl alcohol, carbon dioxide, and aromatic compounds) produced by Lactic Acid Bacteria give tarhana its distinctive flavor and, increase its acidity, and extend its shelf-life [66]. Tarhana also contains vitamins such as niacin, riboflavin, folic acid and thiamine. Lactic acid bacteria strains *L. delbrueckii, L. acidophilus, L. casei, L, plantarum, Lc. lactis, Leuconostoc cremoris, E. faecium, P. pentosaceus* and baker's yeast *S. cerevisiae* are existed in tarhana microflora [49,66]. Tarhana is consumed under different names and in different composition forms in the Middle East and Balkan countries, Greece, Syria, Iraq, Iran, Palestine, Egypt, Jordan and Hungary [49].

1.2. Effects of Probiotics on Health

Most of the health effects associated with probiotic microorganisms are directly or indirectly related to the gastrointestinal tract, which is mediated by the immune system. The intestine is the largest organ in the body from an immunological point of view, and the maturation and development of the immune system from birth depends on the microflora and its composition [67].

Scientific studies have shown that probiotics play an important role in solving or alleviating many serious health problems such as diarrhea and constipation, IBD (inflammatory bowel disease), IBS (irritable bowel syndrome), allergies and eczema, lactose intolerance, *H. pylori* infections, diabetes, obesity, high cholesterol, cancer and cardiovascular diseases [14,20,68,69].

Probiotics can prevent the active proliferation of harmful bacteria by lowering the intestinal pH with the effect of lactic and acetic acids they secrete. In addition, they secrete other antimicrobial substances such as hydrogen peroxide, bio-surfactants, and bacteriocins and prevent their reproduction by damaging pathogens and disrupting the appropriate environmental conditions [24,70]. Bacteriocins show more robust antimicrobial activity against pathogens in acidic environments [71].

Some probiotic strains in the gut, create anaerobic environment mainly ideal for most probiotic bacteria [72]. Probiotics keep the gut safe by secreting exopolysaccharides that prevent pathogen adhesion and biofilm formation [73].

Many studies investigate probiotic bacteria's effects on diabetes and insulin resistance. In the study conducted by Hariri et al. (2015), 40 adult patients with type 2 diabetes (T2D) were treated with *L. plantarum* for 8 weeks, and it was determined that methylation levels, (have a role in the development of T2D), 8-OHDG, (early oxidative stress marker), and the activity of superoxide dismutase enzyme (SOD-free radical inactivating enzyme) decreased [74]. In the study of Tonucci et al. (2015), after administering 45 patients with T2D with *L. acidophilus* and *B. lactis* for 6 weeks, it was determined that HbA1c (gives information about glucose level of the past few months), LDL-cholesterol (known as bad cholesterol) and TC (total cholesterol) levels were decreased [75]. In a study by Barreto et al. (2015), 24 female patients with insulin resistance were found to have significant reductions in glucose and homocysteine levels after 12 weeks of administration with *L. plantarum* [76].

In studies of probiotics on obesity, Kadooka et al. (2013) found a decrease in BMI, abdominal fat, waist and hip circumference values after 12 weeks of *L. gasseri* use in a study on 87 individuals with high body mass index (BMI) [77]. Kadooka et al. (2010), in another study conducted on 210 individuals with high visceral fat area, showed that BMI and vascular blood pressure values of individuals decreased after 12 weeks of consumption of *L. gasseri* [36]. Sharafedtinov et al. (2013) found that BMI and vascular blood pressure values decreased after 3 weeks of administration of *L. plantarum* on 40 obese individuals [78]. Agerholm-Larsen et al. (2000) determined that after administration of *E. faecium* and *S. thermophilus* strains for 8 weeks to 70 obese and overweight individuals, their body mass, systolic blood pressure (high number in blood pressure), and LDL-cholesterol levels decreased [79].

There are many studies on the effects of probiotics in various gastrointestinal disorders. Wang et al. (2004) observed that 59 patients infected with *H. pylori* had an inhibitory effect on the infection after using *B. lactis* and *L. acidophilus* for 6 weeks [80]. Kuipers et al. (2003) found that after using *L. casei* for 6 weeks in 16 patients infected with *Helicobacter pylori*, growth of the pathogen decreased by 64% in the experimental group and by 33% in the control group [81]. Sood et al. (2009), found

in a study conducted on 77 individuals with ulcerative colitis (UC; type of IBD), and found that after 12 weeks of administration of a probiotic mixture called VSL#3 (consisting of 8 strains; *B. lactis(2), B. breve, L. plantarum, L. acidophilus, L. paracasei, L. helveticus* and *S. thermophilus*.), 42.9% recovery was observed in the experimental group, while a 15.7% recovery was observed in the placebo group [82]. Roškar et al. (2017) conducted a study on 44 lactose-intolerant individuals and found that there was a significant reduction in diarrhea and flatulence after 6 weeks of administration of *L. plantarum* and *B. animalis* [83]. Casey et al. (2017) feed swine for 6 days with fermented milk, which contains probiotic bacteria *P. pentosaceus, L. salivaris, L. pentosus* and *L. murine* species. On the 6th day, they infected swine with *Salmonella typhimurium* pathogen. As a result of the study, the number of *Salmonella* found in the feces of infected animals decreased gradually and the general health of the animal's improved day by day [84].

In a study by Basso et al. (2019), 4 children with Crohn's Disease (CD; type of IBD) were given *L. rhamnosus* GG for 6 months and after treatment, clinically significant improvement was observed [85]. In a meta-analysis study, the effects of *L. rhamnosus* GG and *S. boulardii* probiotics on antibiotic-associated diarrhea (AAD) and *Clostridium difficile* disease (CDD) were examined and it was found that both strains were effective on diarrhea and *S. boulardii* was effective on CDD [86].

There are also studies on the effect of probiotics on cancer. Liu et al. (2011) found that after 16 days of *L. plantarum, L. acidophilus* and *B. longum* use in 100 patients having colorectal carcinoma, the patients' intestinal mucosal barrier improved, and the infection complications decreased [87]. Österlund et al. (2007) determined that after 24 weeks of use of *L. rhamnosus* in 150 patients with colorectal cancer, diarrhea and abdominal pain levels and the need for hospitalization decreased in patients [88]. In a study by Orlando et al. (2009), the antiproliferative effect of *L. rhamnosus* GG on gastric and colon cancer cells was investigated. At the end of the experiment, they found that high concentrations of *L. rhamnosus* GG homogenate and cytoplasmic extracts reduced the viability of DLD1 colon cancer cells by 55% and the viability of HGC-27 gastric cancer cells by 65% [89]. Kahouli et al. (2013) discovered that lactic acid bacteria degrade potential carcinogens and inhibit the pro-cancer enzymatic activities of intestinal bacteria by stimulating the immune system [90]. Another study showed that short-chain fatty acids in the supernatants of

probiotic strains play a prominent role in direct inhibition of colon cancer cell growth by various mechanisms [91].

Additionally, according to a study conducted by Zitgovel et al. (2016), two probiotic strains (*Enterococcus hirae* and *Barnesiella intestinihominis*) were found to boost the effects of chemotherapy on 38 advanced lung and ovarian cancer patients. In this study, CTX (cyclophosphamide) was used as a chemotherapeutic agent, and its activity partly depends on intestinal bacteria. However, usage of these kinds of agents might weaken the host immunity and may cause infections, so generally, antibiotics are prescribed along with chemotherapeutics. Hence, they decided to use probiotics to enhance the effectiveness of chemotherapy and maintain the microbiome. As a result of this study, these two probiotic strains (*E. hirae* and *B. intestinihominis*) restored the efficacy of CTX in antibiotics-treated mice, enhanced anticancer immune response, and it was predicted that patients receiving this type of treatment would have more prolonged progression-free survival rates. These types of microorganisms are known as “oncomicrobiotics” [92].

1.2.1 Colonization Resistance and Antimicrobial Activity

Colonization resistance is the protection of the microbiota of the intestine against exogenous pathogenic bacteria, it is more effective than antibiotic protection of the intestine [93]. Probiotic bacteria attach to the intestinal mucosal cells and begin proliferating at that location by colonization. There is less chance to grow in the gut if there is no adhesion. This rule is also valid for pathogens and there is competition for the adhesion of the niche of the mucosa. If the individual has healthy and robust microflora, the chance of adhesion of pathogens to the gut is low [94]. Commensal bacteria support this resistance by secreting metabolites and consolidating intestinal immunity by stimulating the production of antimicrobial and pro-inflammatory components. Also, these bacteria can produce and secrete lethal or inhibitory factors bacteriocins. However, using antibiotics can hinder these mechanisms by damaging intestinal microbiota and favoring the growth of antibiotic-resistant pathogens and opportunistic bacteria [95]. There are two types of colonization resistance: direct and indirect resistance. Direct colonization resistance depends on the inhibition of the growth of pathogens via competition for nutrients and mucosal niches, antimicrobial compounds, organic acids and bacteriocin production and delivering toxic effectors by Type VI Secretion Systems (T6SS). Indirect or immune-mediated colonization

resistance depends on stimulating immune cells and Paneth cells by commensal bacteria and inhibiting the growth of the pathogen via stimulation the expression of antimicrobial and pro-inflammatory molecules. Some probiotic bacteria help maintain colonization resistance in both direct and indirect pathways [93].

Commensal bacteria can secrete antimicrobial molecules, which help to hinder gut infections. These molecules are hydrogen peroxide, organic acids (lactic, acetic and formic acid), diacetyl, acetaldehyde, carbon dioxide and bacteriocins. Organic acids, diacetyl and CO_2 lower the pH of the environment and also cytoplasm of cells. They disrupt the vital structures of pathogenic bacteria and, cause DNA damage and eventually lead to cell death [70,96-98]. Hydrogen peroxide (H_2O_2) and acetaldehyde exerts antimicrobial activity by oxidizing the bacteria's cell wall, damaging the cells' proteins and DNA [70,99,100].

Bacteriocins are bacteria-killing (bactericidal) proteins or peptides secreted by some bacteria and produced as a self-protection compound. Bacteriocins are divided into four groups: class I, class II (IIa, IIb and IIc), class III (IIIa and IIIb) and class IV [14]. Class I bacteriocins (lantibiotics) are short peptide chains (<5 kDa) and mainly contain lanthionine, β-methyllanthionine and some dehydrated amino acids [24]. Nisin is the most well-known bacteriocin produced by *L. lactis* species. This bacteriocin attaches to lipid II on the cell wall and inhibits cell wall synthesis. Then, pores will form on the bacterial membrane, leading to cell death by impairing membrane permeability [101]. Class II bacteriocins (non-lantibiotics) are heat-stable, non-lanthionine containing small peptides (5-10 kDa) and divided into 3 subclasses: IIa synthesized by two or more genes, IIb contains two different peptides, IIc has a circular structure. Pediocin PA-1 produced by *P. acidilactici,* and Enterocin A produced by *E. faecium* are the best examples for Class IIa bacteriocins. These molecules also cause cell death via pore formation by binding the cell wall and membrane of the bacteria and inhibiting peptidoglycan production [102]. Plantaricins produced by *L. plantarum* belong to class I and class II bacteriocins. The mechanism of action depends on damaging the cell envelope, spores and biofilms of specific pathogens. Disruption of membrane integrity leads to leakage of electrolytes, ATP, nucleic acids and proteins [103-105]. Class III bacteriocins are large, heat-sensitive proteins (>30 kDa) and are divided into two classes: IIIa (bacteriolytic) and IIIb (non-lytic). Helveticin J and Enterolysin are examples of class III bacteriocins [14]. Class

IV bacteriocins are large protein complexes containing lipid and carbohydrate structures. Plantaricin S and Leuconocin S are examples, and their mechanism of action depends on disrupting bacterial cell membrane [106].

According to the studies available on literature, strains of *B. longum, B. breve, B. lactis* [107], *B. bifidum, B. infantis* [108], *L. rhamnosus, L. plantarum* and *L. lactis* [109] contribute to direct colonization resistance to *C. difficile* pathogen, *L. rhamnosus, B. lactis* [110], and *B. bifidum* [111] strains participate in direct colonization resistance to *E. coli* pathogen and *L. plantarum, L. fermentum* and *L. paracasei* strains contribute to direct colonization resistance to *Salmonella* pathogen [110].

Probiotics are also contributing to expression of structural proteins in the intestine which helps to formation of monolayer and maintain epithelial barrier [112]. Epithelial cells of the intestine attach and form a monolayer with the participation of junction complexes. Intestinal tissues have three types of junctions: tight junctions (TJ), adherens junctions (AJ) and desmosomes. Tight junctions are the upmost complexes, block the intracellular void and are comprised of transmembrane proteins (claudins, occludins), peripheral membrane proteins (zonula occludens-1 and 2) and regulatory proteins. They sustain the transportation of ions and small compounds. Adherens junctions exist below the TJ complexes and along with the desmosomes, they maintain the integrity of the intestinal barrier with the assistance of stiff adhesion. These organizations in the gut help to prevent the passage of pathogens from the intestine to the circulatory system and other organs [2].

1.2.2 Immunomodulation

Commensal bacteria regulate our immunity via interacting with innate and adaptive immune system components.

Flagellin, which is a protein that exists in the flagellum of the bacteria, can induce CD103+ dendritic cells (DC) via binding to Toll-like Receptor (TLR) 5 and dendritic cells express IL-22 (Interleukin-22) and IL-23. These interleukins will affect the expression of REGIIIγ (regenerating islet-derived protein 3 gamma) by Paneth cells. REGIIIγ is an antimicrobial C-type lectin that binds peptidoglycan structure on Gram-positive bacteria and causes cell death even for antibiotic-resistant pathogens such as Vancomycin Resistant *Enterococcus* (VRE). Lipopolysaccharides (LPS), a

characteristic compound of Gram-negative bacteria outer cell membrane, is known to stimulate immune cells, which TLR4 can detect in stromal cells. These cells induce the expression of REGIIIγ to kill the pathogenic bacteria [93]. Flagellin and LPS-like structures are known as pathogen-associated molecular patterns (PAMPs), and they can be detected by TLRs.

Furthrmore, a type of commensal bacteria, *Bacteroides thetaiotaomicron* can induce the expression of REGIIIγ. The antibiotic-related depletion of commensal bacteria can decrease the expression of REGIIIγ. Expression of REGIIIγ can be restored by stimulating TLRs and this stimulation is partly dependent on myeloid differentiation primary-response 88 (MYD88) proteins [113]. On the downstream of this stimulation pathway, transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) will be activated and induce the expression of pro-inflammatory cytokines (i.e., IL-1β). Also, IRFs (interferon regulatory factors) will be activated and stimulate the expression of type I interferons [114]. Moreover, NOD2 (nucleotide-binding oligomerization domain 2) intracellular receptors of intestinal epithelial cells can recognize muramyl dipeptide (MDP) which belongs to PAMPs and stimulate the expression of the antimicrobial compound of cryptdin by Paneth cells [115].

Another mechanism for immunomodulation depending on the segmented filamentous bacteria (SFB) stimulating the expression of Immunoglobulin A (IgA) by B cells and serum amyloid A (SAA) mediated cell differentiation of T_H17 (T helper 17) cells. IgA will recognize pathogenic epitopes and T_H17 cells express and secrete pro-inflammatory cytokines such as IL-17 and IL-22, which induce the production of antimicrobial peptides [116]. These mechanisms are also included in the indirect (immune-mediated) colonization resistance [93].

1.2.3 Safety of Probiotics

Humans have used probiotics for centuries to produce nutritious and long-lasting products. Especially the use of *Lactobacilli* is quite old. The FDA (Food and Drug Administration) has included some known types of *Lactobacilli* in the GRAS (generally recognized as safe) category and declared that they are safe for use [117,118]. LABs are also used in food safety, due to the production of antimicrobial components. In particular, because of the organic acids (lower pH) and bacteriocins

they produce, they prevent the unwanted growth of pathogens in foods, thus maintaining food safety and extending its shelf-life. Nisin, the first FDA-approved bacteriocin, is frequently used in fermented foods [14]. In the literature, probiotic foods are considered as safe, and there are very few reports and studies on the health problems caused by these foods. The probability of a problem with using probiotics by healthy people is considered close to zero [99]. However, individuals with specific health problems (infections, chronic diseases, allergies, etc.) should be careful about using probiotics. Guidance and approval should be obtained from experts and authorized persons in this field. Nevertheless, even studies investigating the safety of probiotics have found that probiotics are very safe and problems that arise are primarily seen in individuals with health problems [119-122].

Some cases have been reported as side effects of probiotic use. As mild side effects, conditions such as gas formation in the intestines, bloating, diarrhea, and itchy or rash skin can be seen in individuals [119].

As in different point of view, probiotics may have antibiotic resistance and this resistance gene can be transferred to harmful microorganisms. For this reason, these properties should be examined and evaluated very carefully before probiotics are introduced to the market [123]. Since each strain of probiotics has slightly different properties, each new probiotic strain to be used in products and treatments must be carefully tested.

According to two studies conducted by Elinav et al. (2018), probiotic administration might not be as effective as doctors thought. Instead, personalized treatment should be considered since the "one size fits all" therapy approach does not work with probiotics. In the first study, probiotics (SupHerb Bio-25, a mixture of 11 strains belong to: *Lactobacillus*, *Bifidobacterium*, *Lactococcus* and *Streptococcus* genera) and placebo pills were given to two groups of subjects for four weeks. Then, according to endoscopy and colonoscopy results, some subjects resist probiotic colonization while others do not [124]. In the second study, they investigated the prescription of probiotics after antibiotic treatment-related dysbiosis. Subjects were treated with antibiotics, and then they were administered probiotics. Also, they conducted autologous fecal microbiome transplantation (aFMT) in another group. The last group was left to spontaneous recovery. According to the results, probiotic

adhesion and colonization levels are increased after the antibiotic treatment. However, surprisingly, hosts' microbiome and regular gene expression levels before the treatment could not be sustained for months. But in aFMT groups, it took a couple of days [125]. Therefore, the prescription of probiotics should be tailored to individuals, and alternative approaches should be considered.

In a study conducted on *L. rhamnosus* strains with high adhesion to the intestinal cells, it was determined that these strains may cause bacteremia (transmission of bacteria into the circulatory system) by passing over the junction proteins in the mucosa with the advantage of their high adhesion. In the same study, it was determined that *L. casei, L. acidophilus* and *L. gasseri* strains used as positive controls did not show bacteremia risk [126]. In addition, many bacteremia cases related to *L. rhamnosus* strains were investigated in the literature [120]. It has also been shown in the literature that there are many fungemia cases of the *S. boulardii* fungus and 8 of cases associated with diarrhea resulted in death [127].

