DEVELOPMENT OF EDIBLE FILMS CONTAINING PROBIOTICS FOR ORAL AND DENTAL HEALTH

by
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DEVELOPMENT OF EDIBLE FILMS CONTAINING PROBIOTICS FOR ORAL AND DENTAL HEALTH

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ABSTRACT

DEVELOPMENT OF EDIBLE FILMS CONTAINING PROBIOTICS FOR ORAL AND DENTAL HEALTH

The oral cavity is one of the most diverse microbial area of the human body and the most related infections are periodontal diseases, endodontic infections and oral mucosa-related infections which arise due to an increase in the number of pathogenic microorganisms and a decrease in the number of beneficial microorganisms. Probiotics are living microorganisms that positively affect health therefore, probiotic consumption is required to recover the disrupted microbiological balance and prevent related diseases. The aim of the project is to produce edible films containing probiotic bacteria for sustaining oral and dental health. WPI (whey protein isolate), trehalose, and maltodextrin were used individually or as a mixture as coating materials and *L.rhamnosus* was used as a probiotic content which was encapsulated via spray-drying. The results demonstrated that WPI led to the highest recovery and lowest moisture content in encapsulated products. The results further indicated that the dissolution time of edible films prepared with pectin was relatively faster than tested alternatives. The amount of viability in the pectin based edible films containing 10 percent encapsulated product was also analyzed and the results indicated that the films exhibited approximately 10^5 CFU/ml which is roughly comparable to the recommended daily level. The effectiveness of prepared edible film against pathogens found in saliva (*E.coli, S.aeurus, C.albicans, P.aeuroginosa*) was also tested and a significant decrease in the viability of pathogens was detected while there was no significant deterioration in the viability of *L.rhamnosus.*

ÖZET

AĞIZ VE DİŞ SAĞLIĞI İÇİN PROBİYOTİK İÇEREN YENİLENEBİLİR FİLMİN GELİŞTİRİLMESİ

Ağız boşluğu, insan vücudunun en çeşitli mikrobiyal alanlarından biridir. Bu alandaki en sık görülen enfeksiyonlar ise; patojenik mikroorganizmaların sayısındaki artış ve faydalı mikroorganizma sayısındaki azalmaya bağlı olarak ortaya çıkan; periodontal hastalıklar, endodontik enfeksiyonlar ve oral mukoza ile ilişkili enfeksiyonlardır. Probiyotikler; mikrobiyotadaki dengeyi sağlamakta olumlu yönde etki sağlayan canlı mikroorganizmalardır. Bu nedenle bozulan mikrobiyolojik dengeyi geri kazanmak ve buna bağlı hastalıkları önlemek için probiyotik tüketimi gereklidir. Projenin amacı; ağız ve diş sağlığının sürdürülmesi için, probiyotik bakteri içeren yenilebilir filmler üretmektir. Kaplama materyali olarak WPI (whey protein izolatı), trehaloz ve maltodekstrin tek tek veya karışım olarak kullanılmış, probiyotik olarak *L.rhamnosus* tercih edilmiştir. Sprey kurutma cihazı kullanılarak, kapsüllenmiş probiyotik içerikli ürün elde üretilmiştir. Farklı kaplama malzemeleri kullanılarak üretilen enkapsüle ürünler içerisinde kıyaslama yapıldığında; WPI'le kapsüllenmiş ürünlerin en yüksek geri kazanım ve en düşük neme sahip olduğu görülmüştür. Ayrıca; pektin ile hazırlanan yenilebilir filmlerin çözünme süresinin, test edilen diğer alternatiflere nispeten daha hızlı olduğu analiz edilmiştir. Yapılan başka bir çalışmada da, yüzde 10 kapsüllenmiş ürün içeren pektin bazlı yenilebilir filmlerdeki canlılık miktarı araştırılmıştır. Sonuçlara göre, filmlerdeki canlılık miktarının yaklaşık olarak 10^5 CFU/ml ve bu değerin de tavsiye edilen günlük probiyotik tüketim miktarını karşılayabilir seviyelerde olduğu görülmüştür. Ayrıca; hazırlanan yenilebilir filmlerin tükürükte bulunan patojenlere (*E.coli, S.aeurus, C.albicans, P.aeuroginosa*) karşı etkinliği de test edilmiş ve patojenlerin canlılığında önemli bir azalma tespit edilirken, *L.rhamnosus*'un canlılığında önemli bir azalma olmadığı tespit edilmiştir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS / ABBREVIATIONS

