MODULATING *Akkermansia muciniphila* BY PROBIOTICS FOR OBESITY MANAGEMENT

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ABSTRACT

MODULATING *Akkermansia muciniphila* BY PROBIOTICS FOR OBESITY MANAGEMENT

The aim of this thesis is to investigate the potential of lactic acid bacteria (LAB) having probiotic properties to trigger *Akkermansia muciniphila* as anti-obesity candidate. Artisanal strains of LAB were evaluated in terms of stability in gastrointestinal conditions, mucin/mucus non-utilization properties, growth in the presence of mucin, adhesion to the mucin/mucus layer, biofilm formation, lactate production abilities, antimicrobial properties, HT-29 cell binding and mucin/mucus thickness increment abilities.

Among 40 strains, 17 of LAB including 6 olive isolates were selected for their tolerance to gastrointestinal conditions and analyzed for further abilities e.g., mucin promotion potential. Most of them (13 of the 17 bacteria) remained viable in the range of 10^6-10^8 CFU/mL in the simulated gastric juice, and these bacteria continued their viability in the range of 10^5-10^7 CFU/mL in the simulated intestinal fluid.

Besides, 6 reference and 7 *L. bulgaricus* isolates did not utilize the mucin and showed no or negligible growth in the presence of mucin. Additionally, 13 of the 17 bacteria adhered to mucin layer with variable degree of binding between 41.03-65.37%.

Furthermore, variable degree biofilm formation efficiencies can also support their existence and adhesion to intestinal cells. Varying amounts of lactate by 7 LAB were produced (6.51-18.17 g/L) and this ability is another important criteria for supporting mucus pathway. All those strains adhered to HT-29 cell line and they showed significant increase in mucin thickness by comparing the control.

L. plantarum C47 and *Lactobacillus* AK50 isolates were selected as the anti-obesity prototype candidates for their desirable attributes.

ÖZET

*Akkermansia muciniphila'*NIN OBEZİTE KONTROLÜ İÇİN PROBİYOTİKLERLE DÜZENLENMESİ

Bu tezin amacı, probiyotik özelliklere sahip laktik asit bakterilerinin (LAB) anti-obezite adayı olarak *Akkermansia muciniphila'*yı destekleme potansiyelini araştırmaktır. Geleneksel yollarla elde edilen laktik asit bakterileri; gastrointestinal koşullarda stabilite, müsin/mukus kullanmama özellikleri, müsin varlığında büyüme, müsin/mukus tabakasına yapışma, biyofilm oluşumu, laktat üretim yetenekleri, antimikrobiyal özellikleri, HT-29 hücresine bağlanma ve müsin/mukus kalınlığını artırma yetenekleri açısından değerlendirilmiştir.

Gastrointestinal koşullara tolerans gösteren 17 laktik asit bakterisinin 6'sı zeytin izolatı olup 40 LAB suşu arasından seçilmiş ve müsin miktarını artırma potansiyelleri vb. daha ileri yetenekleri için analiz edilmiştir. Bu bakterilerin çoğu (17 bakterinin 13'ü) simüle mide sıvısında 10^6-10^8 CFU/mL aralığında canlı kalmış ve simüle bağırsak sıvısında ise 10^5-10^7 CFU/mL aralığında canlılıklarını sürdürmüştür.

Bununla birlikte, 6 referans ve 7 *L. bulgaricus* izolatı müsini kullanmamış ve müsin varlığında ya hiç büyümemiş ya da ihmal edilebilir büyüme göstermiştir. Buna ek olarak, 17 bakteriden 13'ü müsin tabakasına %41.03-65.37 arasında değişen oranlarda bağlanmıştır.

Ayrıca, değişen düzeylerdeki biyofilm oluşum etkinlikleri de bu bakterilerin varlığını ve bağırsak hücrelerine yapışmasını destekleyebilir. 7 LAB tarafından değişen miktarlarda (6.51-18.17 g/L) laktat üretilmiştir ve bu yetenek, mukus yolunu desteklemek için bir diğer önemli kriterdir. Tüm bu suşlar HT-29 hücre hattına bağlanmış ve kontrol ile karşılaştırıldığında müsin kalınlığında önemli artış göstermiştir.

L. plantarum C47 & *Lactobacillus* AK50 izolatları istenilen özellikleri sağlayarak obezite karşıtı prototip adayı olarak seçilmiştir.

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LIST OF SYMBOLS AND/OR ABBREVIATIONS

A……...……Absorbance

A1……The absorbance values obtained in modified MRS without fermentable carbohydrates

A2……The absorbance values obtained in modified MRS containing glucose or mucin

A_{600}……..The absorbance values at 600 nm

AC……...…….Ascending Colon

ADU……Adnan Menderes University

AMPK……AMP-Activated Protein Kinase

ARS……..Agricultural Research Service

ATCC……American Type Culture Collection

B……...……..Bacteroidetes

BCP……...……...Bromocresol Purple

BMI……...……...Body Mass Index

CFS……...…….Cell Free Supernatant

CFU……...……Colony Forming Unit

cm^2……...……Centimetre $power^{-2}$

CV……...……..Crystal Violet

DC……...……...Descending Colon

dH_2O……...……...Deionized water

DMEM……...Dulbecco's Modified Eagle Medium

dPBS……Dulbecco's Modified Eagle Medium Phosphate Buffered Saline

e.g……...Exempli gratia (for example)

eqn……...……..Equation

etc……...……Et cetera

EtOH……...……Ethanol

F……...……...Firmicutes

FAO……..Food and Agriculture Organization

g……..The relative centrifugal force (RCF)

g……...……...Gram

GI……...…….Gastrointestinal

GIS……...…..Gastrointestinal System

GIT……...…….Gastrointestinal Tract

h……...……Hour

HCl……...……...Hydrochloric acid

HFD……...……High Fat Diet

HMO…….Human Milk Oligosaccharide

HPLC…….High-Pressure Liquid Chromatography

H_2SO_4……...……...Sulfuric acid

ICR……..Institute of Cancer Research

(IZTECH-FED-MFMLCC)……..İzmir Institute of Technology, Department of Food Engineering, Molecular Food Microbiology Laboratory Culture Collection

KCl……...…….Potassium chloride

KH_2PO_4……...Potassium dihydrogen phosphate

L……...……Liter

LAB……...……..Lactic Acid Bacteria

LBP……Live Biotherapeutic Products

LUB……...….Lactate-Using Bacteria

LPS……...……Lipopolysaccharide

M……...…….Molar

MDa……...……...Megadalton

mg……...……Milligram

mL……...……..Milliliter

mm……...……...Millimeter

mM……...……..Millimolar

min……...……Minute

m.o.s……..…….Microorganism

MUB…….....Mucus-binding proteins

MUCIN……Purified porcine gastric mucin type III

MRS……..de MAN, ROGOSA and SHARPE

N……...……...Normal

N_0……Total number of live strains before implementation

N_1……Total number of live strains after gastric juice implementation

N_2……Total number of live strains after intestinal juice implementation

NaCl……...……...Sodium chloride

Na_2HPO_4…….Disodium hydrogen phosphate

NGS……Next Generation Sequencing

NGP……..Next Generation Probiotics

nm……...……...Nanometer

NP……...……Nanoparticle

PAS……...…….Periodic Acid-Schiff

PBS……....Phosphate Buffered Saline

rRNA……...…….Ribosomal RNA

rpm……...…..Revolutions per minute

RPMI……...Roswell Park Memorial Institute

SCFA……...…Short Chain Fatty Acid

TC……...…….Transverse Colon

TSA……...……Tryptone Soy Agar

TSB……...……...Tryptone Soy Broth

TiO_2……...……...Titanium dioxide

v……...……...Volume

w……...……Weight

WHO……World Health Organization

XOS……...……Xylooligosaccharides

%……...……...Percent

℃……...…….Centigrade degree

µg……...……Microgram

µL……...…….Microliter

µm……...……..Micrometer

μ_{max}……...The maximum specific growth rate

µV. s……...……Microvolts second

CHAPTER 1

INTRODUCTION

Obesity in the World is increasing even more according to the data; the probability of being obese today is three times higher for the average adult compared to 1975 (Hallett, 2019). According to 2016 data on obesity (Figure 1.1), which is also a major problem in our country, the percentage of obese adults is 32% (Ritchie and Roser, 2019).

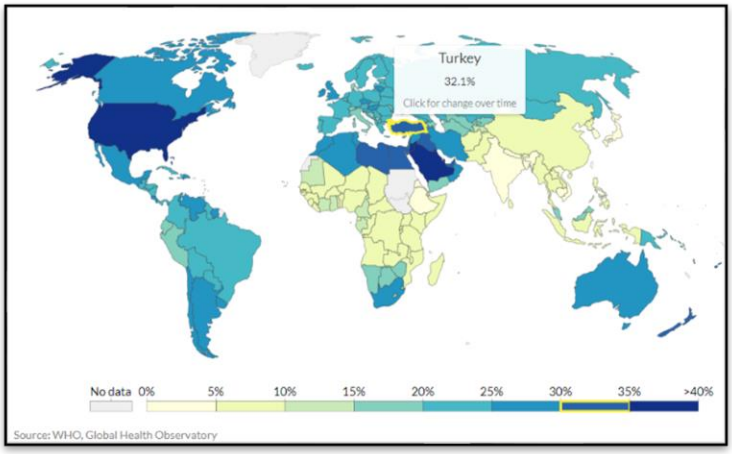


Figure 1. 1. Prevalence of obesity for adults who are defined as obese based on their BMI in 2016, in the world (Source: Ritchie and Roser, 2019).

Obesity and World Situation: If the rising trends of the world's global obesity data continue, the global prevalence of obesity is projected to be over 18% for men and 21% for women by 2025. Moreover, 20% of the world's adult population is projected to be obese by 2030 (Tseng and Wu, 2019).

In our microbiota, *Akkermansia*, which is an effective microorganism against the obesity problem, has a relationship with diet (Anonye, 2017; Naito et al., 2018). *Akkermansia*, a microorganism breaks down mucin, needs other beneficial bacteria (probiotic LAB) that can promote mucin/mucus thickening in the environment for its propogation. The subject of the thesis is based on the assumption that lactic acid bacteria (LAB) having probiotic properties can modulate the microbiota or habitat for the

development of *Akkermansia* group microorganisms, it covers the studies on adhesion, mucin/mucus thickening, antimicrobial properties etc. for the mucin/mucus supporting roles of LAB in the simulated colon environment.

Overweight (25.0-30.0 BMI, WHO) and obesity (> 30 BMI, WHO) (Table 1.1) cause major risks which lead to a range of chronic diseases, e.g., diabetes, cardiovascular diseases and cancer (Fontané et al., 2018; Jiao et al., 2019; Tseng and Wu, 2019). While overweight and obesity were once considered as a problem only in high-income countries, they now appear to be increasing in low- and middle-income countries, especially in urban environments. The abundance of *Akkermansia* in the gut in individuals having high body weight, body mass index (BMI), blood cholesterol level and fasting blood sugar levels, has been suggested that being lower than in the intestines of healthy individuals. In addition, it has been reported that when calorie-restricted diet therapy (dietary treatment) is applied in overweight or obese individuals, a significant increase in *Akkermansia* group microorganisms in the intestine occurs with the elimination of insulin resistance (Naito et al., 2018).

Table 1. 1. The international classification of adult underweight, overweight and obesity according to BMI (Source: The SuRF Report 2, WHO, 2005).

Classification	BMI (kg/m²) Principal cut-off points	BMI (kg/m²) Additional cut-off points
Underweight	<18.50	<18.50
Severe thinness	<16.00	<16.00
Moderate thinness	16.00 – 16.99	16.00 – 16.99
Mild thinness	17.00 – 18.49	17.00 – 18.49
Normal range	18.50 – 24.99	18.50 – 22.99 23.00 – 24.99
Overweight	≥25.00	≥25.00
Pre-obese	25.00 – 29.99	25.00 – 27.49 27.50 – 29.99
Obese	≥30.00	≥30.00
Obese class I	30.00 – 34-99	30.00 – 32.49 32.50 – 34.99
Obese class II	35.00 – 39.99	35.00 – 37.49 37.50 – 39.99
Obese class III	≥40.00	≥40.00

Source: Adapted from WHO, 1995, WHO, 2000 and WHO, 2004

In recent years, studies have often been conducted that problems with obesity can be solved by “modulation of the microbiota”. Historically, gut microbiota has been

associated with obesity in humans and rats using culture-dependent methods in the early 1980s. In the 1990s, application of culture-independent molecular techniques based on 16S ribosomal RNA (rRNA) genes (e.g., competitive PCR) expanded our knowledge towards human gut microbiota, where dominated by Bacteroidetes and Firmicutes bacteria. With the introduction of "next generation sequencing (NGS)" in the early 2000s, more efficient and comprehensive characterization of the microbiota was possible in a shorter time and at a lower cost (Tseng and Wu, 2019). In 2004, *Akkermansia muciniphila* was first isolated from a healthy patient's stool and reported as a joint member of the gastrointestinal tract (GIT) (Dubourg et al., 2017). In the last 15 years, the functions of *A. muciniphila*, which has been included in the new generation super probiotic class, in the gastrointestinal system; the effects on reducing obesity, diabetes, and inflammation continue to be investigated (Naito et al., 2018; Durmaz, 2019; Saarela, 2019).

Research in recent years shows that *A. muciniphila* affects glucose and lipid metabolism and intestinal immunity, and some bioactive components (polyphenols, etc.) can increase the development of *A. muciniphila* in the microbiota. *A. muciniphila* is a Gram-negative, non-sporulating strict anaerob bacterium belonging to the phylum Verrucomicrobia, a common member of the GIT (Collado et al., 2007; Huang et al., 2015; Xavier-Santos et al., 2019). In addition, it also takes its place among mucin-degrading bacteria particularly in the field of metabolic diseases. Besides, next generation probiotics (NGP) or live biotherapeutic products (LBP) represent innovation in the food industry. Strains belonging to the *Bacteroides, Clostridium, Faecalibacterium* and *Akkermansia* groups have been reported as health-related gut bacteria (Saarela, 2019).

CHAPTER 2

MICROBIOTA & OBESITY

2.1. Gut Microbiota

The gut microbiota can stimulate the host's immune system to protect against pathogens. Thereafter gut microbiota plays an important role in establishing the natural immune response and enhancing gut barrier function (Figure 2.1). One of the microbial species in the gut e.g., *A. muciniphila* have a role in immune regulation and increasing intestinal barrier function (Geerlings et al., 2018). Strains of *A. muciniphila* can not be propagated easily and are rarely found. Hence many studies linking *A. muciniphila* to gut health have generally focused on this bacterium during the study of gut microbiota as a remarkable class. They can be considered as next generation probiotics and several *in vivo* studies have also been carried out.

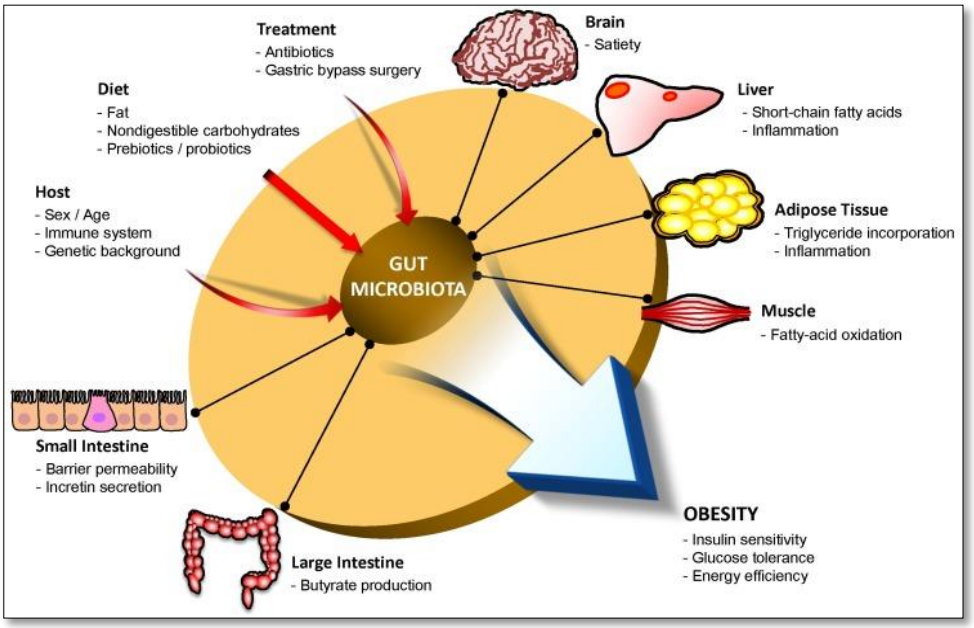


Figure 2. 1. Obesity & gut microbiota
(Source: Yang and Kweon, 2016)

It is known that the growth parameters of *A. muciniphila* growth observed in between the temperatures of 20 to 40 °C and 5.5 and 8.8 pH while the optimum growth temperature and pH are 37 °C and 6.5, respectively. *A. muciniphila* is a mandatory chemoorganotroph that uses mucus as a single source of carbon, nitrogen and energy. By using these, short chain fatty acids (SCFAs) can be produced with acetate, propionate and to a smaller quantities 1,2-propanediol and succinate etc. (Geerlings et al., 2018). *A. muciniphila* is the main producer of propionate in the distal column. It can create acetate, propionate, and ethanol by mucin fermentation (Derrien et al., 2004). Recently, there have been studies for *A. muciniphila* as a propionate-producing bacterium, that is able to prevent adverse effects resulted from high-fat diet-induced obesity, including fat mass gain, adipose tissue inflammation and insulin resistance (Everard et al., 2013; Schneeberger et al., 2015; Van den Abbeele et al., 2018). *A. muciniphila* can sustain its growth even at a low concentration of oxygen in the mucus layer. Mucus layer of large intestines (Figure 2.2) acts as a source of carbon, nitrogen and energy for the use of *A. muciniphila.* Although it is considered to be the most suitable niche for *A. muciniphila* is the mucus layer in the large intestine, mucus is also found in other locations of GIS. Various conditions may vary, such as the type of mucin secreted in the gastrointestinal tract, pH, oxygen concentration, and concentration of bile acids (Geerlings et al., 2018).

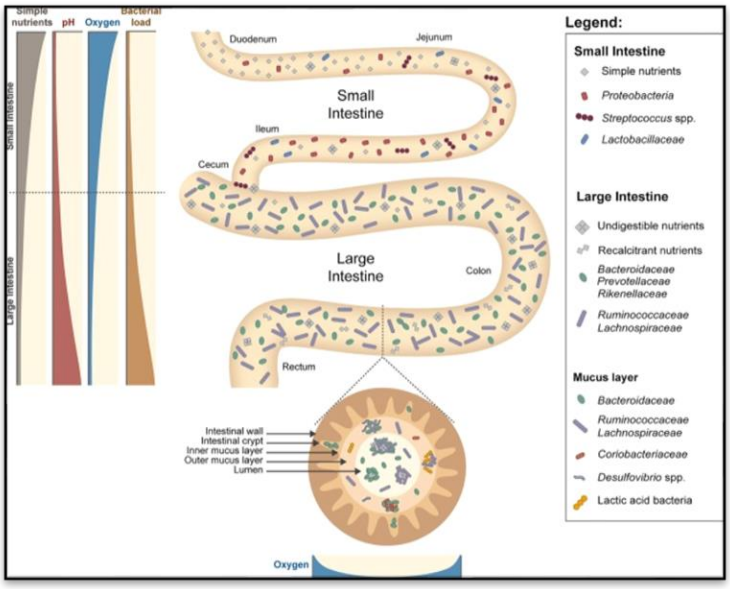


Figure 2. 2. Composition of gut microbiota
(Source: Pereira and Berry, 2017)

2.1.1 The Firmicutes to Bacteroidetes Ratio

The colon, the large intestine, is a complex microbial ecosystem with about 10^{11} cells per gram of the entire intestinal content. Despite the large diversity of strains, 87% (80-90%) of the microbial community in the human column only belonging to two bacterial phylums: Bacteroidetes and Firmicutes (Rios-Covian et al., 2013; Arboleya et al., 2016). Bacteroidetes co-exist with humans (symbionts) and stabilizing the colon ecosystem; e.g., *Bacteroides fragilis.* The class of *Bacteroides* are saccarolytic microorganisms that produce succinic, acetic, lactic and propionic acids, but are also capable of proteolithic fermentation. Bifidobacteria, which often described as probiotic based on health-promoting benefits, accounts for about 3% of the adult human microbiota (Rios-Covian et al., 2013; Zhou, 2017; Xavier-Santos et al., 2019).

In recent periods, after gut microbiota was found to have an impact on host metabolism, research has been focused on explaining which members of the microbiota are more directly associated with weight gain. In this context, firstly, the presence of certain types of microorganisms was associated with the presence of obesity. In animal models, it was concluded that the ratio of Gram-positive bacteria (Firmicutes (F) to Gram-negative bacteria (Bacteroidetes (B), Actinobacteria and Proteobacteria) (F/B) is related to the presence of obesity (Fontané et al., 2018; Naito et al., 2018; Jiao et al., 2019).

Microbiota profile of obese individuals; compared to individuals with normal BMI and anorexic patients, the rate of Bacteroidetes was found to be low while the number of Firmicutes was not differentiated (Armougom et al., 2009). Thus, the high relative ratio of Firmicutes to Bacteroidetes (i.e., F/B ratio) can be considered as a biomarker that apparently points to obesity sensitivity. As for groups of bacteria, comparisons of the gut microbiota of obese and frail people revealed a high rate of Firmicutes and low rates of Bacteroidetes in obesity, and was found to be the opposite profile in people with one year of dietary treatment after gastric bypass (Zhang et al., 2009; Tseng and Wu, 2019).

Obese individuals: ↑ Firmicutes & ↓ Bacteroidetes,
Lean individuals: ↓ Firmicutes & ↑Bacteroidetes

In general, as the predominant phyla Firmicutes, Bacteroidetes and Actinobacteria were found in stool samples obtained from healthy individuals. The less dense filum was observed to be Proteobacteria and Verrucomicrobia (Geerlings et al., 2018).

2.2. Gut Microbiota That Causes Obesity

The gut microbiota as a promoter of obesity (Figure 2.3) contains numerous species. They are described as an "obesity-causing gut microbiota" (Prior et al., 2010) as some species, such as Firmicutes and Bacteroidetes, can contribute to the development of obesity. Firmicutes, together with Bacteroidetes, constitute a significant portion (90%) of the human gut microbiota and are primarily *Ruminiococcus, Clostridium* and *Lactobacillus* (Eckburg et al., 2005; Barlow et al., 2015).

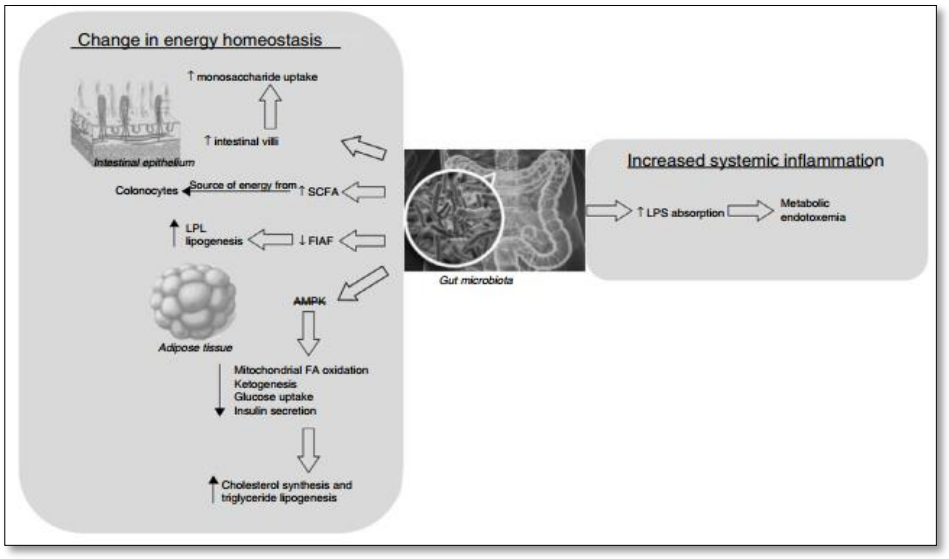


Figure 2. 3. Mechanisms of action of the microbiota as a promoter of obesity (Source: Fontané et al., 2018).

2.3. Development and Aging of the Gut Microbiome

In essence, it has long been thought that the gut microbiota forms immediately after birth (Collado et al., 2016). However, the adult-like structure of the gut microbiota

is emerge the 3rd year of life (Arboleya et al., 2016). Therewithal, depending on the hormones secreted in adolescence, the gut microbiota once again undergoes change and that resulting in differences in the male and female microbiota (Fransen et al., 2017). In adulthood, however, the composition of the gut microbiota is relatively stable. Besides, it is still assumed that it can vary with life events. On the other hand, with the increase of Bacteroidetes in people over the age of 65, the microbial population is changing (Thursby and Juge, 2017).

2.4. Anti-Obesity Gut Microbiota

Yeasts and *Akkermansia muciniphila* can promote weight loss (Tsai et al., 2015; Chelakkot et al., 2018). Yeast supplementation along with bacteriocins of ruminal bacteria; prevented the rise of lipid catabolism and increased weight by enabling gut microbiota modulation in C57BL/6 male mice that fed the Western-style diet (Tsai et al., 2015). *A. muciniphila,* a mucin-degrading bacterium, benefits host metabolism by regulating intestinal barrier integrity by reducing the permeability of Caco-2 cells repaired with lipopolysaccharide (LPS) that a degradation product (Chelakkot et al., 2018). Anti-obesity gut bacteria such as *Bifidobacterium, Lactobacillus,* Bacteroidetes and *A. muciniphila,* all together; can induce weight loss in several ways such as reducing insulin resistance (Figure 2.4) and promoting browning of white fat cells including lowering intestinal permeability, enhancing the integrity of intestinal mucosa with high levels of tight junction proteins, modulating expression of key regulators involved in lipogenesis, alleviating intestinal inflammation with reduced levels of NF-κB, TNF and IL-1β (Cao et al., 2019).

A pilot study in obese children found that the *L. casei* strain in the drink containing Shirota reduced body weight and increased the level of high-density lipoprotein cholesterol. Moreover, a waist circumference of more than 88 cm in women and 102 cm in men indicates that a person has visceral/central obesity (Fox et al., 2007; Rouxinol-Dias et al., 2016). In one study, *L. gasseri* BNR17 was observed to effectively reduce visceral fat mass and waist circumference in obese adults (Kim et al., 2018).

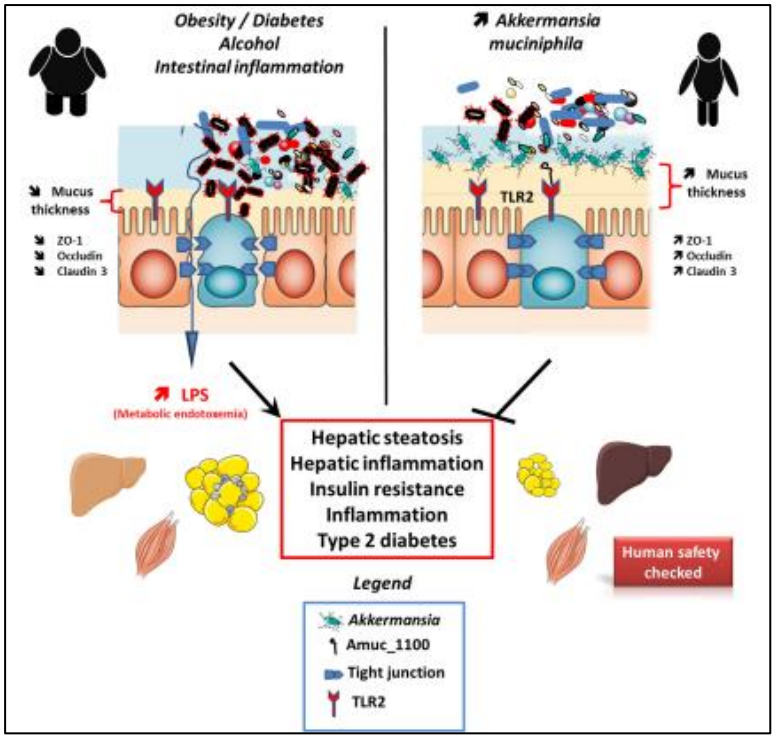


Figure 2. 4. Effects of *A. muciniphila* and derived products on host metabolism

(Source: Cani and de Vos, 2017)

CHAPTER 3

MODULATION OF MICROBIOTA BY TRIGGERING *AKKERMANSIA* WITH PROBIOTICS: A NEW APPROACH TO OVERCOME OBESITY

In this Chapter, considerations on the modulation mechanism of *A. muciniphila* were discussed. Beneficial microorganisms such as probiotic bacteria and certain food ingredients such as polyphenols may increase the abundance of *A. muciniphila* in the gut. Triggering *Akkermansia* affects glucose metabolism, lipid metabolism, and intestinal immunity.

3.1. *Akkermansia muciniphila* «A Next Generation Beneficial Microbe»

A. muciniphila was introduced in the literature as a next generation beneficial microbe. The electromicrogrammes of *A. muciniphila* are presented in the Figure 3.1 and Figure 3.2.