Another potential problem with probiotics is SIBO (small intestine bacteria overgrowth), in which microorganisms living in the large intestine start to grow in the small intestine. Types of microorganisms living in the small and large intestines are mostly different because these organs have different conditions. SIBO is also associated with IBS because the symptoms are similar, and the cause is unknown. In a study, it was determined that SIBO might be associated with the use of probiotic bacteria. After discontinuing probiotic use, improvement in the symptoms of patients was observed [128,129].

1.3. Characterization of Probiotics

Not all foodborne microorganisms are probiotics. In order to be classified as probiotic, microorganisms should bear specific properties, these properties are [13,20,130-132];

i. They should be available for human consumption, and preparations should not be pathogenic or produce toxins.
ii. They must be stable and resistant to low pH and high bile salts because of high acidity in the stomach and high salt content in duodenum fluids.
iii. They show strong adhesion abilities to intestinal epithelial cells and can compete with pathogens to adhere to the intestinal epithelium.

iv. They should be susceptible to antibiotics, not to transfer resistance to pathogens.
v. They should protect their viability during mass production for commercial purposes.
vi. Probiotics should be tested in at least one clinical trial that is accepted by scientific standards.
vii. They should be able to strengthen the immune system against food allergens and pathogens.
viii. They should keep the quality of the food to which it is added.
ix. Probiotics should be able to regulate the digestive system and increase the bioavailability of nutrients.
x. They should have an antagonistic effect on pathogenic bacteria.
xi. They should produce antimicrobial substances.

These features need to be tested, and according to the results, microorganisms should be classified. For this reason, we have performed some tests to distinguish the probiotic properties of our bacterial isolates.

1.3.1 Acid and Bile Salt Tolerance

In order for a bacterial strain to be accepted as a probiotic, it is expected to survive and multiply through extreme conditions of the human digestive system. In order for this to be tested, harsh conditions in the digestive tract must be simulated. It is known that when food is consumed, it generally reaches the end of our stomach between 1 and 3 hours [133]. For this reason, experiments are usually carried out between 1-3 hours [134,135]. Especially, low pH caused by stomach acid and the antimicrobial environment created by bile salts should be mimicked and the viability of bacteria needs to be determined. It is known that the acidity of gastric juice is between pH 2.0 and pH 3.0 due to strong acids such as HCl [136]. Later the stomach, foods are exposed to bile salts, even though bile facilitates the digestion of food, it makes it difficult to survive of the bacteria, because of the potential detergent-like antimicrobial bile salts. PBS or media containing 0.3% and 0.5% bile salts are used in the studies [134,135,137]. It is also difficult to attach to intestinal epithelial cells for Gram-positive bacteria, whose membrane structure is disrupted due to bile salts. In addition, bacteria die due to leakage from the cell membrane and DNA damage caused by bile salts [138].

1.3.2 Identification of Isolates at Species Level by 16S rRNA Sequencing

16S ribosomal RNA is a structure that participates in the 30S ribosomal subunit of prokaryotic microorganisms and contributes to protein synthesis. It binds to the S1 and S21 proteins to initiate translation, and also recognizes the start codon. Additionally, it binds to the Shine-Dalgarno sequence with its complementary regions [139]. It interacts with the 23S rRNA and helps to bind the 30S and 50S subunits to form the 70S ribosomal complex [140]. The gene encoding this rRNA is the 16S rRNA gene, which has approximately 1500 bp length [141]. 16S rRNA gene has highly conserved regions within species, it can be sequenced to construct a phylogenetic tree in evolutionary studies. It contains 9 hypervariable regions (V1-V9), and these regions contain species-specific sequences [142,143]. For the same reasons, species of microorganisms in the human microbiota can be determined by 16S rRNA sequencing. Conserved sequences between hypervariable regions facilitate the design of universal primers suitable for all species. It is much cheaper, simpler and faster than whole genome sequencing. For these reasons, it is often preferred in microbiota analysis studies [139].

1.3.3 Adhesion to Caco-2 Cells

Bacteria must adhere to the intestinal epithelial cells to multiply in the intestine. Microorganisms that can adhere to the intestinal mucosa have the ability to grow and colonize there. In addition, bacteria with adhesion ability can inhibit the invasion and colonization of harmful bacteria in the gut [144]. LABs prevent the adhesion and proliferation of pathogenic bacteria in the intestine through various substances they produce. Secreted exopolysaccharides (EPS) have anti-bacterial, anti-viral, anti-biofilm and anti-inflammatory activities [73]. Also, 2-hydroxyisocaproic acid which produced by LABs, prevents pathogenic bacteria from forming a biofilm [145]. Some factors that determine probiotics' adhesion ability are auto-aggregation and co-aggregation properties. Auto-aggregation is the colonization ability of only probiotic bacteria and is effective in the adhesion of probiotics to intestinal epithelial cells. Co-aggregation, on the other hand, refers to the colonization of probiotics in the intestine along with pathogens. Mechanisms that facilitate the attachment of probiotics to intestinal epithelial cells in auto-aggregation have the opposite effect for pathogens in

co-aggregation and function as a defense mechanism by preventing the adhesion of pathogens [146].

In vitro adhesion assays are usually performed by using the Caco-2, HT-29 and T84 cell lines. Caco-2 cells were derived from colorectal adenocarcinoma cells of a 72-year-old male having cancer and is the most commonly used cells for probiotic studies. Caco-2 cells form a homogeneous monolayer similar to human mature enterocytes in the small intestine, they also form crypts, which are typical structures of the epithelial monolayer [133,144,147].

1.3.4 Antimicrobial Activity Assay

Some of probiotics can produce bacteria-killing substances which is known as bacteriocins. Despite their narrow-spectrum, these compounds might be effective in hindering growth or colonization of antibiotic-sensitive or resistant pathogenic bacteria in gastrointestinal tract. Because of that, favoring the utilization of these bacteriocin-producer bacteria-containing probiotic foods is important. Antimicrobial properties of these probiotic bacteria should be investigated. For this purpose, Kirby-Bauer disk diffusion assay, agar well diffusion assay, minimum inhibitory concentration (MIC) assay, agar and broth dilution test and time-kill assay can be applied [136,148]. Because of simplicity and cost-effectiveness, the most used technique is disk diffusion assay which is known as Kirby-Bauer Test developed in 1940s. While not all bacteria can be tested with this technique, most can [149]. And this technique has the most standardization among others. These standards which have been accepted worldwide, determined by CLSI (Clinical and Laboratory Standards Institute) and EUCAST (The European Committee on Antimicrobial Susceptibility Testing) and these standards are updated periodically [150,151].

Special discs with a diameter of 6 mm are used for the test. Those who want to prepare the discs themselves should use Whatman filter paper. Mueller Hinton Agar (MHA) is a suitable medium for the test, but enriched MHA can be used for fastidious bacteria. Agar should be 4±0.5 mm thick and 25 ml of medium should be used for a 9 cm diameter petri dish. The turbidity of the bacteria to be inoculated on the medium should be equivalent to the 0.5 McFarland standard (with exceptions), corresponding to approximately $1.5x10^8$ cells/ml and absorbance value of approximately 0.1 at 625 nm wavelength [150]. Bacterial inoculation should be done with the help of cotton

swab, and bacteria should be spread in lawn formation to the medium. Discs should be placed on the agar in 15 minutes after incubation of the bacteria and the distance between the discs should be at least 2.5 cm. 15 minutes after the placement of the discs, the media should be placed in 35 ±2°C incubator. Results are obtained 16-20 hours after incubation and the zone of inhibition is measured by a ruler or caliper. Measured diameters should be evaluated according to performance standards declared by CLSI and EUCAST [150-152].

1.4. Aim of The Thesis

The importance given to the consumption of probiotic foods is increasing with the number of studies in this field [5,6]. It has been proven in many studies that the way to have a healthy body is to have a healthy microbiota [2,3,13,14]. The enhancement in the market share of probiotic supplements is one of the indicators of this situation [7,8]. It is significant to characterize the probiotic bacteria content of traditional foods that have not been studied before, which are especially rich in probiotics and consumed frequently for many years by people. In this study, the durability of lactic acid bacteria isolated from boza, pickled beetroot, sourdough, and tarhana foods under high acidity (pH 2.0 and 3.0), high bile salt (0.3%, 0.5%) conditions will be tested, their species will be detected, their adhesion to intestinal cells will be determined, and their antimicrobial properties will be examined.

Chapter 2

Materials and Methods

2.1. Materials

25 bacterial strains isolated from traditional food products (pickled beetroot-PT, einkorn sourdough-ES, Turkish-type boza-TB, Bulgarian-type boza-BB, Tarhana-T,H,N) from our lab culture collection, MRS and M17 medium purchased from Merck KGaA (Germany), phosphate buffered saline (PBS), HCl, NaOH, bile salts purchased from Sigma Aldrich (MO, USA), Caco-2 cells, kindly provided from METU (Banerjee Lab), Dulbecco's Modified Eagle's Medium (DMEM), 0.1% sterile peptone water, 0.025% Trypsin-EDTA solution (Thermo Fisher Scientific, MA, USA), *Escherichia coli* supernatant (ATCC 25922), Luria-Bertani (LB) medium, Bacterial Genomic DNA Miniprep Kit (Axygen, CA, USA), 6 different pathogenic bacterial strains (*Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, *Proteus mirabilis* ATCC 14153), Bacteriocin producer *Enterococcus faecalis* 7-3 bacterial strain and reference pathogenic strain kindly provided by Uludağ University (Sine Özmen Toğay Lab).

2.2. Acid and Bile Salt Tolerance

Low pH in the stomach and high bile salts in the duodenum were simulated using PBS. For the low pH test, the pH of the PBS was adjusted to 2.0 and 3.0 using 1M HCl and NaOH. For the high bile salt test, PBS was adjusted to contain 0.3% and 0.5% bile salt (w/v). Actively growing bacterial cultures are transferred into PBS with pH 2.0 and pH 3.0 and bile salt concentrations of 0.3% and 0.5%, and the control group at pH 7.2 [134,137]. Bacterial suspensions are incubated for 1 and 3h at 37ºC. After the incubation process, 200 µl of 10^{-7} diluted samples were pour plated into

MRS and M17 agar medium and incubated overnight at 37ºC. After the overnight incubation, colonies were counted, and the number of surviving bacteria was determined and compared with the control group. The formula used to find the number of bacteria in the main experimental stock was determined as "Count of Bacterial Colonies x Dilution Factor" formula. And viability of strains determined as “Number of Experimental Group/Number of Control Group” formula. These tests are done on 25 different probiotic strains. All experiments were replicated including the control group. At the end of the experiments, the most promising 10 strains were selected for further studies. These strains and reasons for the selection are shown in Table 3.2.

2.3. Identification of Isolates at Species Level by 16S rRNA Sequencing

To determine species of probiotic strains, DNAs are isolated from 6 of 10 selected strains. MRS N-1, T-2, M17 N-2 and N-3 strains were already identified by Cebeci Aydın et al. (2020) and names of these species are *P. pentosaceus* (MRS N-1), *E. dispar* (MRS T-2), and *E. faecium* (M17 N-2 and N-3) [66].

For isolation, Bacterial Genomic DNA Miniprep Kit (Axygen, CA, USA) is used and isolation performed by following the manufacturers' instructions. 16S rRNA gene is amplified by using SimpliAmp Thermal Cycler (Thermo Fisher Scientific, MA, USA) device. Forward primer (5’-ATCCGAGCTCAGAGTTTGATCCTGGC-3’) and reverse primer (5’-TCAGGTCGACGCTACCTTGTTACGAC-3’) (9699, 9700 Primers, Oligomer, Ankara, Türkiye) used in PCR reaction.

Amplification conditions are; 95ºC for 2 min, then 35 cycles at 95ºC for 1 min, 58ºC for 1 min and 72ºC for 1 min. The purity and concentration of DNA samples measured by NanoDrop (Thermo Fisher Scientific, MA, USA). Agarose gel electrophoresis is applied to check the amplification on the gel. PCR products were sent to Medsantek Company (İstanbul/Türkiye) for sequencing [66].

Table 2. 1 Components and concentrations of PCR for 16S rRNA sequencing.

16S rRNA Amplification (50 µl)	
Components	Amounts (µl)
10X Ammonium Buffer	5
$MgCl_2$ (25 µM)	1
9699 Forward Primer (10 µM)	1.25
9700 Reverse Primer (10 µM)	1.25
dNTP (25 µM)	0.5
Taq Polymerase (500U)	0.75
DNA	Up to 500 ng/µl
Nuclease-free water	Up to 50 µl

2.4. Adhesion to Caco-2 Cells

10 selected strains were used to test the adherence ability of probiotic bacterial cells. $1x10^5$ Caco-2 cells are planted on 24-well plate and incubated at 37ºC containing 5% CO_2 for 16 days for post-confluency. Medium (supplemented DMEM) is changed in every 2-3 days. 1h before the experiment, the medium is washed two times with 0.1% sterile peptone water and replaced with non-supplemented DMEM. At the end of 1h of incubation, cells are washed two times with 0.1% sterile peptone water and replaced with 500 µl non-supplemented DMEM containing approximately $1x10^8$ two days overnight incubated probiotic bacterial cell precipitate (3500g x 10 min) for 2 hours at 37ºC. Then, wells are washed with 0.1% sterile peptone water twice. 100 µl 0.025% Trypsin-EDTA solution was added to each well and incubated for 15 min at room temperature [134]. Lysates are collected and serially diluted with PBS to obtain an adequate number of colonies on agar plates. 200 µl from each diluted sample pour plated on MRS and M17 agar plates and incubated for 48 hours at 37ºC. At the end of the incubation process, colony numbers are counted and the total number of each strain in the main stock is calculated. All experiments were performed in duplicates including the control group [153].

2.5. Antimicrobial Activity Assay

To test the antimicrobial activity of selected probiotic bacteria, disk diffusion assay was performed. As a positive control, kanamycin sulphate (5 mg/ml) and one known bacteriocin producer *Enterococcus faecalis* 7-3 strain and a reference pathogenic strain *S. aureus* ATCC 6538 (kindly provided from Uludağ University) are used [154]. Pathogenic bacterial suspensions (adjusted to 0.5 McFarland standard) *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, *Proteus mirabilis* ATCC 14153 were planted on Luria Bertani (LB) agar plates by using cotton swab and petri plates divided into 6 regions by using marker [150,155]. After solidification of agar, 0.6 cm diameter Whatman paper disks were placed at the center of each agar plate region. Each disk was incubated with 50 µl of probiotic bacterial culture supernatant (precipitated at 10500g for 10 minutes). Empty and MRS broth containing disks were used as negative control. Agar plates were incubated overnight at 37ºC. After the incubation process, the diameters of the zone of inhibition were measured by a ruler to determine antimicrobial activity. All experiments were performed in duplicates including the control groups [148,149].

Chapter 3

Results and Discussions

3.1. Acid and Bile Salt Tolerance

Probiotics are exposed to an environment with high acidity in the stomach and high bile salts in the duodenum while passing through the gastrointestinal tract. In the meantime, it is important for colonization to stay alive and reach the colon. For this reason, in order to determine the growth of the existing isolates in different pH and bile salt environments, acid is adjusted to pH 2.0 and pH 3.0, and bile salt concentrations are adjusted to 0.3% and 0.5% (w/v). All bacteria were incubated for 1 and 3 hours contain control (pH 7.2), pH 2.0, pH 3.0, 0.3% bile salt (w/v), 0.5% bile salt (w/v), respectively.

All results can be seen in Table 3.1 and the graphs of the 10 strains with the highest potential are given in Figures 3.1-3.10. And the reasons for selecting these strains are given in Table 3.2.

For Table 3.1, orange coloration indicate the viability of the bacteria is under 20%, gray coloration indicate the viability of the bacteria is between 20-50%, blue coloration indicate viability of the bacteria is between 50-80%, green coloration indicate the viability of the bacteria is between 80-100%, and dark green coloration indicate the viability of the bacteria is over 100% for this certain condition. Yellow coloration only indicate the control.

According to the results from Table 3.1; 4 of 25 strains showed high viability at pH 2.0 for 1 and 3 hours: MRS ES-7, MRS ES-11, MRS ES-17, and MRS N-1. All 25 strains have shown high viability at pH 3.0 for 1 and 3 hours, especially, 15 strains; MRS ES-2-3-7-11-12-17, MRS PT-2-14-16, MRS N-1, MRS EB-3, MRS T-1, M17 N-2-3-4 showed higher viability than the control groups. 12 of 25 strains; MRS ES-

1-2-3, MRS PT-1-2-14-16, MRS T-1-2-7 and M17 N-1-3 showed no viability at pH 2.0 for 3 hours of incubation. 5 of 25 strains; M17 TB-1-2, M17 BB-7, MRS EB-3, M17 N-4 showed no viability at pH 2.0 for 1 and 3 hours of incubation.

8 of 25 strains have showed viability at 0.3% bile salt for 1 and 3 hours (see Table 3.1): MRS ES-3, MRS PT-14, MRS T-2, M17 H-1, M17 N-3-4, M17 TB-1-2. 7 of 25 strains have shown viability at 0.5% bile salt for 1 and 3 hours; MRS ES-3, MRS T-2, M17 H-1-2, M17 N-3-4, M17 TB-1. Especially, M17 N-3-4 and M17 TB-1-2 strains showed higher viability at 0.3% and 0.5% bile salt conditions than other strains. 10 strains; MRS ES-2-11-17, MRS PT-1-2-16, MRS T-1-7, MRS N-1 and M17 EB3 showed no viability at 0.3% and 0.5% bile salt conditions for 1 and 3 hours of incubation. MRS ES-2 and MRS T-1 showed no viability at 0.3% and 0.5% bile salts for 3 hours of incubation. MRS ES-7 showed viability only at 0.3% bile salt for 1 hour of incubation.

When microorganisms are in a highly acidic environment such as pH 2 and pH 3, cells lose their ability to maintain homeostasis and start proton leakage, which lowers intracellular pH. By affecting cellular metabolic processes, it triggers protein denaturation, disruption of glycolytic enzymes, DNA and membrane damage [156,157]. Thus, the continued flow of protons causes cellular energy depletion and ultimately lead cell to death [158].

At very low pH levels, there is a small but sufficient amount of undissociated HCl in the medium of the cell, and since HCl has no charge, it can easily move across the cell membrane to the cytoplasm. When undissociated HCl passes into the cytoplasm, it can dissociate in cytosol due to the higher pH in the cell, thereby acidifying the cytoplasm [158]. It is unclear how protons get into bacteria since membranes are not permeable to charged particles. It is thought that protons can enter by using membrane protein channels or damaging the lipid bilayer due to the high proton concentrations [156]. Bacteria and yeasts can cope with acidic environments via various mechanisms, including activation of H^+-ATPases, cell membrane remodeling, intracellular proton consumption (glutamate decarboxylation) or alkali production (urease or arginine deiminase activities) [157,159,160].

Table 3. 1 Number of bacteria for acid and bile salt tests.