µl	Volume (microliter)
cfu/gr	Number of bacteria per gram (colony forming units per gram)
cfu/ml	Number of bacteria per ml (colony forming units per milliliter)
cm-1	Wavelength
cp	Viscosity (centipoise)
g/lt	Mass concentration (gram per liter)
h	Time (hour)
l/min	Flow rate (Liter per minute)
mm	Length
T	Temperature (°C)
v/v	Volume concentration of solution (volume/volume)
w/v	Mass concentration of solution (weight/volume)

1. INTRODUCTION

Microbiota is all microorganisms that are beneficial and harmful in the body. In order to lead a healthy life, these microorganisms in the mouth, intestines and vagina must be in balance. However, unconscious antibiotics, antimicrobial products, unhealthy eating habits damage this ecological balance in our body, and this is the basis of many diseases. Probiotics are live microorganisms that, when present in sufficient quantities in our body, positively affect health. For this reason, probiotic consumption is necessary to restore the deteriorated microbiological balance and prevent some diseases.

The ecological system formed by microbiota is also called as microbiome [1]. Harmful and beneficial microorganisms are found in the mouth, intestine and vaginal area need to be in balance. However, using antibiotics [2] and antimicrobial products, stress, unhealthy eating habits affect this ecological balance in our bodies negatively, and as a consequence, many diseases occur. Probiotic consumption is required to recover the disrupted balance which may result in the prevention of some diseases due to microbiological imbalance.

Probiotics are "live microorganisms (bacteria or yeasts), which when ingested or locally applied in sufficient numbers confer one or more specified demonstrated health benefits for the host"(FAO/WHO, 2001). Thus, they are used in the production of functional foods and pharmaceutical products.

The oral cavity is one of the most diverse microbiomes of the human body. Periodontal diseases, endodontic infections, oral mucosa related infections, which are the most common infection in the world, are caused by oral microbiota[3, 4]. These infections increase the number of pathogenic (harmful) microorganisms and decrease the beneficial microorganisms [5]. Studies in the literature suggest that when probiotic added products are consumed; the number of microorganisms causing tooth decay, inflammation and halitosis has been reduced [2, 3, 5–9]. Almost 95 percent of the population suffers from tooth decay and gum disease [3]. Fluoride use and other preventive measures reduce tooth decay, but they do not control infection [10]. These infections increase the number of pathogenic (harmful) microorganisms and a decrease in the number of beneficial microorganisms [5]. For this reason, establishing a balanced microbiome by increasing the number of probiotics

present in the oral environment is seen as a new and promising method for treating/preventing dental and gum disease [3, 11].

In this project, the aim is to develop edible films that will release encapsulated probiotics to the oral cavity in a short time, and contribute to general oral health, once placed on the tongue.

There is a target product that is not being produced in our country; edible films containing probiotic bacteria for general oral and dental health.

Probiotics must be in a high number in the film to be effective. For this reason, it is ensured that the probiotics remain alive during storage. Before producing the edible film, to increase shelf life, firstly, the bacteria will be encapsulated in a protective matrix. Then the encapsulated probiotic goes into an edible film mixture. Once the probiotics in the film release in the simulated mouth conditions, they will be examined.

The most innovative part of the project is that this product will be an edible film. There are two reasons why the product is prepared in the form of an edible film:

(i) In fast living conditions, this product will have the advantage of being consumed without the need for water at any time wanted.

(ii)The edible film is dissolved entirely in the mouth within enough time that the probiotics stay on the teeth and the oral surfaces so that the bacteria will remain in the mouth area. To our best knowledge, there is no equivalent domestic or foreign product on the market.