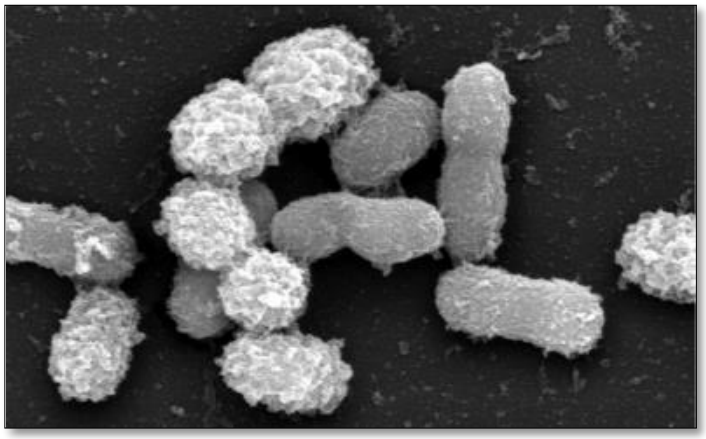


Figure 3. 1. Scanning electronic micrograph of *A. muciniphila* ATCC BAA-835 (bar represents 1 mm) (Source: Derrien et al., 2017)

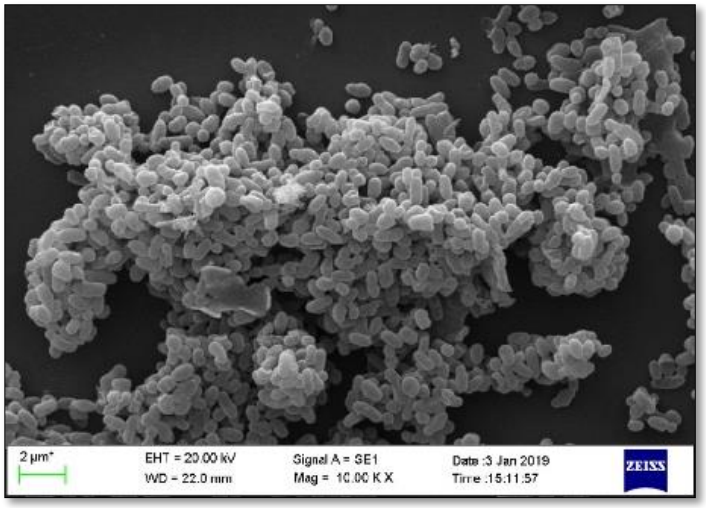


Figure 3. 2. Scanning electronic micrograph of *A. muciniphila* (bar represents 2 µm) (Source: Zhang et al., 2019)

3.2. Probiotic/Prebiotics and *Akkermansia muciniphila*

According to the definition of the World Health Organization; probiotics are live microorganisms that, when taken in adequate amounts, have a beneficial effect on human health (FAO/WHO, 2002). Probiotics, which have disease prevention and therapeutic properties, have an important place in protecting the health of individuals e.g., weight control (Table 3.1); *Lactobacillus* and *Bifidobacterium* are the most common probiotic strains (Parvez et al., 2006). Probiotics can adhere to the intestinal mucosa and competitively eliminate pathogens and maintain their viability (Sharma and Devi, 2014). There are few studies yet examining the effects of probiotic and prebiotic intake on the density of *A. muciniphila*. In a study, the effect of probiotic mixtures on *A. muciniphila* have been examined by *in vitro* intestinal model system, showed that SCFA concentration increased with time in the colon with probiotic supplementation and promoted mucin thickening. It was stated that such an environment contributes to the development of *A. muciniphila*, which degrades mucin (Zhou, 2017).

Table 3. 1. Use of probiotics for weight control: Animal model studies

(Source: Fontané et al., 2018)

Reference	Animals	Strain (dose)	Duration of treatment	Main outcome
Miyoshi et al., 2015[55]	29 mice with diet-induced obesity	*Lactobacillus gasseri SBT2055* (5×10^8 CFU)	24 weeks	↓ Weight and body fat ↓ Leptin
Park et al., 2014[56]	40 mice with diet-induced obesity	*Lactobacillus plantarum LG42* (10^7 CFU and 10^9 CFU)	12 weeks	↓ Weight and body fat
Park et al., 2013[57]	36 mice with diet-induced obesity	*Lactobacillus curvatus HY7601* (5×10^9 CFU) and *Lactobacillus plantarum KY1032* (5×10^9 CFU)	18 weeks	↓ Weight and body fat Modulation of pro-inflammatory genes or fatty acid oxidation-related genes in the liver and adipose tissue
Fåk et al., 2012[58]	39 mice with metabolic syndrome	*Lactobacillus reuteri ATCC PTA 4659, DSM 17938 and L6798* (10^9 CFU)	12 weeks	↓ Weight and body fat (for ATTC PTA 4659 only) ↓ Insulin resistance (fasting insulinaemia)
Kondo et al., 2010[59]	18 mice with diet-induced obesity	*Bifidobacterium breve B-3* (10^8 or 10^9 CFU)	8 weeks	Suppression of weight gain Improved cholesterolaemia, insulinaemia and basal glycaemia
An et al., 2011[60]	36 male rats	*Bifidobacterium pseudocatenulatum SPM 1204, Bifidobacteium longum SPM 1205 and 1207* (10^8–10^9 CFU)	7 weeks	↓ Weight and body fat ↓ Blood pressure
Yin et al., 2010[32]	48 male rats	*Bifidobacteria L66-5, L75-4, M13-4 and FS31-12* (10^8 CFU)	6 weeks	↓ Weight in L66-5 and improved weight gain in M13-4 ↓ Triglyceridaemia and cholesterolaemia

CFU: colony-forming unit; ↓: decrease.

Consumption of prebiotic compounds influence the weight management since SCFA are the main products of their fermentation in the gut, and therefore modulates the release of gastrointestinal satiety hormones (Delzenne and Cani, 2005). This role of the gut microbial community demonstrates the potential of prebiotic compounds to target metabolic diseases (Van den Abbeele et al., 2018). Moreover, polyphenols provide metabolic benefits to the host by alleviating intestinal inflammation to support the presence of *Akkermansia* in the gut microbiota, thus representing a safer alternative. The first evidence of a polyphenol-rich extract obtained from fruits is that it has developed metabolic syndrome by having a prebiotic effect on *Akkermansia* (Anhê et al., 2016). In various host GIT microbiota, LAB and bifidobacteria gain great importance because of their probiotic properties and industrial applications. Among LAB, *L. plantarum* is a diverse and versatile group of bacteria used in a variety of food fermentation processes due to their wide adaptability. They have also proven to survive within the gastrointestinal tract of humans and animals and promote some health benefits (Devi and Halami, 2017).

Adhesion of probiotics to intestinal layer is considered as an important criterion in the application of their beneficial effects to the host, which mainly includes triggering

epithelial-associated signaling molecules by secreting various pro- or anti-inflammatory cytokines, thereby controlling the growth of pathogens (Devi and Halami, 2017). However, it has been found that *in vitro* methods applied to determine the adhesion property for a probiotic bacteria are time consuming, troublesome and not reproducible. Specific mechanisms and methodologies to select potential probiotic bacteria with high adhesion are still lacking (Devi and Halami, 2017).

Lactic acid bacteria strains do not degrade mucins, do not produce DNAse, and are non-hemolytic. Thus, the physiological properties and safety assessment of strains highlight the further use of these strains in the preparation of different probiotic food formulations (Boricha et al., 2019). The beneficial effects of intestinal microbial balance on human health are a valuable probiotic property. Therefore, *Lactobacillus* strains have been characterized by their ability to adhere and colonize the intestinal mucus layer and produce antimicrobial agents. These properties enable strains of probiotics to compete with GIS pathogens and food spoilage pathogens. The antimicrobial effect is due to the production of antimicrobial compounds, namely bacteriocins, organic acids, hydrogen peroxide, etc. (Boricha et al., 2019). Among the probiotic strains, bacteria and probiotic mixtures that bind strongly to mucus (*L. reuteri* etc.) or weakly bound (*S. thermophilus* etc.) have been found to promote mucin thickening. These may provide the necessary mucin source for *A. muciniphila*; in order to provide lactate production, *S. thermophilus* bacteria together with β-galactosidase etc. enzyme systems breaks down this layer and thus it is thought that may support increasing the amount of *Akkermansia* in the intestine.

In summary, it has been observed that bacteria with the ability to adhere to mucus layer and contribute in thickening of the mucus, thus promoting an increment of mucin. On the other hand, it is thought that bacteria such as *S. thermophilus*, which have poor adhesion to mucus, produce lactate by breaking down lactose and thus support the mucus pathway, regulate the mucus environment, stimulate the mucus layer, and may also be a signal that can modulate the colonic epithelium. At this point, the fact that lactate fights pathogens in the environment thanks to its antimicrobial feature supports the improved mucin/mucus environment for *Akkermansia.* Furthermore, the adhesion of lactic acid bacteria to the mucosa in the colon and promoting the secretion of mucus by intestinal epithelial cells (HT-29 cell) is another important aspect that supports the development of *Akkermansia.* It has been reported in studies that probiotics promote mucin thickening, prebiotics affect weight management through SCFAs and target metabolic diseases, while polyphenols provide metabolic benefits to the host to support the presence of

Akkermansia in the intestinal microbiota. Thus, in this suitable habitat, *Akkermansia* produces SCFAs such as acetate and propionate using mucin oligosaccharides. It is thought that propionate produced by this bacterium, which is known to be a propionate producer, can prevent the negative effects of obesity.

3.2.1. Probiotic Effect on Body Weight

Different types of the same genus can have different effects on obesity; some *Lactobacillus* species e.g., *L. plantarum*, *L. sakei* and *L. reuteri* etc. have been reported that encode more bacteriocins by expressing more glucose permease enzymes than *Lactobacillus* which cause weight gain (Drissi et al., 2014). *Lactobacillus* has many subspecies, some of them which are known to be associated with weight gain and inducing obesity. *Lactobacillus* species that promote weight gain were found to be lacking in glucose metabolic enzymes, antioxidant enzymes, and some important enzymes such as dextrin, L-ramnosis, and acetate-synthetases (Drissi et al., 2014). In a meta-analysis conducted based on animal and human models *L. acidophilus* practice causes weight gain in humans; it was also concluded that *L. fermentum* and *L. ingluviei* causes weight gain in animals (Million et al., 2012). Moreover, in the human study, endocarditis patients treated with vancomycin, a common antibiotic, the increase in body mass index was found to be associated with increased *Lactobacillus* (BMI increase of over 10% in 12.2% of patients). Because these LAB lack of the ability to break down fructose or glucose and have stronger replication, recombination and repair activities, the eco-balance of the host gut microbiota can be disrupted (Cao et al., 2019).

Although some *Lactobacillus* are associated with weight gain, most lactobacilli have an anti-obesity effect (Barlow et al., 2015). A meta-analysis, has shown that *L. plantarum* increases weight loss in animals and on the other hand, *L. gasseri* increases weight loss in obese people and animals (Million et al., 2012). *L. acidophilus* 031 CE significantly inhibited the development of obesity and accumulation of lipids in the liver of ICR (Institute of Cancer Research) mice that fed high fat diet (HFD) by reducing triglyceride level and activities of aspartate transaminase and alanin transaminase (Li et al., 2016). Implementation of *L. sakei* OK67 to mice that fed HFD greatly reduced their body fat and weight gain (Lim et al., 2016). Besides that, different *L. sakei* strains have been reported to be able to reduce HFD-induced obesity, colitis and anxiety through the

activation of NF-κB pathways with AMP-activated protein kinase (AMPK). And modulation of SIRT-1 expression via gut microbiota (Cao et al., 2019). In one study, *L. gasseri* BNR17 reduces weight gain, on the other hand, *L. gasseri* L66-5 encouraged weight gain. In this study, *L. rhamnosus* GGMCC was found to be the only strain that had a positive effect on weight loss in humans. Besides, *L. plantarum* and *L. curvatus* HY7601 applied together with *L. plantarum* LG42, *L. gasseri* SBT2055 and KY103 have been reported to have anti-obesity effects in animal models (Rouxinol-Dias et al., 2016).

In the literature review for the detection of LAB strains showing anti-obesity properties, mainly 46 *L. plantarum*, 17 *L. rhamnosus*, 9 *L. casei* strains and with 7 *L. acidophilus,* 6 *L. reuteri*, 5 *L. paracasei*, 4 *L. lactis*, 2 *L. brevis* and 1 *L. delb.* ssp. *bulgaricus* strains from 78 articles, was concluded that they showed anti-obesity properties generally in *in vivo* studies (animal/human experiments) and also in *in vitro* experiments.

When these studies are examined on a species basis, the literature studies on weight control and obesity related to *Lactiplantibacillus plantarum* revealed that 46 different strains with weight loss and anti-obesity properties were detected in 49 of 55 research articles, on the other hand, in 6 of them, 4 different *L. plantarum* strains have been identified that can cause weight gain (Andersson et al., 2010; Bejar et al., 2013; Choi et al., 2019; Wang et al., 2020) especially in chickens (Huang et al., 2020) or 6-month-old babies (Karlsson et al., 2011).

Similarly, when the literature studies on weight gain and obesity related to *Lacticaseibacillus rhamnosus* species were examined, 17 different strains showing weight loss and anti-obesity properties were detected in 20 of 21 articles. Besides, in one of these studies, *L. rhamnosus* GG strain was supported weight gain in 6-month-old infants (Vendt et al., 2006). Essentially, it can be understood that this situation is not associated with obesity, but is a healthy growth indicator with the positive change in the infant microbiota. In addition to this, it was found that the same strain showed anti-obesity properties in 7 different research studies (Kim et al., 2013; Kim et al., 2016; Ji et al., 2018; Kim et al., 2018; Roselli et al., 2018; Ejtahed et al., 2019; Li et al., 2020). It is stated that the inconsistent effects of probiotic *Lactobacillus* on the prevention of obesity may be related to the differences and doses of the bacterial strains used, the duration of administration, and the bioactive compounds of the foods in the diet (Cao et al., 2019).

3.3. Functional Foods and Functional Food Ingredients and Their Place in Turkey and the World

In the industrial sense, it seems that functional formulations containing probiotics and the diversity and production of food products are not widespread in our country but there is a market open to development. For this reason, new product categories with probiotics, consisting of new and different raw materials, have gained importance in terms of the development of the functional food market. Current research, is in the direction of expanding the range of functional products in the market and offering new options in a different variety according to the demands and desires of the consumer. In recent years, functional food ingredients containing probiotics, which have positive effects on intestinal flora, have gained popularity as well as functional foods.

The relationship between food consumption and health is gaining more and more importance every day. Demands for functional food products are showing increase significantly as consumers turn to natural products and functional foods. Functional foods were first introduced by adding calcium and some vitamin-like compounds to foods due to their health benefits. The functional food market in Turkey is rapidly developing, albeit far behind, compared to Europe and the United States. In this context, the importance of probiotic bacteria and their positive effects on human health are increasingly emphasized, and an increase is observed in the consumption of functional products produced with probiotic bacteria. The probiotic content of the foods in question; must contain at least 10^6 to 10^7 CFU/g of living bacteria (Terpou et al., 2019).

In order to support the anti-obesity microbiota, probiotic and/or symbiotic preparations promoting the *Akkermansia* group can be developed. Therefore, new functional foods can be prepared with the addition of these formulations. Moreover, increased lactate production with the help of β-galactosidase etc. enzyme systems can enhance the amount of *Akkermansia.* In addition, more reliable clinical studies on humans are needed to be investigated in order to understand the effects of SCFAs on obesity.

CHAPTER 4

MUCIN/MUCUS LAYER

4.1. Mucus Adhesion Capabilities and Functions of Lactic Acid Bacteria

The mucus layer is the main component of the intestinal barrier in terms of the preservation of the host and its harmonious partners (Nishiyama et al., 2016). The layer of mucus covering the gastrointestinal (GI) channel is the first point of contact between the gut microbiota and host, providing a habitat for the microbiota. Established bacteria can affect the composition, structure and mucus properties of the mucus layer (Kebouchi et al., 2020). Moreover, secretion and collection of mucin and movement of intestinal content creates a liquid environment in the GI channel. Therefore, its adhesion to the mucus surface is one of the critical prerequisites for the colonization of non-mobile organisms in the GI channel; this also gives organisms a competitive advantage in this ecosystem (Nishiyama et al., 2016).

The human gastrointestinal system (GIS) houses a heterogeneous group of microbes formed by 10^{14} different species. Several mucin or mucin-like proteins have been recorded as being expressed with GIS epithelial cells and making it easier for bacteria to adhere through binding sites, thereby increasing colonization. Some adhesins have been extensively studied with fimbriae, pili or cell surface proteins and have been found to be responsible for bacterial adhesion. However, the best example of adhesion is mucus-targeting proteins or mucus-binding proteins (MUB) that well characterized among *Lactobacillus* species (Devi and Halami, 2017). Mucus is involved in conservation, defence and homoeostasis between host and partner. A reduction in mucus thickness leads to inflammatory diseases and therefore, bacteria that promoting mucus formation strengthens the defence. Mucus formation in the gut is a dynamic process provided by goblet cells and sustained by transcription factors (such as KLF4). For example, mucus adhesins expressed on the cell surface of *L. reuteri* strains can show immune-regulating effects in the gut.

4.2. Lactate Production of Lactic Acid Bacteria

Although some LAB have poor adhesion to the mucus, for example, *Streptococcus thermophilus* has been shown to partially support the mucus pathway by producing lactate in the digestive tract (Fernandez et al., 2018). Lactose, by breaking down by *S. thermophilus* etc., lactate is produced that can shape and strengthen bacterial communities found in the gut microbiota in the digestive tract (Veiga et al., 2010). Lactate is a powerful antimicrobial factor that inhibits the growth of pathogens and participates in the nutritional chain between microbial communities; because it supports the growth of bacteria that consume lactate and then produce secondary SCFAs such as acetate, propionate and butyrate, and these products have well-known beneficial activities on the gut (Turpin et al., 2013; Louis and Flint, 2017; Fernandez et al., 2018).

Lactate in the fermentation process, is a key intermediate produced in homofermentative or heterofermentative ways by *Lactobacillus* and/or *Bifidobacterium* (Matsuki et al., 2013; Van den Abbeele et al., 2018). Lactate participates in a reversible reaction with pyruvate that another important component of the fermentation process. In co-culture fermentations, the presence of *Bifidobacterium bifidum* promoted the growth of other strains. Therefore, *B. bifidum/ Bifidobacterium infantis* co-cultures yield acetate, formate and lactate, while *B. bifidum/ Eubacterium hallii* co-cultures formed acetate, formate and butyrate (Bunesova et al., 2018). In this case, lactate is observed as a precursor to the formation of acetate, propionate and butyrate in species such as *Megasphaera* and *Veillonella*, which are among the lactate-utilizing bacteria (LUB). Among these, *Veillonella ratti* produces acetate, propionate and formate while *Desulfovibrio piger* produces acetate. Moreover, *Eubacterium limosum* and *Eubacterium hallii* produce butyrate, while *Propionibacterium avidum* produces propionate (Muñoz-Tamayo et al., 2011; Pham et al., 2017).

In vivo glycolytic activity of *S. thermophilus*; breaking down lactose and the subsequent mechanism for promoting health through lactate production is important (Fernandez et al., 2018). *S. thermophilus* has been shown to regulate the mucus pathway with the production of lactate, which increases the number of goblet cells in the digestive tract of gnotobiotic rats and the amount of KLF4 mucus-inducing transcription factor. A significant increase in KLF4 protein due to the production of lactate by this bacterium has also been reported in HT29-MTX cells. It suggests that the lactate produced may at

least sustain the partial stimulation of KLF4, which is involved in differentiating the cells that provide the secretion. This state indicates that the anti-proliferative effect observed for lactate in *in vivo* and *in vitro* studies is consistent with the stimulation of cell cycle arresting proteins (Rul et al., 2011; Matsuki et al., 2013; Fernandez et al., 2018).

Another study previously reported that the accumulation of lactate produced by a mixture of three lactobacilli (*L. fermentum, L. paraplantarum* and *L. salivarius*) had no effect on mucus production in gnotobiotic rodents (Turpin et al., 2013). Thus, stimulation of the mucus pathway is likely to require lactate and additional factors brought during its intestinal transit by *S. thermophilus.* The inclusion of *S. thermophilus* as subdominant bacteria (10^3 CFU/g faeces) in the complex microbiota environment caused induction of digestion. It is possible that during intestinal transit of *S. thermophilus*, bioactive muco-inducing peptides can be produced in the intestine as well as in yogurt (Fernandez et al., 2018). In studies by Plaisancie et al (2013, 2015) and Bruno et al (2017); that LAB in yogurt hydrolyze milk caseins to form small and functional peptides; thereby some, such as peptide β-CN (casein) (94-123), have been shown to significantly induce the production of *in vitro* and *in vivo* mucus.

4.3. Influence of SCFA e.g., Lactate, Propionate to *Akkermansia*

Lactate; has anti-microbial activity by acidifying the luminal fluid in the intestine and with its indirect mechanisms affecting the expression of colonization factors necessary for the proliferation of pathogens. Remarkably, lactate was determined to reduce transcription of genes needed for *Campylobacter jejuni*'s *in vivo* growth and catabolism (Luethy et al., 2017). A lactate-rich environment within the gut can also increase tolerance to commensal bacteria (Angelin et al., 2017) and weaken pro-inflammatory pathways (Iraporda et al., 2015, 2016). Therefore, lactate has widely beneficial effects that can feed microbial communities within the gut, fight pathogens, mitigate inflammatory processes, and strengthen the gut barrier through mucus production (Fernandez et al., 2018).

Van den Abbeele et al. (2018) investigated how long-term administration of a xylooligosaccharide/polyphenol mixture affects prebiotic potential and immune modulation. Accordingly, the applied mixture correlated with an increase in lactobacilli and bifidobacteria (+ 2.5 log/mL) while leading to a reduction in ammonium and

increased lactate levels towards the top part of the colon. However, an increase in propionate-producing *A. muciniphila* was observed in the proximal colon lumen (up to 7.15 log/mL). Propionate acts as a precursor to gluconeogenesis in the liver and affects cell differentiation with its health-promoting potential effect on gut inflammation and cancer development (Reichardt et al., 2014; Bunesova et al., 2018). Additionally, when looking at microbial community data in recent studies, *A. muciniphila* is virtually absent from the rising column (AC), while a finding has been confirmed that it specifically colonizes distal colon regions (transverse colon (TC) and descending colon (DC)) (Figure 4.1). Furthermore, a distinct protective effect was observed on Caco-2 barrier function and immune-activating properties in the distal column (Van den Abbeele et al., 2018).

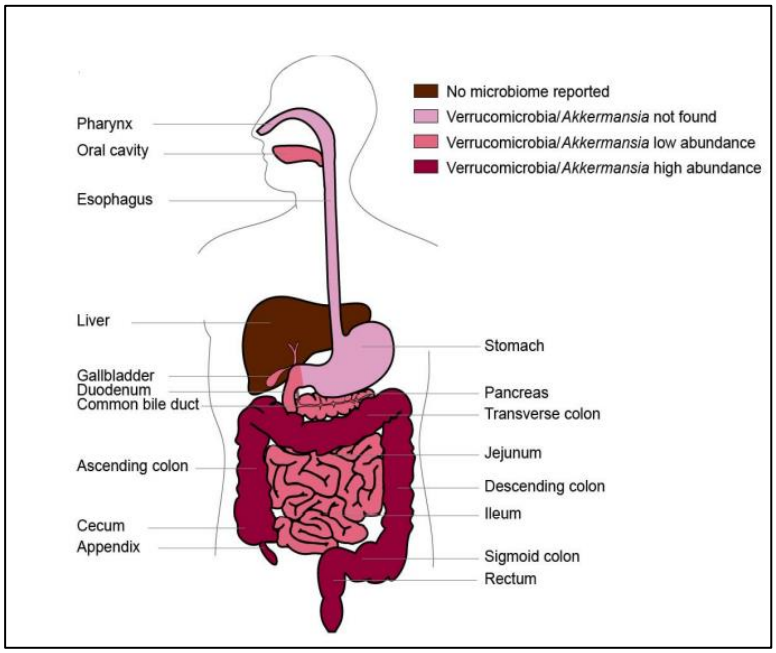


Figure 4. 1. Verrucomicrobia/*Akkermansia* sequences in the human digestive system (Source: Geerlings et al., 2018)

Xylooligosaccharides (XOS) have been shown to selectively stimulate *Bifidobacterium* levels in the human gastrointestinal tract, and polyphenols stimulate the growth of both lactobacilli and bifidobacteria (Dueñas et al., 2015). Both groups can produce high concentrations of lactate, an important metabolite in the human colonic environment, as it exerts potent antimicrobial effects; it also causes the production of

metabolites such as butyrate as it has a number of nutrient/trophic interactions with other bacteria (Van den Abbeele et al., 2018).

In summary, while yogurt bacteria help lactose digestion, especially in lactose intolerant individuals, β-galactosidases complete the lactose breakdown efficiency with these bacteria. Lactose, broken down by bacteria which have poor adhesion to mucus such as *S. thermophilus* etc. enabling the production of lactate in the digestive system, and this is important in terms of the mucus pathway supporting mechanism. Thus, lactate is an intermediate metabolite used as a substrate by members of the microbiota. At this point, lactate can be metabolized by lactate utilizing bacteria (LUB), to form different end products. In a study, it was observed that the xylooligosaccharide/polyphenol mixture led to increased lactate levels towards the upper part of the colon with an increase in lactobacilli and bifidobacteria. However, it was concluded that propionate-producing *A. muciniphila* also increased in the proximal colon lumen. At this point, it is conceivable that the increase in the amount of lactate in the colon positively affected the amount of *Akkermansia* in the environment. As a result, the adhesion of lactobacilli on mucin has attracted attention as one of the critical factors contributing to the lasting beneficial effects of *Lactobacillus* in an ever-changing intestinal environment. Therefore, understanding the interactions between *Lactobacillus* and mucin is crucial in terms of elucidating the survival strategies of LAB in the GI tract (Nishiyama et al., 2016).

4.4. Mucins and their Importance in terms of Microbiota

Mucins are extracellular, high molecular weight glycoproteins (0.5-20 MDa) that form the main components of epithelial mucus layers (Celebioğlu et al., 2017). Mucin glycoproteins are the most common structural components of GI mucus; followed by a complex mixture of lipids, enzymes, nucleic acids, and immunoglobulin A secretion. Mucin secretion is a highly dynamic process and new mucin is constantly produced in colonic goblet cells and has a conversion rate of <1 hour. The muc gene encodes the core protein of mucin, and to date, about 20 different mucin-encoding genes have been discovered in humans and several homologous muc genes have been identified in other animal species (Nishiyama et al., 2016). Mucin is used as a food source by gut bacteria since it is composed of amino acids and oligosaccharides. The interaction of mucins with the microflora plays an important role in normal function. Mucins are modified in various

diseases, and this may be due to excessive mucin peptide or glycosylation (Corfield, 2015). It is stated that there is a negative relationship between the cell number of *A. muciniphila* and the fecal mucin concentration (Collado et al., 2007).

Among the intestinal bacteria known to have a mucolytic activity, *A. muciniphila* is one of the bacteria that has large enzymatic systems for the breakdown of mucin oligosaccharides (Kebouchi et al., 2020). Other gut bacteria, such as *Bifidobacterium bifidum, Bacteroides fragilis* and *Ruminococcus gnavus* also show a mucolytic activity (Png et al., 2010; Katoh et al., 2017; Kebouchi et al., 2020). On the other hand, in the case of *A. muciniphila* BAA-835, different genes encoding putative mucinolytic enzymes have been identified in the genome. However, despite the apparent reciprocal relationship between *A. muciniphila* and the host, mucin functions and their role in mucin degradation are mostly unknown (Kosciow and Deppenmeier, 2020).

Akkermansia muciniphila is involved in the degradation of the oligosaccharide chains of mucins with its enzyme system and it has been reported that the components resulting from this degradation consist of the glycan part, which constitutes approximately 80% of the total molecular mass, N-acetylgalactosamine, N-acetylglucosamine, fucose, galactose and mannose, as well as sialic acid, sulfate, disaccharide and small oligosaccharides (Derrien et al., 2011; Tailford et al., 2015; Kosciow and Deppenmeier, 2020). The purification, characterization and also their activities towards natural mucin glycan chains of 3 β-galactosidase enzymes were investigated to understand the mucin destruction mechanism of *A. muciniphila.* These enzymes are: Amuc_0771, Amuc_0824 and Amuc_1666. During enzyme characterization, various chromogenic and non-chromogenic compounds consisting of different types of sugars and linked by different glycosidic bonds were screened. However, with this approach, the possibility of target functionality of other structures for the three enzymes in mucin glycan chains has not been ruled out (Kosciow and Deppenmeier, 2020).

The fact that *A. muciniphila* is associated with obesity increases the investigation of *Akkermansia* as a super probiotic day by day; however, the safety and dosage of the strains are anticipated to be problematic factors; little is still known about how safe this bacterium is for human consumption. Similar to many other gut bacteria, *A. muciniphila* is also difficult to cultivate in routine clinical laboratories (Saarela, 2019). Therefore, the need for caution in the use of *Akkermansia* as a probiotic treatment for obesity-related inflammatory diseases comes to the fore. As a safer approach, alternative strategies can

be used to increase the gut majority of *Akkermansia*; such as other probiotics and/or extracts rich in polyphenols.

4.5. Lactic Acid Bacteria, HT-29 Cell Culture and *A. muciniphila*

It is known that LAB adhere to the mucosa in the colon and HT-29 cells are used in *in vitro* experiments to examine this phenomenon (Gopal et al., 2001; Wallace et al., 2003; Koller et al., 2008; Pinto et al., 2009). On the other hand, it was found that *A. muciniphila* strongly adhered to Caco-2 and HT-29 colon cells (Reunanen et al., 2015; Geerlings et al., 2018). HT-29 cells are not known to be primarily mucus-forming cells; however, in addition to adhesion studies in which LAB and HT-29 cells are together (Servin and Coconnier, 2003; Kim et al., 2008; Stöber et al., 2010), many studies have reported that lactobacilli increase mucin expression and induce mucin secretion (Deplancke and Gaskins, 2001; Mattar et al., 2002; Ohland and MacNaughton, 2010). Dudik et al. (2020) predicts that pre-incubation of lactobacilli with mucin stimulates their *in vitro* adhesion to HT-29 cells, thus potentially leading to better bioavailability in the gut at the *in vivo* level. In most of these studies, probiotic LAB strains (e.g., *L. plantarum* 299v, *L. rhamnosus* GG, *L. acidophilus* A4) both increased the expression of MUC2 and MUC3 genes (mucin) in HT-29 colon cell cultures and contributing to barrier function and inhibition of pathogens has been observed.

4.6. Thesis Objective

Obesity is increasing more in the world as is a major problem in our country. *Akkermansia muciniphila* is defined as the most effective microorganism in human microbiota against this problem. This bacterium utilizes mucin, and its existence can be promoted by beneficial bacteria that support mucin/mucus thickening.

In this thesis, it is aimed to identify probiotic LAB that can promote mucin/mucus thickening as a potential anti-obesity probiotic candidate, in order to increase the presence of mucin, which is required for the proliferation of the anti-obesity *Akkermansia* group population in the microbiota, in terms of gastrointestinal system (GIS) health.

The overall objective of this thesis is to "select probiotic LAB with the ability to promote mucin/mucus growth in order to create a suitable environment for the development and proliferation of *Akkermansia* in the intestinal environment".