Cell Line	Incubation Conditions	1 Hour Viability	1h Viability (% to control)	3 Hour Viability	3h Viability (% to control)	Viability Change (% 3h to 1h)
MRS ES-2	Control	5,83E+07	Control	4,33E+07	Control	74,2
	pH 2.0	6,75E+06	11,6	0	0	0
	pH 3.0	4,45E+07	76,4	5,98E+07	138,2	134,3
	Bile 0.3%	0	0	0	0	0
	Bile 0.5%	0	0	0	0	0
MRS ES-7	Control	2,05E+08	Control	3,01E+08	Control	146,6
	pH 2.0	1,53E+08	74,4	2,07E+08	68,9	135,7
	pH 3.0	2,65E+08	129,1	2,75E+08	91,3	103,7
	Bile 0.3%	7,25E+06	3,5	2,50E+05	0,1	3,4
	Bile 0.5%	2,50E+05	0,1	0	0	0
MRS ES-11	Control	4,50E+07	Control	4,73E+07	Control	105
	pH 2.0	4,38E+07	97,2	4,18E+07	88,4	95,4
	pH 3.0	3,88E+07	86,1	4,45E+07	94,2	114,8
	Bile 0.3%	0	0	0	0	0
	Bile 0.5%	0	0	0	0	0
MRS ES-17	Control	5,78E+07	Control	6,70E+07	Control	116
	pH 2.0	6,60E+07	114.3	7,33E+07	109,3	111
	pH 3.0	1,60E+08	276.6	1,70E+08	253	106,1
	Bile 0.3%	0	0	0	0	0
	Bile 0.5%	0	0	0	0	0
MRS PT-1	Control	1,57E+08	Control	2,56E+08	Control	162,8
	pH 2.0	1,14E+08	72,2	0	0	0
	pH 3.0	1,87E+08	119,1	1,10E+08	42,9	58,6
	Bile 0.3%	0	0	0	0	0
	Bile 0.5%	0	0	0	0	0
MRS PT-2	Control	2,58E+07	Control	3,35E+07	Control	130,1
	pH 2.0	2,30E+07	89,3	0	0	0
	pH 3.0	4,28E+07	166	3,45E+07	103	80,7
	Bile 0.3%	0	0	0	0	0
	Bile 0.5%	0	0	0	0	0
MRS PT-16	Control	5,45E+07	Control	7,50E+07	Control	137,6
	pH 2.0	4,08E+07	74,8	0	0	0
	pH 3.0	9,65E+07	177,1	8,10E+07	108	83,9
	Bile 0.3%	0	0	0	0	0
	Bile 0.5%	0	0	0	0	0

Table 3. 1 Continued

Cell Line	Incubation Conditions	1 Hour Viability	1h Viability (% to control)	3 Hour Viability	3h Viability (% to control)	Viability Change (% 3h to 1h)
MRS N-1	Control	5,75E+06	Control	4,50E+06	Control	78,3
	pH 2.0	1,63E+07	282,6	4,50E+06	100	27,7
	pH 3.0	2,35E+07	408,7	2,40E+07	533,3	102,1
	Bile 0.3%	0	0	0	0	0
	Bile 0.5%	0	0	0	0	0
M17 N-1	Control	3,19E+08	Control	1,81E+08	Control	56,8
	pH 2.0	1,93E+07	6	0	0	0
	pH 3.0	2,84E+08	88,9	6,75E+07	37,2	23,8
	Bile 0.3%	5,75E+06	1,8	1,75E+06	1	30,4
	Bile 0.5%	3,25E+06	1	1,75E+06	1	53,8
M17 N-2	Control	1,32E+08	Control	1,72E+08	Control	130,3
	pH 2.0	7,33E+07	55,5	5,00E+05	0,3	0,7
	pH 3.0	2,99E+08	226,5	2,82E+08	164	94,3
	Bile 0.3%	8,48E+07	64,2	6,33E+07	36,8	74,6
	Bile 0.5%	7,93E+07	60	6,05E+07	35,2	76,3
M17 N-3	Control	4,15E+07	Control	5,85E+07	Control	141
	pH 2.0	5,25E+06	12,7	0	0	0
	pH 3.0	8,73E+07	210,2	8,50E+07	145,3	97,4
	Bile 0.3%	2,78E+07	66,9	1,25E+07	21,4	45
	Bile 0.5%	1,18E+07	28,3	1,00E+07	17,1	85,1
M17 N-4	Control	1,71E+08	Control	1,83E+08	Control	107
	pH 2.0	0	0	0	0	0
	pH 3.0	2,52E+08	147,4	1,47E+08	80,3	58,3
	Bile 0.3%	2,35E+08	137,4	1,75E+08	95,6	74,5
	Bile 0.5%	1,06E+08	61,7	2,83E+07	15,4	26,8
MRS T-2	Control	3,20E+07	Control	1,95E+07	Control	60,9
	pH 2.0	4,75E+06	24,4	0	0	0
	pH 3.0	2,60E+07	81,3	3,50E+06	17,9	13,5
	Bile 0.3%	2,03E+07	103,8	2,50E+05	0,8	1,2
	Bile 0.5%	3,25E+06	16,7	0	0	0
M17 H-1	Control	2,32E+08	Control	1,52E+08	Control	65,5
	pH 2.0	1,75E+06	0,8	5,75E+06	3,8	328,6
	pH 3.0	2,41E+08	103,9	9,00E+07	59,2	37,3
	Bile 0.3%	3,40E+07	14,7	3,78E+07	24,8	111
	Bile 0.5%	2,50E+07	10,8	2,18E+07	14,3	87

Table 3. 1 Continued

Cell Line	Incubation Conditions	1 Hour Viability	1h Viability (% to control)	3 Hour Viability	3h Viability (% to control)	Viability Change (% 3h to 1h)
M17 H-2	Control	3,82E+08	Control	1,07E+08	Control	28
	pH 2.0	5,00E+07	13,1	5,25E+06	4,9	10,5
	pH 3.0	4,07E+08	106,5	7,35E+07	68,7	18,1
	Bile 0.3%	4,98E+07	13	7,50E+06	7	15,1
	Bile 0.5%	7,25E+06	1,9	4,00E+06	3,7	55,2
M17 TB-1	Control	2,71E+08	Control	2,96E+08	Control	109,2
	pH 2.0	0	NA	0	0	0
	pH 3.0	6,90E+08	254,6	3,32E+08	112,2	48,1
	Bile 0.3%	1,93E+08	71,2	1,30E+08	43,8	67,1
	Bile 0.5%	2,00E+08	73,8	1,18E+08	39,9	59
M17 TB-2	Control	8,78E+07	Control	8,70E+07	Control	99,1
	pH 2.0	0	0	0	0	0
	pH 3.0	2,13E+08	242,7	8,80E+07	101,1	41,3
	Bile 0.3%	1,29E+08	146,4	5,05E+07	58	39,3
	Bile 0.5%	7,03E+07	80,1	1,63E+07	18,5	23,1
MRS ES-1	Control	1,12E+08	Control	4,33E+07	Control	38,5
	pH 2.0	6,20E+07	55,2	0	0	0
	pH 3.0	9,00E+07	80,2	4,55E+07	105,2	50,6
	Bile 0.3%	7,15E+07	63,7	0	0	0
	Bile 0.5%	6,50E+07	57,9	0	0	0
MRS ES-3	Control	1,32E+08	Control	8,43E+07	Control	63,8
	pH 2.0	6,10E+07	46,2	0	0	0
	pH 3.0	2,89E+08	218,9	4,40E+08	522,3	152,2
	Bile 0.3%	7,53E+07	57	1,70E+07	20,2	22,6
	Bile 0.5%	9,15E+07	69,3	1,90E+07	22,6	20,8
MRS ES-12	Control	4,28E+07	Control	3,05E+07	Control	71,3
	pH 2.0	1,00E+06	2,3	7,50E+05	2,5	75
	pH 3.0	4,90E+07	114,6	7,35E+07	241	150
	Bile 0.3%	2,75E+06	6,4	1,25E+06	4,1	45,5
	Bile 0.5%	1,00E+06	2,3	5,00E+05	1,6	50
MRS T-7	Control	1,43E+08	Control	9,33E+07	Control	65,2
	pH 2.0	2,25E+06	1,6	0	0	0
	pH 3.0	2,47E+08	172,7	1,41E+08	150,7	56,9
	Bile 0.3%	5,00E+05	0,3	0	0	0
	Bile 0.5%	5,00E+05	0,3	0	0	0

Table 3. 1 Continued

Cell Line	Incubation Conditions	1 Hour Viability	1h Viability (% to control)	3 Hour Viability	3h Viability (% to control)	Viability Change (% 3h to 1h)
M17 BB-7	Control	2,29E+08	Control	1,51E+08	Control	65,9
	pH 2.0	0	0	0	0	0
	pH 3.0	8,45E+07	36,9	1,05E+08	69,5	124,3
	Bile 0.3%	1,08E+07	4,7	5,75E+06	3,8	53,5
	Bile 0.5%	4,75E+06	2,1	5,75E+06	3,8	121,1
MRS EB-3	Control	1,61E+08	Control	2,08E+08	Control	129,2
	pH 2.0	0	0	0	0	0
	pH 3.0	1,91E+08	118,6	3,70E+08	177,9	193,7
	Bile 0.3%	0	0	0	0	0
	Bile 0.5%	2,50E+05	0,2	0	0	0
MRS PT-14	Control	6,00E+07	Control	3,95E+07	Control	65,8
	pH 2.0	2,75E+06	4,6	0	0	0
	pH 3.0	9,90E+07	165	8,50E+07	215,2	85,9
	Bile 0.3%	1,05E+07	17,5	4,25E+06	10,8	40,5
	Bile 0.5%	1,50E+06	2,5	1,50E+06	3,8	100
MRS T-1	Control	2,70E+07	Control	2,88E+07	Control	106,5
	pH 2.0	3,00E+06	11,1	0	0	0
	pH 3.0	4,00E+07	148,1	5,35E+07	186,1	133,8
	Bile 0.3%	5,00E+05	1,9	1,25E+06	4,3	250
	Bile 0.5%	2,50E+05	0,9	2,50E+05	0,9	100

Bile acid and salts can disrupt the structure of bacterial cell membranes, denature proteins, chelate metals such as iron and calcium, and cause DNA damage [161]. To tolerate bile acids and salts, probiotic microorganisms can synthesize required proteins, produce protective biopolymers, hydrolyze bile salts using a specific enzyme (BSH), change the composition of the cell membrane, efflux bile via membrane channel proteins and produce stress response proteins [162-164].

Apart from tolerating bile salts, deconjugation of bile salts is an important modification and one of the criteria for the selection of bacteria as a probiotic. Bile Salt Hydrolase (BSH), which catalyzes the deconjugation of glycine or taurine-linked bile salts to release free bile acids. This feature has evolved as a mechanism to protect the cell from harms of bile salts [165]. The difference between conjugated and unconjugated bile acids is the presence of glycine or taurine conjugation, which

lowers the pKa, increases water solubility and decreases lipophilicity, therefore conjugated bile salts can cause more damage to cellular components [166]. Deconjugation of bile salts may be a mechanism that reduces the toxicity of bile [161,167]. In addition, it has been suggested that deconjugation of bile salts plays a role in lowering blood cholesterol levels since conjugated bile salts aid the absorption of food cholesterol in the bowel and deconjugated bile acids are absorbed in low levels and eventually disposed in the feces [163,168,169]. Glycine or taurine is released during the deconjugation process and can provide energy for intestinal microorganisms [170].

In other similar studies in the literature, Manini et al. (2016), isolated *P. pentosaceus* strains from white bran sourdough showed 31.4% viability in 2 hours incubation at pH 2.5 and 49.9% viability in 0.3% Oxgall medium in 24 hours of incubation (37°C) [171]. According to Pinto et al. (2020), *P. pentosaceus* strains isolated from cheese showed 93.7% viability in a 2 hour incubation (37°C) at pH 2.5, while *P. pentosaceus* strains isolated from Cambodian fermented fish showed 96.9% viability under the same conditions [165]. In our study, it was known that MRS N-1 strain belongs to the *P. pentosaceus* species and showed 100% viability compared to the control group (pH 7.2) in 3 hour incubation at pH 2.0 (37°C). At pH 3.0, it showed much more viability than the control group in 3 hours of incubation. It is a significant difference to see such strong vitality at pH 2.0 and 3.0 conditions for *P. pentosaceus*, whose optimal pH conditions are known to be between 5.5-6.5 [172,173]. These results show that bacteria are highly adapted to acidic environments. On the other hand, N-1 strain showed no viability in 0.3% and 0.5% bile salt, indicating that the bile tolerance mechanisms in the strain were absent or ineffective.

Shehata et al. (2016), found that isolates of *L. bulgaricus, L. paracasei, L. rhamnosus* strains from boza (Egypt) showed 77% viability in 3 hours of incubation (37°C) at pH 2.0 [174]. Alkalbani et al. (2019) determined that the *E. durans* strain isolated from dried fish showed 81.8% viability after of 2h incubation at pH 2.0 [175]. Li et al. (2020), on the other hand, determined that the *E. durans* strain isolated from the duodenum showed 80.1% viability after of 4h incubation at pH 3.0 and showed 56.3% viability in 0.3% Oxgall for 4h [176]. In our study, TB-2 and BB-7 strains isolated from Turkish and Bulgarian-tpye boza which belong to *E. durans* and *E. faecium* respectively, did not show any viability at pH 2.0, while BB-7 strains showed

69.5% viability compared to the control group in 3 hours incubation at pH 3.0. TB-1 and TB-2 strain showed over 100% viability under the same conditions. In the same study conducted by Shehata et al, three strains showed 70.1-82.5% viability in 3 hours of incubation (37°C) in 0.3% Oxgall [174]. In our study, TB-1-2 and BB-7 strains showed 65.2%, 21.2% and 0.04% viability respectively in the same conditions (3h 37°C) in 0.3% bile salt.

Vasiee et al. (2014) isolated LABs (54 strains) from Iranian tarhana, only two *L. fermentum* and one *L. brevis* strains was found to be able to survive at pH 2.5. In the same study, it was found that *L. fermentum* and *P. pentosaceus* strains can tolerate low pH values. In the same study, two *L. fermentum* and one *L. plantarum* strains were found to survive in the presence of 0.4% bile salt [177]. In a master thesis study conducted by Ateş (2019), *P. pentosaceus* (13 strains), *L. brevis* (2 strains), *E. faecalis* (5 strains), *E. faecium* (2 strains) and *L. fermentum* (4 strains) isolated from tarhana were found to grow in the presence of 0.3% bile salt for 4 hours [178]. Petrovic et al. (2020), showed *E. faecium* (21 strains) isolated from Sokobanja sausage viable for 2 hours of incubation at pH 3.0 [179]. In this study, M17 N-2 and N-3 strains which belong to *E. faecium* species isolated from tarhana, showed no viability under pH 2 for 3h of incubation, but N-2 showed 55.5% viability under pH 2 for 1h of incubation. They showed 164% and 145% viability at pH 3 for 3h of incubation and this may indicate that low pH might favor the growth of bacteria or neutral pH (7.2) may inhibit the growth of the bacteria. For viability in bile salts, N-2 did not show any viability in bile salt medium, but N-3 showed 47.4% and 20% viability in 0.3 and 0.5% bile salt medium respectively.

In the study conducted by Tokatlı et al. (2015), 25 of 39 bacterial strains of *L. brevis, L. plantarum* and *P. ethanolidurans* species isolated from traditional pickles (Çubuk) showed viability between 33% and 85% for 4 hours of incubation at pH 2.5 (37°C). Bile salt tolerance of these 25 strains was measured in 0.3% Oxgall presence for 4 hours at 37°C, and 22 strains were found to have viability between 45% and 99%. Especially all 5 strains of *L. brevis* species showed viability between 94-99% [60].

In the study by Çakır et al. (2020), all 13 probiotic strains belonging to *L. plantarum, L. brevis, L. fermentum, L. curvatus, P. acidilactici* species isolated from

einkorn sourdough showed viability between 78% and 93% at pH 2.5 and all strains showed viability between 85% and 94% in 0.3% bile salt [180]. In the study performed by Doğan and Özpınar (2017), all 35 strains of *L. plantarum, L. brevis, E. faecium* and *L. paraplantarum* species isolated from boza, cheese, kefir and raw milk were viable at pH 2.5, while 8 strains were viable in 0.3% Oxgall [181]. In our study, ES-3, ES-7, PT-14 strains which belong to *L. plantarum* species, ES-3 and PT-14 strains showed no viability under pH 2 for 3h, but ES-3 showed 46.2% viability for 1h of incubation. And ES-7 strain showed 68.9% viability at pH 2 for 3h of incubation. And all three strains showed 522%, 91.5% and 215% viability, respectively. This may indicate growth of these *L. plantarum* strains favored by the low pH levels and maybe growth of bacteria is inhibited under neutral pH conditions (pH 7.2). According to the studies in the literature, their optimal growth pH is between 5.5-6.2 [182]. And previously explained studies about the *L. plantarum* strains support the idea that they can live well under low pH conditions.

According to study conducted by Succi et al. (2017), strains of L. plantarum can adopt low pH levels (pH 3.0 and 3.5) and show great viability [183]. And their viability in 0.3% bile salt are 20%, 0% and 10.8%, in 0.5% bile salt are 22.6%, 0%, 4% respectively. According to these results, it can be conferred bile salt tolerance of our isolates of *L. plantarum* species remains at low levels. For ES-11 strain which belongs to *L. brevis* species, viability at pH 2 was 68.9%, pH 3 was 91.5%, 0.3-0.5% bile salt was 0%.

According to the literature information, tolerance of *L. brevis* under low pH and high bile salts is high. However, in our study, although the acid tolerance was high, the bile salt tolerance was low [60,181].

No available studies on the probiotic properties of *Enterococcus dispar* were found in the literature.

As a result of the evaluations, 10 strains (MRS ES-3-7-11, MRS PT-14, MRS N-1, MRS T-2, M17 BB-7, M17 N-2-3, M17 TB-2) showing high viability under high acidic and bile salt conditions were selected for further studies.

Table 3. 2 Selected cell lines for further studies and reasons for selection.