Almost 95percent of the population suffers from tooth decay and gum diseases [3]. Because the single best matching probiotics usually occupy a particular area in the mouth cavity. There is a limited area in the mouth flora for each ecological environment (such as fissure). This area is occupied by the largest number of organisms in the saliva. If a species has already occupied the settlement areas on the tooth, new opportunistic organisms are not accepted here, and they cannot be a part of the plaque [4]. For this reason, a high number of probiotic bacteria may prevent the establishment of pathological microorganisms.

Few products in the world contain probiotics for oral health. Examples include Pro-t-action, PerioBiotic, EvoraPlus and EvoraPro, and EvoraKids. The essential criteria in probiotic

products are choosing the right species for the target users and delivering these bacteria to the target region in high numbers and alive.

Based on recent studies in the literature and limited commercial products, this project aims to develop edible films capable of releasing encapsulated probiotic bacteria in the oral cavity within a short time frame. There was no similar domestic or foreign product in our market research. The formulation of the product in the form of an edible film is the innovative aspect of the process, and essentially there are two reasons for choosing such a design:

(i) The product will be accessible, comparably more comfortable and will be in a form such that the consumer can comfortably carry,

(ii) The film form design of the product will allow enough time for the probiotics to stick to the teeth and mouth surfaces so that the bacteria will remain in the oral cavity for a prolonged period.

2. LITERATURE SURVEY

The most important criteria for probiotic bearing products are selecting bacteria for the right purpose and delivering corresponding viable bacteria to the target area in considerably higher quantities. Considering these two criteria, this project is; to develop edible films that contain encapsulated probiotic bacteria for oral health. As stated before, in order for probiotics to be effective, the population should be at high concentrations and bear excellent viability.

Consequently, probiotics should be protected from environmental and process effects, leading to enhanced survival rates. For this purpose, the most commonly used method is encapsulation [12, 13]. Therefore, the bacteria will first be encapsulated in a protective matrix, and then the release of probiotics added to the developed films will be examined in simulated oral conditions.

The health benefits of probiotic bacteria have been studied for a long time. Literature studies are mostly on probiotics' arrival to the intestines alive and in high numbers, where they colonize and positively affect the host's health. Therefore, most of the commercial probiotic food products and food supplements are designed for this purpose.

Studies have shown that many health problems, from obesity to heart disease, from intestinal disorders to depression, from lowering cholesterol to food allergies, can be prevented or treated using probiotics [14]. Therefore, the number of probiotic-added food products and food supplements increases day by day [15, 16]. The global probiotic market (for animal and human consumption), which is now 35 billion dollars, will be around USD 64.02 Billion by 2022 billion dollars [17]. In our country, the needs of this newly developing sector continue.

Scientific studies in recent years have shown that probiotics are also important in terms of oral and dental health [5–9, 11, 18, 19]. In these studies, different probiotic bacteria were added in carriers such as chewing gum, cheese, yogurt, mouth rinse, tablet, lozenge, capsules, or without placing them in any carriers, and these probiotics are:

(i) on *Streptococcus mutans (MS)* bacteria causing tooth decay,

(ii) on *Candida albicans mushroom* causing fungal infections,

(iii) on bacteria that cause bacterial gingivitis and

(iv) investigated the effects on bacteria causing the halitosis.

There are limited commercial products in the world that contain probiotics for oral health. Examples include Pro-t-action, PerioBiotic, EvoraPlus and EvoraPro, and EvoraKids.

Studies showed that *L.rhamnosus , L.reuteri, Bifidobacteria bacteria* are sufficient on *MS* while; *L.reuteri, L.salivarius, S.salivarius, S.mitis, S.sanguis, W.cibaria*, and *L.acidophilus* bacteria are capable of detriment the number of *C.albicans*. Additionally, *W.cibria* and *S.salivarius* are shown to reduce the halitosis [19–22]. In these studies, the probiotics used possessed significant differences in the adhesion capacities activity in the mouth and tooth surface [3, 11]. It is further shown that the activity is also dependent on the dose and the strain of the probiotic used. For this reason, to provide the probiotics remain alive during storage, during the filmmaking phase, and storage, the bacteria need to be encapsulated in a protective matrix, all of which will then be incorporated into the developed films.