In this context, it is planned to examine the mucin/mucus adhesion capabilities of LAB, determine the antimicrobial effects of LAB, and prepare the mucin layer as a suitable niche for the *Akkermansia* group. It has been hypothesized that the lactate produced by LAB, which has poor adhesion to the mucus, can be used as a substrate for the production of SCFAs such as propionate, which support the growth of anti-obesity microorganisms such as *Akkermansia*. In this thesis study, the following studies were carried out in order to answer the question of "Can a suitable environment (habitat, niche) be provided for the development and proliferation of *Akkermansia* with the mucin/mucus adhesion abilities and other properties of LAB?".

1. Identification of lactic acid bacteria resistant to gastrointestinal conditions
2. Determination of mucin degradation abilities of LAB and their growth characteristics in the presence of mucin/mucus (selecting those that do not degrade mucin)
3. Determination of adhesion of LAB to mucin/mucus due to its promoting potential in mucin/mucus thickening
4. Determination of biofilm-forming abilities of LAB due to their promoting potential in mucin/mucus thickening
5. Determination of lactate production capacity of LAB due to their promoting potential in mucin/mucus thickening
6. Supporting the theory of promoting *Akkermansia* group growth by inhibiting pathogenic microorganisms with its antimicrobial effect as well as the mucin/mucus thickening role of lactate production,
7. Determination of the effect of selected probiotic LAB on mucin thickening by *in vitro* cell culture (HT-29) studies.

CHAPTER 5

MATERIALS AND METHODS

5.1. Materials

In this study, in order to provide a simulated environment that can support the development of *Akkermansia muciniphila*, species of LAB have been emphasized as they have an important place in anti-obesity studies (Section 3.2.1).

Commercial species of *Lactiplantibacillus plantarum* NRRL-B 4496, *Lactobacillus acidophilus* NRRL-B 1910, *Lacticaseibacillus casei* NRRL-B 441, *Lacticaseibacillus rhamnosus* NRRL-B 442, *Lactobacillus pentosus* NRRL-B 227, *Lactiplantibacillus plantarum* DSM 1954 and *Streptococcus thermophilus* CCM 4757, were obtained from the ARS Culture Collection (NRRL, USA).

LAB strains were obtained from the culture collection of İzmir Institute of Technology, Department of Food Engineering, Molecular Food Microbiology Laboratory (IZTECH-FED-MFMLCC).

Another LAB to be used as probiotic bacteria isolated from olive samples were obtained from the culture collection of Adnan Menderes University, Department of Food Engineering, Microbiology Laboratory.

According to the literature research, 40 LAB were selected among the reference and isolates in the culture collection. They were evaluated in terms of stability in gastrointestinal conditions, not degrading mucin/mucus, growth in the presence of mucin, adhesion to the mucin/mucus layer, biofilm formation amounts, lactate production abilities, antimicrobial properties and HT-29 cell binding levels and by focusing on the relevant strain group at each step, the phase of observing (quantitative) and measuring (qualitative) mucin/mucus thickness was reached. Thus, with this study, it was aimed to determine the anti-obesity probiotic prototype candidate.

5.2. Methods

In this section, the studies carried out on the following subjects are described respectively: Growth and preparation of bacterial cultures, stability of LAB in simulated gastrointestinal conditions, determination of mucin degradation ability of isolates and growth ability in the presence of mucin, bacterial adhesion on mucin, biofilm formation**,** sugar fermentation tests, lactate production, antimicrobial properties and HT-29 cell culture & LAB: Qualitative & quantitative determination of mucin layer.

5.2.1. Growth and Preparation of Bacterial Cultures

In this section, bacterial cultures used and methodologies for their growth conditions, resuscitation from stock culture and preparation of cell suspension have been described.

5.2.1.1. Bacterial Cultures and Growth Conditions

Among the LAB, 6 reference and 1 isolate bacterium resistant to simulated gastrointestinal conditions (and capable of binding to mucus) were determined to be used in preliminary studies. In addition, 33 LAB strains that have been identified and isolated from our traditional fermented foods were used in the following studies.

Based on the literature review studies, among the LAB to be used as probiotic bacteria, which species that show the most anti-obesity feature, which does not degrade mucin (*L. acidophilus, L. rhamnosus*) (Zhou et al., 2001; Monteagudo-Mera et al., 2012), strong adhesion to mucin/mucus (*L. pentosus, L. plantarum*) (Shekh et al., 2016; Montoro et al., 2018), high lactate production ability (*L. acidophilus, L. casei* and *L. plantarum*) (Matsuki et al., 2013; da Silva Sabo et al., 2017; Moens et al., 2017) were identified for preliminary study. "*L. plantarum* NRRL-B 4496, *L. acidophilus* NRRL-B 1910, *L. rhamnosus* NRRL-B 442, *L. casei* NRRL-B 441, *L. pentosus* NRRL-B 227 and *L. plantarum* DSM 1954" strains were selected from the culture collection of İzmir Institute of Technology, Department of Food Engineering, Molecular Food Microbiology Laboratory (IZTECH-FED-MFMLCC) which stored in glycerol stocks at -80 ˚C. These

strains were resuscitated by subculturing twice for 18-24 hours at 37 ºC and 42 ºC using MRS and M17 media (Table A.1). Thus, preliminary studies have been carried out on growth from stock culture, determining dilution intervals, measurement of bacterial density (OD=0.5) with McFarland, inoculating and counting on MRS and M17 agar, determination of cell densities by spectrophotometer and creation of standard graphs between the measured Absorbance (A) values and cell density (A)/CFU (Colony forming unit).

5.2.1.2. Resuscitation from Stock Culture

Among the LAB in our collection kept in glycerol stocks at -80 °C, *L. plantarum* NRRL-B 4496, *L. acidophilus* NRRL-B 1910, *L. casei* NRRL-B 441, *L. rhamnosus* NRRL-B 442, *L. pentosus* NRRL-B 227, *L. plantarum* DSM 1954 strains and *S. thermophilus* CTY24 were selected, subcultured twice at 37 °C and 42 °C for 18-24 hours using MRS and M17 media, and resuscitated forms were used as inoculum in the trials.

5.2.1.3. Preparation of Cell Suspension

The prepared cell suspensions are planned to be used in the following studies. 10 mL of each of the resuscitated cells was taken, centrifuged (2500 x g, 10 min, 4 °C) and washed twice with 10 mL of equal volume of phosphate buffered saline (PBS, 0.1 M, pH 7, 0.85% NaCl). They were then resuspended in 1.5 mL of phosphate buffer (0.1 M, pH 7) to prepare the cell suspension (approximately 10^9-10^{10} CFU/mL). Prepared cell suspensions were used in the following studies.

5.2.2. Stability of LAB in Simulated Gastrointestinal Conditions

Simulated gastric and intestinal fluid solutions were prepared as described by Okuklu, B. (2014) and Bengoa et. al. (2018) with some modifications. Simulated gastric juice was prepared as described by Guo et al. (2009) and Okuklu, B. (2014). The pH of the PBS medium (Table B.4) was adjusted to 3.0 with 1 N HCl and sterilized at 121 °C for 15 minutes. Pepsin solution was prepared at a final concentration of 3 g/L, sterilized

by microfiltration (0.22 µm) and then mixed with the PBS solution. The activated test culture was centrifuged at 2500 g for 10 minutes at 4 °C. Cells washed twice in 0.85% (w/v) saline solution were resuspended in sterile saline solution. Cell suspensions (1 mL) were inoculated into PBS (pH 3.0, 9 mL) supplemented with pepsin and incubated at 37 °C for 1.5 hours (1.5-2-2.5). After incubation, the total number of viable cells was determined by the pour plate method and the survival rates were calculated using the following equation 5.1:

$$\text{Survival rate (\%)} = (\log \text{CFU } N_1 / \log \text{CFU } N_0) \times 100 \quad \text{(eqn. 5.1)}$$

(N_1= total number of live strains after gastric juice implementation, N_0= total number of live strains before implementation)

Simulated intestinal juice was prepared as described by Bengoa et al. (2018). 0.1% w/v pancreatin and 0.15% w/v bovine bile salts were added to PBS by filter sterilization (0.22 µm). One milliliter of the culture was transferred to 9 mL of PBS (pH 7.2-8) (Table B.4) containing pancreatin and bovine bile salts. Intestinal transit tolerance was determined by incubation of bacterial cultures in intestinal fluid at 37 °C for 3 h (3-3.5-4). Afterwards, as a result of incubation of pour plate cultivation using MRS and M17 agar for 48 h at 37 °C, survival rates were calculated using the following equation 5.2:

$$\text{Survival rate (\%)} = (\log \text{CFU } N_2 / \log \text{CFU } N_0) \times 100 \quad \text{(eqn. 5.2)}$$

(N_2= total number of live strains after intestinal juice implementation, N_0= total number of live strains before implementation)

5.2.3. Determination of Mucin Degradation Ability of Isolates and Growth Ability in the Presence of Mucin

In this section, methodologies for mucin degradation ability of isolates have been explained together with the growth ability of isolates in the presence of mucin.

5.2.3.1. Mucin Degradation Ability of Isolates

The experiment was carried out as stated by Turpin et al. (2012). Briefly, glucose (20.00 g/L) and porcine/large pig gastric mucin type III (3.00 g/L) were combined with modified MRS (Okuklu B., 2014) (Table B.1, Table B.2). 5 µL of the overnight bacterial cultures were applied as spot on MRS and M17 solid media that containing mucin. Plates were incubated at 37 °C for 72 hours without shaking and then stained with Amido black (3 g/L) in acetic acid (3.5 M) for 30 minutes. Then, plates were washed with acetic acid (1.2 M) until the site of mucin lysis (colorless halo) was revealed. Mucin lysis was detected by observing the colorless rings around the spotting bacterial cultures. Mucin degradation activity is expressed in Figure 6.10 and Figure 6.11 by the diameter size of the colorless rings in the mucin lysis region.

5.2.3.2. Growth Ability of Isolates in the Presence of Mucin

The experiment was carried out as stated by Turpin et al. (2012). Revived bacteria 2% (v/v) were incubated in modified MRS broth (Okuklu B., 2014) containing 20.0 g/L glucose and porcine gastric mucin for 24 h at 37 °C. At the end of the incubation, the absorbance values (A_{600}) were determined by measuring. Results are expressed by subtracting from the absorbance values obtained in modified MRS (Table B.1) containing glucose or mucin (A2) to the absorbance values obtained in modified MRS medium without fermentable carbohydrates (A1) (Sugar-free-control) (A2-A1).

At this point, by screening the mucin degradation capabilities of the LAB that bind to mucin/mucus, those that do not degrade mucin were selected for subjecting to the next methods.

5.2.4. Bacterial Adhesion on Mucin

In this section, experimental methods “preparation of plates for adhesion and bacterial binding assays” have been described to assess the adhesion properties of bacterial cultures.

5.2.4.1. Preparation of Plates for Adhesion

This method is based on the Carasi et al. (2014). Briefly, partially purified porcine gastric mucin type III (hereinafter MUCIN) was dissolved in PBS at pH 7.0, and then 100 µL of 3 g MUCIN/L solution was added to each well and fixed to 96 well sterile polystyrene plates (Sánchez et al., 2009). Plates were incubated for 1 hour at 37 °C, followed by overnight (16-18 h) at 4 °C. A second incubation was performed with the same solution for 2 h at 37 °C to minimize the number of empty binding sites in the polystyrene microtiter plates. Finally, the wells were washed twice with 200 µL of PBS. Four parallels were applied to determine the adhesion of the analyzed strain.

5.2.4.2. Bacterial Binding Assays

The prepared LAB cell suspensions were resuspended in 1.5 mL of phosphate buffer (0.1 M, pH 7) and the optical density was adjusted to OD_{550} = 1.0 (~ 10^9 CFU/mL).100 µL of the prepared bacterial cell suspensions were added to each well. After the plates were incubated for 2 h at 37 °C, the wells were washed 6 times with 200 µL of sterile PBS to remove unbound bacteria then they were treated with 200 µL of 5 mL/L Triton X-100 solution (Table A.2) at 37 °C for 30 minutes to decompose the adherent bacteria (Carasi et al., 2014; Bengoa et al., 2018). The number of bound LAB was determined by dilution of 100 µL of bacterial suspension from each well with PBS and then seeding on MRS and M17 agar plates. Colony counts were performed by serial dilutions for the sample in each well, inoculated as a pour plate, and counted after 48 h at 37 °C.

5.2.5. Biofilm Formation

The method specified in the Couvigny et al. (2015) study was applied with some modifications. Biofilm formation was determined by the quantitative microplate method based on crystal violet staining for 7 LAB strains that resistant to simulated gastrointestinal conditions. Each well of a 96 well cell culture plate containing 0.2 mL of MRS/M17 broth was inoculated with 10 µL of overnight grown bacterial culture samples

and incubated at 37 °C for 18 h. Next, the 96 well cell culture plate was inverted emptied and 50 µL of 1% crystal violet (CV) was added to each well and the plates were incubated at room temperature for 15 minutes. The wells of the microtiter plates were washed three times with 0.2 mL of deionized water to remove unbound cells and residual dye, and then dried at 37 °C for approximately 1 h. Then, 200 µL of EtOH used for microbiological analysis was added to each well and it was waited for about an hour for the CV dye to dissolve. This process was repeated 3 times and the samples in the wells that dissolved in the last step were transferred to a new, sterile 96 well plate by using pipette. Biofilm formation was determined by absorbance measurement at 590 nm in the Varioskan device. Determination of biofilm formation was performed in four parallel and three independent replicates.

5.2.6. Sugar Fermentation Tests and Microbial Metabolic Activity

The biochemical fermentation properties of LAB were investigated. In order to determine the ability of LAB strains to decompose mucin sugars, they were weighed at 10% for each carbon source (galactose, mannose, lactose, glucose, sucrose, xylose, fructose, rhamnose, fucose, N-acetylglucosamine, N-acetylgalactosamine) to be tested. Then these sugars were dissolved, and then passed through a 0.20 µm filter and prepared into sterile falcon tubes by filter sterilization. On the other hand, modified MRS broth (without sugar, with BCP) was prepared (pH: 6.2-6.6) (Table B.3). Then, 5 mL of each bacterium that was revived overnight was taken and centrifuged at 5000 rpm, 4 °C, for 10 minutes. After washing twice with 5 mL of modified MRS, the same volume (5 mL) of cell suspension was obtained. 160 µL of cell suspensions prepared with modified MRS was added to each well of the 96 well plate. 40 µL of 10% sugar solutions were added to them. At this point, while glucose was the positive control, modified MRS medium was added to the wells for the negative control. The microtiter plates were incubated for 24 h at 37 °C to observe growth (Fernandez et al., 2018).

5.2.7. Determination of Lactate Production

The lactate concentration in the culture supernatant was measured by HPLC equipment. 1.5 mL was taken from the bacteria that were revived overnight (5 mL) and transferred to sterile eppendorfs. After the samples were centrifuged at 14000 x g in a refrigerated centrifuge at 4 °C for 15 minutes, 1 mL of cell supernatant from each was homogenized with 4 mL of dH_2O and diluted 5 times. Then, approximately 1-1.5 mL of culture supernatant for each bacterium was transferred to vials by passing through a 0.20 µm filter and placed sequentially in the HPLC equipment. For standard solutions, a 2 g/L lactate solution was prepared using lithium lactate. This prepared standard was passed through a 0.20 µm filter and transferred to the vial in approximately 1.5 mL. Then, standards of 1 g/L, 0.5 g/L and 0.25 g/L were also prepared for the standard curve plot. Analyzes were carried out in HPLC device using Aminex-hpx-87H column, flow rate of 0.6 mL/min, temperature of 65 °C and analysis time of 15 minutes, and 5 mM H_2SO_4 solution was used as mobile phase. At the end of the study, data are expressed as g/L sample (Pham et al., 2016).

5.2.8. Determination of Antimicrobial Properties

The antimicrobial activity of LAB was determined using agar spot test, well diffusion method and disk method (Arena et al., 2016). For this purpose, pathogenic microorganisms *E. coli* 3008, *E. coli* RSHM 4024 (ATCC 25922), *L. innocua* NRRL-B 33314, *S. aureus* RSKK 1009 and *B. cereus* ATCC 14579 were used in the experiments. TSB (Tryptone Soy Broth) was used as growth medium for these bacteria in the experiments.

5.2.8.1. Agar Spot Test

In this method, overnight cultures of *Lactobacillus* genus were applied to MRS agar as 5 µL spot application, cultures of *Streptococcus* genus were applied to M17 agar with 5 µL spot application and they were incubated at 37 °C for 24 h. Pathogen cultures grown overnight were mixed with TSB soft agar (0.6% (w/v)) at a ratio of 1/100 and

poured onto colonies developed on MRS and M17. After drying, the plates were placed for incubation at 37 °C and 42 °C for 24 h, and the diameters of the inhibition zones were measured at the end of the time. In another method, 100 µL of pathogenic bacteria diluted 100-fold (*B. cereus* 10-fold) was taken into 20 mL TSA and spread well with sterile L-baguette and waited for them to dry. 5 µL spots from LAB that were revived overnight were applied on them. After drying, the plates were placed for incubation at 37 °C for 24 h, and the diameters of the inhibition zones were measured at the end of the time.

5.2.8.2. Well Diffusion Method

In this method, overnight cultures were centrifuged at 4470 x g at 4 °C for 20 minutes, cell-free supernatants were obtained by filter sterilization (0.20 µm). 50 µL of supernatants was added to wells of 6 mm diameter that drilled with sterile cork borer in 25 mL MRS and M17 agar plates. Plates were incubated at 4 °C for approximately 3 h, allowing the cell-free supernatant to diffusion into the medium. Pathogenic bacteria grown overnight were inoculated at 1% (v/v) fresh TSB soft agars and poured onto MRS and M17 agar plates that containing cell-free supernatant. All plates were removed for incubation at 37 °C for 24 h, and the diameters of the inhibition zones were measured at the end of the period. In another method, 100 µL of pathogenic bacteria diluted 100-fold (*B. cereus* 10-fold) was taken into 20 mL TSAs and spread well with sterile L-baguette and waited for them to dry. Six-millimeter diameter wells were opened with sterile cork borer. 50 µL of LAB were added to the wells. Plates were incubated at 4 °C for approximately 3 h, allowing the cell-free supernatant to diffusion into the medium. And then, all plates were removed for incubation at 37 °C for 24 h, and the diameters of the inhibition zones were measured at the end of the period.

5.2.8.3. Disc Method

100 µL of pathogenic bacteria diluted 100-fold (*B. cereus* 10-fold) was taken into 20 mL TSA and spread well with sterile L-baguette. After drying, the blank discs were immersed in overnight grown cultures of the LAB with sterile tweezers and placed in

pathogen spread plates. After drying again, the plates were placed for incubation at 37 °C for 24 h, and the diameters of the inhibition zones were measured at the end of the time.

5.2.9. Determination of Mucin/Mucus Thickening Effect of Selected Strains for Lactate Production and Other Characteristics

The mucin/mucus thickening effect of the strains determined in terms of the above-mentioned properties (tolerance to gastrointestinal conditions, mucin degradation, mucin adhesion, biofilm formation, antimicrobial tests) along with lactate production was investigated. Here, the most significant elimination was observed in the tolerance to gastrointestinal conditions. In this experiment performed with LAB that meet these conditions at varying levels and 6 reference and 1 isolate bacterium were first studied.

5.2.9.1. Cultivation Conditions of LAB

Overnight grown cultures of 7 LAB strains were inoculated at 1% in MRS and M17 broth media and grown at 37 °C for 24 h (Dudik et al., 2020). 1 mL of bacterial cultures were taken from 2 mL of medium overnight grown of LAB culture and transferred to sterile eppendorfs. They were centrifuged at 4900 g, 5.5 min, at 4 °C. After washing twice with 1 mL of PBS, a cell suspension was prepared with 200 µL of sterile PBS.

5.2.9.2. Cultivation Conditions of Cell Line HT-29

HT-29 cell (ADU Food Engineering Laboratories Cell Culture Collection, kindly gifted by Assist. Prof. Olcay BOYACIOĞLU) were grown in DMEM medium (and RPMI) (4.5 mg glucose mL^{-1}, 1000 U penicillin mL^{-1}, 1000 µL streptomycin mL^{-1}, 10% fetal bovine serum, 0.3 mg glutamine mL^{-1}) at 37 °C in an atmosphere of 5% CO_2 using 12 well plates. The initial number of cells inoculated into each well was approximately 1.2×10^5 / 1.12 cm^2. The culture medium was changed every two days. Antibiotics in the medium were removed 24 h before the experiments (Dudik et al., 2020).

5.2.9.3. HT-29 Cell Culture in the Presence of Lactic Acid Bacteria

HT-29 cells were cultured for 14 days as indicated above. Bacteria names were coded in three parallels for 7 bacteria on two 12 well plates that had reached to 80% HT-29 cell density. First, excess medium (DMEM) was removed from the plates. 500 µL of DMEM PBS (dPBS) was added to each well twice, and the excess dPBS was removed. Then, 500 µL of penicillin-free DMEM was added to each well. 10 µL of LAB cell suspensions dissolved in PBS were added to them in 3 parallels from each bacterium. Adhesion of bacteria to HT-29 cells was checked under the microscope and it was observed that they adhered. LAB cell suspensions were incubated with HT-29 cell in 12 well plates for 1.5-2 h at 37 °C incubator (Dudik et al., 2020).

At this time, in order to determine the number of bacteria at t=0, serial dilution was followed by inoculation on MRS and M17 agars. At the end of the 2 h incubation period; in order to remove non-adherent bacteria, 1 mL of 0.1 M, pH= 7.34 g/L 25 °C PBS (microbiological) was added to each well and washed 3 times. Then, 100 µL of Trypsin EDTA (Table A.2) was added to each well, incubated at 37 °C for approximately 2 min. And then, the wells were washed with 150 mL of PBS to terminate trypsin activity. After washing, 1.5-2 mL of bacteria & cell in PBS was taken from the 12 well plate to sterile eppendorf. They were centrifuged at 4900 g, 5.5 min, at 37 °C. Afterwards, the supernatant, PBS & trypsin medium (excess DMEM) was removed. 200 µL of sterile PBS was added to each eppendorf so that bacterial count could be counted. Then, in order to determine the number of bacteria at t=2, serial dilution was performed and then inoculation on MRS and M17 agars.

In addition, 7 bacteria and a control were encoded without parallel in one 12 well plate that had reached 80% HT-29 cell density to be able to observe under the microscope (Sharma and Kanwar, 2017). This 12 well plate was subjected to the same treatments as the other 2 plates until washing process in the first part of the experiment. After washing, Giemsa dye was added to each well at a ratio of 1:20 to cover the surface (600 µL). It was incubated at room conditions for 20 minutes. The wells were washed two times with 1 mL of sterile PBS to remove excess dye. Next, images of the bacteria were obtained from the microscope.

5.2.9.4. Qualitative Determination of Mucin Layer

The amount and thickness of mucin produced by HT-29 cells in the presence and absence of LAB were first examined under a light microscope by applying the Periodic Acid-Schiff (PAS) staining method and the steps of this method are given below.

5.2.9.4.1. PAS Staining

The PAS staining method was applied as follows by modifying Ning et al. (2012) study for the purpose of examining mucin glycoproteins under light microscopy. Prepared samples were first kept in 0.5% periodic acid for 5 minutes and then washed with distilled water for 5 minutes. In the next step, they were treated in Schiff's reagent (Table A.2) for 10 minutes and then washed with dH_2O for 5 minutes. After washing, counterstaining was applied in Harris hematoxylin (Table A.2) for 1 minute and then washed with dH_2O for 5 minutes. Afterwards, they were kept in 1% acid alcohol for 1 minute and then kept in 26% ammonia water for 10 minutes (until the color of the samples turned blue) and then washed in dH_2O for 5 minutes. Finally, they were dried using 70%, 95% and 99.9% EtOH for increasing alcohol application. At the last stage, some xylene was added to each well, and the samples were cleaned and left to dry for one day. Thus, mucin glycoproteins were observed in purple pink (magenta) color with a light microscope. The mucin thickness in the samples, in which mucin was observed in the microscope by PAS staining, was determined by the quantitative method given below.

5.2.9.5. Quantitative Determination of Mucin Layer

The mucin glycoprotein was determined using the Schiff's reagent method as follows by modifying Leonard et al. (2010); Akbari et al. (2017) and Limage et al. (2020) studies. First of all, the medium was removed to determine the mucin-forming properties of epithelial cells in the presence of bacteria. Cells were lysed in PBS containing 1% Triton X-100 in 1 mL. In the experiment that performed in 12 well plates, 15 µL (total 30 µL) of cell lysate taken from two parallel wells for each sample was transferred to 96 well plates as 3 parallels that containing 120 µL of 0.5% periodic acid for each cell. Then, the

samples were incubated for 2 h at 37 °C. Meanwhile, before the experiment, periodic acid solution was prepared by adding 10 µL of 50% periodic acid to 10 mL of 7% acetic acid. After incubation, 100 µL of Schiff's reagent was added on each well and the samples were incubated for 30 minutes under dark room conditions. The amount of mucin was determined by absorbance measurement at 555 nm using a microplate reader. A calibration curve with a linear range of 10-600 µg/mL was created using porcine stomach mucin type III (Leonard et al., 2010) (Figure 5.1, Figure 5.2).

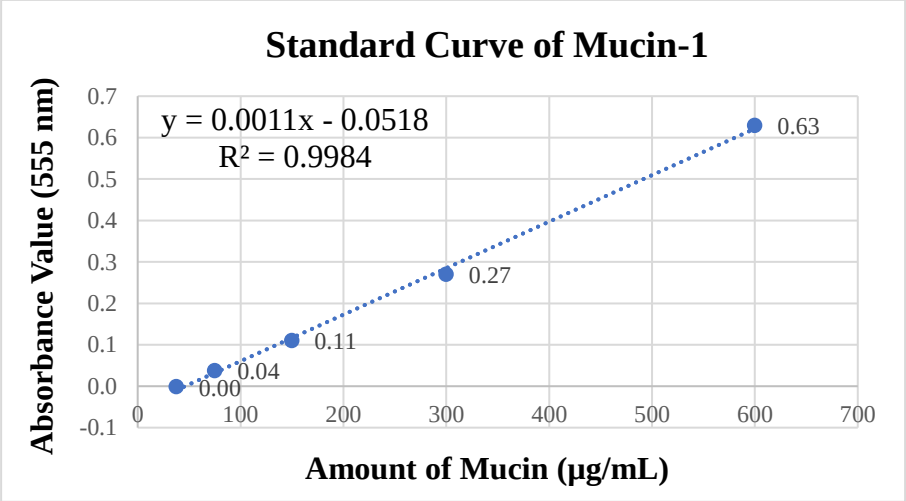


Figure 5. 1. A standard curve plot generated with using mucin for 8 LAB

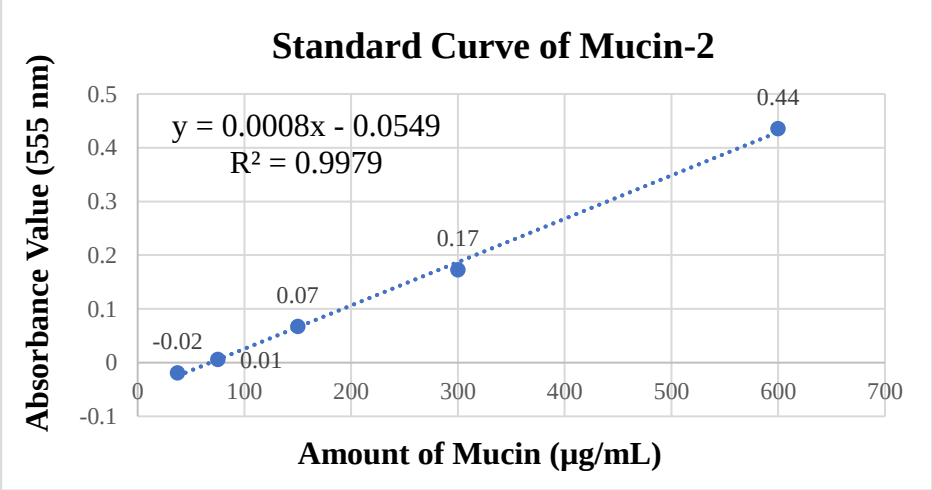


Figure 5. 2. A standard curve plot generated with using mucin for 7 LAB

CHAPTER 6

RESULTS AND DISCUSSION

6.1. Results

In this section, the data and results obtained on the following subjects are evaluated respectively: Growth and preparation of bacterial cultures, stability of LAB in simulated gastrointestinal conditions, determination of mucin degradation ability of isolates and growth ability in the presence of mucin, bacterial adhesion on mucin, biofilm formation, sugar fermentation tests, lactate production, antimicrobial properties and HT-29 cell culture & LAB: Qualitative & quantitative determination of mucin layer.

6.1.1. Growth and Preparation of Bacterial Cultures

Experiments were carried out by making 5, 10, 15, 25 and 50 fold dilutions and by determining the dilution ranges with 5 reference bacteria that selected at the beginning. These bacteria are respectively; *Lactiplantibacillus plantarum* NRRL-B 4496 (Figure 6.2, Table C.1), *Lactobacillus acidophilus* NRRL-B 1910 (Table C.2), *Lacticaseibacillus casei* NRRL-B 441 (Table C.3), *Lacticaseibacillus rhamnosus* NRRL-B 442 (Table C.4) and *Lactobacillus pentosus* NRRL-B 227 (Table C.5). The Absorbance/CFU graphs obtained as a result of trials for these 5 bacteria are shown in Figure 6.1, Figure 6.3, Figure 6.4, Figure 6.5, Figure 6.6. The numbers of all bacteria included in the subsequent experiments were slightly increased in the final concentration of the bacterial suspensions prepared before the experiments, and these bacterial numbers obtained before each experiment with the counts made at t=0 were shown in tables.