Cell Line	Identified Species	Reason of Selection
MRS ES-3	*L. plantarum*	High acidity tolerance
MRS ES-7	*L. plantarum*	High acidity tolerance
MRS ES-11	*L. brevis*	High acidity tolerance
MRS PT-14	*L. plantarum*	High acidity tolerance
MRS N-1	*P. pentosaceus*	High acidity tolerance
MRS T-2	*E. dispar*	High bile salt tolerance
M17 BB-7	*E. faecium*	Increasing viability at pH 3.0 for 3h
M17 N-2	*E. faecium*	High acidity and bile salt tolerance
M17 N-3	*E. faecium*	High acidity and bile salt tolerance
M17 TB-2	*E. durans*	High acidity and bile salt tolerance

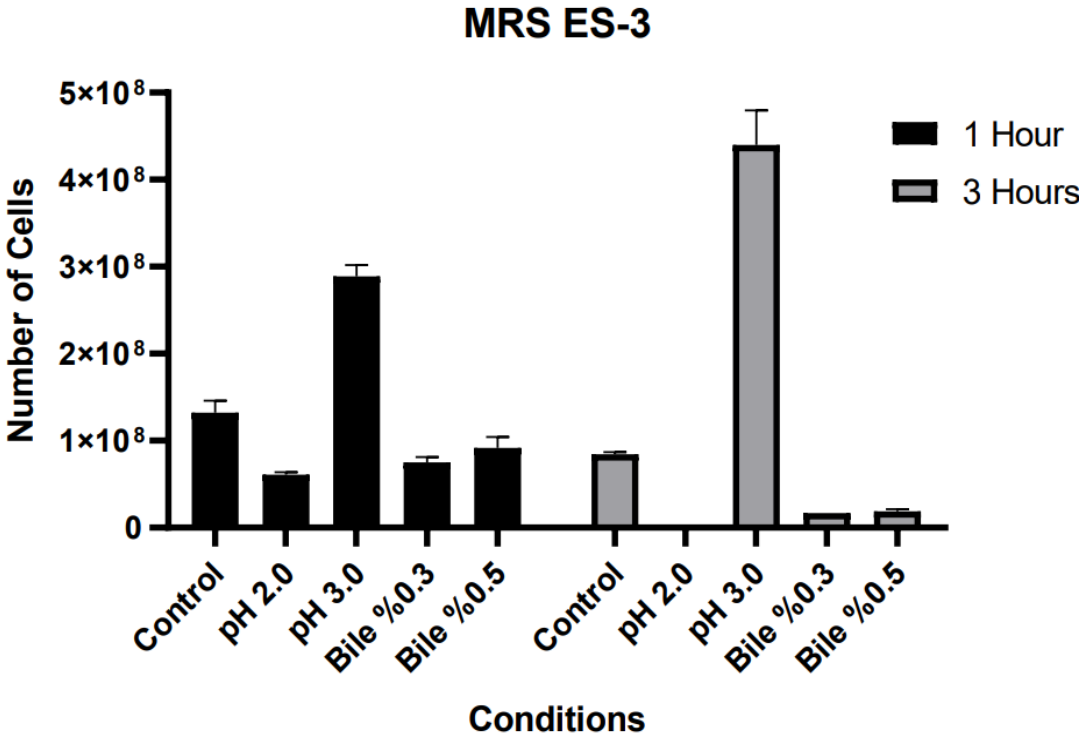

Figure 3. 1 Acid and bile salt tolerance tests for MRS ES-3 (*L. plantarum*).

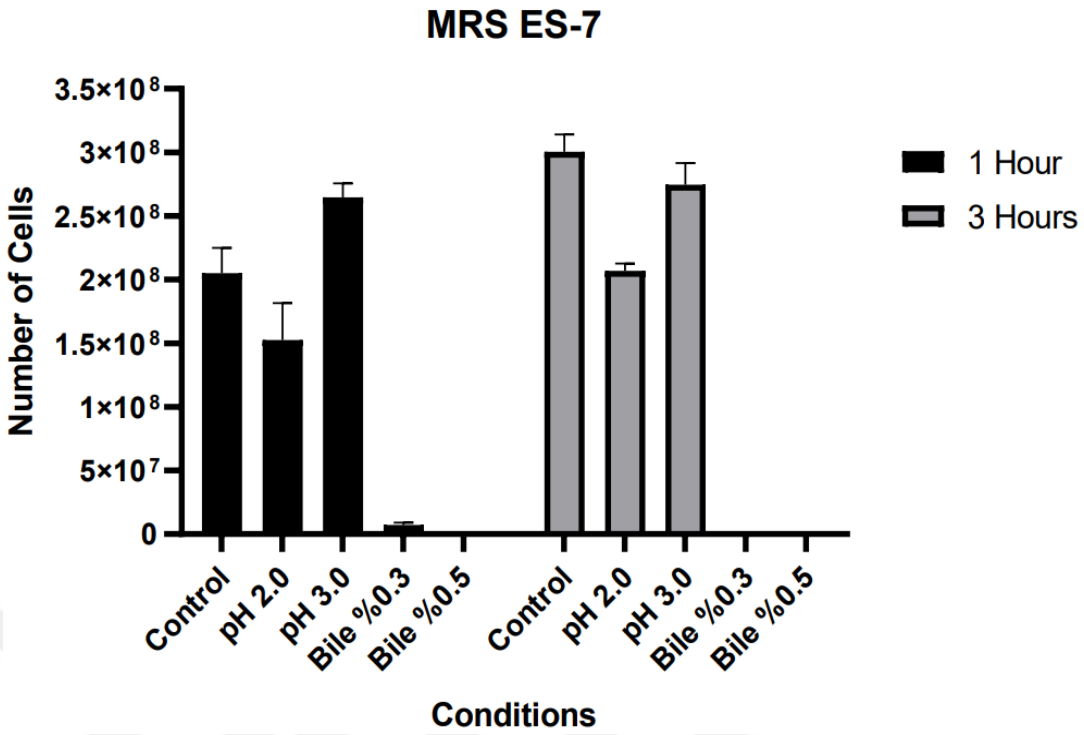

Figure 3. 2 Acid and bile salt tolerance tests for MRS ES-7 (*L. plantarum*).

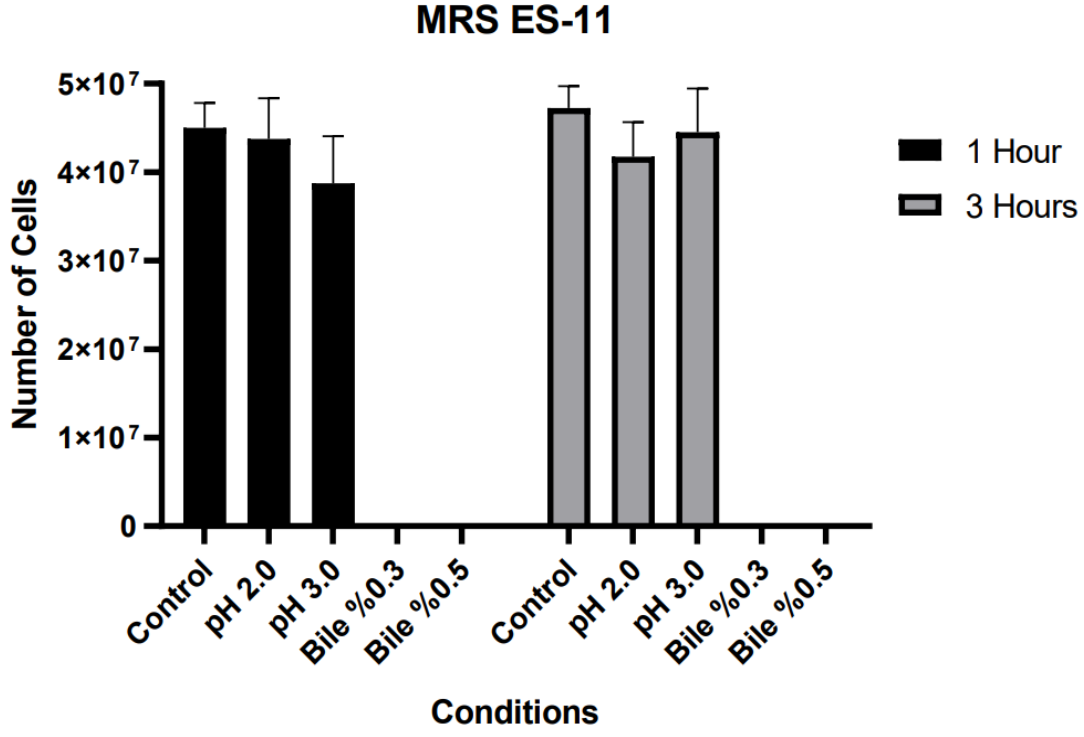

Figure 3. 3 Acid and bile salt tolerance tests for MRS ES-11 (*L. brevis*).

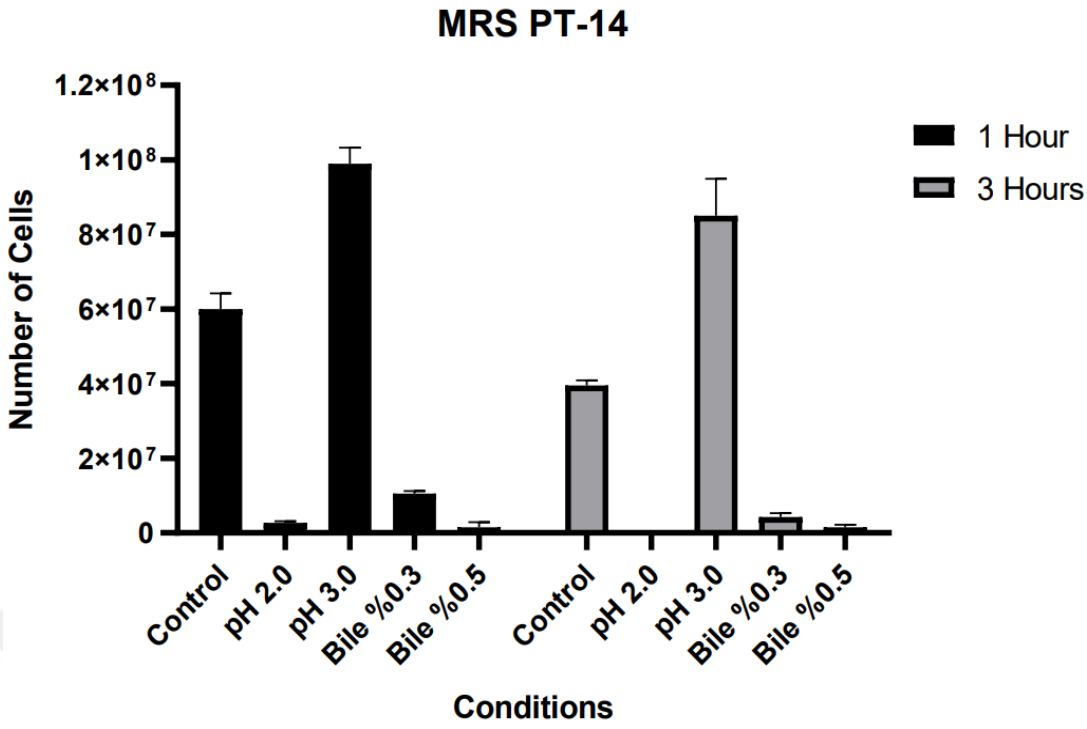

Figure 3. 4 Acid and bile salt tolerance tests for MRS PT-14 (*L. plantarum*).

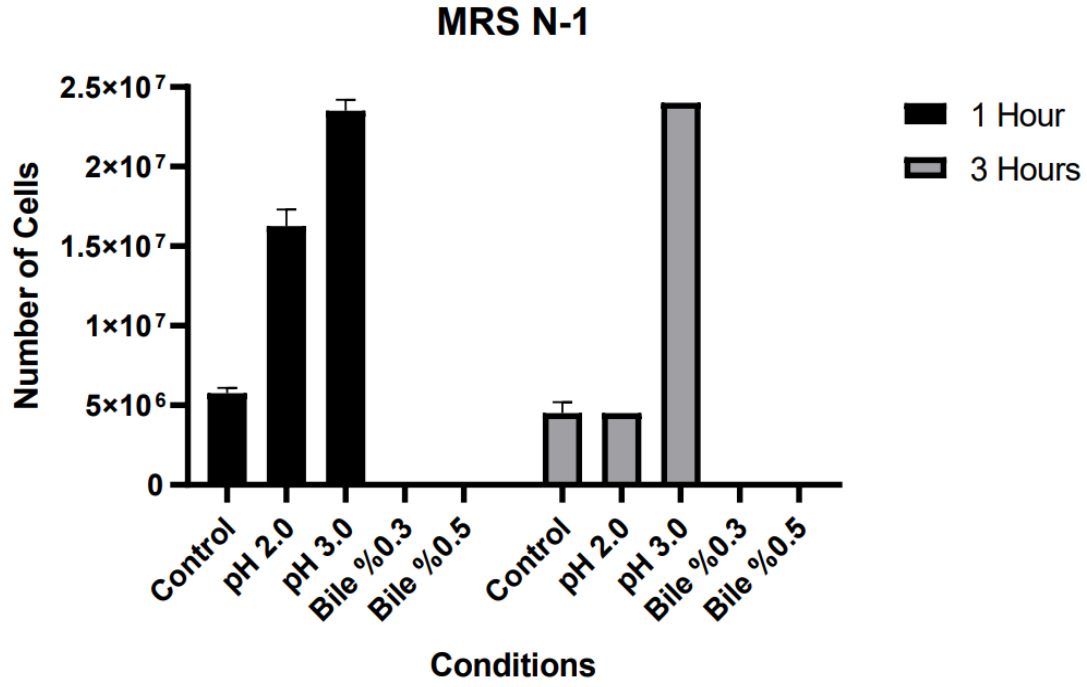

Figure 3. 5 Acid and bile salt tolerance tests for MRS N-1 (*P. pentosaceus*).

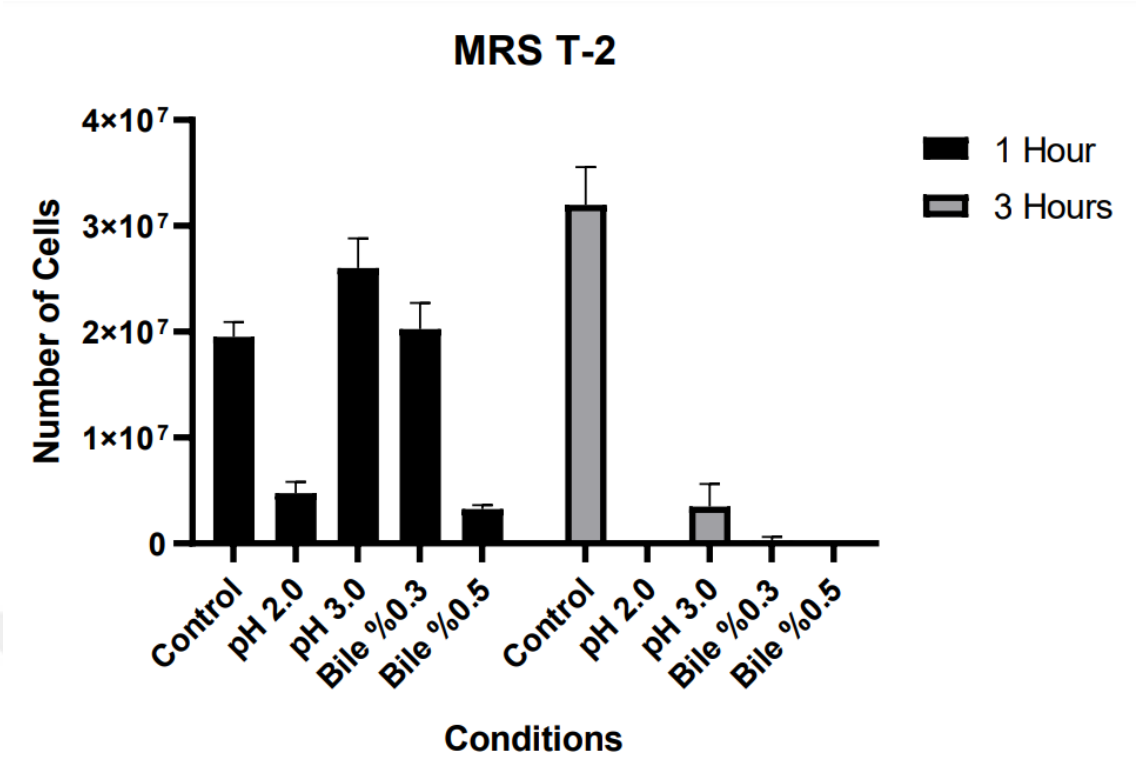

Figure 3. 6 Acid and bile salt tolerance tests for MRS T-2 (*E. dispar*).

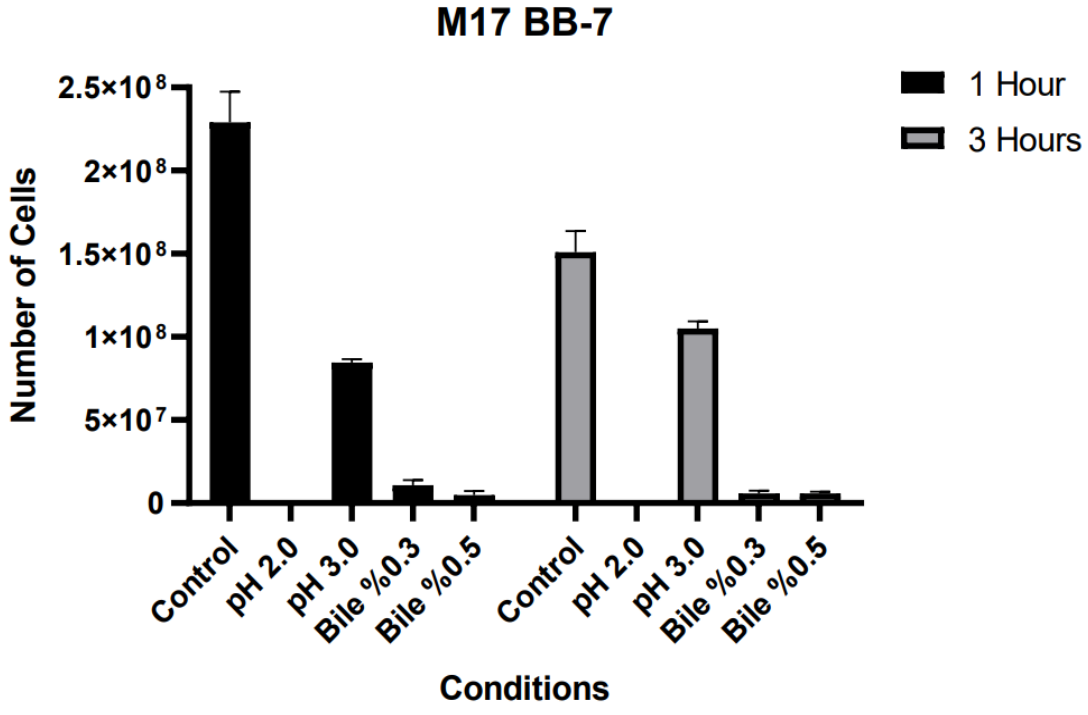

Figure 3. 7 Acid and bile salt tolerance tests for M17 BB-7 (*E. faecium*).