For many years, probiotic bacteria have been encapsulated using different wall materials and technologies [12, 13]. To protect bacteria from external influences by trapping them in a protective matrix or capsule and achieving their targeted release. This protection stage is crucial as probiotics' health benefits are only possible if many viable bacteria reach the target site (mouth or intestine).

The survival of probiotic organisms under harsh conditions encountered during food processing or through the gastrointestinal system is still a big challenge for researchers and producers. Many wall materials have been tested to produce innovative matrices that can maintain probiotic bacteria's viability at an applicable rate during the spray-drying process, storage, and passage through the gastrointestinal tract.

In the spray drying method to be used for the encapsulation, the activated bacteria is mixed with the wall solution, fed to the dryer, and powdered by the effect of heat at the outlet of the nozzle while being atomized [23].

Spray drying is the evaporation of moisture from an atomized liquid feed by mixing the liquid spray and drying gas (typically air or nitrogen). The mixture sprayed can be a solution or suspension. The drying proceeds until the desired moisture level is reached in the powder particles and has separated from the drying gas.

The physicochemical characteristics of wall materials, their density, the size of the produced particle, the outlet/inlet temperature, and the process's flow rate are essential parameters that determine the probiotic viability, the shelf-life duration, and the yield of the product [24, 25]. For this reason, the microencapsulation process was optimized by drying the coating material individually with different inlet temperatures, flow, and aspiration rates. According to the achieved outlet temperature, product yield, and humidity values, functional conditions were retained for *L.rhamnosus GG*'s microencapsulation.

In this project, probiotic encapsulation was performed via spray-drying, one of the most effective encapsulation approaches. Spray drying is one of the most preferred methods, as it is facile, fast, reproducible, and possess a low operating cost, high production capacity, and ease of scalability [15, 26–28]. Another reason for choosing this method among different encapsulation methods is that the encapsulated probiotics should have a small particle size and narrow size distribution as they will be incorporated into a thin film. It is also possible to obtain the powder form's encapsulated product (10-400μm) via spray drying.

As the wall material, combinations of starch, maltodextrin, and arabic gum, which are most suitable for this technique, will be used. Another reason for selecting these materials is that the matrix is readily cleaved by the amylase enzyme in the mouth leading to the bacteria's release.

In process optimization, the wall material components, the viscosity, the density, the nozzle diameter, and the temperature and flow rate of the process parameters are essential. Generally selected materials for the walls are starch derivatives and arabic gums best suited to the formed microparticles' spherical shape. Rapid and repeatable spray drying has advantages such as low operating costs, high production capacity, and ease of passing to the industrial scale. A high temperature can still be seen as a disadvantage because it damages the probiotic's cells during the encapsulation [13]. However, good results can be obtained by optimizing the system's inlet and outlet temperatures and adding agents (skimmed milk, disaccharides, inulin...) to protect bacteria against heat before drying [12, 29]. For this reason, active probiotics are mixed with trehalose (disaccharide, not harmful to the teeth). It is protected against heat, then added to different wall materials (starch, maltodextrin, and arabic gum combinations) and prepared for encapsulation.

As well as the process parameters, the nature of the selected probiotic bacterium, strain (heat tolerance), and the wall material to be used is also essential [12, 15, 27, 30–32].

The most commonly used probiotics are *lactobacillus* and *bifidobacterium* groups [8, 27, 30]. In studies, *Lactobacillus GG* effectively protects dental health [33]. It protects the health of the mouth by preventing damage from harmful bacteria formed in the mouth [33].