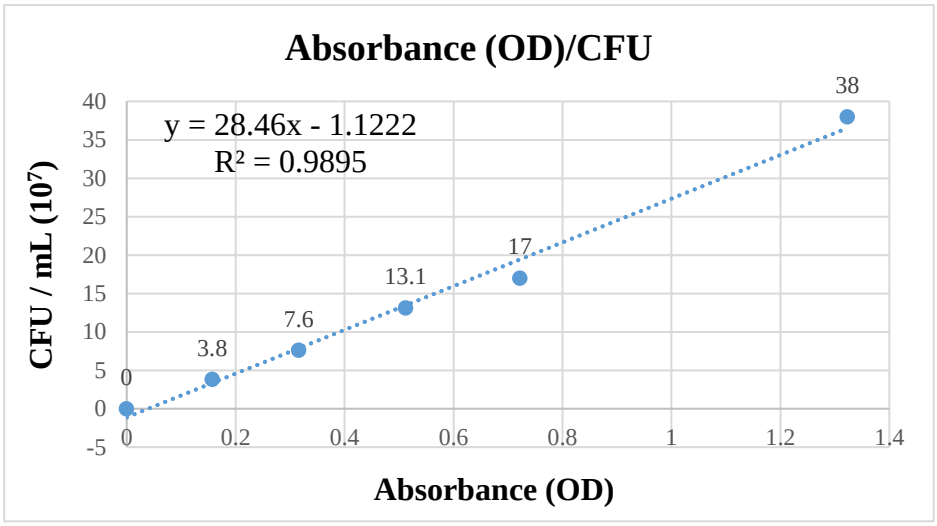


Figure 6. 1. Absorbance (OD)/CFU graph of *Lactiplantibacillus plantarum* NRRL-B 4496

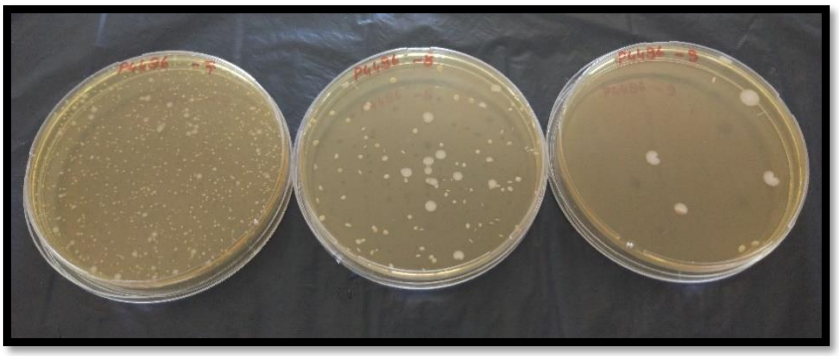


Figure 6. 2. *Lactiplantibacillus plantarum* NRRL-B 4496

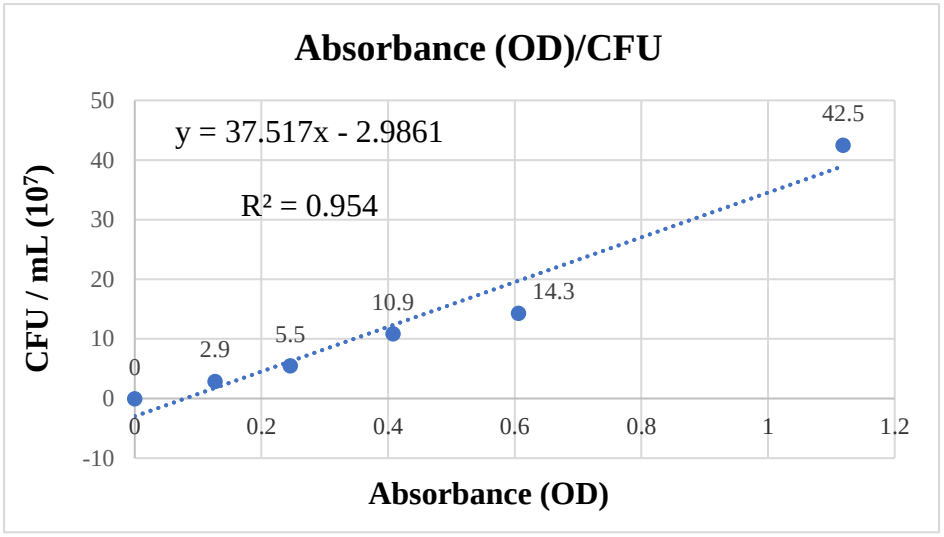


Figure 6. 3. Absorbance (OD)/CFU graph of *Lactobacillus acidophilus* NRRL-B 1910

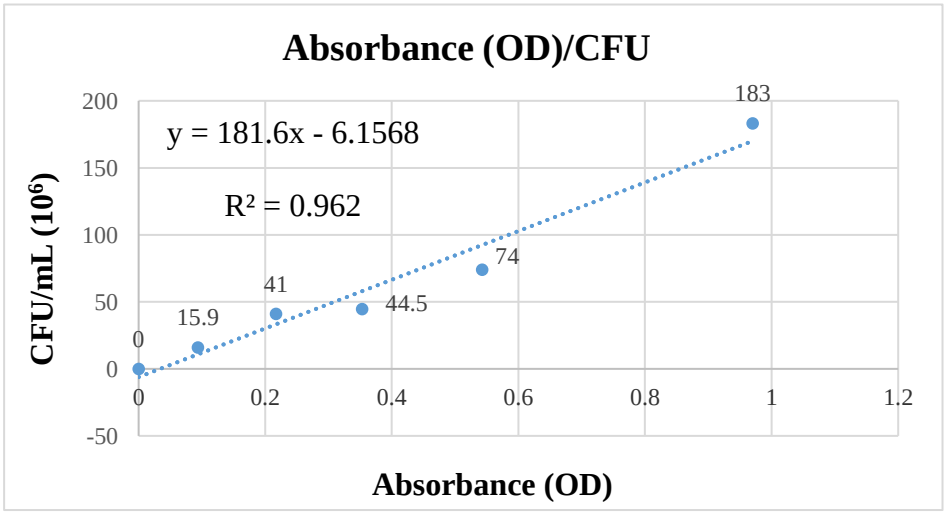


Figure 6. 4. Absorbance (OD)/CFU graph of *Lacticaseibacillus casei* NRRL-B 441

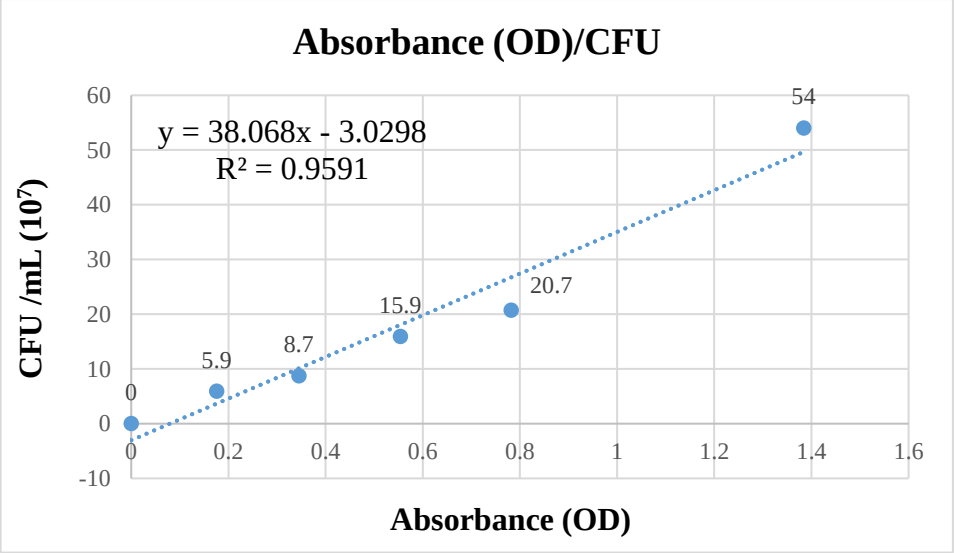


Figure 6. 5. Absorbance (OD)/CFU graph of *Lacticaseibacillus rhamnosus* NRRL-B 442

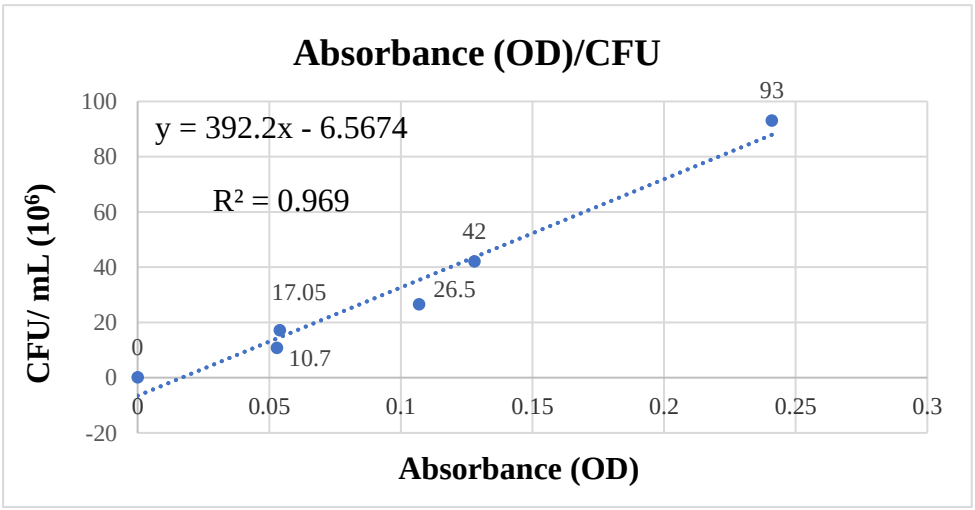


Figure 6. 6. Absorbance (OD)/CFU graph of *Lactobacillus pentosus* NRRL-B 227

6.1.2. Stability of LAB in Simulated Gastrointestinal Conditions

Based on literature research, the probiotic LAB among our collection were selected according to their lactate producing and mucin degradation ability, anti-obesity properties, and strong adhesion capacity on mucin/mucus layer. These species are respectively; *L. plantarum*, *L. acidophilus*, *L. casei* (Figure 6.7) *L. rhamnosus*, *L. pentosus*, *L. paracasei*, *L. lactis* and *S. thermophilus*. Studies were carried out with a total of 40 bacteria isolated from different sources including 7 reference, 13 yogurt, 9 cheese, 7 olives (green/black), 3 pickles and 1 breast milk as shown in Table 6.1. (A total of 10 of these bacteria belonging to olive and pickle samples have not been identified yet.)

Table 6. 1. Lactic acid bacteria used in the experiment of stability of LAB in simulated gastrointestinal conditions

Bacteria No:	**Bacteria Name:**	**Source of Isolation:**	**Bacteria Code:**
1	***L. plantarum* NRRL-B 4496**	Reference	P. 4496
2	***L. acidophilus* NRRL-B 1910**	Reference	A. 1910
3	***L. casei* NRRL-B 441**	Reference	C. 441
4	***L. rhamnosus* NRRL-B 442**	Reference	R. 442
5	***L. pentosus* NRRL-B 227**	Reference	P. 227
6	***L. plantarum* DSM 1954**	Reference	DSM 1954
7	***S. thermophilus* CCM 4757**	Reference	CCM 4757
8	***S. thermophilus* AS95**	Human breast milk	T. AS95
9	***S. thermophilus* C74**	Yogurt	T. C74
10	***S. thermophilus* C77a**	Yogurt	T. C77a
11	***S. thermophilus* C95-1**	Yogurt	T. C95-1
12	***S. thermophilus* C95-2**	Yogurt	T. C95-2
13	***S. thermophilus* C97-2**	Yogurt	T. C97-2
14	***S. thermophilus* CTY24**	Yogurt	T. CTY24
15	***S. thermophilus* CTY41**	Yogurt	T. CTY41
16	***S. thermophilus* CTY44**	Yogurt	T. CTY44
17	***S. thermophilus* UN5**	Yogurt	T. UN5
18	***S. thermophilus* UN9**	Yogurt	T. UN9

(cont. on next page)

Table 6. 1. (cont.)

19	***S. thermophilus* UN19**	Yogurt	T. UN19
20	***S. thermophilus* UIB31**	Yogurt	T. UIB31
21	***S. thermophilus* UIN9**	Yogurt	T. UIN9
22	***L. paracasei* C8**	Cheese	PR. C8
23	***L. paracasei* C15**	Cheese	PR. C15
24	***L. plantarum* C47**	Cheese	PL. C47
25	***L. plantarum* D1**	Cheese	P. D1
26	***L. lactis* A1**	Cheese	L. A1
27	***L. lactis* A19**	Cheese	L. A19
28	***L. lactis* A20**	Cheese	L. A20
29	***L. lactis* A22**	Cheese	L. A22
30	***L. lactis* A23**	Cheese	L. A23
31	**LAB. O. AK6**	Olive	O. AK6
32	**LAB. O. AK8**	Green Olive Brine	O. AK8
33	**LAB. O. AK16**	Black Olive Brine	O. AK16
34	**LAB. O. AK22**	Black Olive (Sele type)	O. AK22
35	**LAB. O. AK23**	Black Olive (Sele type)	O. AK23
36	**LAB. O. AK42**	Black Olive Brine	O. AK42
37	**LAB. O. AK50**	Dark Green Olive Brine	O. AK50
38	**BT10**	Pickle	P. BT10
39	**BT14**	Pickle	P. BT14
40	**BT40**	Pickle	P. BT40

In this study, it is aimed that LAB remain highly viable (at least 10^5,10^6 CFU/mL) in simulated gastrointestinal conditions by determining LAB resistant to gastric and intestinal conditions.

In the experiments, 17 of the 40 bacteria used showed viability in intestinal conditions with a number of 10^4 and above, and 13 of them showed viability of 10^5-10^6 CFU/mL and above. 4 out of 23 other bacteria showed resistance in stomach conditions but could not survive or that could only survive up to 10^4 CFU/mL in intestinal conditions. The data obtained for 17 bacteria are given in Table 6.2.

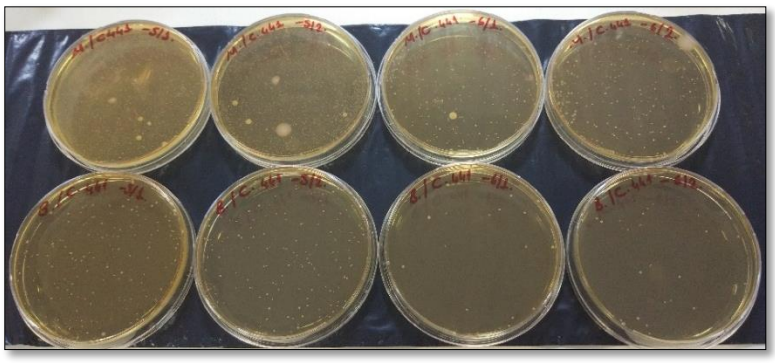


Figure 6. 7. *Lacticaseibacillus casei* NRRL-B 441 after simulated gastrointestinal fluid

Table 6. 2. Counting results obtained after simulated gastric and intestinal fluid with number of bacteria at t=0 for 17 bacteria that used in the experiment

Bacteria No:	**Bacteria Name:**	**Number of Bacteria at t=0 (CFU/mL)**	**Logarithmic Number of Bacteria at t=0**	**Number of Bacteria After Simulated Gastric Fluid (CFU/mL)**	**Logaritmic Number of Bacteria After Simulated Gastric Fluid**	**Percentage Survival of LAB After Simulated Gastric Fluid (%)**	**Number of Bacteria After Simulated Intestinal Fluid (CFU/mL)**	**Logaritmic Number of Bacteria After Simulated Intestinal Fluid**	**Percentage Survival of LAB After Simulated Intestinal Fluid (%)**
1	***L. plantarum* NRRL-B 4496**	1.36×10^{10}	**10.13**	2.45×10^{8}	**8.39**	**82.82**	2.09×10^{7}	**7.32**	**72.26**
2	***L. acidophilus* NRRL-B 1910**	1×10^{9}	**9**	1.82×10^{7}	**7.26**	**80.67**	7.25×10^{5}	**5.86**	**65.11**
3	***L. casei* NRRL-B 441**	5.2×10^{9}	**9.72**	3.29×10^{8}	**8.52**	**87.65**	2.17×10^{7}	**7.34**	**75.51**
4	***L. rhamnosus* NRRL-B 442**	1×10^{9}	**9**	4.32×10^{7}	**7.64**	**84.89**	1.08×10^{6}	**6.03**	**67**
5	***L. pentosus* NRRL-B 227**	1×10^{8}	**8**	1.65×10^{6}	**6.22**	**77.75**	0	**0**	0
6	***L. plantarum* DSM 1954**	1.01×10^{10}	**10**	3.49×10^{8}	**8.54**	**85.4**	2.8×10^{7}	**7.45**	**74.5**

(cont. on next page)

Table 6. 2. (cont.)

7	***S. thermophilus* UIN9**	7.7×10^9	**9.89**	1.19×10^7	**7.08**	**71.59**	7.1×10^5	**5.85**	**59.15**
8	***L. paracasei* C15**	4.9×10^9	**9.69**	9.5×10^6	**6.98**	**72.03**	4.95×10^6	**6.69**	**69.04**
9	***L. plantarum* C47**	7.2×10^9	**9.86**	4.29×10^8	**8.63**	**87.53**	3.09×10^7	**7.49**	**75.96**
10	***L. plantarum* D1**	8×10^9	**9.9**	7.14×10^8	**8.85**	**89.39**	5.96×10^7	**7.78**	**78.59**
11	***L. lactis* A19**	1.1×10^{10}	**10.04**	7.15×10^6	**6.85**	**68.23**	8.5×10^4	**4.93**	**49.1**
12	**LAB. O. AK6**	4.1×10^9	**9.61**	3.25×10^6	**6.51**	**67.74**	3.5×10^4	**4.54**	**47.24**
13	**LAB. O. AK8**	1.23×10^9	**9.09**	1×10^5	**5**	**55**	1.5×10^4	**4.18**	**45.98**
14	**LAB. O. AK16**	4.4×10^9	**9.64**	2.4×10^8	**8.38**	**86.93**	5.4×10^5	**5.73**	**59.44**
15	**LAB. O. AK22**	1.22×10^{10}	**10.09**	7.35×10^6	**6.87**	**68.09**	5.9×10^5	**5.77**	**57.19**
16	**LAB. O. AK23**	5.4×10^9	**9.73**	$\sim 10^8$	**8**	**82.22**	1.11×10^7	**7.05**	**72.46**
17	**LAB. O. AK50**	6.6×10^9	**9.82**	5×10^7	**7.7**	**78.41**	7.55×10^5	**5.88**	**59.88**

According to Table 6. 2, the pre-experiment initial numbers for the bacteria that used in the experiment varied between 10^8-10^{10} CFU/mL is seen. 13 of the 17 bacteria remained viable in the range of 10^6-10^8 CFU/mL in the simulated gastric juice, and these bacteria continued their viability in the range of 10^5-10^7 CFU/mL in the simulated intestinal fluid.

On the other hand, bacteria that were able to survive in the range of 10^5-10^6 CFU/mL in the simulated gastric fluid but could not survive in the simulated intestinal fluid (*L. pentosus* NRRL-B 227) or that could only survive up to 10^4 CFU/mL bacteria that *L. lactis* A19, O. AK6 and O. AK8 were added to the experiment in order to compare the adverse conditions.

In the Figure 6.8, the initial numbers of 17 bacteria and the bacterial counts obtained after simulated gastric and intestinal conditions are shown on the log base. Accordingly, the initial values for 17 bacteria ranged from 8-10.13 log, while the values for 13 bacteria after simulated gastric fluid ranged between 6.87-8.85 log. After simulated intestinal fluid, the changing value range for these bacteria is 5.73-7.78 log.

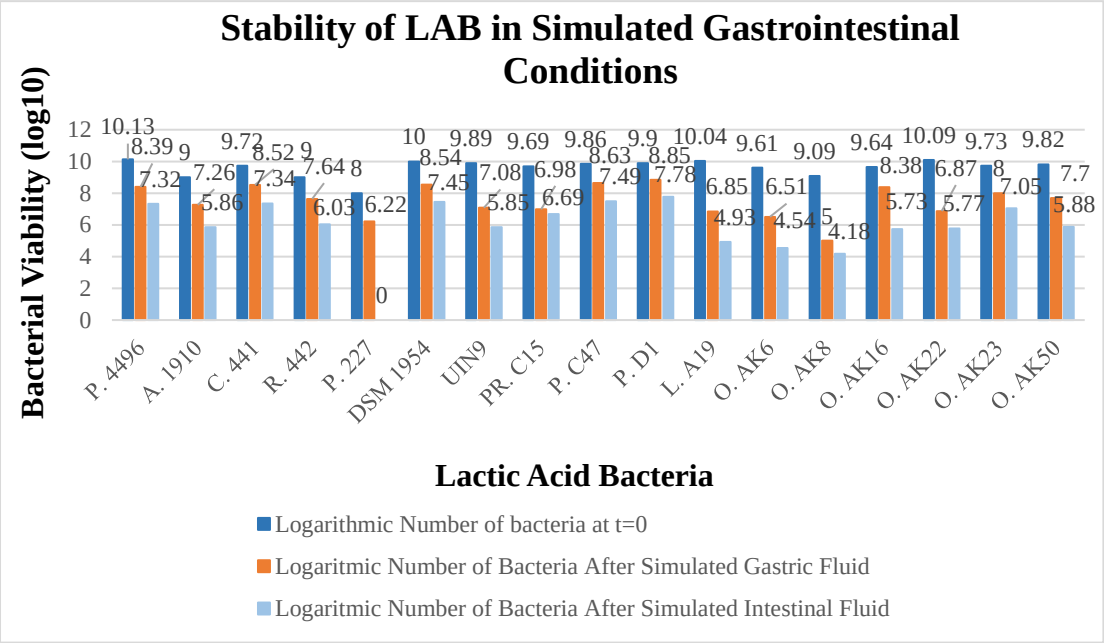


Figure 6.8. Stability of LAB in simulated gastrointestinal conditions (log10)

In the Figure 6.9, the bacterial counts obtained after simulated gastric and intestinal conditions of 17 bacteria are shown on the percentage. Accordingly, the percentage values for 13 bacteria after simulated gastric fluid ranged between 68.09-89.39%. After simulated intestinal fluid, the percentage value range for these bacteria is 59.15-78.59%.

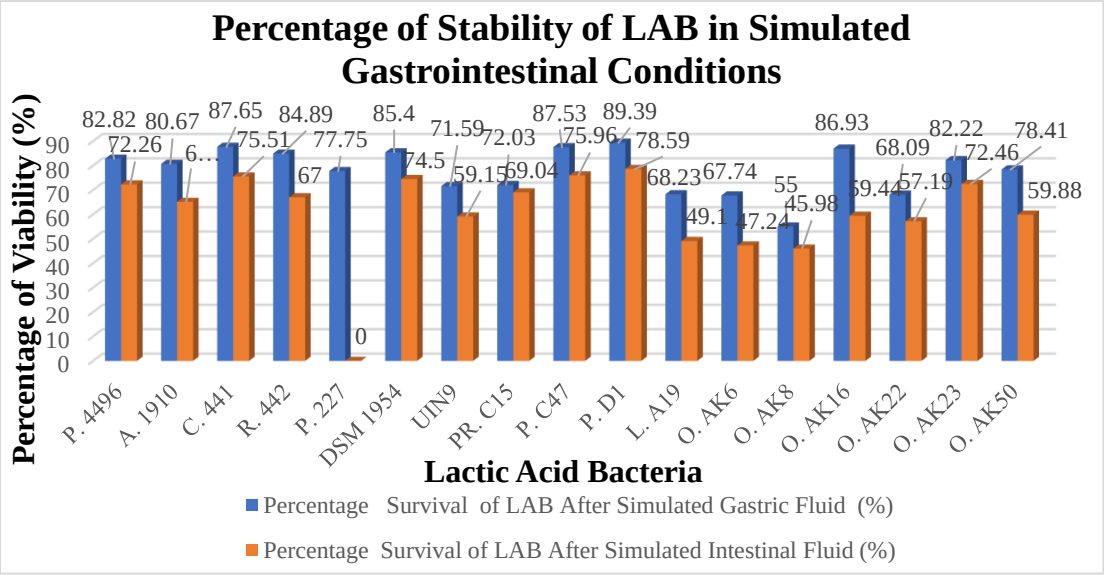


Figure 6. 9. Percentage of stability of LAB in simulated gastrointestinal conditions

Grimoud et al. (2010), Okuklu, B. (2014) and Bengoa et al. (2018) analyzed the intestinal tolerances of *L. paracasei, Lactobacillus*, and *Streptococcus* species isolated from different sources such as kefir, yogurt, and newborn faces. In these studies, while the initial bacterial counts were between 10^7-10^8 CFU/mL, the number of bacteria that remained viable after intestinal conditions was approximately 10^3-10^6 CFU/mL. The percentage of viability varies between 37.6-79.8%.

At the end, the data obtained at this method step appear to show similarities to the results in the papers contained in the literature.

6.1.3. Determination of Mucin Degradation Ability of Isolates and Growth Ability in the Presence of Mucin

In this section, the data and results obtained on the following subjects are evaluated respectively: Mucin degradation ability of isolates and growth ability of isolates in the presence of mucin.

6.1.3.1. Mucin Degradation Ability of Isolates

In this method, 6 reference and 7 isolates LAB were used among total 13 LAB strains. These are respectively: *L. plantarum* NRRL-B 4496, *L. acidophilus* NRRL-B 1910, *L. rhamnosus* NRRL-B 442, *L. casei* NRRL-B 441, *L. pentosus* NRRL-B 227, *L. plantarum* DSM 1954 as reference strains and *L. bulgaricus* UZ22, *L. bulgaricus* DT54, *L. bulgaricus* DT62B, *L. bulgaricus* UIIN24, *L. bulgaricus* UIIN26, *L. bulgaricus* UZ12, *L. bulgaricus* UIN42 as isolate strains.

In this study, it was aimed to select LAB that do not degrade mucin and show no lysis zone into the mucin containing medium. In the Figure 6.10 and Figure 6.11, there are photographs of *L. rhamnosus* NRRL-B 442 and *L. casei* NRRL-B 441 samples for spot application applied in the presence of glucose and mucin in the medium. It was observed that they did not form a lysis zone in the presence of mucin. The 13 LAB strains used in the experiment did not in any way form a clear zone of lysis around colonies with negative mucinolytic activity after administration. With this test result, it can be interpreted that LAB do not degrade mucin.

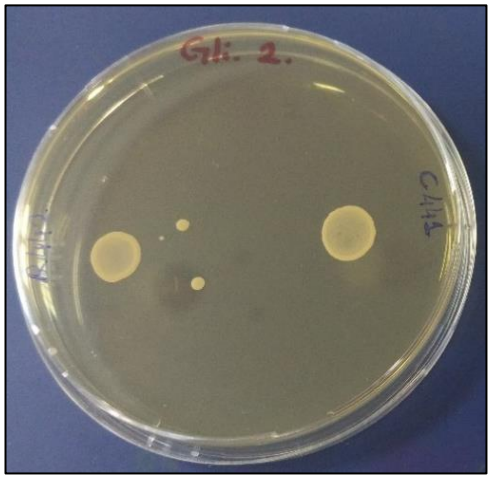
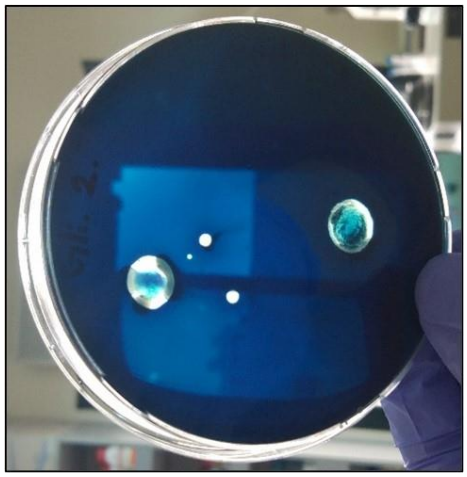
a)  b) 

Figure 6. 10. Spot application with *L. rhamnosus* NRRL-B 442 (left) and *L. casei* NRRL-B 441 (right) in the presence of glucose before the experiment (a), and after the experiment (b)

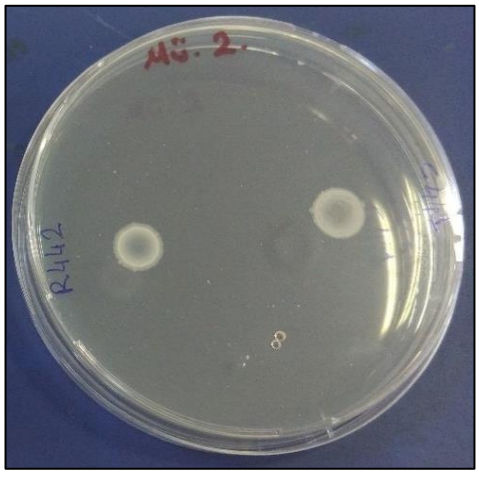
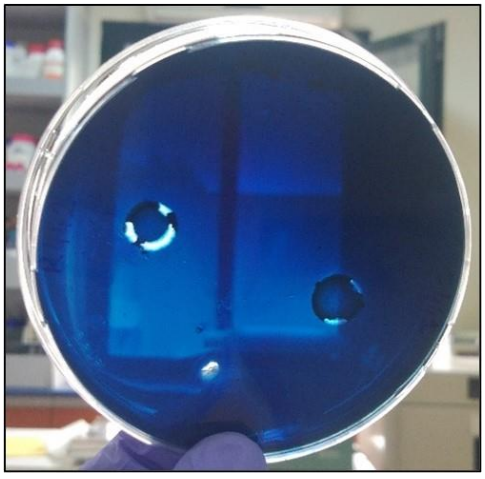
a)  b) 

Figure 6. 11. Spot application with *L. rhamnosus* NRRL-B 442 (left) and *L. casei* NRRL-B 441 (right) in the presence of mucin before the experiment (a), and after the experiment (b)

6.1.3.2. Growth Ability of Isolates in the Presence of Mucin

In this experiment, it was aimed to select LAB that do not use mucin as a carbon source or have limited absorbance values for LAB growth when only mucin is present as a carbon source.