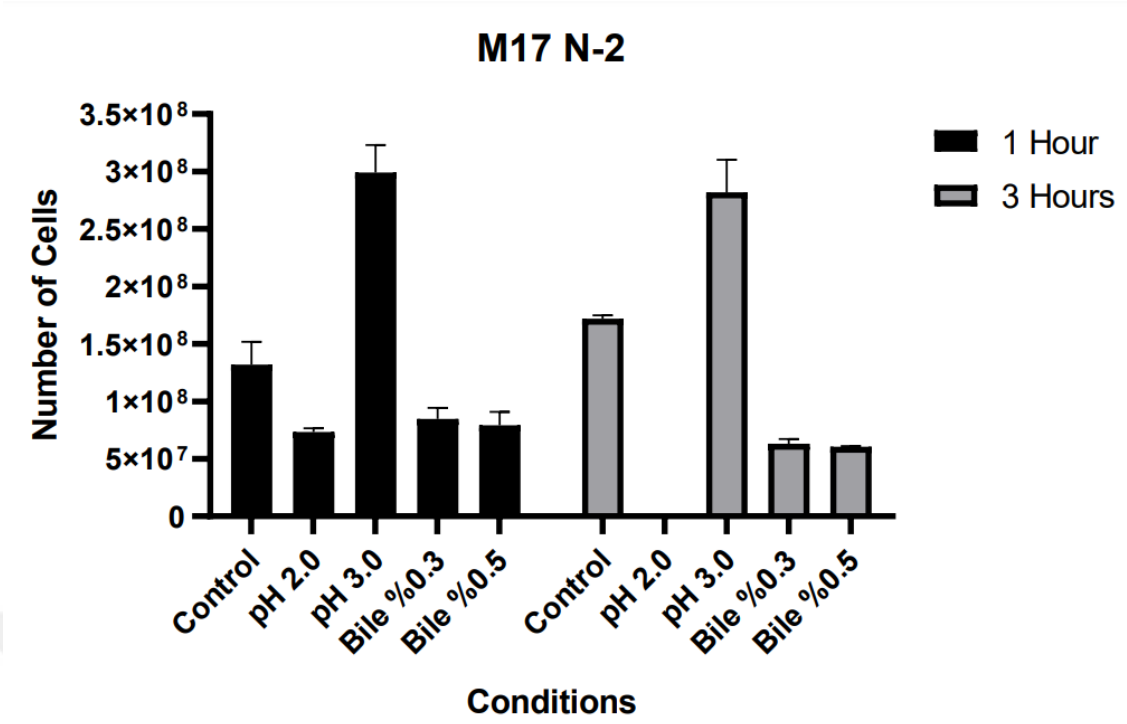

Figure 3. 8 Acid and bile salt tolerance tests for M17 N-2 (*E. faecium*).

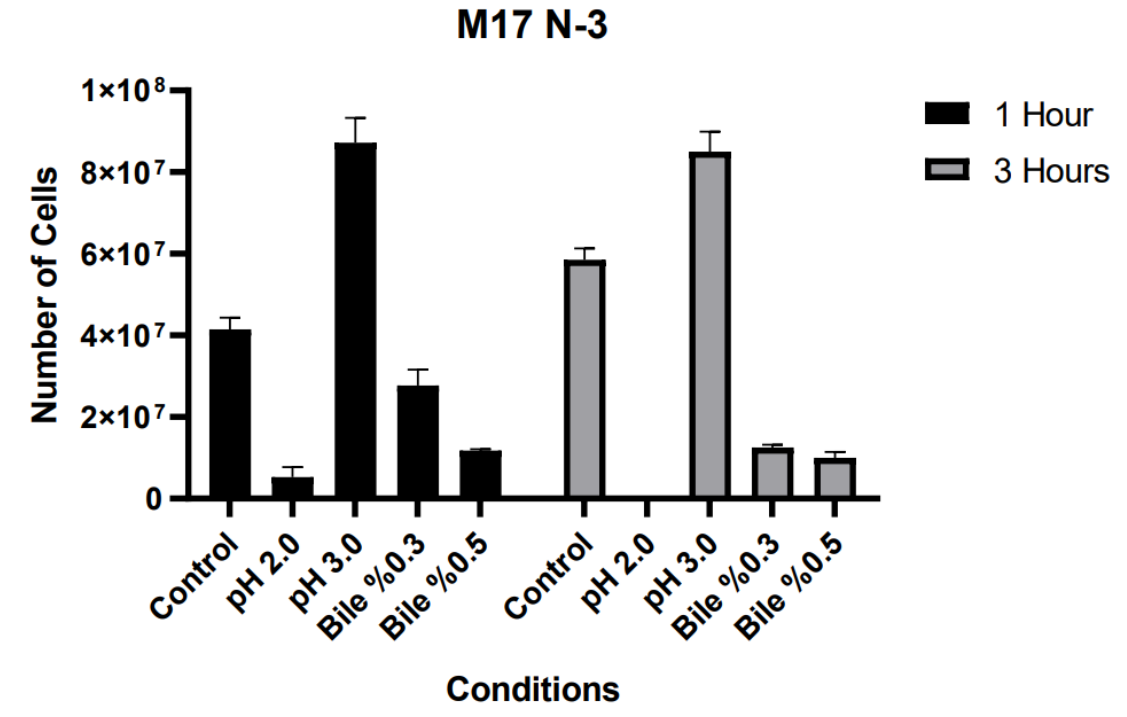

Figure 3. 9 Acid and bile salt tolerance tests for M17 N-3 (*E. faecium*).

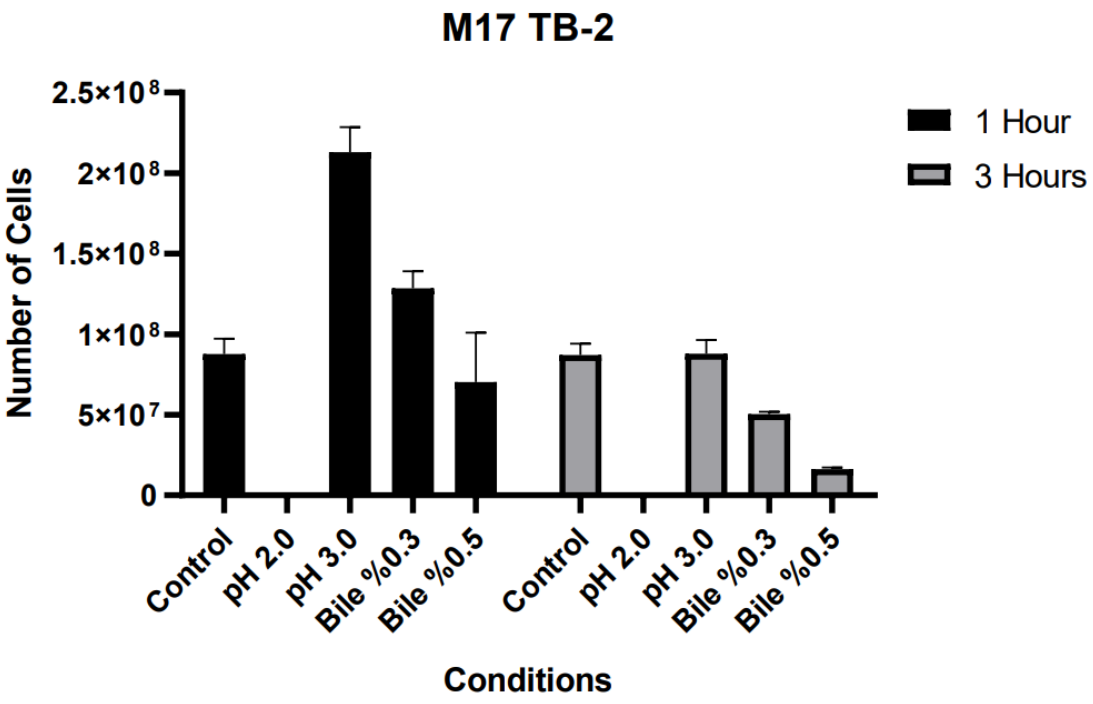

Figure 3. 10 Acid and bile salt tolerance tests for M17 TB-2 (*E. durans*).

3.2. Identification of Isolates at Species Level by 16S rRNA Sequencing

10 strains are selected for further assays because of their resistance to acid and bile salt tolerances. These strains are; MRS ES-3, MRS ES-7, MRS ES-11, MRS PT-14, MRS N-1, MRS T-2, M17 BB-7, M17 N-2, M17 N-3 and M17 TB-2. 6 of 10 samples' (MRS ES-3, ES-7, ES-11, PT-14, M17 BB-7, TB-2) DNA were isolated and amplified by PCR via 9699 and 9700 forward and reverse primers. DNA concentrations were measured by NanoDrop. Concentrations were, 170 ng/µl for ES-3, 140 ng/µl for ES-7, 210 ng/µl for ES-11, 170 ng/µl for PT-14, 150 ng/µl for BB-7, 170 ng/µl for TB-2. To check the amplification, agarose gel electrophoresis is performed. Relevant wells and bands can be seen in Figure 3.11, left to right; MRS ES-3, ES-7, ES-11, PT-14, M17 BB-7, TB-2. The upper third band of reference ladder corresponds to 1500 bp.

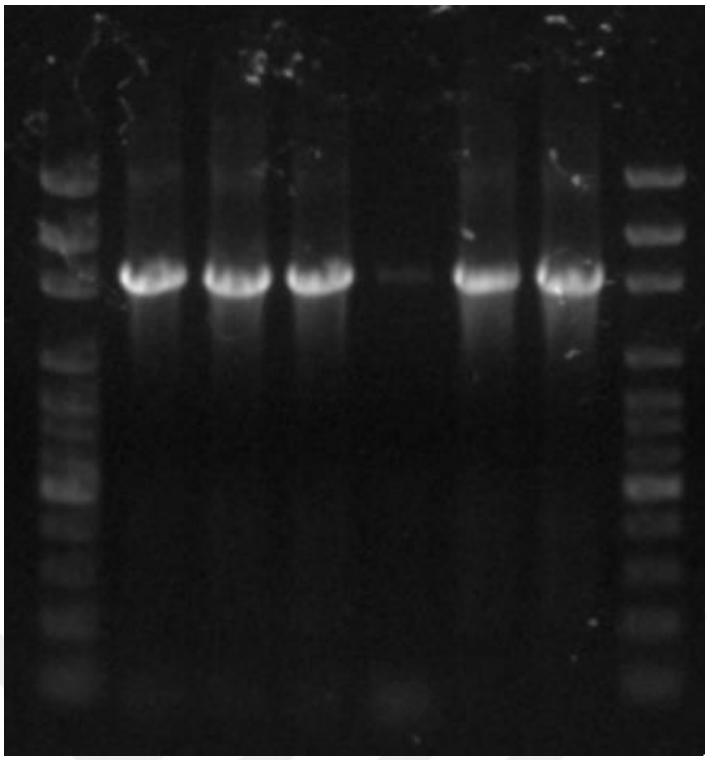

Figure 3. 11 1500 bp PCR-products of 6 probiotic strains.

PCR products were sent to Medsantek (Istanbul, Türkiye) for sequencing. The sequences were subjected to NCBI BLASTn for identification at species level. They were identified as shown in Table 3. 3. The DNA sequences and BLASTn results are provided in the appendix.

Table 3. 3 Identification and similarity rates of 16S rRNA alignments for each strain.

Strain	Identified Species	Percent Identity
MRS ES-3	*Lactiplantibacillus plantarum* (*L. plantarum*)	99.31%
MRS ES-7	*Lactiplantibacillus plantarum* (*L. plantarum*)	99.00%
MRS ES-11	*Levilactobacillus brevis* (*L. brevis*)	99.23%
MRS PT-14	*Lactiplantibacillus plantarum* (*L. plantarum*)	97.86%
M17 BB-7	*Enterococcus faecium* (*E. faecium*)	99.29%
M17 TB-2	*Enterococcus durans* (*E. durans*)	99.49%

In this study, it was determined that, isolates of einkorn sourdough MRS ES-3, and ES- 7 strains belong to *L. plantarum*, and strain ES-11 belong to *L. brevis* species, M17 TB-2 and BB-7 strains isolated from Turkish and Bulgarian-type boza belong to *E.durans* and *E. faecium* species, and finally MRS PT-14 strain from pickled beetroot belong to the *L. plantarum* species.

According to the studies in the literature, strains isolated from tarhana so far include *L. brevis, L. plantarum, L. pentosus* [184], *Lb. kunkeei Lb. delbrueckii*, baker's yeast *S. cerevisiae, L. lactis, L. acidophilus, L. casei, Leuconostoc cremoris* [49,66], *P. acidilactici, P. pentosaceus, E. faecium*, and *S. thermophilus* species [185]. Strains belonging to the species *L. plantarum, L. brevis, L. rhamnosus, L. pentosus, L. paracasei, L. lactis, L. acidophilus* [49,181], *P. pentosaceus, P. acidilactici, S. cerevisiae* were identified among the strains isolated from boza [56,186,187]. The strains isolated from sourdough in the literature were determined to belong to *L. plantarum, L. brevis, L. L. acidophilus, L. sanfranciscensis, L. casei, L. delbrueckii, Leu. Lactis, L. lactis* species [61,62,64,184]. Lastly, strains isolated from fermented beetroot determined to belong to *L. rhamnosus, L. paracasei, L. casei* species, and among the strains isolated from traditional pickles, belonging to *L. brevis, L. plantarum, L. pentosus, L. acidophilus, L. fermentum, P. pentosaceus* species [59,60,188].

3.3. Adhesion to Caco-2 Cells

Colonization resistance is important for gut health because adherent cells can grow and colonize on the gut effectively. Adherent probiotics show beneficial effects as long as they grow and colonize on mucosa, and at the same time, they hinder the infection by preventing the adhesion and colonization of pathogenic bacteria to the mucosa through competitive adhesion. Adhesion assay was performed because of the importance of colonization on the gut.

According to the results, MRS ES-3, MRS N-1, MRS T-2, M17 BB-7, M17 N-2 and M17 N-3 showed higher adhesion to Caco-2 cells than other strains (>37%). Especially, MRS N-1 and M17 N-2 showed over 85% adhesion to Caco-2 cells. Results of the adhesion assay can be examined in Table 3.4 and Figure 3.12.

Table 3. 4 Number of bacteria and survival rates for adhesion assay.

Cell Line	Identified Species	Control	Adhesion	Adhesion Rate
MRS ES-3	*L. plantarum*	$8{,}00x10^7$	$5{,}20x10^7$	65.0%
MRS ES-7	*L. plantarum*	$1{,}00x10^8$	$2{,}25x10^7$	22.5%
MRS ES-11	*L. brevis*	$1{,}35x10^8$	$2.05x10^7$	15.1%
MRS PT-14	*L. plantarum*	$7{,}05x10^7$	$2{,}65x10^7$	37.6%
MRS N-1	*P. pentosaceus*	$2{,}90x10^7$	$2{,}55x10^7$	87.9%
MRS T-2	*E. dispar*	$3{,}38x10^7$	$1{,}34x10^7$	39.7%
M17 BB-7	*E. faecium*	$4{,}38x10^7$	$2{,}57x10^7$	58.6%
M17 N-2	*E. faecium*	$7{,}65x10^7$	$6{,}95x10^7$	90.9%
M17 N-3	*E. faecium*	$1{,}16x10^8$	$4{,}34x10^7$	37.4%
M17 TB-2	*E. durans*	$8{,}35x10^7$	$1{,}13x10^7$	13.5%

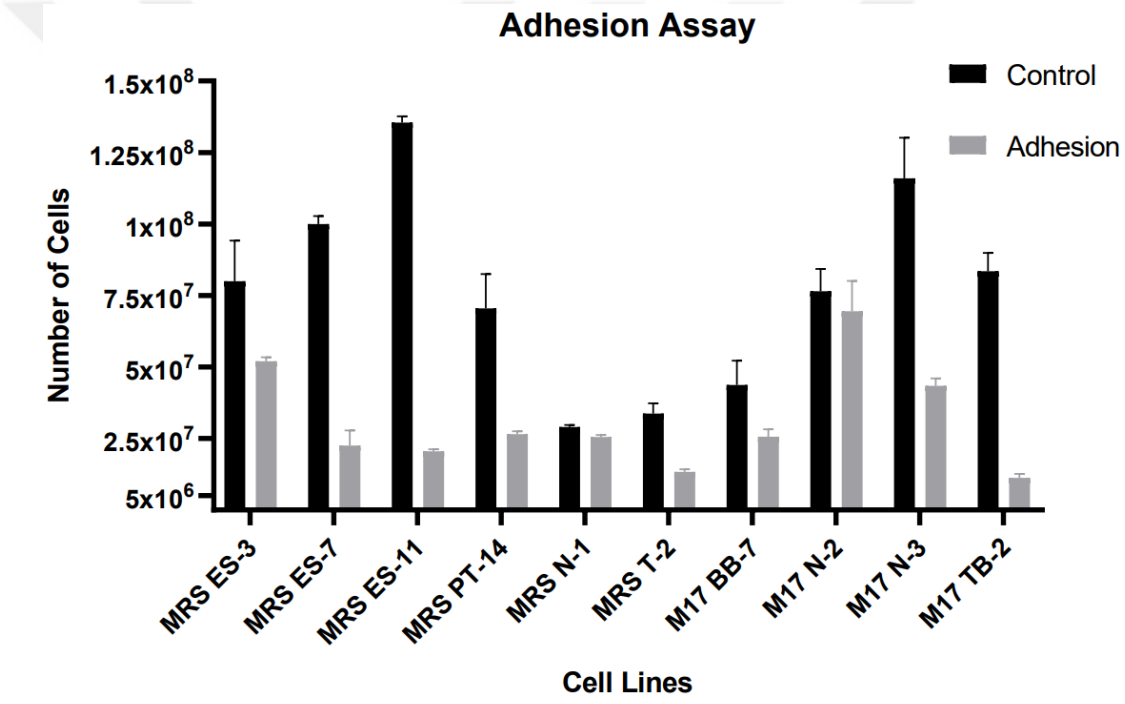

Figure 3. 12 Adhesion of potential probiotics on Caco-2 cell line.

For probiotics to survive and multiply in the intestine, they must colonize and adhere to intestinal epithelial cells [136]. There are several theories to explain this attachment. These are the hydrophobic interactions that occur between probiotic bacteria and the intestinal epithelium [189], the binding of mucin-binding proteins (MucBP) in the bacterial cell membrane to the mucin proteins in the mucosa of the intestinal epithelium [190], their attachment to the mucosa thanks to the pilus structure found in some bacteria such as *Bifidobacteria* [191]. Fibronectin-binding and surface-

layer proteins embedded in the bacterial cell wall [192] and extracellular polysaccharides synthesized by some bacteria are thought to be factors that assist in binding to the intestinal mucosa [193]. In this way, probiotic bacteria adhere to the intestinal epithelium and contribute positively to general health by secreting important metabolites such as bio-film formation inhibitor-EPS, vitamins, organic acids, amino acids, and bacteriocins. At the same time, they also reduce the possibility of infection by preventing pathogens from attaching and colonizing the areas to which they are attached [144].