In the studies performed on the spray dryer device, it was observed that the different encapsulated bacteria with the highest viability were obtained when the coating material prepared at 25-30 percent w/v provided an output temperature of 70°C [27, 34, 35]. The main factors affecting the encapsulation are inlet and outlet temperatures [27]. Also, probiotics lose their viability at high temperatures [27, 36]. So, the aim is to protect from the harmful effects of heat and increase the viability rate to reduce the output temperature.

In the studies, the disaccharide group was used to reduces the harmful effects of heat and prevents the loss of probiotic's viability. In the disaccharide group, whey protein, trehalose, sucrose were used, and it was observed to increase the viability of the probiotics [36–38]. Trehalose and WPI addition reduced losses during the encapsulation process [15].

Many studies have shown that pectin –alginate, agar sucrose, and glycerol are used in edible film formation. [39–42]. Pectin is a polysaccharide obtained from plant cell walls. It takes the form of pectin gel in acidic conditions and is generally used to thicken foods. Alginate is a polysaccharide derived from brown seaweed. A complex carbohydrate is a compound consisting of a combination of many monosaccharide molecules. It is called a polysaccharide (starch, cellulose, etc.). Monosaccharides are single and straightforward sugars that cannot be broken down into smaller units with water.

The aim was to determine the appropriate inputs to obtain a homogeneous, easily removable, bubble-free edible film. Two important points were emphasized while preparing the edible film mix. The first one was to ensure good crosslinking to obtain a homogeneous film. the second is; the aim was to ensure that the amount of viability in the film was at the daily recommended 10^6 levels[43].

One of the most critical issues is maintaining the viability of the produced capsule in storage conditions. For this reason, in the studies, the level of viability is analyzed after waiting 45-60 days at 4C and 24C temperatures [36, 38, 44].

The amount of moisture is one of the main factors that reduces probiotic viability [27]. For this reason, a minimum moisture of (3.5-4 percent) is targeted in the capsules obtained.

In previous studies, it has been observed that the exposure time influences the viability of the probiotic, too. Therefore,

(i) The parameters are also be checked in laboratory studies via spray dryer [34].

(ii) The formulation of an edible film and the addition of probiotic bacteria

(iii) Probiotic release kinetics in the simulated oral environment. Formulated edible films containing encapsulated probiotics will be exposed to simulated human saliva. The number of bacteria passing from the films to the solution will be monitored over time by spectrophotometry [45, 46].

3. PRODUCTION OF THE ENCAPSULATED PRODUCT

3.1.MATERIALS AND METHODS

3.1.1. Materials

Maltodextrin (MD) MP 960048, whey protein isolate (protein >95 percent) (WPI), Davisco Foods International, and Trehalose (Cargill, Germany) were used as coating materials either individually or in a mixture. Trehalose (Tr) was used to protect *Lactobacillus rhamnosus GG* from the effect of heat damage as auxiliary methods, too.

Lactobacillus rhamnosus GG (53103, ATCC, Germany) was chosen for its efficiency in maintaining a healthy oral cavity [8, 27, 30] as a core material. The identity of the isolated strain *L.rhamnosus GG* was verified with a Matrix-Assisted Laser Desorption Ionisation – Time Of Flight MALDI-TOF mass spectrometry (Microflex lt-sh, Bruker Brand, Germany). *L. plantarum 299v* (14917, ATCC, Germany) was used to compare stress differences with *L. rhamnosus GG*. The growth was made on MRS agar (7543A, Acumedia, UK)), conservation was made from an overnight culture in MRS broth (7406A, Acumedia, UK)) and bacteria was incubated in incubators (IFA 110, ESCO, USA). MRS broth was used as a pre-culture medium for the inoculum preparation.

The micro-encapsulation of *L.rhamnosus GG* was carried via a spray dryer (Unopex B15 2017-081662, Bakon, Turkey) with a 0,7 mm nozzle.

Fourier Transform Infrared (FTIR) spectrometer (IS10, Thermo, USA) is a tool used to determine the functional groups and chemical bond structures in a material. Different dried microcapsules (with and without lactic acid bacteria) were analyzed via FT-IR spectrometer. KBr (Merck104907) was used as a baseline.