Table 6.3 shows the absorbance and turbidity values measured in the presence of mucin and glucose in the medium for 13 bacteria belonging to the species of *L. plantarum, L. acidophilus, L. rhamnosus, L. casei, L. pentosus,* and *L. bulgaricus.*

Table 6. 3. Absorbance and turbidity values of LAB in the presence of mucin & glucose

Bacteria No.	**Bacteria Name:**	**Bacteria Code:**	**Turbidity of the Revived Bacteria (MRS/M17)**	**Modified MRS with Glucose**		**Modified MRS with Mucin**		**Modified MRS Without Mucin and Glucose**	
				Absorbance	**Turbidity**	**Absorbance**	**Turbidity**	**Absorbance**	**Turbidity**
1	***L. plantarum* NRRL-B 4496**	P4496	10	2.30	10	0.6	3.1	0.56	2.6
2	***L. acidophilus* NRRL-B 1910**	A1910	10	2.25	10	0.61	3.3	0.56	2.8
3	***L. rhamnosus* NRRL-B 442**	R442	6	2.42	10	0.97	4.7	0.92	4.6
4	***L. casei* NRRL-B 441**	C441	7.7	1.92	8.3	1.20	5.2	1.20	5.1
5	***L. pentosus* NRRL-B 227**	P227	10	2.42	10	1.00	5.2	0.90	4.4
6	***L. plantarum* DSM 1954**	DSM 1954	10	2.28	10	0.62	3.1	0.54	2.9
7	***L. bulgaricus* DT54**	DT54	7.8	0.9	4.5	0.10	1.2	0.07	0.8
8	***L. bulgaricus* DT62B**	DT62B	7.7	1.42	6.1	0.09	1.1	0.07	0.8

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Table 6. 3. (cont.)

9	***L. bulgaricus* UIN42**	UIN42	8.9	1.20	5.6	0.12	1.3	0.12	1
10	***L. bulgaricus* UIIN24**	UIIN24	6.6	1.69	7.2	0.59	3.4	0.60	3
11	***L. bulgaricus* UIIN26**	UIIN26	8	1.76	7.4	0.15	1.4	0.11	1
12	***L. bulgaricus* UZ12**	UZ12	8.5	0.92	4.3	0.16	1.4	0.18	1.3
13	***L. bulgaricus* UZ22**	UZ22	7.5	1.12	5	0.15	1.3	0.13	1

Table 6.4 shows the calculation of absorbance values of modified MRS media containing glucose and mucin for 13 bacteria. Thus, growth data for each bacterium in the presence of mucin and glucose were obtained as the absorbance values shown in this table. These data appear more prominently in the Figure 6.12.

Table 6. 4. Calculation of absorbance values that amount of mucin & glucose for LAB

Lactic Acid Bacteria			**Modified MRS With <u>Glucose</u> -Modified MRS Without Mucin & Glucose**		**Modified MRS With <u>Mucin</u> -Modified MRS Without Mucin & Glucose**	
Bacteria No:	**Bacteria Name:**	**Bacteria Code:**	**Calculation:**	**Result of Glucose**	**Calculation:**	**Result of Mucin**
1	***L. plantarum* NRRL-B 4496**	P4496	2.30-0.56 = 1.74	**1.74**	0.60-0.56 = 0.04	**0.04**
2	***L. acidophilus* NRRL-B 1910**	A1910	2.25-0.55 = 1.69	**1.69**	0.61-0.56 = 0.05	**0.05**
3	***L. rhamnosus* NRRL-B 442**	R442	2.42-0.92 = 1.5	**1.5**	0.97-0.92 = 0.05	**0.05**
4	***L. casei* NRRL-B 441**	C441	1.92-1.20 = 0.72	**0.72**	1.20-1.20 = 0	**0**
5	***L. pentosus* NRRL-B 227**	P227	2.42-0.90 = 1.52	**1.52**	1.00-0.90 = 0.1	**0.11**

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Table 6. 4. (cont.)

6	***L. plantarum* DSM 1954**	DSM 1954	2.28-0.54=1.74	**1.74**	0.62-0.54 = 0.08	**0.08**
7	***L. bulgaricus* DT54**	DT54	0.90-0.07 = 0.83	**0.83**	0.10-0.07 = 0.03	**0.03**
8	***L. bulgaricus* DT62B**	DT62B	1.42-0.07 = 1.348	**1.35**	0.09-0.07 = 0.02	**0.02**
9	***L. bulgaricus* UIN42**	UIN42	1.20-0.12 = 1.084	**1.08**	0.12-0.12 = 0	**0**
10	***L. bulgaricus* UIIN24**	UIIN24	1.69-0.60 = 1.09	**1.09**	0.59-0.60 = -0.01	**-0.01**
11	***L. bulgaricus* UIIN26**	UIIN26	1.76-0.11 = 1.65	**1.65**	0.15-0.11 = 0.04	**0.04**
12	***L. bulgaricus* UZ12**	UZ12	0.92-0.18 = 0.741	**0.74**	0.16-0.18 = -0.02	**-0.02**
13	***L. bulgaricus* UZ22**	UZ22	1.12-0.13 = 0.99	**0.99**	0.15-0.13 = 0.02	**0.02**

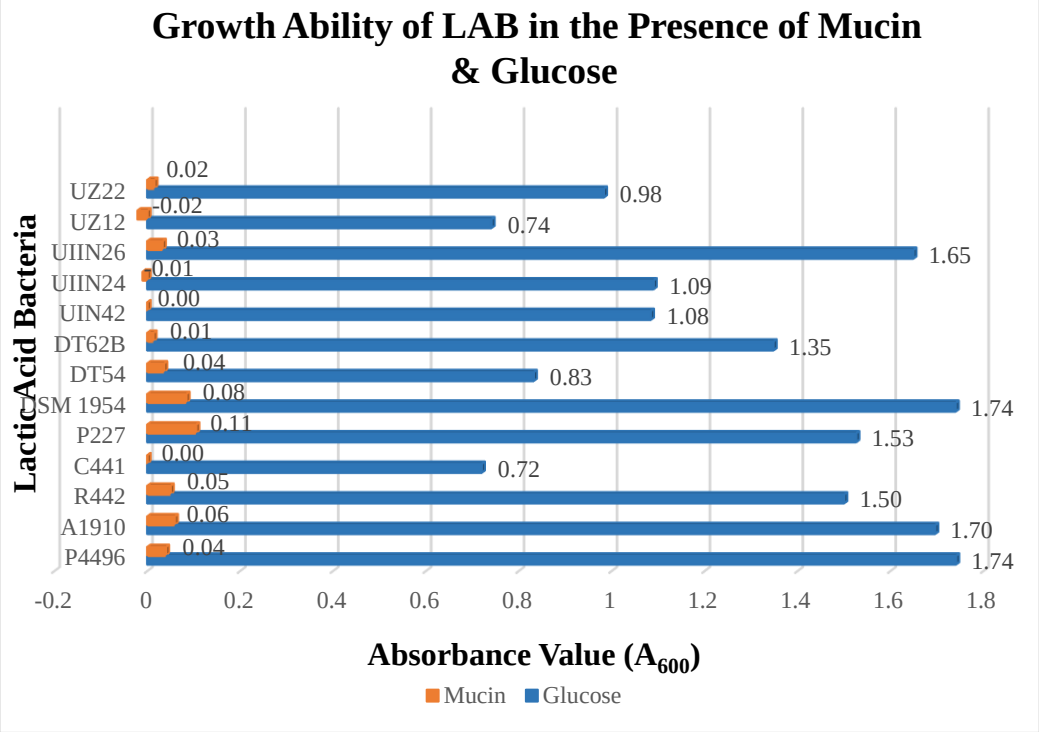


Figure 6. 12. Growth ability of LAB in the presence of mucin & glucose

As a result of the experiment performed with 13 bacteria belonging to the species of *L. plantarum, L. acidophilus, L. rhamnosus, L. casei, L. pentosus,* and *L. bulgaricus;* glucose absorbance values vary between 0.72-1.74, while mucin absorbance values vary between 0-0.11 at 600 nm and these values show no or negligible growth in all strains.

In the study of Zhou et al. (2001), showed mucin degradation ability of several LAB isolates of *L. rhamnosus* HN001, *L. acidophilus* HN017, *B. lactis* HN019. As a result of the experiment, LAB strains did not form a lysis zone whether there was glucose into the culture medium they were cultivated. Therefore, it can be said that they do not show mucinolytic activity.

In other study of Turpin et al. (2012), mucin degradation ability of 30 strains of *Lactobacillus, Leuconostoc, Pediococcus* bacteria isolated from traditional fermented plant foods in tropical countries has been researched. As a result of this experiment performed on petri dishes, when all LAB used were examined, no lysis zone was observed in any of them.

In another study of Abouloifa et al. (2020) mucin degradation ability of 5 *L. brevis*, 2 *L. pentosus* and 7 *L. plantarum* isolates selected from traditional fermented Moroccan green olives has been investigated. In this experiment, these LAB strains have not lysis zone was observed.

6.1.4. Bacterial Adhesion on Mucin

In order to perform the bacterial adhesion on mucin experiment; studies were carried out with a total of 17 bacteria belong to species of *L. plantarum*, *L. acidophilus*, *L. casei*, *L. rhamnosus*, *L. pentosus*, *L. paracasei*, *L. lactis* and *S. thermophilus* isolated from different sources including 6 olives (green/black), 4 cheese and 1 yogurt bacteria and 6 reference as shown in Table 6.5.

In this experiment, the aim was to determine the LAB with good adhesion behavior due to their promoting potential in mucin/mucus thickening. In this case the number of bacteria that binds to mucin was expected to ensure the criteria of the range of 10^4-10^8 CFU/mL.

Table 6. 5. The number of bacteria at t=0 and the number of bacteria obtained after the binding experiment

Bacteria No:	Bacteria Name:	Bacteria Code:	Number of Bacteria at t=0 (CFU/mL)	Logarithmic Number of Bacteria at t=0	Number of Bacteria After Adhesion to Mucus	Log Number of Bacteria After Adhesion to Mucus	Percentage of Mucus Adhesion (%)
1	***L. plantarum* NRRL-B 4496**	P. 4496	1.11×10^{10}	**10.05**	2.53×10^{5}	**5.4**	**53.73**
2	***L. acidophilus* NRRL-B 1910**	A. 1910	6.6×10^{9}	**9.82**	1.57×10^{5}	**5.2**	**52.95**
3	***L. rhamnosus* NRRL-B 442**	R. 442	5×10^{9}	**9.7**	9.55×10^{3}	**3.98**	**41.03**
4	***L. casei* NRRL-B 441**	C. 441	6.8×10^{9}	**9.83**	1.84×10^{5}	**5.26**	**53.51**
5	***L. pentosus* NRRL-B 227**	P. 227	3.8×10^{9}	**9.58**	5.38×10^{5}	**5.73**	**59.81**
6	***L. plantarum* DSM 1954**	DSM 1954	9.8×10^{9}	**9.99**	3.38×10^{6}	**6.53**	**65.37**
7	***S. thermophilus* UIN9**	T. UIN9	1.69×10^{10}	**10.23**	3.45×10^{6}	**6.54**	**63.93**
8	***L. paracasei* C15**	PR. C15	6.2×10^{9}	**9.79**	6.3×10^{5}	**5.8**	**59.24**
9	***L. plantarum* C47**	PL. C47	1.54×10^{10}	**10.19**	3.6×10^{4}	**4.56**	**44.75**
10	***L. plantarum* D1**	PL. D1	8.8×10^{9}	**9.94**	3.7×10^{5}	**5.57**	**56.04**
11	***L. lactis* A19**	LC. A19	1.38×10^{10}	**10.14**	1.54×10^{6}	**6.19**	**61.05**
12	**LAB O. AK6**	O. AK6	2.36×10^{9}	**9.37**	2×10^{3}	**3.3**	**35.22**
13	**LAB O. AK8**	O. AK8	1.42×10^{9}	**9.15**	2.47×10^{4}	**4.39**	**47.98**
14	**LAB O. AK16**	O. AK16	5.3×10^{9}	**9.72**	3.33×10^{4}	**4.52**	**46.5**
15	**LAB O. AK22**	O. AK22	8.7×10^{9}	**9.94**	3.49×10^{4}	**4.54**	**45.67**
16	**LAB O. AK23**	O. AK23	1.02×10^{10}	**10.01**	8.79×10^{4}	**4.94**	**49.35**
17	**LAB O. AK50**	O. AK50	6.2×10^{9}	**9.79**	1.29×10^{5}	**5.11**	**52.2**

According to the data shown in Table 6.5, the pre-experiment initial numbers for the bacteria used in the experiment varied in between 10^{9}-10^{10} CFU/mL.

In the Figure 6.13, the initial numbers of 17 bacteria and the final bacterial counts after adhesion test are shown as log baseline. Accordingly, the initial values for 17 bacteria ranged from 9.15-10.23 log, while the values for 13 bacteria after adhesion test ranged between 3.98-6.54 log.

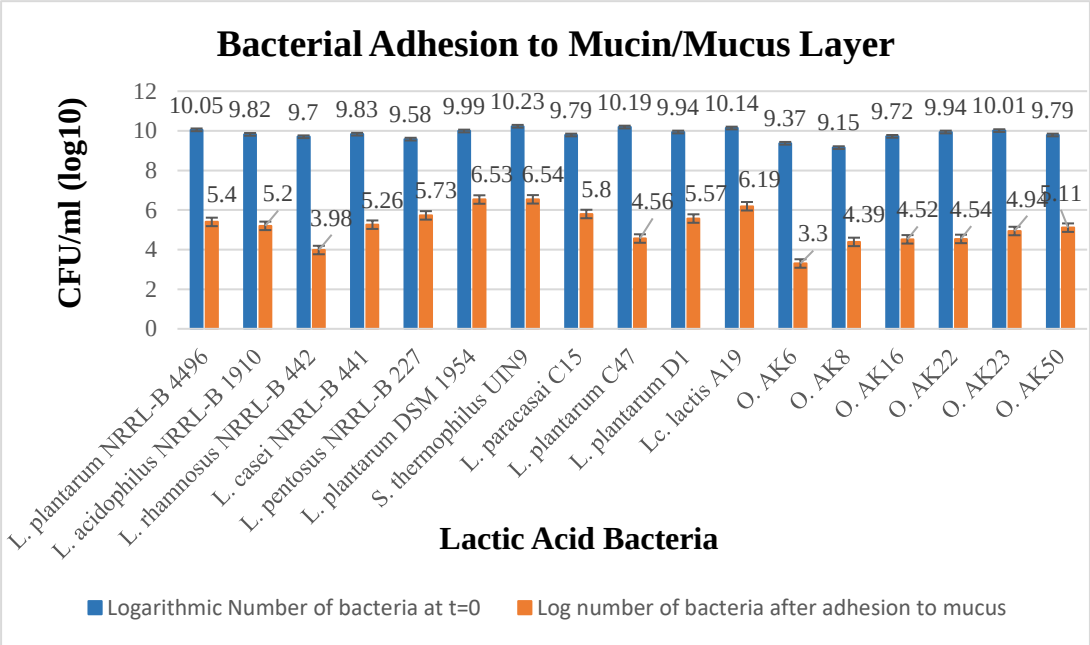


Figure 6. 13. Bacterial adhesion to mucin/mucus layer of LAB

In the Figure 6.14, the percentage of adhesion values for 13 bacteria after adhesion test ranged between 41.03-65.37% is seen.

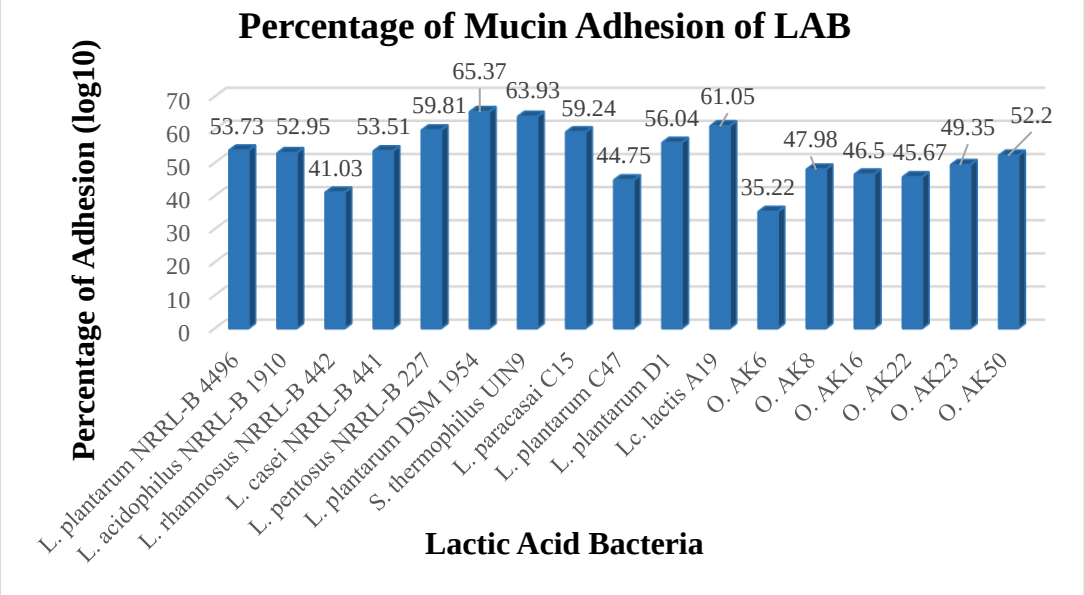


Figure 6. 14. Percentage of mucin adhesion of LAB

In this study, the adhesion ability of 17 LAB to mucin/mucus layer was investigated. As a result of this experiment was *L. plantarum* DSM 1954 (65.37%) has the highest adhesion ability and *L. rhamnosus* NRRL-B 442 (41.03%) has the lowest adhesion ability (Figure 6.13, Figure 6.14). In addition, it can be said that the 2nd and 3rd bacteria with the highest mucin binding from other bacteria are *S. thermophilus* UIN9 (63.93%) and *L. paracasei* C15 (59.24%).

According to obtained data, for 13 of the 17 bacteria selected to be used in the experiment, the initial bacterial count was 10^9-10^{10} CFU/mL, while the number of bacteria bound was between 10^5-10^6 CFU/mL, and the mucin/mucus adhesion percentages varied between 41.03-65.37%.

In the study of Carasi et al. (2014), while the initial bacterial count of 7 strains of *L. kefiri* isolated from kefir was 10^8 CFU/mL, it changed to 10^5-10^6 CFU/mL after mucus adhesion. In this case, the mucus adhesion percentage range was 62.5-75%.

In other study of Valeriano et al. (2014), the mucus binding percentages for *L. mucosae* LM1, *L. mucosae* PF01, *L. mucosae* LGG bacteria were 75.51%, 60.15% and 53.79%, respectively.

In another study of Bengoa et al. (2018), for *L. paracasei* 83124 isolated from kefir, the initial number was 10^8 CFU/mL, while the amount of adhesion to the mucin/mucus layer was 1.67×10^6, that is, 77.75% binding, on the other hand, for *L. paracasei* 8339, the initial number was 10^8 CFU/mL and the amount of adhesion to the mucin/mucus layer was 1.50×10^5, which corresponds to 64.75% binding.

When the results of this study are compared with the literature values; it is seen that the mucin binding percentages obtained for 17 different LAB in this study are similar results in the literature studies.

6.1.5. Biofilm Formation

In order to show the biofilm forming capabilities of LAB, experiments were carried out with 6 reference and 1 isolate strains: *L. plantarum* NRRL-B 4496, *L. acidophilus* NRRL-B 1910, *L. rhamnosus* NRRL-B 442, *L. casei* NRRL-B 441, *L. pentosus* NRRL-B 227, *L. plantarum* DSM 1954 as reference and *S. thermophilus* UIN9 as isolate strains.

In this experiment, it was aimed to determine the biofilm forming abilities of LAB due to their stimulating potential in mucin/mucus thickening and accordingly, if the absorbance values obtained at 590 nm are above 0.5 this is an indicator of high biofilm formation (Figure 6.15). A total of 3 replications were carried out with 4 parallel experiments for each bacterium.

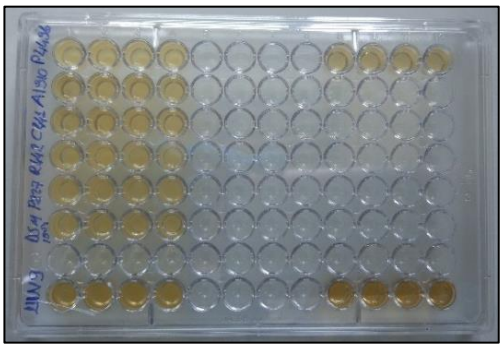
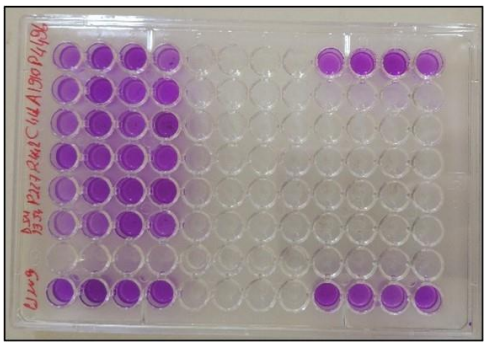
a)  b) 

Figure 6. 15. Liquid appearance on the 96 well plate at the beginning (a), and end of the experiment (b)

In the first experiment, *S. thermophilus* UIN9 and *L. pentosus* NRRL-B 227 have 3.26 and 2.25 absorbance values, respectively; they were higher than the controls. In the second experiment, *L. acidophilus* NRRL-B 1910 has 1.76 absorbance value and *S. thermophilus* CTY44 has 1.48 absorbance value, and they were also higher than the controls. In the 3rd experiment, *S. thermophilus* UIN9 reached to a higher absorbance value (1.16) value than the control.

The experiment was repeated 3 times in 4 parallels for 6 reference *L. plantarum* NRRL-B 4496, *L. acidophilus* NRRL-B 1910, *L. rhamnosus* NRRL-B 442, *L. casei* NRRL-B 441, *L. pentosus* NRRL-B 227, *L. plantarum* DSM 1954 in total, and *S. thermophilus* UIN9 isolate were tested 2 times in 4 parallels.

The average of the absorbance values obtained for the reference and isolate bacteria samples was taken and shown in the Table 6.6 and Figure 6.16.

Accordingly, it was concluded that *S. thermophilus* UIN9 strain was the yogurt isolate with the highest biofilm forming capacity among these 7 bacteria. In addition, it can be said that the other 6 bacteria have the capacity to form biofilms in varying amounts, albeit in a smaller amount than the control.

Table 6. 6. Biofilm formation results obtained at absorbance value of 590 nm

Bacteria No:	Bacteria Name:	Bacteria Code:	Absorbance Value (590 nm):	Number of Experiments:
1	***L. plantarum* NRRL-B 4496**	P4496	1.13	3
2	***L. acidophilus* NRRL-B 1910**	A1910	1.55	3
3	***L. rhamnosus* NRRL-B 442**	C441	1.10	3
4	***L. casei* NRRL-B 441**	R442	0.65	3
5	***L. pentosus* NRRL-B 227**	P227	1.05	3
6	***L. plantarum* DSM 1954**	DSM 1954	0.87	3
7	***S. thermophilus* UIN9**	T. UIN9	2.21	**2**
CONTROL	**CONTROL**	MRS B.	2.05	3
		MRS B. + EtOH	1.47	2
		M17 B.	1.14	3
		M17 B. + EtOH	0.69	2
		dH_2O	-0.05	2
		EtOH	-0.03	3

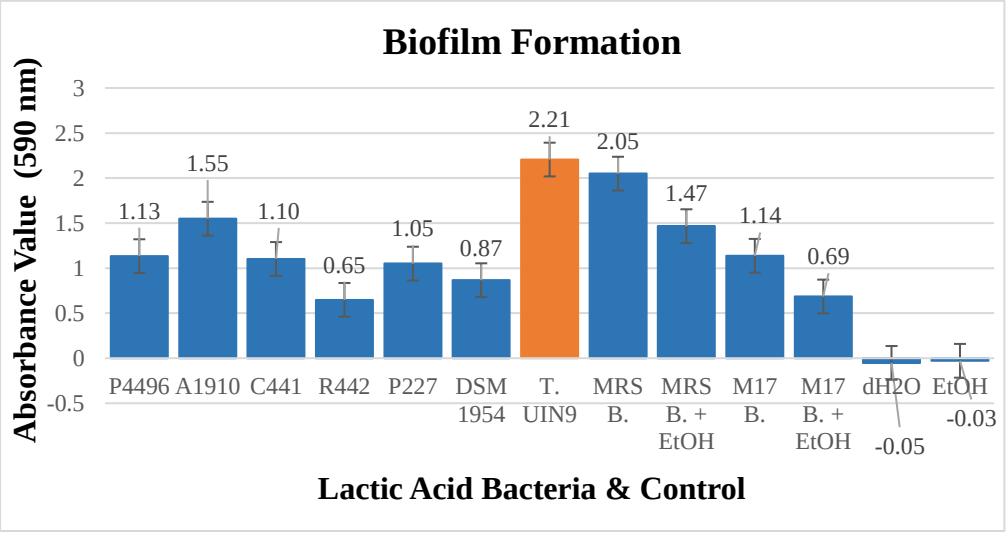


Figure 6. 16. Biofilm formation results obtained at absorbance value of 590 nm

In the Couvigny et al. (2015) study, the biofilm forming ability of 23 isolates of *S. thermophilus* were investigated. All strains showed similar growth rates. Only the

JIM8232 strain produced more biofilms than the other 4 biofilm-forming strains. In this experiment, this strain was chosen as a strong biofilm forming positive control. On the other hand, LMD-9 strain is an example of poor biofilm producing strains. Since most of the 23 bacteria used in the experiment produced biofilms similar to LMD-9 strains, it was concluded that strains of this species generally formed weak biofilms.

In the another study Gómez et al. (2016), the biofilm forming ability of 8 isolates of LAB were investigated. Accordingly, all strains used in the experiment were biofilm producers after 48 h of incubation at 30 °C in MRS medium. The highest biofilm formations are respectively: *L. lactis* 368 (1.65), *Lactobacillus helveticus* 352 (1.38) and *L. lactis* 94 (1.10), and these values are seen to be above 1. Based on the their OD values, the strains were grouped as follows: Non-biofilm producers (OD ≤ ODC (control)), weak (ODC <OD ≤ 2 × ODC), moderate (2 × ODC <OD ≤ 4 × ODC), or strong biofilm producers (4 × ODC <OD). As a result, the LAB used in this experiment were strong biofilm producers, except for *Weissella viridescens* 113, which had moderate biofilm formation.

In the another study Kostelac et al. (2021), the biofilm forming ability of 2 LAB were investigated. Based on the OD values obtained after 48 h cultivation, biofilm formation of *L. plantarum* M2 and *L. plantarum* KO9 respectively: 0.3381 and 0.4255 at 540 nm. According to the OD values grouped as in the Gómez et al. (2016) study, the LAB used in this experiment were strong biofilm producers.

6.1.6. Sugar Fermentation Tests and Microbial Metabolic Activity

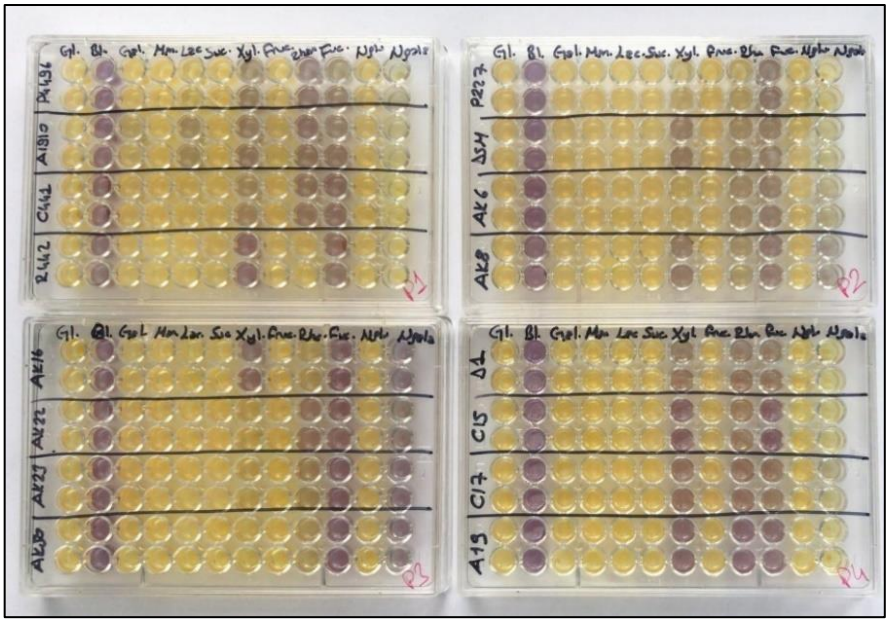
In this sugar test experiment, studies were carried out with a total of 17 bacteria belong to species of *L. plantarum, L. acidophilus, L. casei*, *L. rhamnosus, L. pentosus*, *L. paracasei*, *L. lactis* and *S. thermophilus* isolated from different sources including 6 olives (green/black), 4 cheese and 1 yogurt isolate and 6 reference as shown in Table 6.7.

Eleven sugars; glucose, galactose, mannose, lactose, sucrose, xylose, fructose, rhamnose, fucose, N-acetylglucosamine and N-acetylgalactasomine were used in the experiments and the changing of medium color from purple to yellow indicated sugar fementation ability (Figure 6.17).

Before incubation, 160 µL of each bacterial suspension was first added to the 96 well plates. Afterwards, 40 µL of each 10% sugar solution that prepared before was added

on them and the prepared modified MRS media (with BCP, sugar-free) were initially purple in color, and it is expected to turn yellow as an indicator of the sugars used.

a)



b)

Figure 6. 17. Plate view for 16 LAB (a) and T. UIN9 (b) after incubation

According to the transformation of the medium from purple to yellow (Figure 6.17, Table 6.7), it seems that while P4496 almost did not use xylose, rhamnose, fucose, it used N-acetylgalactosamine in a very small amount and used the other sugars in the experiment fully. Additionaly, A1910 almost did not use lactose, xylose, rhamnose and fucose, it used N-acetylgalactosamine partially and used the other sugars in the experiment fully. Then, C441 almost did not use xylose, rhamnose and fucose, and used the other sugars in the experiment fully. Finally, R442 did not use xylose and fucose and used the other sugars in the experiment fully.