In studies from the literature, Vasiee et al. (2020) detected *Pediococcus* strains isolated from fermented cereal-milk products showed adhesion between 4-16% after one hour of incubation with Caco-2 cells [146]. In our study, MRS N-1 strain from *P. pentosaceus* species showed 87.9% adhesion after 2 hours of incubation with Caco-2 cells. The survival rate was found by dividing the total number of bacteria in the experimental group by the total number of bacteria in the control group. This result shows that one or more of the above-mentioned binding mechanisms may be valid for strain N-1.

Rao et al. (2019) found that the *L. plantarum* strain isolated from pickles showed 65% adhesion to Caco-2 cells after 1 hour of incubation [194]. Pino et al. (2019) determined that *L. plantarum, L. rhamnosus, L. paracasei, L. pentosus* strains isolated from ripened cheese showed 3-19% binding with Caco-2 cells after 90 min incubation [195].

According to Todorov et al. (2008), 8 strains of *L. plantarum* (3), *L. paracasei* (2), *L. rhamnosus* (2) and *L. pentosus* (1) isolated from boza were incubated with Caco-2 cells for 24 hours. They found that all 8 strains showed adhesion between 0.3-9 [58]. Li et al. (2020) found that 3 strains of *Enterococcus* isolated from wild boar showed 34-64% binding to Caco-2 cells after 1 hour of incubation [176].

Park et al. (2019) determined that the *L. plantarum* strain isolated from sourdough showed four times more adhesion than the *L. rhamnosus* GG control strain after incubation with Caco-2 cells for 2 hours [196]. In our study, MRS ES-3, ES-7 and PT-14 strains of *L. plantarum* species showed 65%, 22.5%, 37.6% adhesion to Caco-2 cells respectively. Our strains of *L. plantarum* showed different rates of

adhesion in-between and higher than other studies, indicating they are different strains, and their adhesion mechanisms differ.

Wang et al. (2020) found that 7 strains of *E. faecalis* isolated from the feces of healthy infants showed 10.6%-54.9% adhesion to Caco-2 cells after 2 hours of incubation [197]. In our study, M17 BB-7, N-2 and N-3 strains from *Enterococcus faecium* species showed 58.6%, 90.9% and 37.4% adhesion with Caco-2 cells, respectively, after 2 hours of incubation. Especially, N-2 strain shows a high potential as probiotic bacteria with its high adhesion ability. All 3 strains of *E. faecium* showed different rates and this shows they are different than each other and their adhesion mechanisms are different.

In a study conducted by Xu et al. (2005) 8 probiotics from *B. longum* B6, *L. acidophilus, L. brevis, L. casei, L. rhamnosus* GG, *P. acidilactici* species are tested by incubation with Caco-2 cells for 2 hours of incubation and only above 20% of *B. longum* B6 and *L. rhamnosus* GG strains were found to adhere to Caco-2 cells [153]. Nowak et al. (2022) detected all 20 strains of *L. plantarum* (5), *L. casei* (1), *P. pentosaceus* (3) and *P. acidilactici* (5) species isolated from flowers, honey and pollen showed 55-90% binding after 2 hours of incubation with Caco-2 cells [144]. Argyri et al. (2013) determined 11 strains of *L. pentosus* (4), *L. plantarum* (3), *L. paracasei* (2), *L. casei* (1), *L. rhamnosus* GG (1) isolated from fermented olives adhere to Caco-2 cells between 30.5-74% after 4 hours of incubation [137].

Fonseca et al. (2020) determined that the *L. brevis* strain isolated from the traditional beverage caium showed 0.6% adhesion after 90 minutes of incubation with Caco-2 cells [198]. However, in the study of Nowak (2022), *L. brevis* strain showed 77.3% adhesion after 2 hours of incubation with Caco-2 cells [144]. In our study, the ES-11 strain of *L. brevis* species showed 15.1% adhesion to Caco-2 cells. This may indicate that *L. brevis* strains might not be suitable for adhesion to Caco-2 cells.

Zhou et al. (2021) found that the *E. durans* strain isolated from a healthy Chinese baby showed 38.5% adhesion to Caco-2 cells after 2 hours of incubation [198]. In our study, TB-2 strain from *E. durans* showed 13.5% adhesion to Caco-2 cells under the same conditions. According to that result, our *E. dispar* strain might be ineffective regarding to adhesion of Caco-2 cells. There is no available literature information about adhesion features of *Enterococcus dispar* species.

3.4. Antimicrobial Activity Assay

For the antimicrobial activity assays 10 potential probiotic strains were selected. Additionally, kanamycin sulphate 5 mg/ml was used as a positive control, sample 2 was *E. faecalis* 7-3 cell-free supernatant as positive control of *S. aureus* ATCC 6538 [154], samples 7 and 14 were empty disk and MRS broth as negative control and they are both placed at the center of agar plates. Samples 3, 4, 5, 6, 8, 9, 10, 11, 12 and 13 were cell-free supernatants of MRS ES-3, ES-7, ES-11, PT-14, N-1, T-2, M17 BB-7, N-2, N-3 and TB-2 respectively. The diameter of the disk is 6 mm.

According to the results (see Table 3.5, Table A.1 and Figures 3.13-3.17), positive control kanamycin showed greater antimicrobial activity than other samples. Cell-free supernatant of EF 7-3 strain showed moderate antimicrobial effect on *S. aureus* ATCC 6538 pathogens. Among our cell-free supernatant of probiotic strains, only MRS ES-3 (*L. plantarum*) showed limited antimicrobial activity on *S. aureus* ATCC 6538 and *K. pneumoniae* ATCC 4352 pathogens.

Table 3. 5 Zone of inhibition diameters of antimicrobial activity assay.

	Zone of Inhibition (ZOI) Diameters (mm)				
Number and Type of Sample	***S. aureus* ATCC 6538**	***E. coli* ATCC 25922**	***K. pneumoniae* ATCC 4352**	***P. mirabilis* ATCC 14153**	***S. epidermidis* ATCC 12228**
1 Kan	15,5	18	18,5	17	23
2 EF 7-3	14	8	11	10	9
3 ES-3	9,5	7,5	9,5	9	8
4 ES-7	7	7	8	8	8
5 ES-11	7	7	7	8	8
6 PT-14	7	7	7	7,5	8
7 Empty	0	0	0	0	0
8 N-1	7	7	7,5	7	8,5
9 T-2	7	8	7,5	7	9
10 BB-7	0	0	0	0	0
11 N-2	0	0	0	0	0
12 N-3	0	0	0	0	0
13 TB-2	0	0	0	0	0
14 MRS	0	0	0	0	0

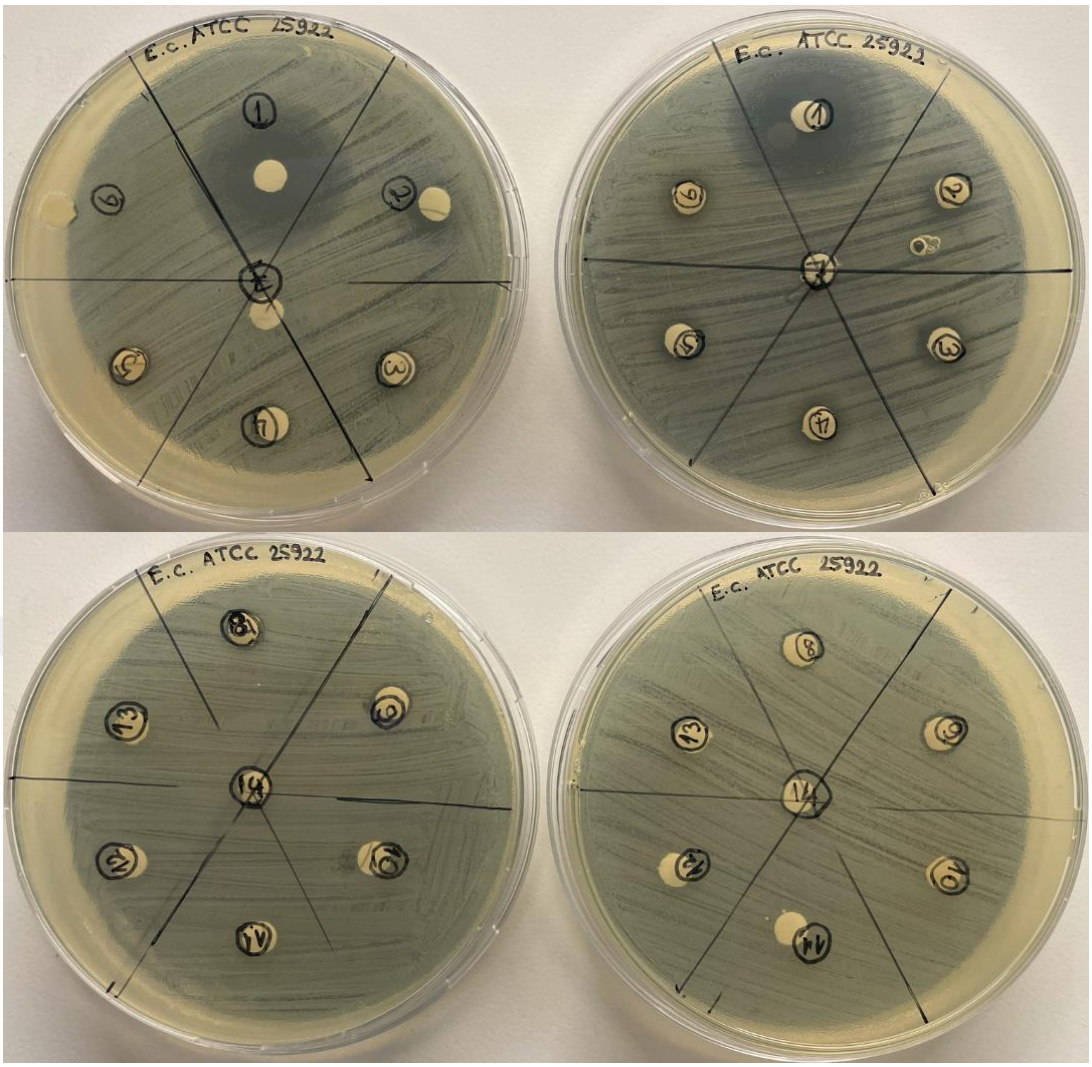

Figure 3. 13 Digital image of antimicrobial activity assay results against *E. coli* ATCC 25922.

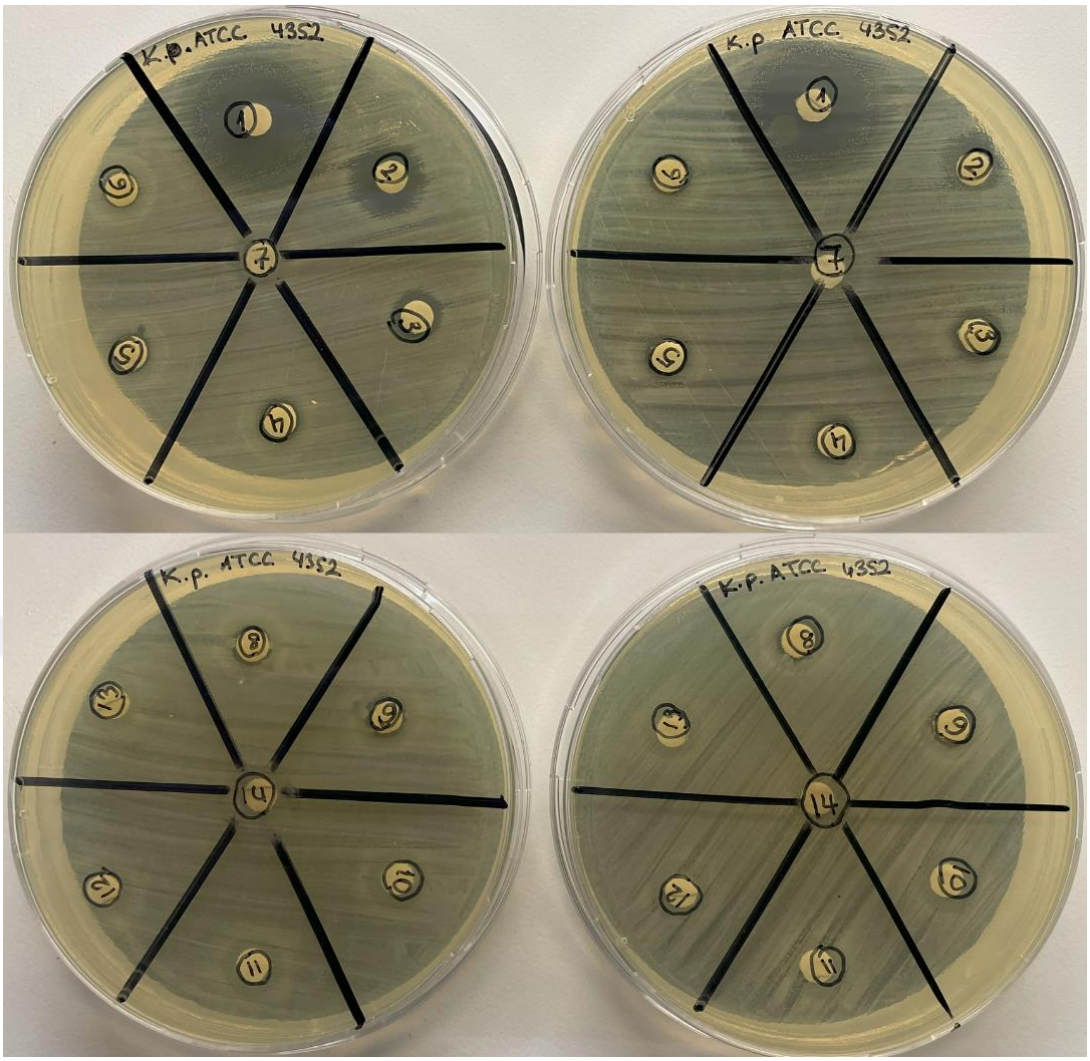

Figure 3. 14 Digital image of antimicrobial activity assay results against *K. pneumoniae* ATCC 4352.

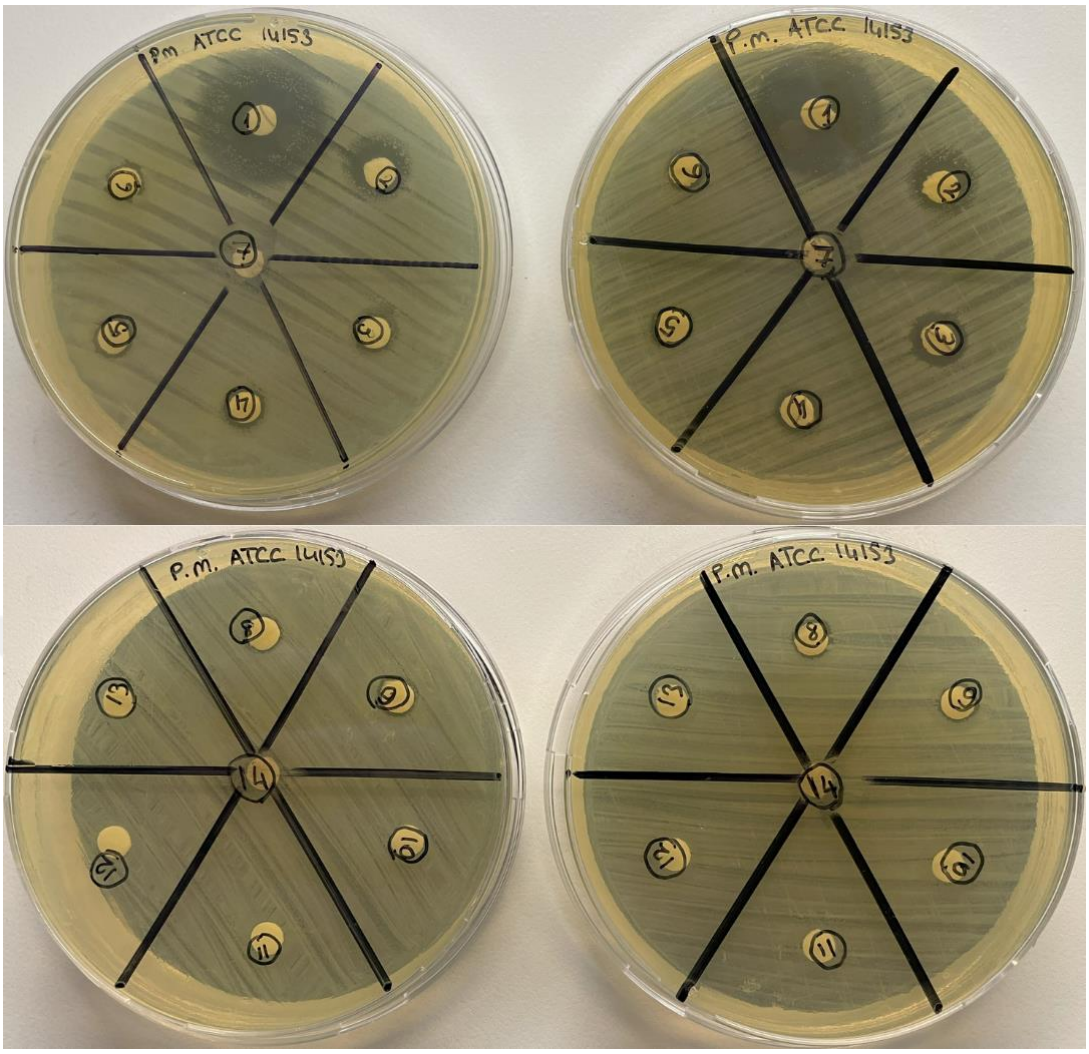

Figure 3. 15 Digital image of antimicrobial activity assay results against *P. mirabilis* ATCC 14153.