The encapsulated bacteria's viability after the spray drying process and storage was assessed using tetrazolium-based colorimetric assay (MTS Kit, CellTiter 96® AQueous Non-Radioactive for Cell Proliferation Assay, Promega, USA). Absorbance for MTS assays was measured using a microplate scanning spectrophotometer (xMark, Bio-Rad, USA) at a wavelength of 490 nm.

Scanning electron microscopy (SEM) (EVO 40, Zeiss, Germany) was used to observed the encapsulated particle's morphology.

The glass transition temperature (Tg) of the encapsulated product used in the production of edible films is measured using differential scanning calorimetry, (DSC)(Setaram, DSC131, France).

The sensitive scale (EX324, OHAUS, USA) was used to weigh, the humidity analyzer (V20F, Mettler Toledo, USA) was used to measure humidity. Viscosities were measured via the viscosimeter with LV2 spindle (DV3, Brookfield Brand, USA)

3.1.2. Preperation of the Spray Drying Solutions

15g of Tr was dissolved in 100ml of DSW (distilled water) and autoclaved for 15min at 121°C to prepare a 15 percent trehalose solution (w/v). 15g of WPI was dissolved in 100ml of DSW (distilled water) and autoclaved for 15min at 121°C to prepare a 15 percent WPI solution (w/v).

In preparing 15 percent (Trehalose + WPI) (w/v), 7.5g of both products were made in a 50ml batch as described above. Then, they were mixed until they reached at room temperature.

In preparing 15 percent (Maltodextrin+WPI) (w/v), 7.5g of both products were prepared in 50ml as before mentioned being mixed when they reached at room temperature.

Lactobacillus rhamnosus GG was recovered by isolation on MRS agar at 37°C and 5 percent CO_2. After that, conservation was made from an overnight culture in MRS broth, and mixed with glycerol 50 percent (v/v) and kept at -80°C. Finally, for short-term preservation, the probiotic was inoculated with MRS agar into a 15 ml falcon tube and incubated for 48 hours, then kept at 4°C for daily use.

Lactobacillus plantarum 299v used to compare the stress after spray dryer, was cultured at 37°C and 5 percent CO_2 for 48h on MRS agar and overnight in MRS broth, too.

3.1.3. The Spray Drying Process

During the drying process, as an output temperature, the goal was to achieve a 60°C to 65°C output temperature, because bacterial viability decreases above 70°C, and to not exceed 3.5-4 percent of humidity value via testing different inlet temperatures, flow, and aspiration rates.

WPI solution was prepared by rehydration of whey protein isolate at 15 percent (w/v) in 100 ml of DSW. Rehydration was carried at as described by Guerin *et al.* [47]. The solution was briefly stirred at 750 rpm for two hours at room temperature 25°C, then overnight at 4°C. After rehydration, WPI solutions were denatured by heating at 78°C for 10 min and cooled at 4°C. Maltodextrin and trehalose solutions were prepared at 15 percent (w/v) in 100ml of DSW before being autoclaved at 121°C for 15 min. The feeding solution was prepared by mixing the wall material solution 100ml with an overnight culture (beginning of the stationary phase) of LAB strains to a initial concentration of 10^{11}CFU/ml.

The studies were continued with different materials until the target viability of 6-7 log CFU/gr was reached. The encapsulated product's viability rate was analyzed immediately after production and incubated in incubators at 4°C / 24°C temperature for 30/45 days.

3.1.4. Viability of Cells

After the spray drying process, to determine the probiotic bacteria's survival rate via direct culture method, 1 g spray-dried powders were rehydrated with 9 mL DSW sterile solution. Afterward, they were serially diluted and spread plated in triplicate on MRS agar. Plates were incubated at 37°C for 48-72h then examined for colony number recording.

A Gram+ coloration was prepared for each sample before and after the spray drying process to observe the bacterial dispersion's encapsulation impact.