Table 6. 7. Sugar fermentation test results on 17 bacteria with 11 different sugar solutions

Bacteria/Sugar:	Glucose	Galactose	Mannose	Lactose	Sucrose	Xylose	Fructose	Rhamnose	Fucose	N-acetylglucosamine	N-acetylgalactasomine
P4496	+	+	+	+	+	- (±)	+	- (±)	- (±)	+	+ (±)
A1910	+	+	+	- (±)	+	- (±)	+	- (±)	- (±)	+	+ (±)
C441	+	+	+	+	+	- (±)	+	-(±)	- (±)	+	+
R442	+	+	+	+	+	-	+	+	-	+	+
P227	+	+	+	+	+	+ (±)	+	+ (±)	-	+	+
DSM 1954	+	+	+	+	+	- (±)	+	+ (±)	- (±)	+	+ (±)
AK6	+	+	+	+	+	+	+	+ (±)	-	+	- (±)
AK8	+	+	+	+	+	-	+	+ (±)	-	+	-
AK16	+	+	+	+	+	-	+	+ (±)	-	+	-
AK22	+	+	+	+	+	+	+	- (±)	-	+	-
AK23	+	+	+	+	+	+	+	+ (±)	-	+	-
AK50	+	+	+	+	+	+	+	+ (±)	-	+	-
PL. D1	+	+	+	+	+	- (±)	+	- (±)	- (±)	+	+
PR. C15	+	+	+	+	+	-	+	- (±)	+	+	+
PL. C47	+	+	+	+	+	- (±)	+	- (±)	- (±)	+	+ (±)
LC. A19	+	+	+	+	+	-	+	-	-	+	+
T. UIN9	+	+	+	+	+	-	+	- (±)	-	+	+

+ (strong positive results), + (±) (weak positive),

- (±) (weak negative), - (strong negative)

In second plate, P227 did not use fucose and it used xylose and rhamnose partially and used the other sugars in the experiment fully. Then, DSM 1954 almost did not use xylose and fucose, and it used rhamnose ve N-acetylglucosamine partially and used the other sugars in the experiment fully. And then, AK6 did not use fucose, and this bacterium almost did not use N-acetylgalactosamine and it used rhamnose partially and used the other sugars in the experiment fully. AK8 did not use xylose, fucose and N-

acetylgalactosamine, and it used rhamnose partially and used the other sugars in the experiment fully.

In third plate, AK16 did not use xylose, fucose and N-acetylgalactosamine, and it used rhamnose partially and used the other sugars in the experiment fully. Then, AK22 did not use fucose and N-acetylgalactosamine, and almost did not use rhamnose and used the other sugars in the experiment fully. And then, AK23 and AK50 did not use fucose and N-acetylgalactosamine, and it used rhamnose partially and used the other sugars in the experiment fully.

In another plate, PL. D1 almost did not use xylose, rhamnose and fucose and used the other sugars in the experiment fully. Then, PR. C15 did not use xylose and this bacterium almost did not use rhamnose and used the other sugars in the experiment fully. And then, PL. C47 almost did not use xylose, rhamnose and fucose and this bacterium used N-acetylgalactosamine partially and used the other sugars in the experiment fully. LC. A19 did not used xylose, rhamnose and fucose, and used the other sugars in the experiment fully. In last plate, T. UIN9 did not used xylose and fucose, and this bacterium almost did not use rhamnose, and used the other sugars in the experiment fully.

Three of the 11 sugars used in the sugar fermentation test are mucin sugars. These are: Fucose, N-acetylglucosamine and N-acetylgalactosamine.

In this experiment with 17 LAB, they all seem to have made full use of one of the mucin sugars, N-acetylglucosamine. On the other hand, the olive isolates AK6, AK8, AK16, AK22, AK23 and AK50 did not use both fucose and N-acetylgalactosamine. Although this results obtained from qualitative analysis (Figure 6.17), these bacteria seems to have desirable property.

In addition to this, 16 LAB exactly used the lactose. A1910 almost did not use this sugar. Among these, those that bind slightly to mucin (e.g., R442 & AK6) can also support the mucus pathway by breaking down lactose and producing lactate.

Moreover, mucin bacterial binding test results for these 6 bacteria are listed as follows, from high to low: AK50, AK23, AK8, AK16, AK22 and AK6 (Figure 6.14).

Bacteria with positive results in these two experiments are; AK8, AK16, AK22, AK23 and AK50. While these bacteria show strong bacterial binding to mucin, they also contribute to the development of *Akkermansia* by not consuming 2 of the 3 mucin sugars that *Akkermansia* needs for mucin degradation.

In the study Fernandez et al. (2018), the growth rates of *S. thermophilus* LMD-9 and LMG18311, which have weak mucus binding, were examined in the presence of

galactose, mannose, fucose, N-acetylglucosamine, N-acetylgalactosamine, or lactose. As a result of this experiment, it was concluded that these two bacteria only grew in the presence of lactose at a rate of 0.27 and 0.30 μ_{max}, respectively, but did not grow in the presence of mucin sugars.

In the other study Valeriano et al. (2014), the sugar metabolism profile of *Lactobacillus mucosae* LM1, which showed good adhesion, aggregation and coagulation abilities, was evaluated. In this study, it has been shown that *L. mucosae* LM1 metabolizes various sugars (e.g., ribose, lactose etc.), and uses only glucose and galactose of the present glycoside sugar components. D-fucose, L-fucose and N-acetylglucosamine sugars were not utilized from this bacterium. In addition, no genes related to mucin degradation activity were found in the *L. mucosae* LM1 genome.

In the another study Schwab and Ganzle (2011), investigated the ability of 6 LAB to digest HMO components. This strains are: *L. plantarum* FUA3112, *L. acidophilus* FUA3191, *L. fermentum* FUA3177, *L. reuteri* FUA3148, *L. mesenteroides* FUA3143 and *S. thermophilus* FUA3194. Lactose and glucose were used wholly or partially by 6 LAB. *L. plantarum*, *L. acidophilus*, and *S. thermophilus* produced lactate as major metabolite from glucose or lactose. *L. reuteri*, *L. fermentum*, and *L. mesenteroides* produced lactate and acetate or ethanol. 6 LAB formed lactate and acetate when grown with N-acetylglucosamine as sole carbon source. And, the *L. plantarum* and *L. reuteri* metabolized this sugar during their growth. More than 5% of the available fucose was used only by *L. plantarum* and *L. acidophilus*. And generally, fucose utilization was low or absent in all LAB.

When the results of this study are compared with the literature; it is seen that different LAB strains did not grow in the presence of mucin sugars or they have no genes related to mucin degradation activity. So, thesis results are similar in the literature studies.

6.1.7. Determination of Lactate Production

In this experiment, it was aimed to determine lactate production capacities of LAB due to their promoting potential in mucin/mucus thickening and find the highest lactate producers in the 24 h. The lactate production analysis was performed using HPLC device and data for lactate standard curve showed in Figure D.1 and Table D.1.

The lactate peak areas obtained for each bacterium are divided by the lactate peak area obtained for 1 g/L standard solution (Figure D.2), then multiplied by the dilution factor (5). The data obtained from this calculation are given in Table 6.8. For example, the lactate peak area is 496121.15 for the P4496 (Figure 6.18), while the peak area is 136.487 for the 1 g/L standard solution. The amount of lactate produced here was 18.17 g/L.

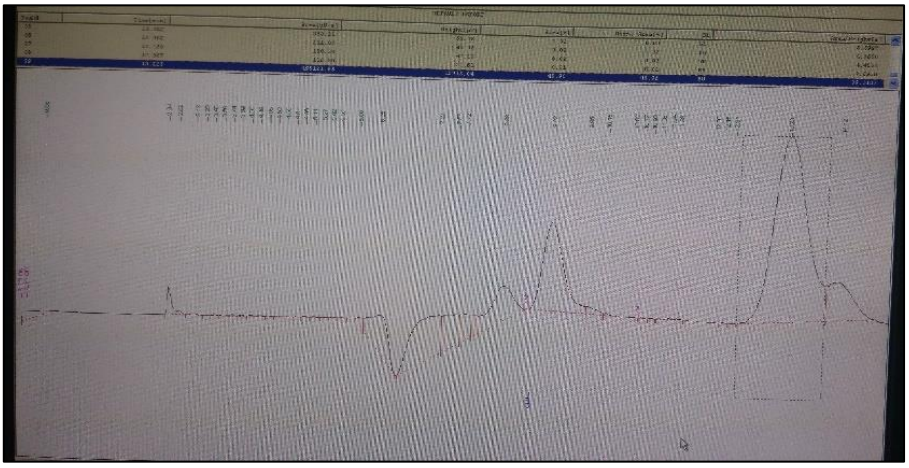


Figure 6. 18. Peak area of lactate production of *L. plantarum* NRRL-B 4496

Lactate production amounts for 6 reference and 1 isolate LAB are shown in Table 6.8 and Figure 6.19 as g/L. Accordingly, the isolate with the lowest lactate production out of 7 bacteria is T. UIN9 with 6.51 g/L, while the bacterium with the highest lactate production capacity with 18.17 g/L is P4496. Lactate production amounts for the other 5 bacteria vary between 11.28 and 17.83 g/L.

However, the peak area observed in about 9-9.5 minutes on the charts was interpreted as glucose. The remaining glucose amounts for 6 reference bacteria were calculated, with the knowledge that approximately 225.00 peak areas were observed at 1 g/L for the glucose standard from previous experiments. Two different sugars were observed in the T. UIN9 isolate and their standards were not known during this experiment, so they were not included in the calculations. C441, A1910, P227, R442, DSM 1954 and P4496.

Table 6. 8. Data for lactate analysis

Bacteria Code:	y = 156976x - 12689	x	Peak Area of LAB:	Peak Area of 1 g/L Standard Solution:	Amount of Lactate Production (g/L)
P4496	496121.65= 156976x - 12689	3.24	496121.65	136487	**18.17**
A1910	346740.54 = 156976x - 12689	2.29	346740.54	136487	**12.7**
C441	307894.08 = 156976x - 12689	2.04	307894.08	136487	**11.28**
R442	461236.96 = 156976x - 12689	3.02	461236.96	136487	**16.9**
P227	422287.34 = 156976x - 12689	2.77	422287.34	136487	**15.47**
DSM 1954	486840.4 = 156976x - 12689	3.18	486840.4	136487	**17.83**
T. UIN9	177785.97 = 156976x - 12689	1.21	177785.97	136487	**6.51**

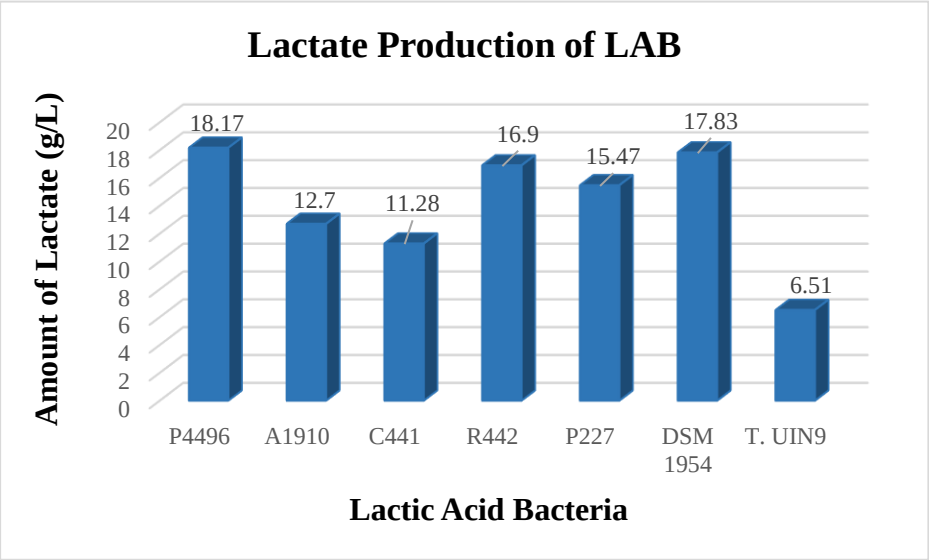


Figure 6. 19. Lactate production quantities of LAB

In this Table 6.9 and Figure 6.20, the amount of lactate for 6 of the 7 selected bacteria and the remaining glucose amounts are shown. According to this, the highest lactate productions are respectively P4496, DSM 1954, R442, P227, A1910 and C441.

Table 6. 9. Amount of lactate production & glucose consumption (Remained)

Bacteria Code & Standard Solutions:	Peak Area for Lactate (µV.s):	Time (min.):	Peak Area for Glucose (µV.s):	Amount of Lactate Production (g/L):	Amount of Glucose Consumption (Remained) (g/L):
P4496	496121.65	13.23	153.025	**18.17**	**3.4**
A1910	346740.54	13.23	376.350	**12.7**	**8.36**
C441	307894.08	13.24	427.322	**11.28**	**9.5**
R442	461236.96	13.21	183.542	**16.9**	**4.08**
P227	422287.34	13.21	281.754	**15.47**	**6.26**
DSM 1954	486840.4	13.23	184.912	**17.83**	**4.11**
T. UIN9	177785.97	13.25		**6.51**	
2 g/L	304663.8	13.23			
1 g/L	136487	13.183			
0.5 g/L	65402.2	13.18			
0.25 g/L	31353	13.15			

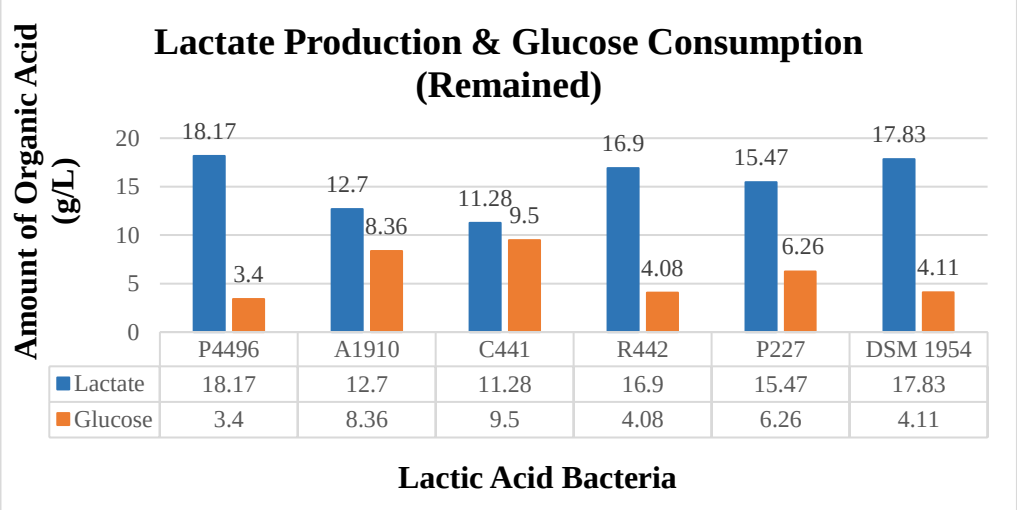


Figure 6. 20. Lactate production & glucose consumption (Remained)

When the literature studies are examined; in Hu et al. (2019) study, while the amount of lactate produced by *L. plantarum* S11 isolated from yogurt was 26.4 g/L, *L. plantarum* P1 isolated from Urum was~20.0 g/L and *L. plantarum* M7 obtained from the fermentation of millet sample was~23 g/L.

In the Vodnar et al., (2010) study, lactate production was 6.08 g/L for *L. plantarum*, 6.16 g/L for *L. casei*, 6.17 g/L for *Bifidobacterium breve*, 7.09 g/L for *B. infantis* and 7.12 g/L for mix (*L. plantarum, L. casei, B. breve* and *B. infantis*).

Additionally, in Nagarajan et al. (2020) study, a lactate value of 29.6 g/L was obtained for *L. plantarum* 23. In another the study De Simone et al. (2021), a lactate value was obtained for *L. plantarum* UFG 121 as 16.93 and 14.70 for *L. plantarum* PAN01.

As can be seen from 4 different literature studies, lactate amounts ranging from 6.08 to 29.6 g/L were obtained. Lactate amounts in this thesis between 11.28-18.17 g/L for C441, A1910, P227, R442, DSM 1954 and P4496, respectively. So, these results appear to show similarities.

6.1.8. Determination of Antimicrobial Properties

In this experiment, it was aimed to investigate the antimicrobial properties of LAB to support the theory of promoting the growth of the *Akkermansia* group by inhibiting pathogenic microorganisms through its antimicrobial effect as well as the mucin/mucus thickening role of lactate production. In this context, it is aimed to observe clear zones of inhibition over 8 mm for at least 1 pathogenic microorganism.

The numbers of 5 different pathogenic microorganisms used in the experiment are shown in Table 6.10.

Table 6. 10. Determination of pathogen bacteria number (after the 2nd passage)

Pathogen:	***E. coli* 3008**	***L. innocua* NRRL-B 33314**	***B. cereus* 14579**	***E. coli* RSHM 4024 (ATCC 25922)**	***S. aureus* RSKK 1009**
Bacteria Number:	6.3×10^7	2.49×10^7	4.24×10^6	3.6×10^8	2.23×10^8

6.1.8.1. Agar Spot Test

Six reference strains of *Lactobacillus* were selected, including 2 *L. plantarum*, *L. acidophilus, L. casei, L. rhamnosus, L. pentosus* and 1 isolate strain of *S. thermophilus*.

All strains were screened for antibacterial action against *2 E. coli*, *L. innocua*, *B. cereus*, and *S. aureus* using agar spot test with two different methods. Agar spot test was more successful than the other method and showed a wide range of inhibition zones against pathogens.

Agar spot test was applied in two different ways. In the first method, after the spot application, the pathogen bacteria spread on the LAB and the results are shown in Figure 6.21 and Figure 6.22.

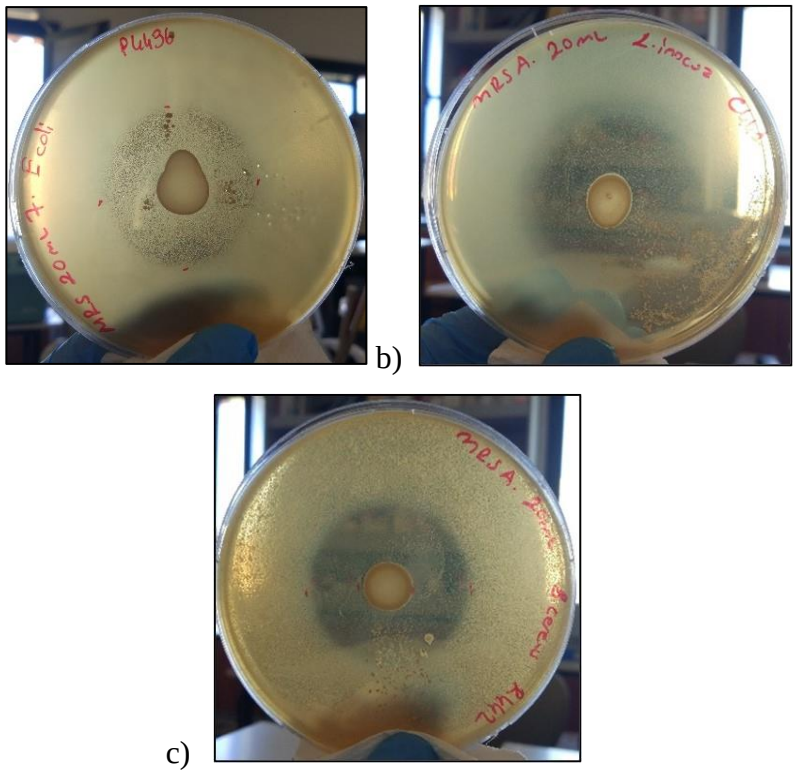


Figure 6. 21. *L. plantarum* NRRL-B 4496-*E. coli* 3008 (a), *L. casei* NRRL-B 441-*L. innocua* 33314 (b), *L. rhamnosus* NRRL-B 442-*B. cereus* 14579 (c) with agar spor test

In the second method, spot application was made after the pathogen bacteria spread on the media. However, while *L. plantarum* NRRL-B 4496, *L. casei* NRRL-B 441 and *L. pentosus* NRRL-B 227 showed resistance to *L. innocua* 33314 pathogens in this experiment, they could not show resistance to other pathogens and other LAB could not show resistance to 4 pathogens used in the experiment. In this situation, it can be interpreted that the first spread of the pathogenic bacteria does not give the LAB the

opportunity to grow, since pathogen microorganisms have a dominant effect in the environment.

Figure 6.21 and Figure 6.22 show inhibition zone diameter of agar spor test for 7 LAB on 5 selected pathogens respectively; *E. coli* 3008, *L. innocua* NRRL-B 33314, *B. cereus* 14579, *E. coli* RSHM 4024 (ATCC 25922) and *S. aureus* RSKK 1009.

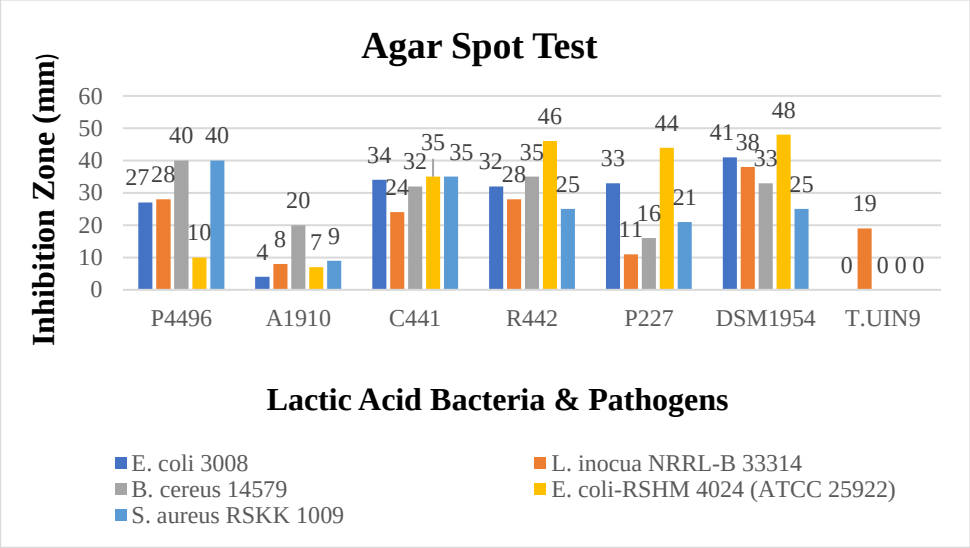


Figure 6. 22. Antimicrobial activity of 7 LAB on 5 pathogens with agar spor test

In this method, it was determined that inhibitory effect of selected LAB was observed on these pathogens, but *S. thermophilus* UIN9 only inhibited the *L. innocua* NRRL-B 33314.

According to the data in the Figure 6.22, the inhibition zones of LAB on 5 pathogens vary in the following ranges: 10-40 mm for P4496, 4-20 mm for A1910, 24-35 mm for C441, 28-46 mm for R442, 11-44 mm for P227, 25-48 mm for DSM 1954 and 0-19 mm for T. UIN9. In addition, not more than 8 mm from the data obtained: Inhibition zones of A1910 on *E. coli* 3008 (4 mm) and *E. coli* RSHM 4024 (ATCC 25922) (7 mm).

6.1.8.2. Well Diffusion Method

Six reference strains of *Lactobacillus* were selected, including 2 *L. plantarum, L. acidophilus, L. casei, L. rhamnosus, L. pentosus* and 1 isolate strain of *S. thermophilus*.

All strains were screened for antibacterial action against *2 E. coli*, *L. innocua*, *B. cereus*, and *S. aureus* using well diffusion test with two different methods. First method was successful than other method and collected different inhibition zones according to pathogens.

Well diffusion method was applied in two different ways. In the first method, wells of 6 mm width were opened and prepared LAB suspensions were added to these wells. Then, the pathogenic bacteria were added to the nutrient medium in the petri dish by spreading method and the results are shown in Figure 6.23. In the second method, wells of 6 mm width were opened after the pathogen bacteria spread on the media. In this situation, it can be interpreted that the first spread of the pathogenic bacteria does not give LAB the opportunity to grow, since pathogen microrganisms have a dominant effect in the environment.

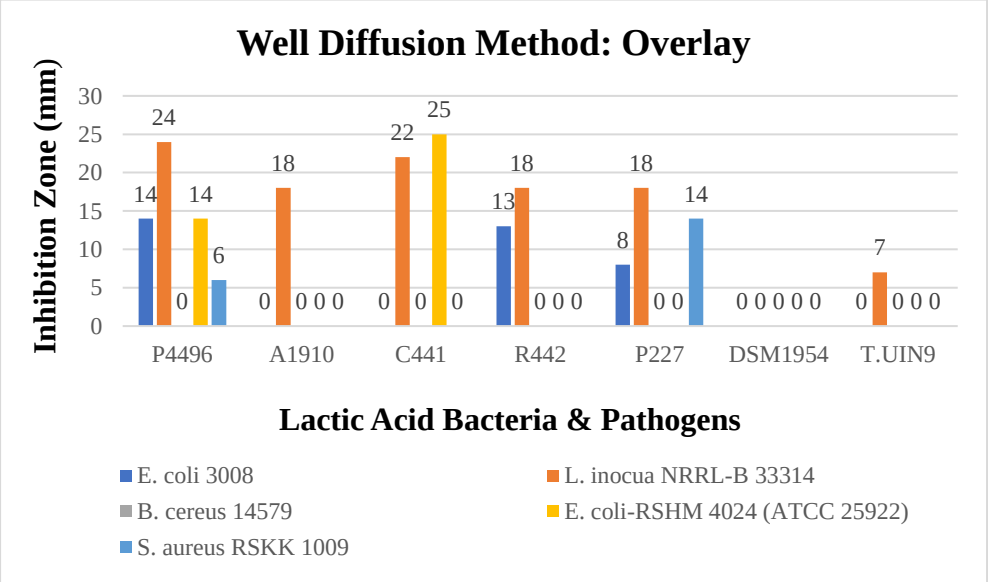


Figure 6. 23. Antimicrobial activity of 7 LAB on 5 pathogens with well diffusion method

Figure 6.23 shows inhibition zone diameter of well diffusion method for 7 LAB on 5 selected pathogens respectively, *E. coli* 3008, *L. innocua* NRRL-B 33314, *B. cereus* 14579, *E. coli* RSHM 4024 (ATCC 25922) and *S. aureus* RSKK 1009.

In this method, the inhibitory effects of selected LAB on these pathogens were determined; *L. plantarum* DSM 1954 could not inhibit these 5 pathogens with this method

and *S. thermophilus* UIN9 and *L. acidophilus* NRRL-B 1910 only inhibited the *L. innocua* NRRL-B 33314.

According to the data in the Figure 6.23, the inhibition zones of LAB on pathogens vary in the following ranges: 0-24 mm for P4496, 0-18 mm for A1910, 0-25 mm for C441, 0-18 mm for R442, 0-18 mm for P227, and 0-7 mm for T. UIN9. In addition, not more than 8 mm from the data obtained: Inhibition zones of P4496 on *S. aureus* RSKK 1009 (6 mm); inhibition zones of T. UIN9 on *L. innocua* NRRL-B 33314 (7 mm).

6.1.8.3. Disc Method

This method is the most widely used antimicrobial test in the literature. In this method, P4496, A1910, C441 and P227 bacteria seem to be able to maintain their viability against varying amounts of *L. innocua* NRRL-B 33314 and *B. cereus* 14579. While R442 and DSM 1954 could only survive against *L. innocua* NRRL-B 33314, T. UIN9 could not fight against these selected pathogens (Figure 6.24).

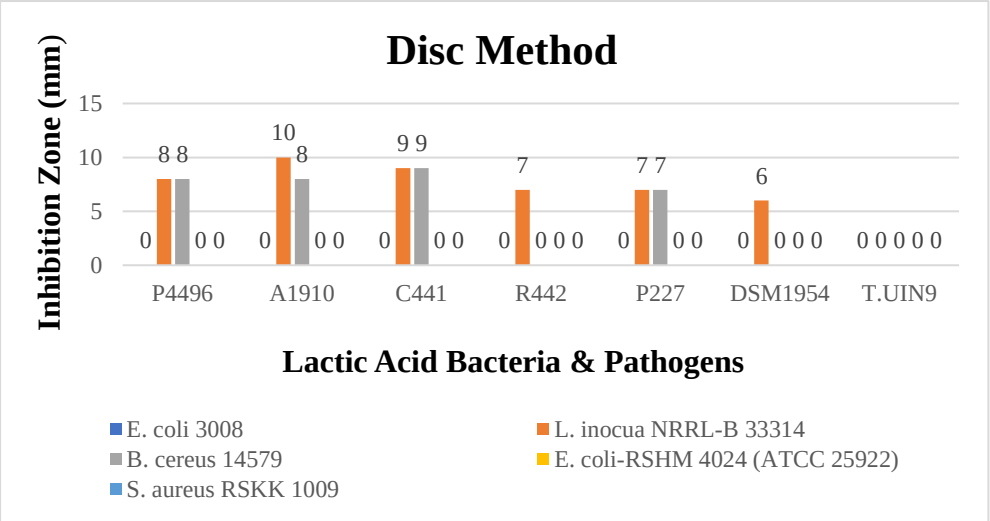


Figure 6. 24. Antimicrobial activity of 7 LAB on 5 pathogens with disk method

According to the data in the Figure 6.24**,** the inhibition zones of LAB on 5 different pathogens vary in the following ranges: 0-8 mm for P4496, 0-10 mm for A1910, 0-9 mm for C441, 0-7 mm for R442, 0-7 mm for P227, and 0-6 mm for DSM 1954. In

addition, not more than 8 mm from the data obtained: Inhibition zones of R442 on *L. innocua* NRRL-B 33314 (7 mm); inhibition zones of P227 on *L. innocua* NRRL-B 33314 (7 mm) and *B. cereus* 14579 (7 mm) pathogens.

In this experiment, 5 pathogenic bacteria were used, of which 3 were extracellular and 1 was intracellular pathogen (Bhunia, 2018). Considering the results obtained with 3 different methods for *L. innocua*, which is a intracellular pathogen, inhibition zones of 7 LAB changing between 6-38 mm.

When the 3 methods applied in antimicrobial activity experiments are compared: It is seen that the results obtained with the agar spot test showed stronger inhibitions and found consistent than the results of the other two tests.

Studies have shown that obesity causes inflammation. In order to reduce this effect, the selection of LAB that inhibit pathogens to a large extent is important for support of *Akkermansia*, an anti-obesity bacterium.