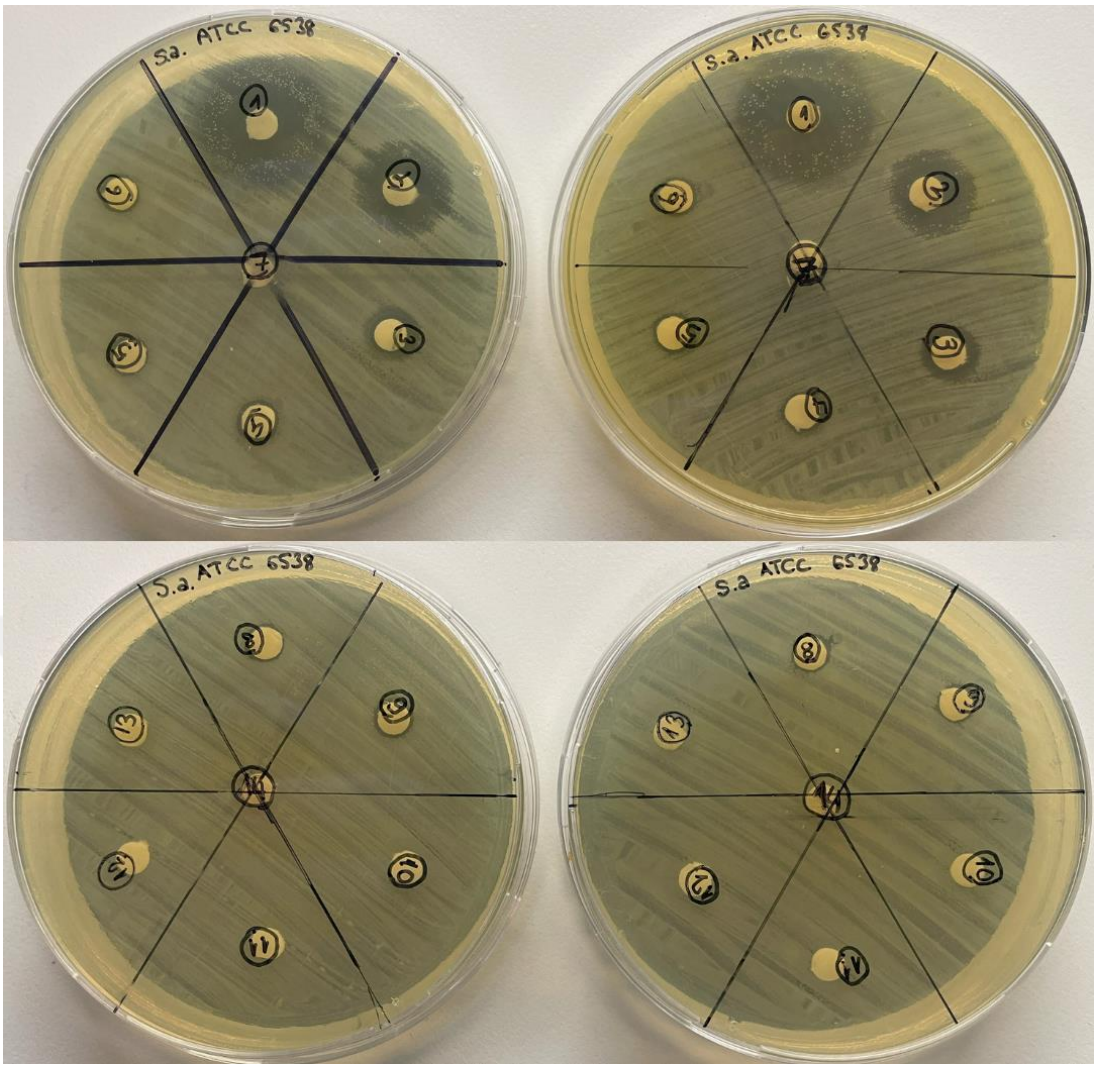

Figure 3. 16 Digital image of antimicrobial activity assay results against *S. aureus* ATCC 6538.

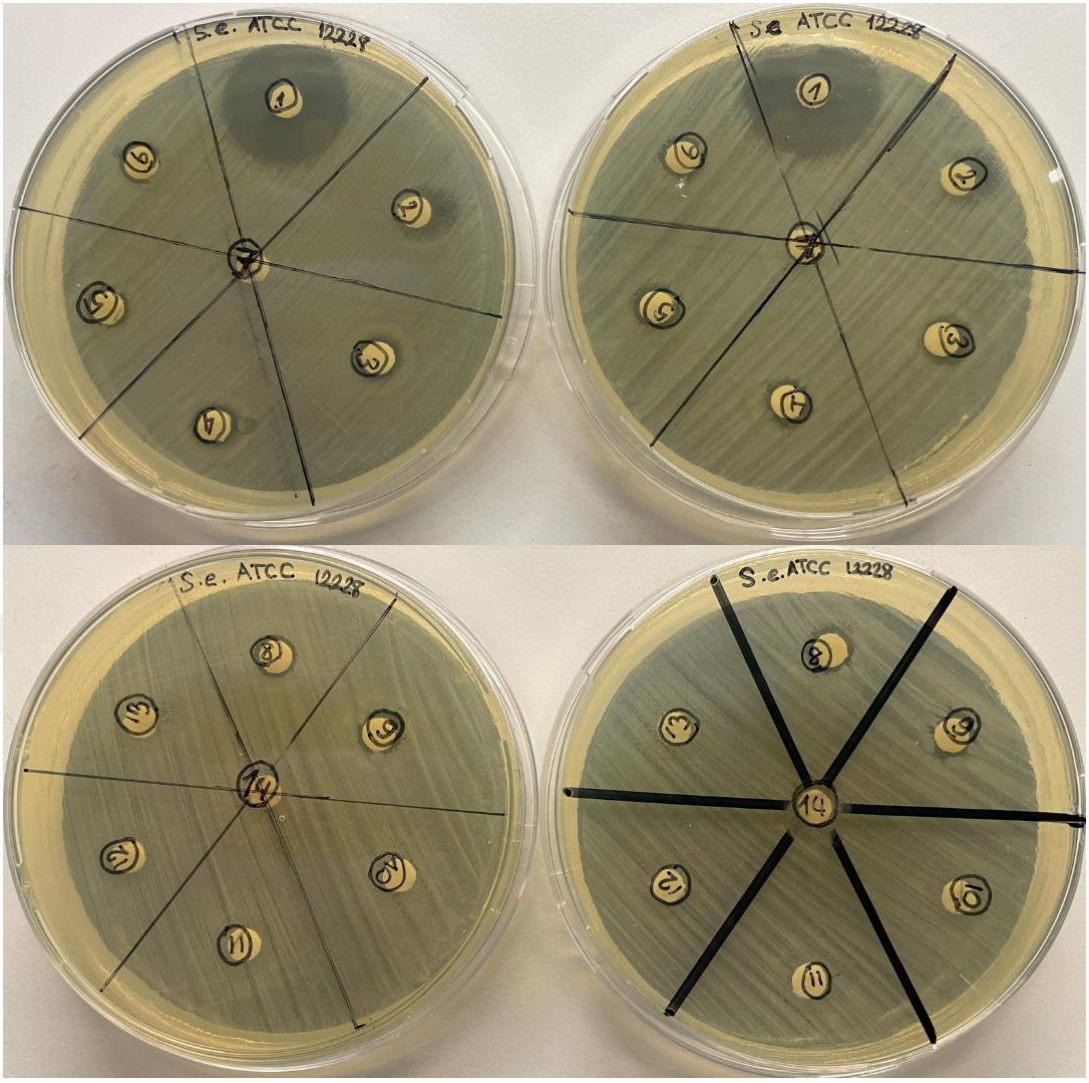

Figure 3. 17 Digital image of antimicrobial activity assay results against *S. epidermidis* ATCC 12228.

Probiotic bacteria produce two groups of substances with antimicrobial properties. The first group is non-bacteriocin structures, most common examples are organic acids, hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde, and 3-hydroxy fatty acids [70]. Hydrogen peroxide and acetaldehyde shows antimicrobial activity by oxidizing the bacterial cell wall and disrupting the essential proteins in the cell molecular structure [70,99,100]. Organic acids (lactic and acetic acids), diacetyl and CO_2 show antimicrobial effects by lowering the pH of the environment with their ionized forms and by directly lowering the cytoplasmic pH with their non-ionized forms [70,96-98]. These acids are also used as additives to preserve ready-made fermented foods [199]. By passing through the cell membrane, lactic acid causes the acidification of the cytoplasm, change of the membrane pH gradient, and the decrease in the production of energy necessary for the survival of the cells, hence leading to

cell death [200]. The second group is peptide or protein-structured bacteriocins produced and secreted by bacteria. Bacteriocins, which are not as broad-spectrum as antibiotics, are generally effective on a limited class of bacteria close to them, and the bacteria that produce bacteriocins are immune to their own bacteriocins. Yet, some bacteriocins have strong effects on food pathogens [113].

One of the common mechanisms of action of bacteriocins is the destruction of pathogens by pore formation or inhibition of cell wall synthesis [201]. For example, nisin attaches to cell wall structure lipid II, and inhibits cell wall synthesis of spore-forming bacillus. The complex assembles and precipitates these peptides to form a pore in the bacterial membrane, thereby causing cell death by impairing membrane permeability of the cells [101,113].

Kanamycin (5mg/ml) and cell-free supernatant of bacteriocin-producing *E. faecalis* 7-3 (EF 7-3) strain were used as control group in our study [154]. Although kanamycin showed antimicrobial activity on all pathogens, it showed a lower than expected activity on pathogens except *S. epidermidis* ATCC 12228 (15<r<19 mm). Likewise, strain EF 7-3 showed only moderate effect on *S. aureus* ATCC 6538 pathogens [154]. Based on these results, it is seen that pathogens have a certain level of resistance against antibiotics. According to the standards published by CLSI, the zone of inhibition created by the disc containing 30 µg kanamycin should be between 17-25 mm for *E. coli* ATCC 25922 strain, and 19-26 mm for *S. aureus* ATCC 25923 strains [202]. According to these values, it is not unexpected that the inhibition zones formed by cell-free supernatants are narrow.

The bacteriocin production and antimicrobial potential of other strains belonging to the same species used in our study have been explained in some studies. For instance, plantaricin is a bacteriocin produced by some strains of the *L. plantarum* species and has inhibitory effects on certain bacterial strains [187,203,204]. Pediocin is a bacteriocin produced by strains of *Pediococci* such as *P. pentosaceus* and *P. acidilactici* [205]. Enterocin is a bacteriocin produced by strains of *Enterococci* and has antimicrobial properties [206]. In addition, there are studies on bacteriocin production and antimicrobial potential of strains of the *L. brevis* species [207,208].

In this study, antimicrobial activity of cell-free supernatant obtained from 10 different strains was studied on 5 pathogen strains.

In the study conducted by Atlama (2021), the antimicrobial activity of strains of *E. faecium* (4), *L. plantarum* (2), *P. pentosaceus* (2) isolated from tarhana on *E. coli* and *S. aureus* pathogens was investigated. It was found that they were effective on the pathogen *E. coli* OH157:H7 (16<r<24 mm), while the same strains were found to be ineffective on the pathogen *S. aureus* ATCC 6538 [185]. In the study conducted by Ateş (2019), antimicrobial activities of *P. pentosaceus* (14), *L. brevis* (2) and *E. faecium* (3) strains isolated from tarhana were examined and it was observed that all of them had low level activities (r<11 mm) [178]. In our study, it was determined that only ES-3 strain of *L. plantarum* species had limited effect, while other strains of *L. plantarum* (ES-7 and PT-14) were found to be ineffective on pathogens. This antimicrobial activity may not be solely due to bacteriocin production, it may also be related to H_2O_2 (hydrogen peroxide) or organic acid production. Although there are similar studies in the literature, it is not possible to establish a direct correlation between the results because different bacterial strains are isolated from different nutrients.

In Başdoğan's (2020) study, strains of *L. plantarum* (6) and *L. brevis* (2) isolated from various traditional pickles had no antimicrobial effect on *E. coli* OH157:H7 and *S. aureus* ATCC 6538 pathogens (7< r<12 mm) was detected [209]. In the study conducted by Petrovic et al. (2020), antimicrobial activities of *E. faecium* (21) strains isolated from Sokobanja sausage were determined on *E. coli*, *Pseudomonas* and *Li. monocytogenes* pathogens and inhibition zones were 12-35 mm, 20-30 mm and 16-30 mm, respectively [179]. In our study, it was observed that M17 BB-7, N-2 and N-3 strains belonging to *E. faecium* species had no antimicrobial activity on pathogens. Due to the fact that different strains were tested on different pathogen strains, there were differences in the results in that manner. Likewise, the bacteriocin-producing strain *E. faecalis* 7-3 also showed moderate antimicrobial activity on the *S. aureus* ATCC 6538 pathogen. MRS N-1 (*P. pentosaceus*) and T-2 (*E. dispar*) strains isolated from tarhana, and M17 TB-2 (*E. durans*) strains isolated from boza did not show any effect on pathogens. Although these bacteria do not show antimicrobial activity, they may have indirectly contributed to the antimicrobial activity by triggering the immune response in the body. In addition, the bile salts they processed to deconjugated form are harmless to their own species but may inhibit the growth of pathogenic bacteria.

Chapter 4

Conclusions and Future Prospects

4.1. Conclusions

This thesis unveiled the probiotic potential of four traditional food products (tarhana, pickled beetroot, Turkish and Bulgarian-type boza, and einkorn sourdough) via several tests. 25 lactic acid bacterial isolates were studied in acid and bile salt tolerance experiments, and MRS ES-3-7-11, MRS PT-14, MRS N-1, MRS EB-3, MRS T-1-2, M17 N-3, M17 TB-2 strains showed growth at pH 3.0 compared to the control groups. M17 N-3-4 and M17 TB-1-2 strains showed higher viability in 0.3% and 0.5% (w/v) bile salt media than other strains. As a result of these experiments, 10 strains with higher probiotic potential were selected for further experiments. According to 16S rRNA sequencing, MRS ES-3, ES-7, and PT-14 belong to *L. plantarum*, MRS ES-11 belongs to *L. brevis*, M17 BB-7 belongs to *E. faecium*, and M17 TB-2 belongs to *E. durans* species. In adhesion assays to Caco-2 cells, MRS ES-3, MRS N-1, MRS T-2, M17 BB-7, M17 N-2, and M17 N-3 strains showed higher adhesion ability than other strains. MRS N-1 and M17 N-2 showed over 85% adhesion. Lastly, MRS ES-3 strain showed antimicrobial activity on *S. aureus ATCC 6538* and *K. pneumoniae ATCC 4352* pathogens, while the other strains showed no antimicrobial effect on any pathogen.

4.2. Societal Impact and Contribution to Global Sustainability

This thesis aims to improve public health by investigating the potential of probiotic-rich traditional fermented foods (tarhana, einkorn sourdough, Turkish and

Bulgarian-type boza, and pickled beetroot) and popularizing the consumption of these foods. Increasing the consumption of such fermented probiotic-containing foods will make it easier and inexpensive to reach a better level of public health by solving or mitigating the health problems experienced by individuals having unhealthy microbiota. Many probiotic microorganisms have been discovered so far, and some of them have started to be sold commercially as tablets ready for use by the community. Mainly, *Bifidobacteria* and *Lactobacilli* belonging to the Lactic Acid Bacteria (LAB) family have been studied, and their benefits have been proven in many studies [210,211]. It has also been proven that probiotics have positive and significant effects not only on the digestive system but also on the immune system and chronic diseases [2]. In addition, some probiotics have high adhesion ability to intestinal cells and pathogen inhibitory activity, and consuming these probiotics as a food supplement may help individuals heal without side effects. With the popularization and enrichment of uncomplicated traditional diets, it will be much easier to have a healthy body.

Furthermore, nutrients obtained by fermenting the foods grown in the local regions will prevent the decrease in biodiversity. Promoting the consumption of traditional foods will contribute to developing the local economy and providing sustainable agriculture. In addition, research and development (R&D) studies will be carried out in order to produce these products in a better quality and valuable way, and the way for scientific development in these regions will be paved. With the consumption of traditional foods, the waste that may occur in the production of supplemental probiotics will also decrease. In these ways, it will be easier to implement the UN Sustainable Development Goals "Good Health and Well-Being" (SDG 3) and "Reduced Inequalities" (SDG 10) [212].

Lastly, within the framework of the 11th Development Plan, and in the field of Competitive Production and Efficiency, in the Priority Sectors field of Pharmaceuticals and Medical Devices, with purpose 362 and policies 363-364; society will be able to access these supplements more efficiently and cheaper by increasing the competitiveness of our country in the international market and by introducing these probiotic bacteria, which are easy to obtain and produce, to the market as supplements [213].

4.3. Future Prospects

Future studies will examine the effects of probiotic bacterial strains on intestinal organoids and the outcomes of their *in vivo* administration on mice. Additionally, how the inflammation that is triggered in Caco-2 cells will be changed by probiotic bacteria administration will be examined by real-time PCR amplification of the mRNA isolates. Furthermore, additional detailed information about probiotic candidate strains can be obtained by performing tests such as BSH activity test, growth in the presence of pepsin and pancreatin, competitive adhesion assay with pathogens, short-chain fatty acid test, and phenol tolerance test. Lastly, to determine the cytotoxicity effects of cell-free supernatants of these strains on cancer cells, MTT assay can be performed.

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APPENDIX

- 16S ribosomal RNA sequencing result for MRS ES-3 (*L. plantarum*) strain:

AAGTCGAACGAACTCTGGTATTGATTGGTGCTTGCATCATGATTTACATTTG
AGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGG
GGGATAACACCTGGAAACAGATGCTAATACCGCAAACAACTTGGACCGCAG
GGTCCGAGCTTGAAAGATGGCTTCGGCTATCATTTTTGGATGGTCCCGCGGC
GTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCC
AACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCC
TACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGA
GCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTA
AAGAAGAACATATCTAAGAGTAACTGTTCAGGTATTGACGGTATTTAACCA
GAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCA
AGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGT
CTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGGAA
ACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCG
TAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACT
GACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTA
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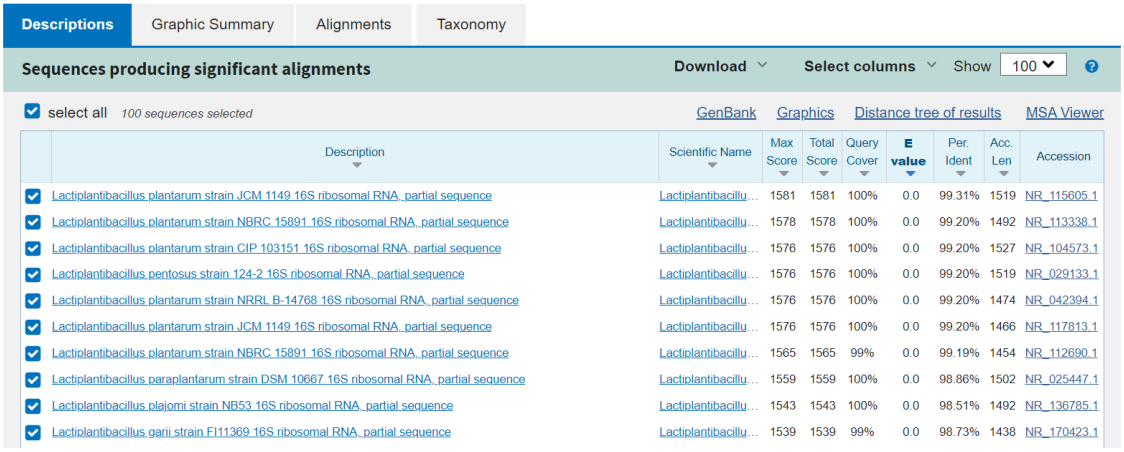

Figure A. 1 BLASTn result and top 10 most similar strains for MRS ES-3.