One colony of *L.rhamnosus* was seeded from an MRS agar 48h culture to a MRS broth. Then decimal serial dilutions were spread plated on MRS agar to determine the seeded colonies (T0). After that, spread plating of decimal serial dilutions was carried out every hour to follow the CFU evolution incubation time via direct culture method.

The viability of bacteria is always evaluated by the direct culture method on agar media, also called colony plate counting [47, 48]. Although culture-depending methods are always used, they are still far from giving reliable information about cells' viability since it enables them to detect viability but non-cultivable (VBNC) bacteria. The VBNC state is induced naturally in cells by environmental stresses such as high temperature and humidity like in the spray-drying process [49]. Under this state, indeed, bacteria may not grow on the routine bacteriological media they used to develop colonies, but they are still alive and able to show metabolic activity [49]. The use of bacterial physiological indicators state, should be more sensitive to estimate bacterial survival. The starter cultures' viability is traditionally quantified by the colony plate counting method, which evaluates cells' ability to grow in an appropriate medium. Hence, the population of cultivable bacteria is determined. However, under stressed conditions, different industrial processes are used to produce dried biomass, many species of bacteria enter into a physiologically VBNC state [49, 50]. Although they are alive in the VBNC state, bacteria cannot develop colonies on the routine bacteriological media they would generally grow [49]. Therefore, cell viability evaluation based on forming colonies may lead to underestimating viable cells and microencapsulation efficiency. The aim was to detect the viable but non-culturable state within encapsulated bacteria under different spray-drying conditions and during two months of storage at room temperature.

Additionally, culture-dependent methods lack the resolving power to provide real-time results, and bacteria may occur in chains and clumps, resulting in underestimating true bacterial counts [51, 52].Thus, more sensitive and rapid methods have been developed to monitor probiotic survival, such as quantitative real-time PCR [53] fluorescent in situ hybridization (FISH), and flow cytometry [54, 55]. However, these methods' industrial adoption failed due to their lack of cost-efficiency and scope for the extension of product applications [55]. In this context, an easy colorimetric non-culture method, based on the bacterial ability to reduce MTS was optimized to assess the VBNC state's level within the encapsulated LAB under different conditions to evaluate the microencapsulation process's efficiency. Encapsulation by the spray drying method was performed on mesophilic lactic acid bacteria (LAB) reference strains: *L.rhamnosus GG* and *L. plantarum 299v*.

The acid produced by LAB inhibits tetrazolium salt reduction. Uses of tetrazolium salts have been reported as producing lactic acid bacteria (LAB). In this respect, the ability of *L.rhamnosus GG* and *L. plantarum 299v* to reduce MTS was firstly verified. Among various

methods using enzymatic activity to monitor microbial population density, assays based on bio-transformations of tetrazolium salts (TS) have gained much popularity.

The MTS assay protocol is based on reducing the MTS tetrazolium compound by viable mammalian cells (and cells from other species) to generate a colored formazan dye soluble in cell culture media. It is carried out with NAD(P)H-dependent dehydrogenase enzymes in metabolically active cells. The formazan dye is quantified by measuring the absorbance at 490-500 nm. The ability of bacteria to reduce tetrazolium salts depends on the salt type and the bacterial species. For example, Gram-positive bacteria form water-soluble formazan better than Gram-negative bacteria. Therefore, it reduces tetrazolium salts better than gram-negative bacteria [56]. Thus, the TS assays must be conducted under correctly optimized conditions for a reliable absorbance value that is directly proportional to the number of viable cells [57]. The reduced tetrazolium salt's color intensity, formed by reducing activity of bacteria, was proportional to the number of actively growing bacteria in the culture. Therefore, the correlation between the MTS absorbance unit at OD490 to logarithmic colony-forming units on MRS agar media was performed in initial experiments. No coloration was observed with dead bacteria in negative controls, which means that MTS reduction is related to viable bacteria only.

To this intent, the trials were made to optimize an MTS colorimetric based assay to assess the viability of two *Lactobacillus* strains (*L.rhamnosus GG* and *L. plantarum 299v*) in the different matrix after spray drying. The VBNC state of encapsulated bacteria will be estimated through the difference of bacteria enumeration results between the colony plate counting method and the new optimized assay. The sensitivity of both methods in evaluating bacteria losses and the microencapsulation efficiency under different conditions were also be compared.