In the Arena et al. (2016) study, the possible antimicrobial activity of 79 *L. plantarum* strains were investigated against 7 pathogenic bacteria. These are: *E. coli* O157:H7, *L. monocytogenes*, *Salmonella enteritidis* and 4 strains of *S. aureus.* This experiment in which the agar spot test and well diffusion method was applied ensures evidence that several of the screened *L. plantarum* strains possess an important ability to contrast various pathogenic microorganism, which can contaminate food and are responsible for diseases in people.

In the Gaudana, et al. (2010) study, *L. rhamnosus* GG, *L. rhamnosus* CS25, *L. plantarum* CS23, *L. plantarum* ATCC 8014, *L. fermentum* ASt1, *L. delbrueckii* M were examined against 5 pathogens. These pathogens are: *E. coli* S5, *Pseudomonas aeruginosa* (ATCC 25 668), *Salmonella typhi* and *Shigella dysentery* as Gram-negative and *S. aureus* (ATCC 6538) as Gram-positive. The *L. plantarum* ATCC 8014 and *L. rhamnosus* CS25 had the highest inhibitory activity against both Gram-positive and Gram-negative bacteria, followed by *L. delbrueckii* M and *L. plantarum* CS23. 6 LAB tested showed a strong inhibition towards *E. coli* S5.

In the Choi et al. (2021) study, 10 LAB isolated from kimchi were examined against 3 pathogens. These 10 LAB included 3 strains (KCTC 13302, 13372, 13374) of *Leuconostoc mesenteroides*, 5 strains (KCTC 33131, 21004, 33133, 13093 KCCM 43246) of *L. plantarum*, one strain (KCTC 13090) of *L. paracasei*, and one strain (KCTC 12976) of *L. kimchicus*. On the other hand, 3 pathogens are *Escherichia coli* including enteropathogenic *E. coli* EPEC, enterotoxigenic *E. coli* ETEC, and *E. coli* O157:H7 were

used. As a result, cell free supernatant (CFS) of these LAB had less inhibitory effects on *E. coli* O157:H7 than EPEC and ETEC. Overall, CFS produced by *Leuconostoc mesenteriodes* (KCTC 13374), *L. plantarum* (KCTC 33133), *L. plantarum* (KCTC 13093), and *L. paracasei* (KCTC 13090) showed bactericidal abilities against pathogenic *E. coli* in this study.

When the results of this study are compared with the literature values; it is seen that different LAB strains showed a strong inhibition zones and bactericidal abilities against pathogenic bacteria. Therefore, thesis results are found similar in the literature studies.

6.1.9. Determination of Mucin/Mucus Thickening Effect of Selected Strains According to Lactate Production and Other Characteristics

In this experiment, it was aimed to determine the effect of selected probiotic LAB on mucin thickening by *in vitro* cell culture studies in order to select the anti-obesity probiotic candidates.

In the qualitative measurements, it is aimed to observe the purple pink (magenta) color under light microscope with the PAS staining method.

In the mucin quantitative test, it is aimed to determine the mucin amount in the linear range of 10-600 µg/mL and to obtain at least 5 prototypes as a result of these experiment.

6.1.9.1. HT-29 Cell Culture in the Presence of Lactic Acid Bacteria

In this study, firstly, the binding status of LAB to HT-29 cells was observed and the number of bacteria before the experiment for 6 reference and 1 isolate. And then, the number of attached bacteria on HT-29 cell culture were obtained as a result of 2 h incubation in 37 ºC, 5% CO_2 atmosphere incubator. The census results obtained are shown in Table 6.11 and Figure 6.25.

Table 6. 11. Number of bacteria and percent adhesion of LAB on HT-29 cell culture

Bacteria Code:	Initial Number of LAB: (B0) (t=0)	Final Number of LAB: (B1) (t=2 h)	Percent Adhesion of LAB: (B1/B0) * 100
P4496	2.08×10^8	4.4×10^7	**21.15**
A1910	4.9×10^6	8.5×10^5	**17.35**
C441	7.45×10^7	1.07×10^7	**14.36**
R442	7.04×10^7	1.14×10^6	**1.62**
P227	9.02×10^7	5.5×10^6	**6.10**
DSM1954	1.02×10^8	1.76×10^7	**17.25**
T. UIN9	1.47×10^8	1.04×10^7	**7.07**

In the first stage of bacterial binding experiments with HT-29 cell, P4496, A1910 and DSM 1954 bacteria from the 7 bacteria used showed high binding, while T. UIN9, P227 and R442 had low binding percentages.

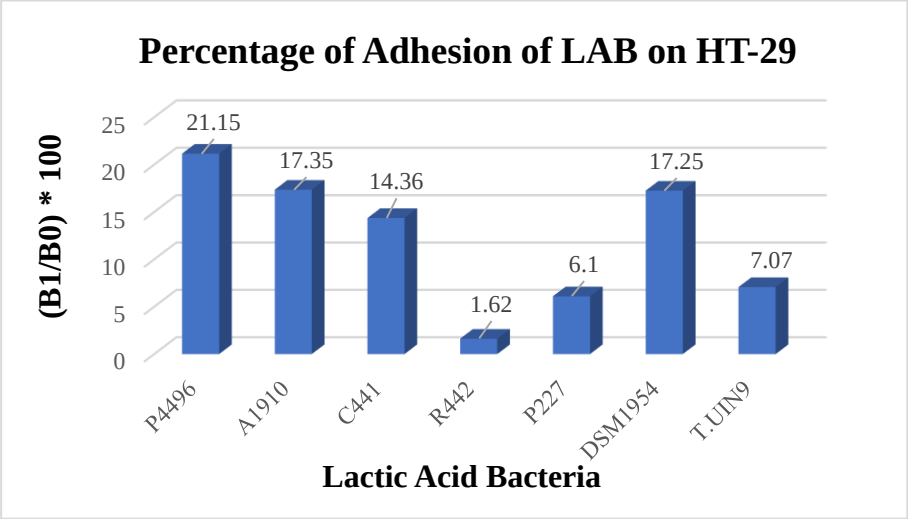


Figure 6. 25. Percent adhesion of LAB on HT-29 cell culture

6.1.9.2. Qualitative Determination of Mucin Layer

In order to perform the mucin qualitative analysis experiment; studies were carried out with a total of 17 bacteria belong to species of *L. plantarum*, *L. acidophilus*,

L. casei, L. rhamnosus, L. pentosus, L. paracasei, L. lactis and *S. thermophilus* isolated from different sources including 6 olives (green/black), 4 cheese and 1 yoghurt isolate and 6 reference as shown in Table 6.2.

HT-29 cells are not known to be primarily mucus-forming cells; however, in addition to adhesion studies in which LAB and HT-29 cells are together, it has been stated in many studies that lactobacilli increase mucin expression and ensure mucin secretion. Dudik et al. (2020) predicts that pre-incubation of lactobacilli with mucin stimulates their *in vitro* adhesion to HT-29 cells, potentially leading to better bioavailability in the gut at the *in vivo* level (Section 4.5). Therefore, mucin (50 µg/mL) was added to the medium 24 h before the experiments, and the results are given in the figures below.

It is thought that LAB such as *S. thermophilus*, which have poor adhesion to the mucus, produce lactate by breaking down lactose and thus support the mucus pathway, regulate the mucus environment, stimulate the mucus layer, and may also be a signal that can modulate the colonic epithelium. At this point, the fact that lactate fights pathogens in the environment thanks to its antimicrobial feature supports the improved mucin/mucus medium for *Akkermansia*. Due to the mucin/mucus thickening role and other positive effects of lactate production, the changes observed with the addition of lactate at two different concentrations to the cell culture medium in these experiments are given in the figures below.

This experiment was performed on 12 well plates. There are 6 control groups: HT-29 cell culture, HT-29 cell culture + mucin (50 µg/mL), HT-29 cell culture + lactate (20 mM), HT-29 cell culture + lactate (50 mM), HT-29 cell culture + mucin (50 µg/mL) + lactate (20 mM) and HT-29 cell culture + mucin (50 µg/mL) + lactate (50 mM). In the experiment, 2 parallels were applied for each control and bacteria.

Figure 6.26, Figure 6.27, Figure 6.28, Figure 6.29, Figure 6.30 and Figure 6.31, images obtained for the *L. plantarum* NRRL-B 4496 (P4496), *L. casei* NRRL-B 441 (C441) and *L. rhamnosus* NRRL-B 442 (R442) as a result of the PAS staining method applied for the qualitative analysis of mucin. Left side (a): Control group, right side (b): Images obtained when LAB was added.

According to Figure 6.26, on the left is the image for the HT-29 cell, on the right is the image obtained with *L. plantarum* NRRL-B 4496 added to the HT-29 cell. The purple pink (magenta) color, which indicates the presence and density of mucin, appears to increase when this bacterium is added into the medium.

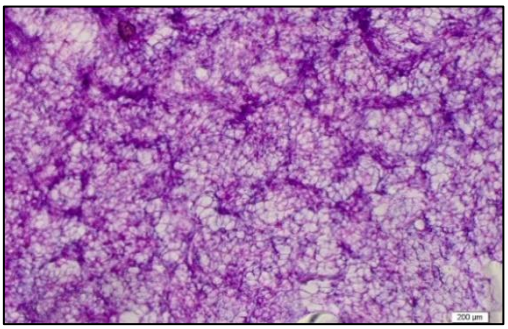
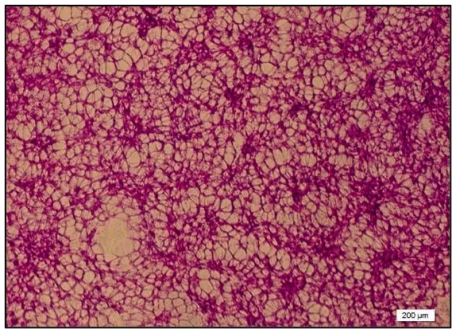
a)  b) 

Figure 6. 26. HT-29 cell (CNT-1) (a), HT-29 cell + LAB (P4496) (b)

According to Figure 6.27, on the left is the image of mucin (50 µg/mL) added to the HT-29 cell, and on the right is the image obtained with *L. rhamnosus* NRRL-B 442 with the mucin added to the HT-29 cell. When this bacterium is added into the medium, the purple pink (magenta) color, which expresses the presence and density of mucin, seems to increase with the effect of the added mucin.

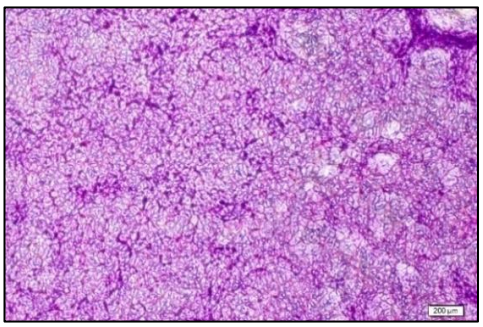
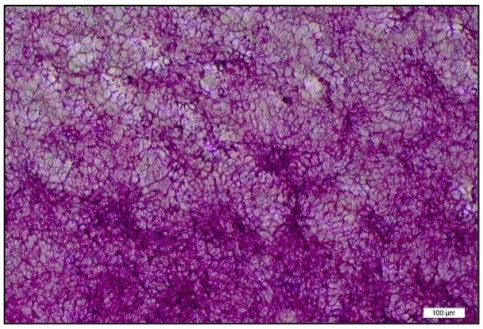
a)  b) 

Figure 6. 27. HT-29 cell + mucin (CNT-2) (a), HT-29 cell + mucin + LAB (R442) (b)

According to Figure 6.28, on the left is the image for lactate (20 mM) added to the HT-29 cell a little, and on the right is the image obtained with the *L. casei* NRRL-B 441 with lactate (20 mM) added to the HT-29 cell. In this PAS staining method, the magenta color, which expresses the presence and density of mucin in the medium, appears to increase slightly with the effect of the added lactate when this bacterium is added.

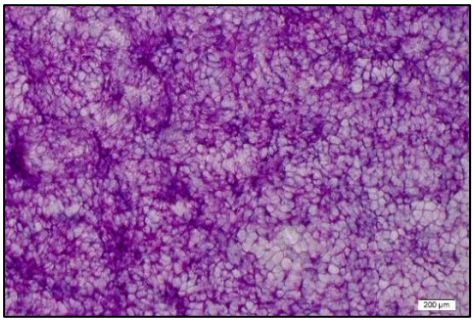
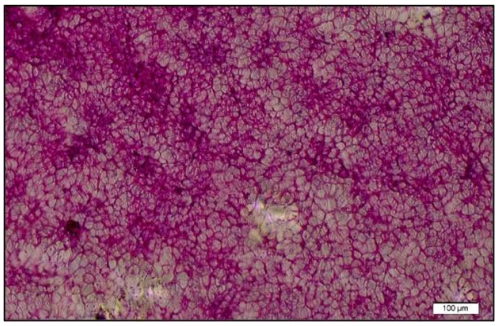
a)  b) 

Figure 6. 28. HT-29 cell + lactate (20 mM) (CNT-3) (a), HT-29 cell + lactate (20 mM) + LAB (C441) (b)

According to Figure 6.29, on the left is the image for lactate (50 mM) added a little to the HT-29 cell, and on the right is the image obtained with the *L. rhamnosus* NRRL-B 442 with lactate (50mM) added to the HT-29 cell. In this PAS staining method, the magenta color, which expresses the presence and density of mucin in the medium, appears to increase significantly with the effect of the added lactate when this bacterium is added.

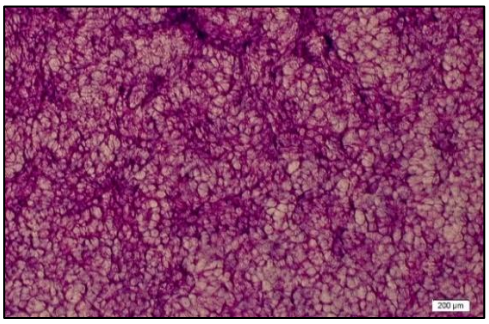
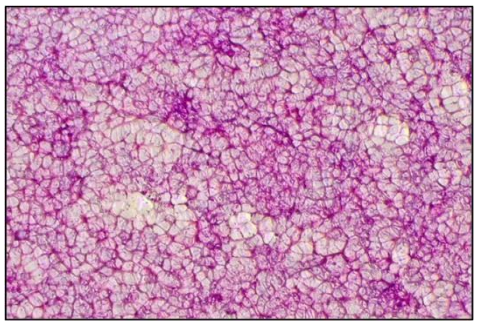
a)  b) 

Figure 6. 29. HT-29 cell + lactate (50 mM) (CNT-3) (a), HT-29 cell + lactate (50 mM) + LAB (R442) (b)

According to Figure 6.30, on the left is the image of lactate (20 mM) added to the HT-29 cell as well as some mucin added to the HT-29 cell, while on the right is the image obtained with the *L. plantarum* NRRL-B 4496 with mucin (50 µg/mL) and lactate (20 mM) added to the HT-29 cell. In this PAS staining method, when the purple pink (magenta) color, which expresses the presence and density of mucin in the medium, was

added to this bacterium, the presence of mucin with the added lactate created a more significant difference than the binary combinations.

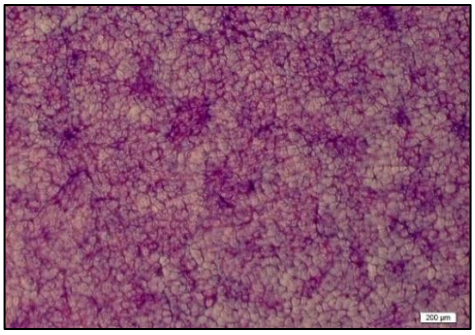
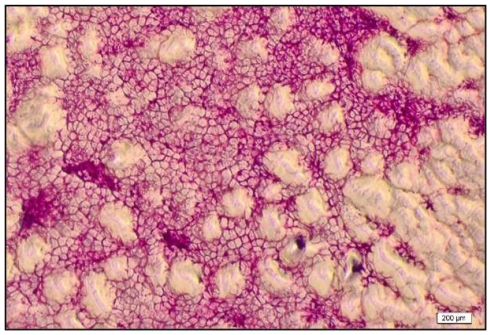
a)  b) 

Figure 6. 30. HT-29 cell + mucin + lactate (20 mM) (CNT-3) (a), HT-29 cell + mucin + lactate (20 mM) + LAB (P4496) (b)

According to Figure 6.31, on the left is the image for lactate (50 mM) added to the HT-29 cell with some mucin, while on the right is the image obtained with the *L. casei* NRRL-B 441 with mucin and lactate (20 mM) added to the HT-29 cell. In this PAS staining method, when this bacterium was added with purple pink (magenta) color, which expresses the presence and density of mucin in the medium, the presence of mucin with the effect of the added lactate at a higher concentration made a more significant difference than the binary combinations.

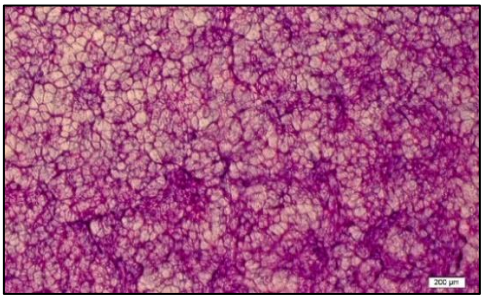
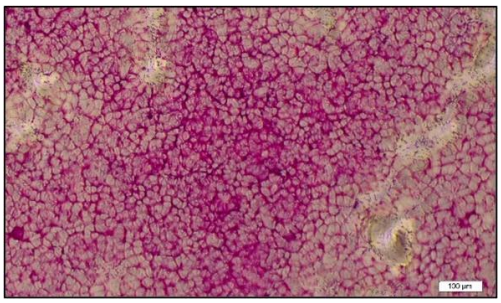
a)  b) 

Figure 6. 31. HT-29 cell + mucin + lactate (50 mM) (CNT-3) (a), HT-29 cell + mucin + lactate (50 mM) + LAB (C441) (b)

6.1.9.3. Quantitative Determination of Mucin Layer

This experiment was carried out with a total of 15 bacteria, 8 LAB in the first time and 7 LAB in the second time. For this reason, calibration curves prepared separately as mucin standard curve 1 and mucin standard curve 2 are included in the Figure 5.1 and Figure 5.2.

In the experiment, a total of 30 µL cells taken from both parallel wells of the 12 well plate was transferred to the 96 well plate in 3 parallels (30 µL each). Afterwards, the average absorbance values obtained in the Varioscan device at an absorbance value of 555 nm for each bacterium are shown in the figure below.

According to this Figure 6.32, the binding of LAB to the HT-29 cell culture was examined, the bacteria in the first group appears to be C47, which is equivalent to the control. In 2nd group, there are 5 bacteria with a higher absorbance value than the control and these are: AK23, AK50, DSM 1954, AK8 and AK16.

According to this Figure 6.33, the binding of LAB to mucin (50 µg/mL) added to the HT-29 cell was examined, the bacteria with a higher absorbance value than the control in the first group appears to be R442, PR. C15 and PL. C47. In 2nd group, there are 2 bacteria appears to be AK8 and AK16, which is equivalent to the control. And, 3 bacteria with a higher absorbance value than the control and these are: AK50, AK6 and AK23.

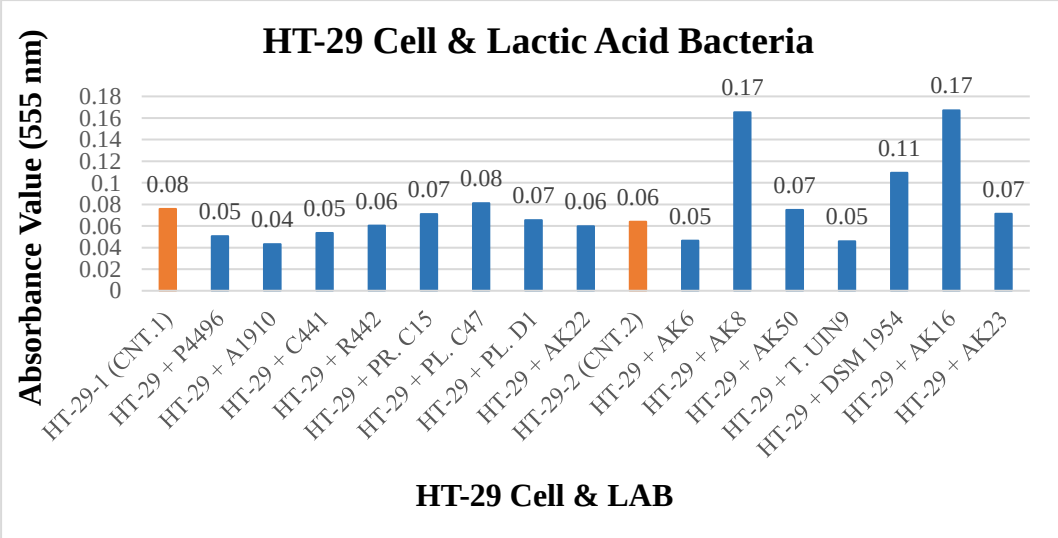


Figure 6. 32. HT-29 cell & LAB

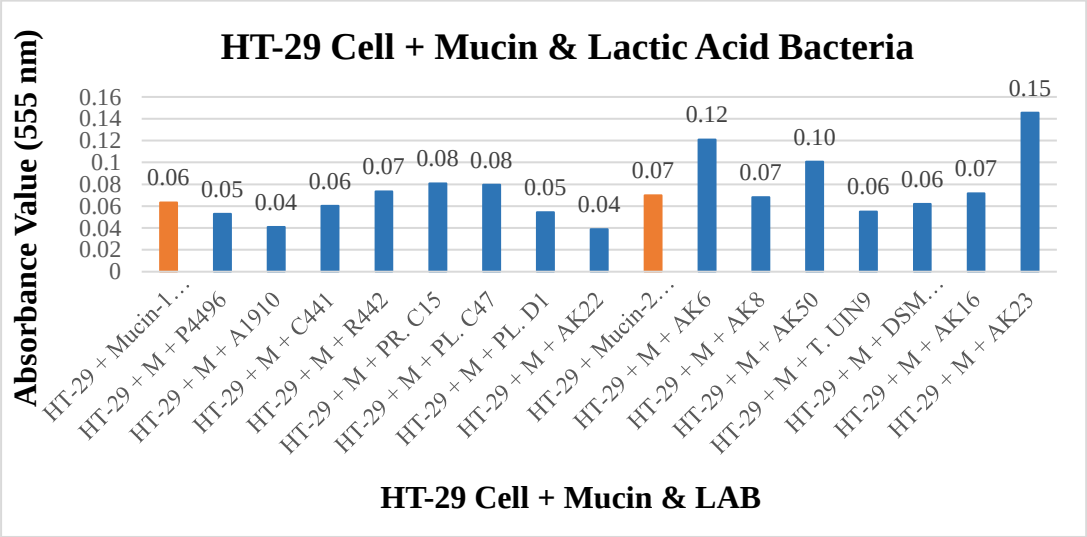


Figure 6. 33. HT-29 cell + mucin (50 µg/mL) & LAB

According to this Figure 6.34, the binding of LAB to lactate (20 mM) added to the HT-29 cell was examined, the bacterium with a higher absorbance value than the control in the first group appears to be only R442. In 2nd group, there are 2 bacteria appears to be AK50 and DSM 1954, which is equivalent to the control. And, only 1 bacterium with a higher absorbance value than the control and this is: AK23.

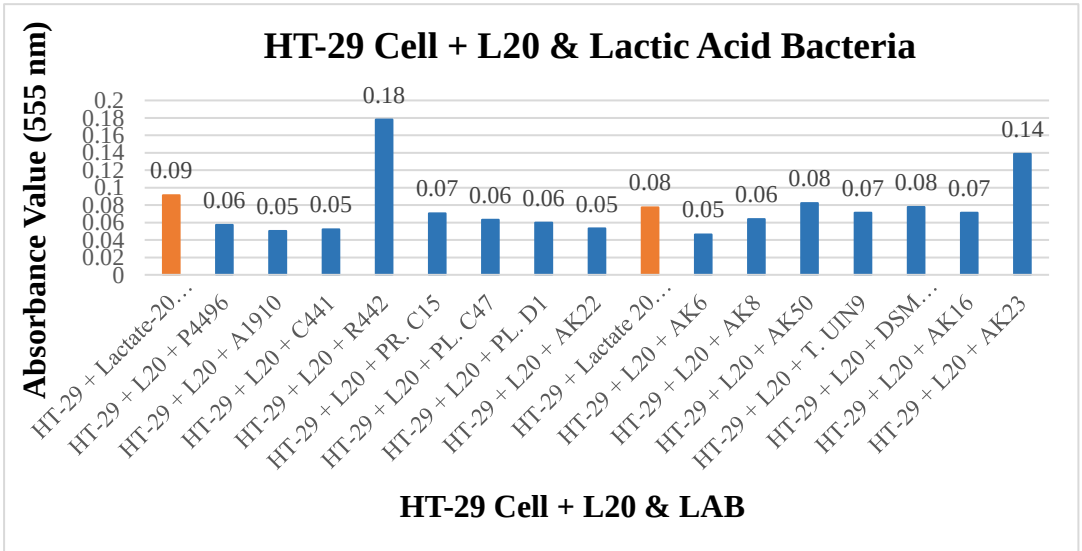


Figure 6. 34. HT-29 cell + lactate (20 mM) & LAB

According to this Figure 6.35, the binding of LAB to lactate (50 mM) added to the HT-29 cell was examined, there is 1 bacterium appears to be PL. C47, which is equivalent to the control. And, the bacterium with a higher absorbance value than the control in the first group appears to be only R442. In 2nd group, there is 1 bacterium appears to be AK6, which is equivalent to the control. And, only 2 bacteria with a higher absorbance value than the control and these are: AK16 and AK8.

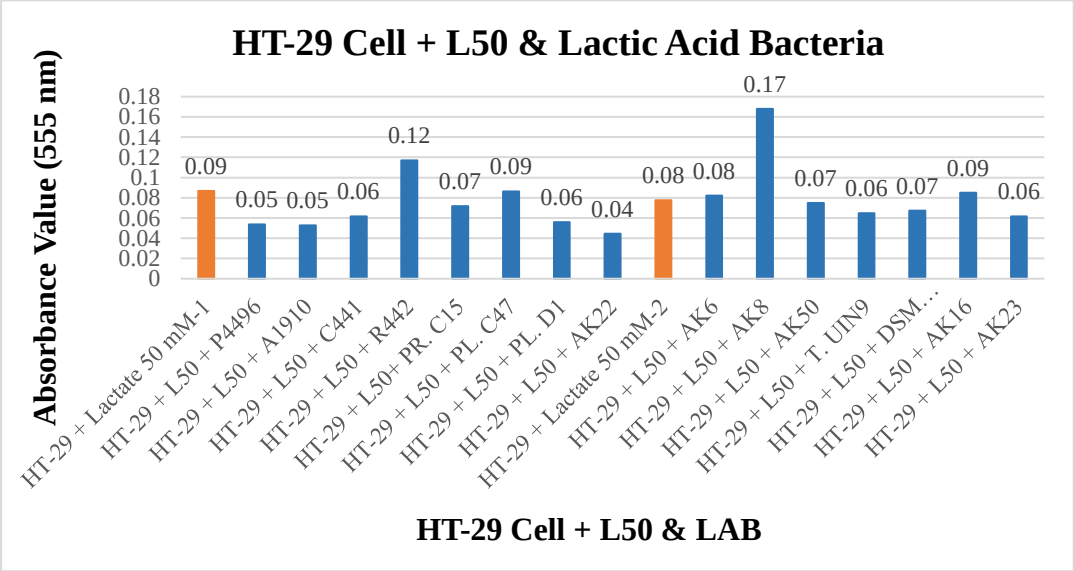


Figure 6. 35. HT-29 cell + lactate (50 mM) & LAB

According to this Figure 6.36, the binding of LAB to mucin + lactate (20 mM) added to the HT-29 cell was examined, the bacteria with a higher absorbance value than the control in the first group appears to be R442 and PL. C47 as equivalent value. In 2nd group, there is 1 bacterium appears to be AK16, which is equivalent to the control. And, only 1 bacterium with a higher absorbance value than the control and this is: AK50.

According to this Figure 6.37, the binding of LAB to mucin + lactate (50 mM) added to the HT-29 cell was examined, there are 3 bacteria appeared to be P4496, A1910 and AK22, which are equivalent to the control. And, the bacteria with a higher absorbance value than the control in the 1st group appeared to be PR. C15 and PL. D1 as equivalent value and PL. C47 has higher value than others. In 2nd group, there are 2 bacteria appeared to be AK8 and AK16, which is equivalent to the control. And, 3 bacteria with a higher

absorbance value than the control and these are: AK23 and AK50 as equivalent value and DSM 1954 has higher value than others.