- 16S ribosomal RNA sequencing result for MRS ES-7 (*L. plantarum*) strain:

GCCTAATACATGCAAGTCGAACGAACTCTGGTATTGATTGGTGCTTGCATCA
GATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTG
CCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGCAAAACA
ACTTGGACCGCAGGGTCCGAGCTTGAAAGATGGCTTCGGCTATCATTTTTGG
ATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCA
ATGATACGTAGCCAACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACA
CGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACG
AAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAA
AACTCTGTTGTTAAAGAAGAACATATCTAAGAGTAACTGTTCAGGTATTGAC
GGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGG
CGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATC
GGAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGC
GGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAAGCGGCTGTCT
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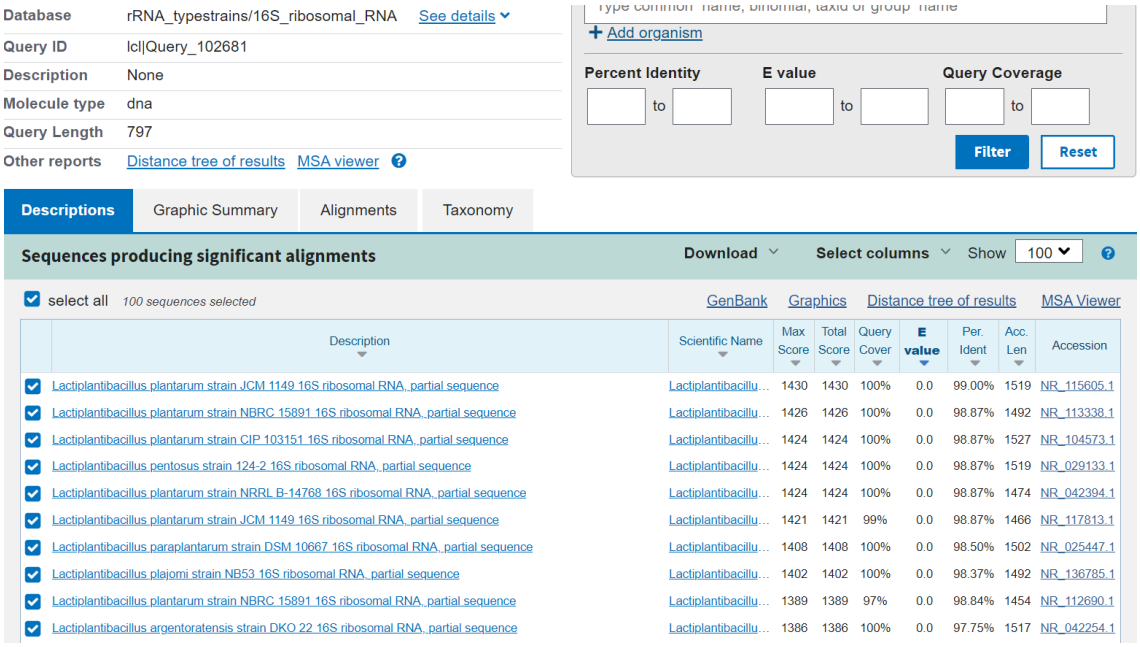

Figure A. 2 BLASTn result and top 10 most similar strains for MRS ES-7.

- 16S ribosomal RNA sequencing result for MRS ES-11 (*L. brevis*) strain:

CAATGAAGCGAGTGGCGAACTGGTGAGTAACACGTGGGGAATCTGCCCAGA
AGCAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACAACAAAAT
CCGCATGGATTTTGTTTGAAAGGTGGCTTCGGCTATCACTTCTGGATGATCC
CGCGGCGTATTAGTTAGTTGGTGAGGTAAAGGCCCACCAAGACGATAATAC
GTAGCCAACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCA
AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCT
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TTGTTAAAGAAGAACACCTTTGAGAGTAACTGTTCAAGGGTTGACGGTATTT
AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
TGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTT
TAAGTCTGATGTGAAAGCCTTCGGCTTAACCGGAGAAGTGCATCGGAAACT
GGGAGACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGG
AATGCGTAGATATATGGAAGATCACCAGTGGCGAAGGCGGCTGTCTAGTCT
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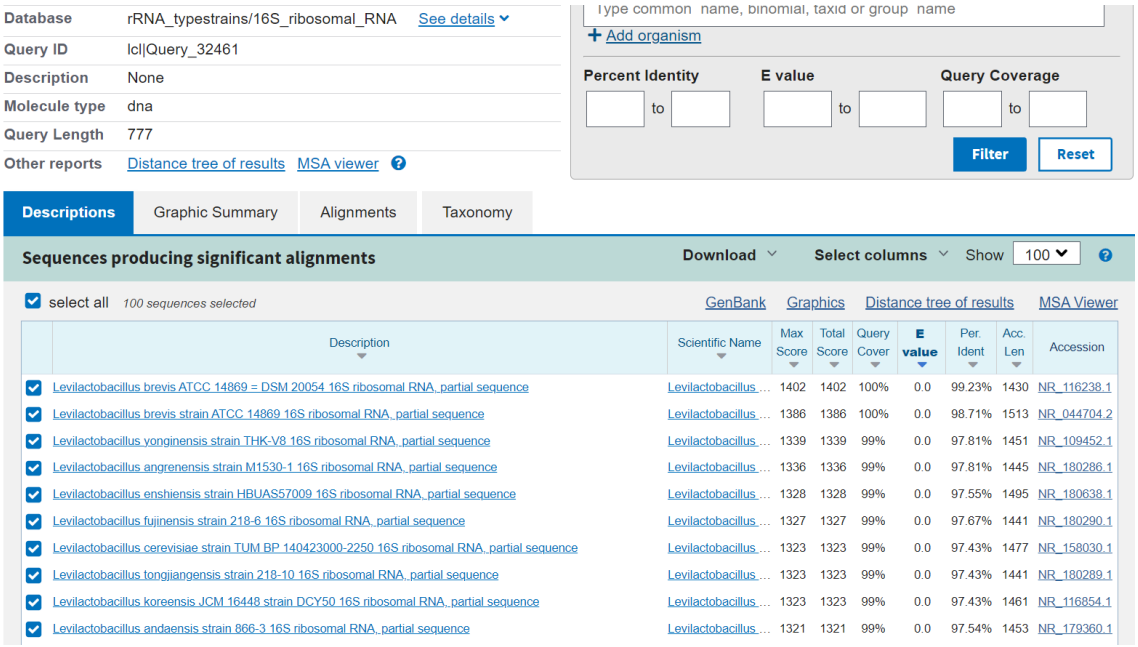

Figure A. 3 BLASTn result and top 10 most similar strains for MRS ES-11.

- 16S ribosomal RNA sequencing result for MRS PT-14 (*L. plantarum*) strain:

GTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACAC
GTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAA
TACCGCATAACAACTTGGACCGCAGGGTCCGAGTTTGAAAGATGGCTTCGG
CTATCATTTTTGGATGGCCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGG
CTCCCCATGGCAATGATCCGTACCCGACCTGAGAGGGTAATCGGCCACATT
GGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTT
CCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGG
TTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTG
TTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAAGCGTTGTCCGGATTTATTGGGCGT
AAAAGCGAGCGCAGGCGGttttttAAGTCTGATGTGAAAGCCTTCGGCTCAACC
GAAGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAAAAGAGGACAGTGG
AACTCCATGTGTAGCGGTGAAAATGCGTAGATATATGGAAGAACACCAGTG
GCGAAGGCGGCTTGTCTGGTCTTGTAACTGACGCTGAGGCTCGAAGGTATG
GGTAGCAAACAGGATTAGATTCCCCTGGTAGTCCATACCGTAAACGATGAA
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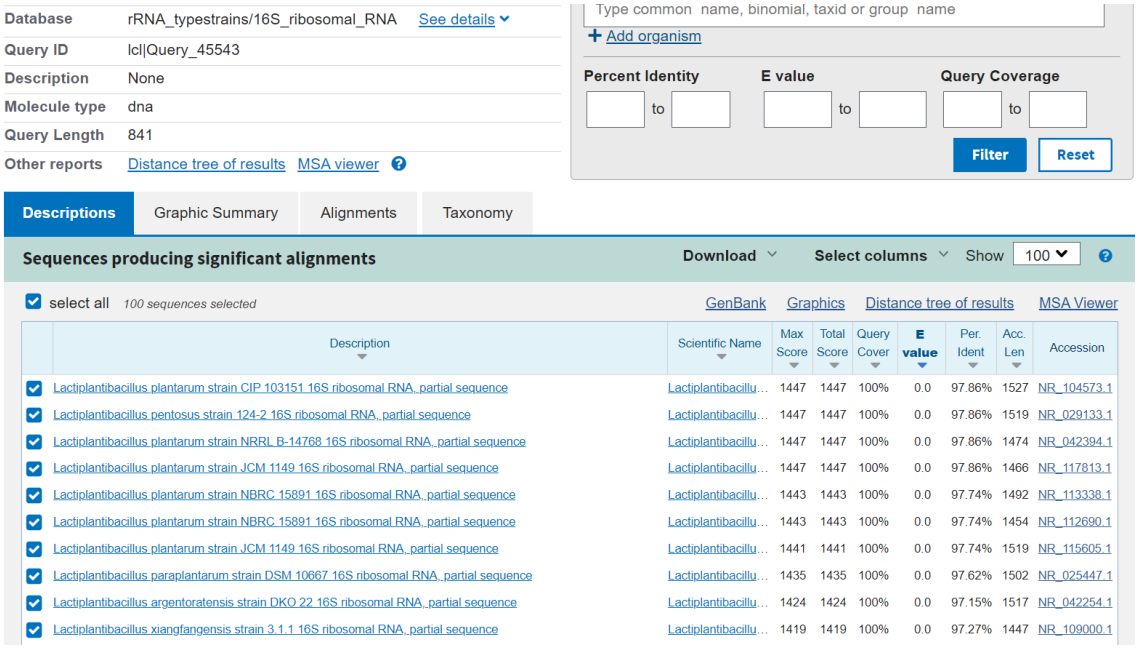

Figure A. 4 BLASTn result and top 10 most similar strains for MRS PT-14.

- 16S ribosomal RNA sequencing result for M17 BB-7 (*E. faecium*) strain:

ACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGCTTCTTTTTCCACCGG
AGCTTGCTCCACCGGAAAAAGAGGAGTGGCGAACGGGTGAGTAACACGTG
GGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTGCTAATACC
GTATAACAATCAAAACCGCAGGGTTTTGATTTGAAAGGCGCTTTCGGGTGTC
GCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCA
CCAAGGCCACGATGCATAGCCAACCTGAGAGGGTGATCGGCCACATTGGGA
CTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGC
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GATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAACTGTTCA
TCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGC
CGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGC
GAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGG
GAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAAT
TCCATGTGTAGCGGTGAAATGCGTAGATTATGGAGGAACACCAGTGGCGAA
GGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAGCGTGGGGAGCAA
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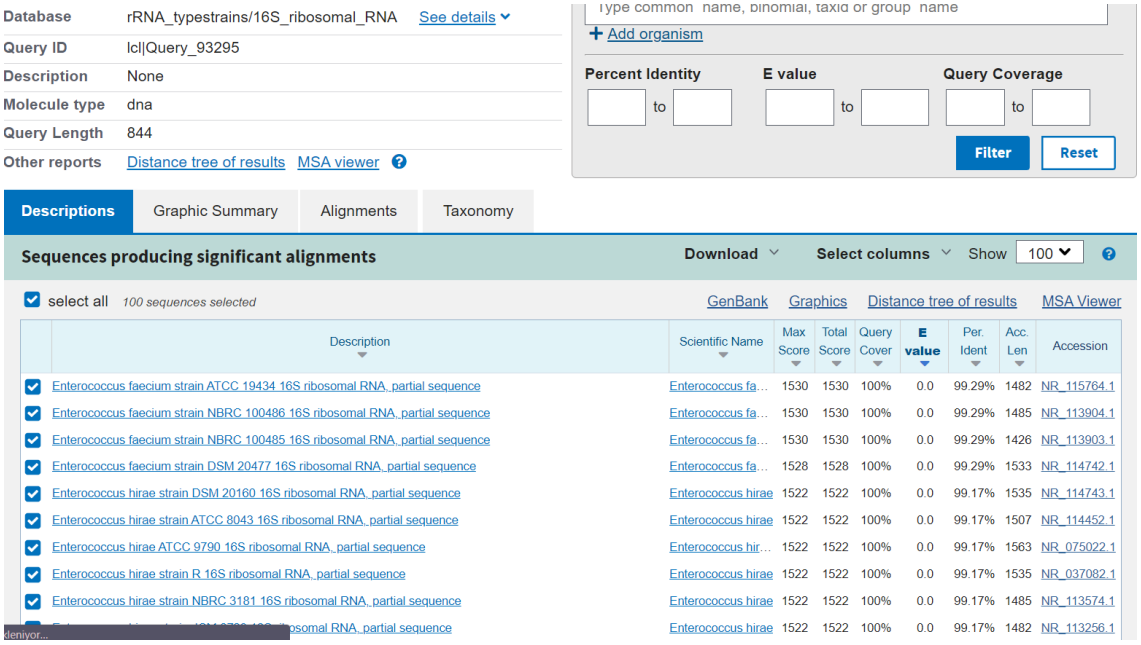

Figure A. 5 BLASTn result and top 10 most similar strains for M17 BB-7.

- 16S ribosomal RNA sequencing result for M17 TB-2 (*E. durans*) strain:

ACGCTGGCGGCGTGCCTAATACATGCAAGTCGTACGCTTCTTTTTCCACCGG
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TATAACAATCGAAACCGCAGGGTTTTGATTTGAAAGGCGCTTTCGGGTGTCG
CTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCAC
CAAGGCCACGATGCATAGCCAACCTGAGAGGGTGATCGGCCACATTGGGAC
TGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCA
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ATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAACTGTTCAT
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GCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCG
AGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGG
AGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATT
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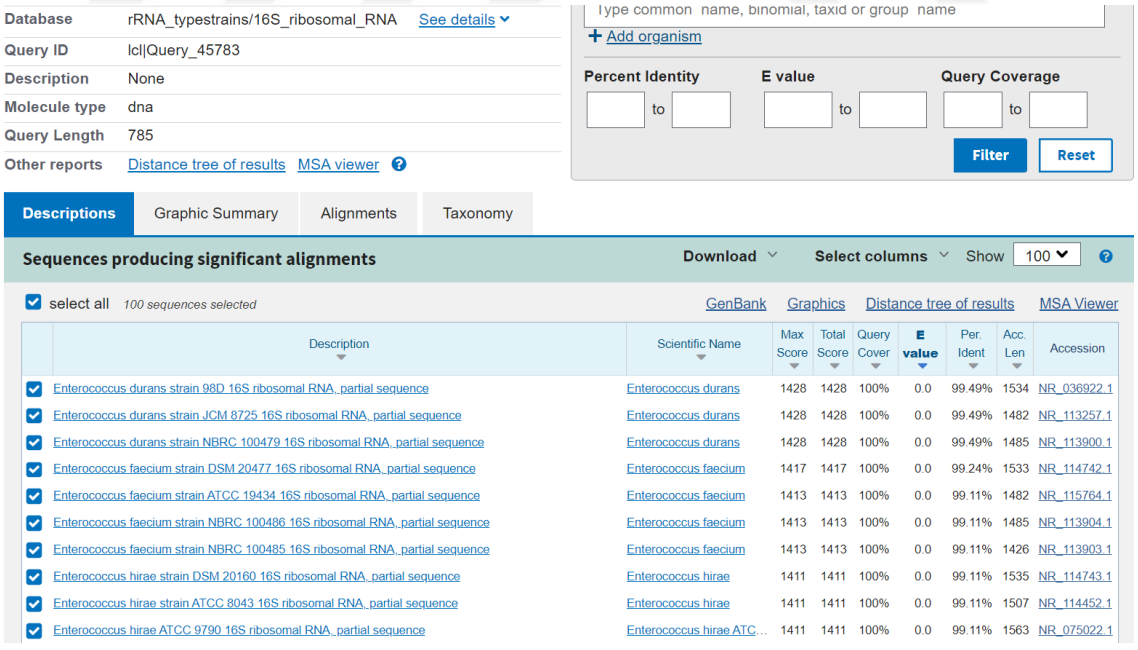

Figure A. 6 BLASTn result and top 10 most similar strains for M17 TB-2.

Table A. 1 Conversion of the zone of inhibition diameters of the antimicrobial activity test to plus (+) and minus (–) signs; (–) means r = 0 mm, (+) means r < 9 mm, (++) means r < 12 mm, (+++) means r < 16 mm and (++++) means r > 16 mm.

	Zone of Inhibition (ZOI) Diameters (mm)				
Number and Type of Sample	***S. aureus* ATCC 6538**	***E. coli* ATCC 25922**	***K. pneumoniae* ATCC 4352**	***P. mirabilis* ATCC 14153**	***S. epidermidis* ATCC 12228**
1 Kan	+++	++++	++++	++++	++++
2 EF 7-3	+++	+	++	++	++
3 ES-3	++	+	++	++	+
4 ES-7	+	+	+	+	+
5 ES-11	+	+	+	+	+
6 PT-14	+	+	+	+	+
7 Empty	-	-	-	-	-
8 N-1	+	+	+	+	+
9 T-2	+	+	+	+	++
10 BB-7	-	-	-	-	-
11 N-2	-	-	-	-	-
12 N-3	-	-	-	-	-
13 TB-2	-	-	-	-	-
14 MRS	-	-	-	-	-

CURRICULUM VITAE

2014 – 2020	B.Sc., Genetics and Bioengineering, İstanbul Bilgi University, İstanbul, TÜRKİYE
2021 – Present	M.Sc., Bioengineering, Abdullah Gül University, Kayseri, TÜRKİYE

WORK EXPERIENCES

Dec 2020 – Present	Research Assistant, Abdullah Gül University, Faculty of Life and Natural Sciences, Molecular Biology and Genetics Department, Kayseri, TÜRKİYE
Oct 2020 – Sep 2022	TUBITAK BIDEB 2210-A – Researcher, Abdullah Gül University, Kayseri, TÜRKİYE