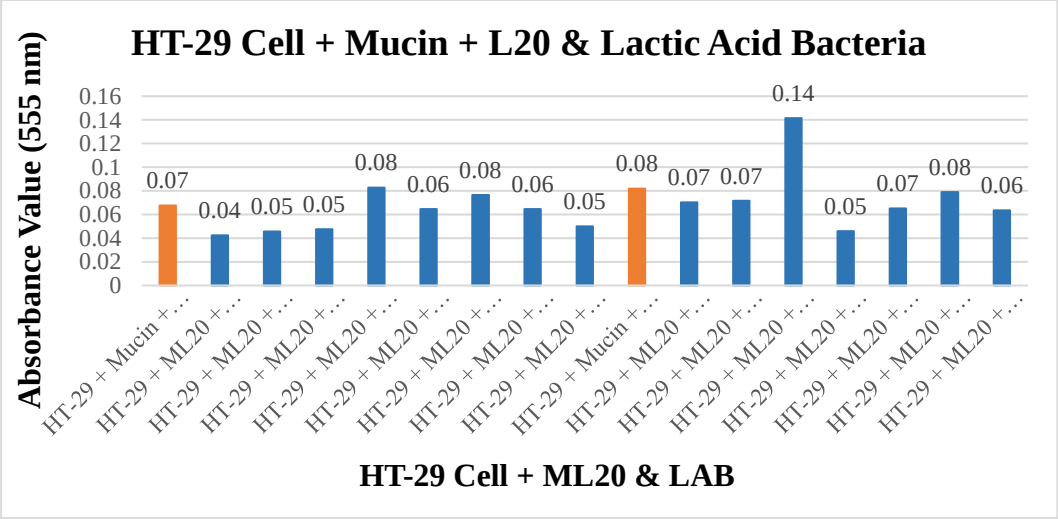


Figure 6. 36. HT-29 cell + mucin (50 µg/mL) + lactate (20 mM) & LAB

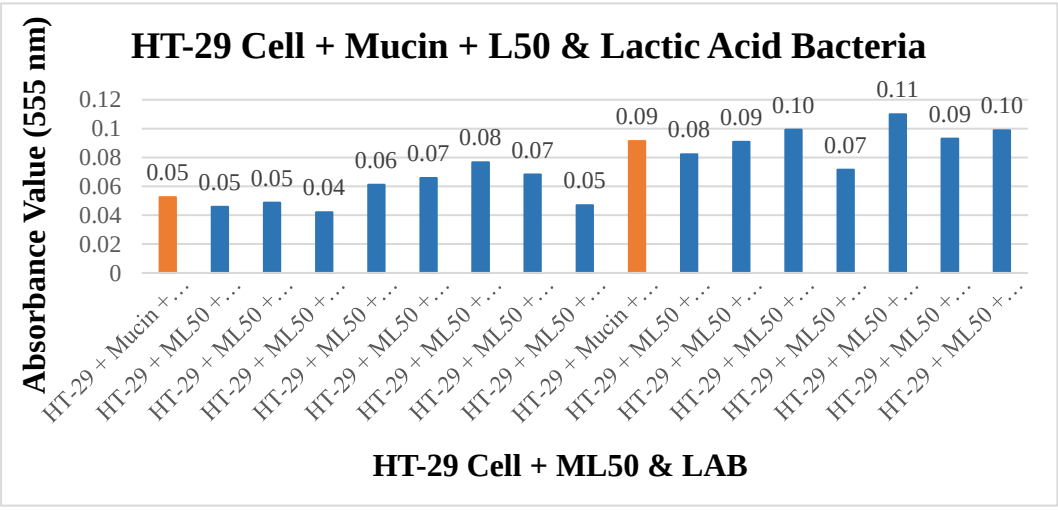


Figure 6. 37. HT-29 cell + mucin (50 µg/mL) + lactate (50 mM) & LAB

In this Table 6.12, the absorbance values obtained when different concentrations of lactate (20 mM & 50 mM) and mucin (50 µg/mL) are added to the HT-29 cell in addition to LAB, are expressed in µg/mL mucin using the equations in the mucin standard curve 1 (Figure 5.1) and mucin standard curve 2 (Figure 5.2) graphs.

Table 6. 12. Average mucin/mucus thickness data for 15 LAB

Cell + Bacteria:	**Ave. Mucus Thickness (µg/mL)**	**Cell + Bacteria:**	**Ave. Mucus Thickness (µg/mL)**	**Cell + Bacteria:**	**Ave. Mucus Thickness (µg/mL)**
HT-29 + Mucin-1 (CNT.1)	**104.66**	**HT-29 + Mucin + Lactate-20 mM-1**	**108.55**	**HT-29 + Mucin + Lactate-50 mM-1**	**94.8**
HT-29 + M + P4496	95.26	HT-29 + ML20 + P4496	85.62	HT-29 + ML50 + P4496	88.82
HT-29 + M + A1910	84.29	HT-29 + ML20 + A1910	88.54	HT-29 + ML50 + A1910	91.28
HT-29 + M + C441	101.85	HT-29 + ML20 + C441	90.37	HT-29 + ML50 + C441	85.36
HT-29 + M + **R442**	**113.85**	HT-29 + ML20 + **R442**	**122.41**	HT-29 + ML50 + **R442**	**102.59**
HT-29 + M + **PR. C15**	120.5	HT-29 + ML20 + PR. C15	105.91	HT-29 + ML50 + PR. C15	106.96
HT-29 + M + **PL. C47**	**119.46**	HT-29 + ML20 + **PL. C47**	**116.53**	HT-29 + ML50 + **PL. C47**	**116.71**
HT-29 + M + PL. D1	96.54	HT-29 + ML20 + PL. D1	105.83	HT-29 + ML50 + **PL. D1**	**109.01**
HT-29 + M + AK22	82.53	HT-29 + ML20 + AK22	92.41	HT-29 + ML50 + AK22	89.68
HT-29 + Mucin-2 (CNT.2)	**155.99**	**HT-29 + Mucin + Lactate 20 mM-2**	**170.98**	**HT-29 + Mucin + Lactate 50 mM-2**	**182.81**
HT-29 + M + **AK6**	**219.78**	HT-29 + ML20 + AK6	156.55	HT-29 + ML50 + AK6	171.27
HT-29 + M + AK8	154.01	HT-29 + ML20 + AK8	158.26	HT-29 + ML50 + AK8	182.1
HT-29 + M + **AK50**	**194.38**	HT-29 + ML20 + AK50	**245.54**	HT-29 + ML50 + **AK50**	**192.73**
HT-29 + M + T. UIN9	137.56	HT-29 + ML20 + T. UIN9	125.92	HT-29 + ML50 + T. UIN9	157.96
HT-29 + M + DSM 1954	146.12	HT-29 + ML20 + DSM 1954	149.93	HT-29 + ML50 + **DSM 1954**	**206.12**
HT-29 + M + **AK16**	**158.14**	HT-29 + ML20 + AK16	167.4	HT-29 + ML50 + **AK16**	**184.82**
HT-29 + M + **AK23**	**250.56**	HT-29 + ML20 + AK23	148.06	HT-29 + ML50 + **AK23**	**192.2**

According to this Figure 6.38, the binding of LAB to mucin added to the HT-29 cell was examined, there are 3 bacteria appeared with a higher mucin thickness than the control in the first group appeared to be R442, PL. C47 and PR. C15. In 2nd group, 4 bacteria with a higher mucin thickness than the control and these are respectively: AK16, AK50, AK6 and AK23.

According to this Figure 6.39, the binding of LAB to mucin and lactate (20 mM) added to the HT-29 cell was examined, there are 2 bacteria appeared with a higher mucin

thickness than the control in the first group appears to be PL. C47 and R442. In 2nd group, only 1 bacterium with a higher mucin thickness than the control and this is: AK50.

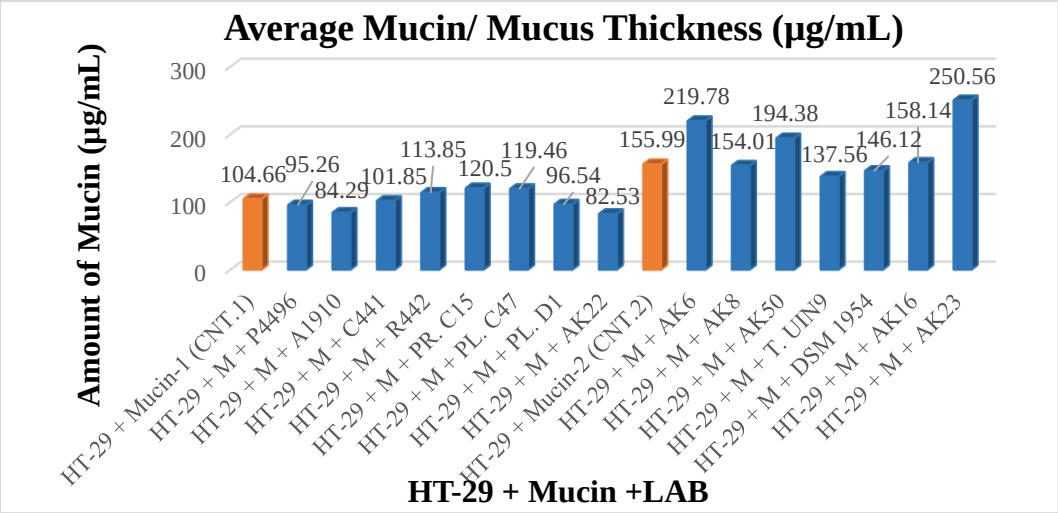


Figure 6. 38. Mucin/mucus thickness (µg/mL) of HT-29 cell + mucin (50 µg/mL) + LAB

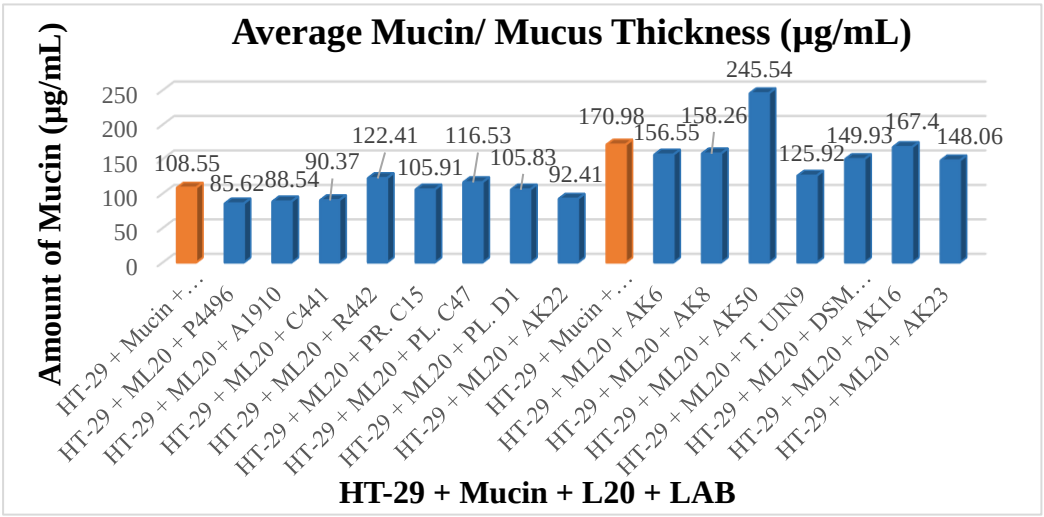


Figure 6. 39. Mucin/mucus thickness (µg/mL) of HT-29 cell + mucin (50 µg/mL) + lactate (20 mM) + LAB

According to this Figure 6.40, the binding of LAB to mucin (50 µg/mL) and lactate (50 mM) added to the HT-29 cell was examined, there are 4 bacteria appeared with a higher mucin thickness than the control in the first group appeared to be R442, PL.

D1, PR. C15 and PL. C47. In 2nd group, 4 bacteria with a higher mucin thickness than the control and these are respectively: AK16, AK23, AK50 and DSM 1954.

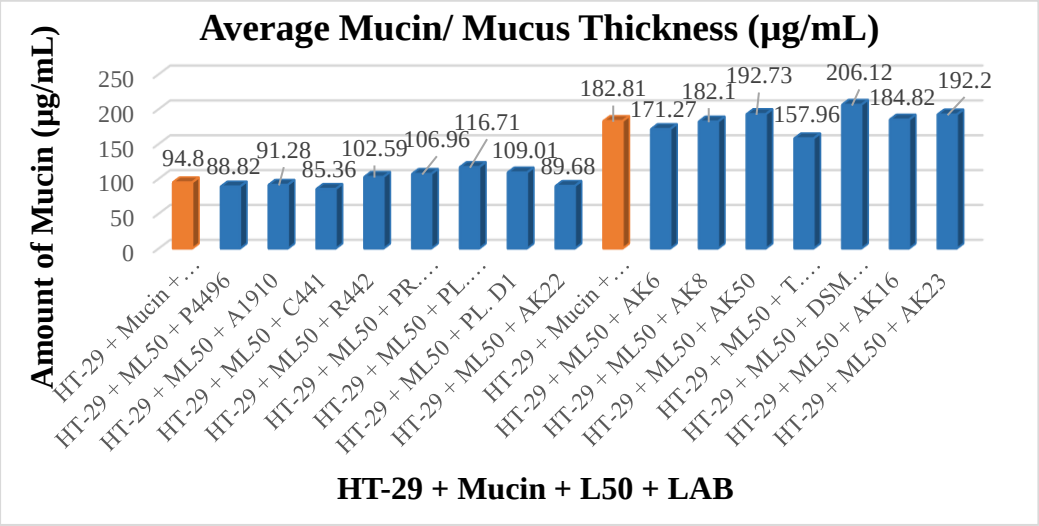


Figure 6. 40. Mucin/mucus thickness (µg/mL) of HT-29 cell + mucin (50 µg/mL) + lactate (50 mM) + LAB

According to the results obtained from these three control groups, there were two bacteria in all three groups with higher mucin formation than the control. These are: PL. C47 and AK50.

Limage et al. (2020) performed the study about quantification of neutral mucins based on the PAS staining method after exposure to TiO_2 NPs and/or bacteria. Results from the PAS staining experiment showed that including neutral and/or sialic acid mucosubstance secretions were notable in the presence of bacteria, gastric digestion, intact TiO_2 NPs, and after concomitant exposure to undigested TiO_2 NPs and *L. rhamnosus* demonstrated a essential increase.

In the Wrzosek et al. (2013) study, *Bacteroides thetaiotaomicron* and *Faecalibacterium prausnitzii* were investigated as functional members of the microbiome effecting host metabolism to construct a simplified microbiota model. The numbers of goblet cells stained with PAS were significantly higher in *B. thetaiotaomicron* than control after 2 and 30 days incubation. *B. thetaiotaomicron,* an acetate producer, enhanced expression of mucus-related genes, goblet cell differentiation, and the ratio of sialylated to sulfated mucins in mono-associated rats. *B. thetaiotaomicron* & *F.*

prausnitzii, which are metabolically complementary, modulate the intestinal mucus barrier by modifying goblet cells and mucin glycosylation *in vivo.*

In the Akbari et al. (2017) study, the potential of Cruciferin/Calcium (Cru/Ca) and Cruciferin/ Chitosan (Cru/Cs) nanoparticles were assessed for oral drug delivery. In this purpose, the scope and proportion of particle uptake in Caco-2 cells and Caco-2/HT29-MTX-E12 co-culture was evaluated. An amount of mucin was detected in Caco-2 line, but the concentration of mucin in the Caco-2/HT29 co-culture was remarkably higher than that of Caco-2 culture. Mucin concentration in this Caco-2/HT29 co-culture enhanced over time and reached to maximum after 21 days incubation.

When the results of this study are compared with the literature values; it is seen that different LAB strains modulate the intestinal mucus barrier, mucin glycosylation, mucosubstance secretions and, mucin concentration enhanced over time were notable in the presence *in vivo* and *in vitro* HT-29 cell culture studies. So, thesis results are similar in the literature studies.

Consequentially, *L. plantarum* C47 and *Lactobacillus* AK50 showed resistance to simulated gastric and intestinal conditions, showed binding in the desired range in the "Mucin Bacterial Binding" experiment and increased the amount of mucin at significant values in the above 3 control groups in cell culture experiments. In addition to these experiments, the common bacteria with positive results in sugar fermentation tests is AK50, an olive isolate. Bacteria that have reached this point by showing positive results from these experiments have been obtained as anti-obesity prototype candidates.

CHAPTER 7

CONCLUSION

There have been a few research in the literature related to understanding of the modulation of *Akkermansia* population in microbiota by anti-obesity probiotic effects and/or mechanism. Thus, this thesis study is predictive in nature so as how to determine "Adhesion to Induction" characteristics, increase the variety of probiotics in colon microbiota, and decrease the ratio of microbial community related with obesity. In order to predict the anti-obesity potential of probiotics, understanding the importance of adhesion properties onto mucin/mucus layer, the effect of lactate on mucin/mucus layer thickening by *in vitro* HT-29 cell culture for LAB. Within this frame, this thesis is an original study that can contribute to the scientific literature, with the *L. plantarum* C47 and *Lactobacillus* AK50 isolates being found as anti-obesity prototype candidates.

In this thesis, a suitable niche was created for *Akkermansia muciniphila*, a new generation probiotic, and 2 anti-obesity prototype candidate LAB isolated from cheese and olives were determined. At this point, it can be argued that this environment prepared for *Akkermansia* may also have positive effects on *Bacteroides, Clostridium* and *Faecalibacterium*, which are among the new generation probiotics. Thus, the proliferation of new generation super probiotics as a group fighting against metabolic disease, autoimmune disease, cardiovascular disease, gastrointestinal disorders and cancer etc. becomes important.

In addition, the *L. plantarum* C47 and *Lactobacillus* AK50 obtained as a result of the experiment may have beneficial effects for leaky gut syndrome, inflammation and cardiovascular diseases, besides its anti-obesity effect. In addition, it can be deduced that other beneficial bacteria may adhere to the mucin layer and promote biofilm formation.

In the light of the data obtained from the cell culture studies performed in *in vitro* conditions, future studies can be better planned for *Akkermansia* in *in vivo* experiments.

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APPENDIX A

CHEMICALS

Table A. 1. Chemicals for microbiological experiments

No:	Material Name:	Brand & Code:
1	MRS Broth	Merck,1.10661.0500
2	M17 Broth	Biolife, 4017202, 500 g
3	Agar-agar	Applichem, A0949,1000,1 KG
4	Peptone-Trypticase Peptone (Pancreatic Digest of Casein)	211921, BD
5	Yeast Extract granulated	Merck, 1.03753.0500
6	NaCl	Applichem, A2942, 1000, 1 KG
7	KCl	Sigma, 12636
8	Na_2HPO_4	Applichem, A2943, 1000, 1 KG
9	KH_2PO_4	Riedel-de Haen, 04243, 1 kg
10	HCl	Merck, 1.00314.2500
11	Pepsin	Merck, 1.07185.0100
12	Pancreatin from porcine pancreas	Sigma, P7545-100 G
13	Bile Salts	Oxoid, LP0055
14	D (+) Glucose ($C_6H_{12}O_6$)	Applichem, A3636, 1000
15	Meat Extract	Merck, 1.03979.0500
16	Ammonium citrate tribasic	Sigma-aldrich, A1332-100 G
17	Sodium acetate trihydrate($C_2H_3NaO_2.3H_2O$)	Applichem, A5268, 0500
18	Potassium phosphate dibasic trihydrate (K_2HPO_4)	Sigma, P5504-500 G
19	Magnesium sulfate heptahydrate ($MgO_4S.\ 7H_2O$)	Applichem, A6287, 0500
20	Manganese (II) sulfate monohydrate ($MnSO_4.H_2O$)	Appliche, A1038, 0500
21	Acetic acid	Merck, 1.00056.2500
22	Amido Black	Merck, 1.01167.0025
23	Mucin from porcine stomach type III	Sigma, M1778-10G

Table A. 2. Chemicals for cell culture experiments

No:	Material Name:	Brand & Code:
1	DMEM High Glucose (4.5 g/l) with L-glutamine with Sodium Pyruvate	Capricorn Scientific, DMEM- HPA, 500 mL
2	RPMI 1640 with L-glutamine with 25 mM HEPES	Capricorn SCI, RPMI-HA, 500 mL
3	PENICILLIN-STREPTOMYCIN SOLUTION	Capricorn SCI, 100 mL
4	Fetal Bovine Serum Advanced	Capricorn SCI, FBS-11B, 100 mL
5	PBS Dulbecco	Biochrom, L 1825,500 mL
6	PBS pH 7.4 (1X)	Gibco, 10010-015, 500 mL
7	Trypsin /EDTA (0.25% Trypsin, 0.1% EDTA)	WISENT MULTICELL, 325-043 CL, 500 mL
8	Trypan Blue Solution 0.5%	Biological Industries,03-102-1B,100 mL
9	Periodic acid	Carlo Erba Sigma 409182 - 25 g
10	Schiff's reagent for microscopy	MERCK - M109033.0500 - 500 mL
11	Hematoxylin Solution, Harris Modified	MERCK - M109253.0500 - 500 mL
12	Ammonium hydroxide solution, 26% NH_3	Sigma-Aldrich, UN 2672, l L
13	ETHANOL ABSOLUTE ≥ 99.9%	ISOLAB, 920.026.2500-2.5 L
14	Xylene	Sigma-Aldrich, 16446-2.5L
15	Triton X-100	Sigma-Aldrich, X100-100ML
16	Acetic acid	Merck, 1.00056.2500
17	HCl	Merck, 1.00314.2500
18	Porcine stomach mucin type III	Sigma, M1778-10g

APPENDIX B

MEDIA

Table B. 1. Modified MRS broth for mucin degradation & growth ability on mucin

No:	Chemical Names	g/L
1	Mucin from porcine stomach type III	3
2	D (+) Glucose ($C_6H_{12}O_6$)	20
3	Peptone-Trypticase Peptone (Pancreatic Digest of Casein)	10
4	Meat Extract	8
5	Yeast Extract granulated	4
6	Triammonium citrate	2
7	Sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$)	5
8	Magnesium sulfate heptahydrate ($MgO_4S.\ 7H_2O$)	0.2
9	Mangonous sulfate tetrahydrate	0.05
10	Tween 80	1 mL
11	Dipotassium hydrogen phosphate	2

Table B. 2. Modified MRS agar for mucin degradation & growth ability on mucin

No:	Chemical Names	g/L
1	Mucin from porcine stomach type III	3
2	D (+) Glucose ($C_6H_{12}O_6$)	20
3	Peptone-Trypticase Peptone (Pancreatic Digest of Casein)	10
4	Meat Extract	8
5	Yeast Extract granulated	4
6	Triammonium citrate	2
7	Sodium acetate trihydrate($C_2H_3NaO_2.3H_2O$)	5
8	Magnesium sulfate heptahydrate ($MgO_4S.\ 7H_2O$)	0.2
9	Mangonous sulfate tetrahydrate	0.05
10	Tween 80	1 mL
11	Dipotassium hydrogen phosphate	2
12	Agar	15

Table B. 3. Modified MRS broth medium for sugar tests

No:	Chemical Names	g/L
1	Peptone-Trypticase Peptone (Pancreatic Digest of Casein)	10
2	Meat extract	8
3	Yeast extract	4
4	Triammonium citrate	2
5	Sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$)	5
6	Magnesium sulfate heptahydrate	0.2
7	Manganous sulfate tetrahydrate ($MgO_4S.\ 7H_2O$)	0.05
8	Tween 80	1 mL
9	Dipotassium hydrogen phosphate	2
10	Bromecresol Purple	0.04

According to Table B.1, B.2 and B.3; these ingredients were dissolved in deionized water by stirring. And then, pH of the medium was adjusted between 6.2-6.6, and this medium was sterilized by autoclaving at 121 °C for 15 min.

Table B. 4. PBS Medium

No:	Chemical Names	g/L
1	NaCl	8
2	KCl	0.2
3	Na_2HPO_4	1.64
4	KH_2PO4	0.24
5	Deionized water	1000 mL

These ingredients were dissolved in deionized water by stirring. Then, pH of the medium was adjusted between 7.0-7.2, and this medium was sterilized by autoclaving at 121 °C for 15 min.

APPENDIX C

GROWTH AND PREPARATION OF BACTERIAL CULTURES

Table C. 1. *Lactiplantibacillus plantarum* NRRL-B 4496

Sample No:	**Dilution factor**	**Dilution factor**	**Bacteria + Peptone Water**	**Mcfarland Turbidity Value**	**Spectrophotometre-Absorbance (600 nm)**	**Results of Number of Bacteria (CFU/mL)**
1	5	0.20	2 mL LAB + 8 mL PW	5.6	1.32	3.8×10^8
2	10	0.10	1 mL LAB + 9 mL PW	3.3	0.72	1.7×10^8
3	15	0.07	0.67 mL LAB + 9.33 mL PW	2.4	0.51	1.31×10^8
4	25	0.04	0.4 mL LAB + 9.6 mL PW	1.5	0.32	7.6×10^7
5	50	0.02	0.2 mL LAB + 9.8 mL PW	0.8	0.16	3.75×10^7
		0.00			0	
			***L. plantarum* NRRL-B 4496**	10	2.26	

Table C. 2. *Lactobacillus acidophilus* NRRL-B 1910

Sample No:	**Dilution factor**	**Dilution factor**	**Bacteria + Peptone Water**	**Mcfarland Turbidity Value**	**Spectrophotometre-Absorbance (600 nm)**	**Results of Number of Bacteria (CFU/mL)**
1	5	0.20	2 mL LAB + 8 mL PW	4.8	1.12	4.25×10^8
2	10	0.10	1 mL LAB + 9 mL PW	2.8	0.61	1.43×10^8
3	15	0.07	0.67 mL LAB + 9.33 mL PW	2	0.41	1.09×10^8
4	25	0.04	0.4 mL LAB + 9.6 mL PW	1.2	0.25	5.5×10^7
5	50	0.02	0.2 mL LAB + 9.8 mL PW	0.8	0.13	2.9×10^7
		0.00		0	0	
			***L. acidophilus* NRRL-B 1910**	9.3	2.20	

Table C. 3. *Lacticaseibacillus casei* NRRL-B 441

Sample No:	Dilution factor	Dilution factor	Bacteria + Peptone Water	Mcfarland Turbidity Value	Spectrophotometre-Absorbance (600 nm)	Results of Number of Bacteria on MRS Agar Plate (CFU/mL)
1	5	0.20	2 mL LAB + 8 mL PW	4.3	0.97	1.83×10^8
2	10	0.10	1 mL LAB + 9 mL PW	2.7	0.54	7.4×10^7
3	15	0.07	0.67 mL LAB + 9.33 mL PW	1.9	0.35	4.45×10^7
4	25	0.04	0.4 mL LAB + 9.6 mL PW	1.2	0.22	4.1×10^7
5	50	0.02	0.2 mL LAB + 9.8 mL PW	0.7	0.09	1.59×10^7
		0.00		0	0	
			***L. casei* NRRL-B 441**	8.6	1.98	

Table C. 4. *Lacticaseibacillus rhamnosus* NRRL-B 442

Sample No:	Dilution factor	Dilution factor	Bacteria + Peptone Water	Mcfarland Turbidity Value	Spectrophotometre-Absorbance (600 nm)	Results of Number of Bacteria (CFU/mL)
1	5	0.20	2 mL LAB + 8 mL PW	5.9	1.38	5.4×10^8
2	10	0.10	1 mL LAB +9 mL PW	3.8	0.81	4.45×10^8
3	15	0.07	0.67 mL LAB + 9.33 mL PW	2.7	0.55	1.59×10^8
4	25	0.04	0.4 mL LAB + 9.6 mL PW	1.7	0.35	8.7×10^7
5	50	0.02	0.2 mL LAB + 9.8 mL PW	0.9	0.18	5.9×10^7
		0.00		0	0	
			***L. rhamnosus* NRRL-B 442**	10	2.26	

Table C. 5. *Lactobacillus pentosus* NRRL-B 227

Sample No:	**Dilution factor**	**Dilution factor**	**Bacteria + Peptone Water**	**Mcfarland Turbidity Value**	**Spectrophotometre-Absorbance (600 nm)**	**Results of Number of Bacteria (CFU/mL)**
1	5	0.20	2 mL LAB + 8 mL PW	1.3	0.24	9.3×10^7
2	10	0.10	1 mL LAB +9 mL PW	0.7	0.13	4.2×10^7
3	15	0.07	0.67 mL LAB + 9.33 mL PW	0.4	0.11	2.65×10^7
4	25	0.04	0.4 mL LAB + 9.6 mL PW	0.3	0.05	1.71×10^7
5	50	0.02	0.2 mL LAB + 9.8 mL PW	0	0.05	1.07×10^7
		0.00		0	0	
			***L. pentosus* NRRL-B 227**	4.3	0.90	

APPENDIX D

DETERMINATION OF LACTATE PRODUCTION

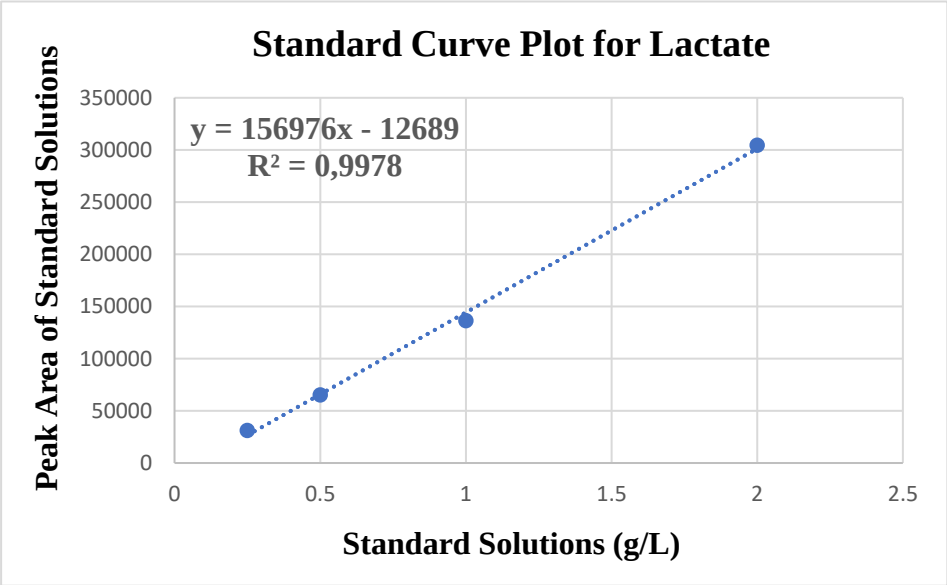

Figure D. 1. Lactate standard curve plot for HPLC analysis

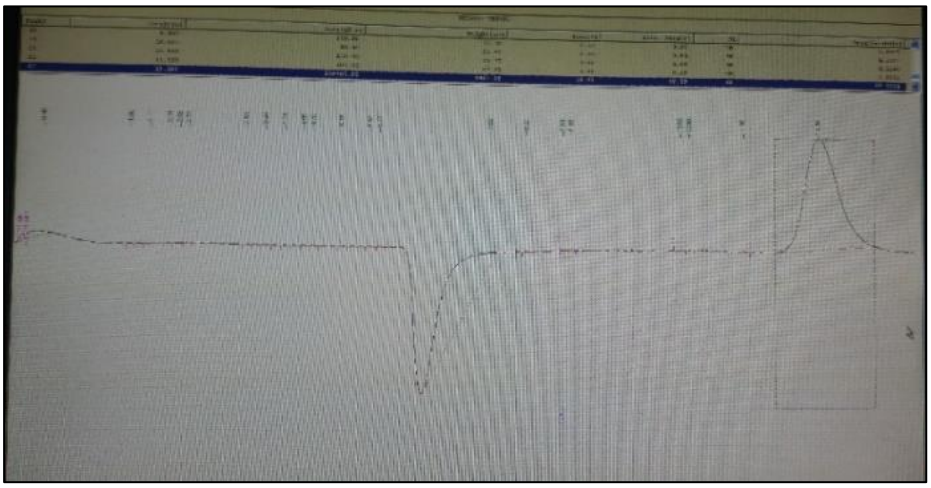

Figure D. 2. Peak area of 1 g/L lactate solution for standard curve