3.1.5. Morphology of the Particles

The encapsulated particle’s morphology issued from the spray drying system was observed with scanning electron microscopy. Carbon double-sided adhesive stubs of the SEM device was used to analyze via SEM.[52] The samples were coated on the SEM stubs by sputtering method. The coating was done with iridium (Q150T Turbo-Pumped Sputter Coater,

ProSciTech Pty Ltd, Queensland, Australia) for 2 min on three sides (around 10 nm thickness per side).

3.1.6. Spectrometric Analysis of the Particles

Fourier Transform Infrared (FTIR) spectrometer is a tool used to determine the functional groups and chemical bond structures in a material. The resulting spectrum shows each material's absorption and molecular transmission characteristics because each functional group has a different vibrational energy. The infrared radiation area is generally divided into near IR regions (14290-4000 cm-1), far IR (700-200 cm-1), and middle-IR (4000-666 cm-1). The most used areas for limited investigation are in the middle-IR region [58].

FT-IR was used to investigate the potential interaction between wall materials (WPI and Trehalose) and *Lactobacillus rhamnosus*.

The primary purpose is to understand whether there is any deterioration in physicochemical properties and to directly determine its relevance to bacterial viability during storage. Suppose, any relationship between changes in the absorption spectrum and bacterial viability can be detected. In that case, FT-IR spectroscopy can be announced for the first time as a useful, non-destructive, and rapid tool for monitoring the probiotic life shell in complex matrices.

3.1.7. Glass Transition Temperature of the Particules

Determining the glass transition temperature is also important, as it is essential for the stability of the encapsulated materials during storage; The glass transition temperature (Tg) of the encapsulated product used in the production of edible films was measured using differential scanning calorimetry, (DSC) [59]. The encapsulated product exhibits a long shelf life when stored below the glass transition temperature since deterioration due to bacterial proliferation and chemical reactions are minimal. The low permeability of the carrier material at temperatures below the glass transition temperature helps prevent oxygen and preserve core materials.

3.1.8. Humidity, Product Yield and Viscosity Measurement

Product yield is obtained by calculating the amount of solid capsule material obtained during spray drying to the total amount of solid component delivered to the spray dryer chamber as a percentage.

The collected encapsulated powder and initial ingredients were weighed with sensitive scales and humidity at 105°C and 130°C were measured with a humidity analyzer.

The prepared solutions' viscosities were measured via the viscosimeter at 100 rpm to prevent the spray dryer nozzle from clogging.

3.1.9. Statistical Analysis

All the experiments were conducted in triplicate (n = 3), and the results were expressed as mean ± standard deviation. Mean and standard deviation values were calculated from data obtained with triplicate trials. Data analysis was carried out two-way analysis of variance using Matlab (R2021b, Mathworks, USA). Matlab code can be found in Appendix.

3.2.RESULTS AND DISCUSSION

During the encapsulation process, different inlet temperatures and flow rates were tested, and the survival percentages of the probiotics and moisture content of the encapsulated product were determined. The most suitable wall material's amounts, inlet temperature, and flow rate for the selected probiotics' encapsulation were determined with the optimization study to be performed.

The target values were approached by coating material/materials individually; then, the experiments were continued. A suitable coating material was mixed with the core material, then the drying and encapsulation process was done via a spray dryer device. The aim was to ensure the probiotics' formation with high viability in capsules produced by the encapsulation method.

The results were used the output temperature, the number of viable bacteria in grams, and the humidity. With this development study, formulation and process parameters were determined with the highest number of viable bacteria (minimum 10^6 – 10^7 CFU/gr product) and lowest humidity (maximum 4 percent) per mass of the product. Thus, the initial core material and coating material mixture at different ratios (15 percent single or mixed) were prepared according to viability result.

The encapsulated product's viability rate was analyzed immediately after production and incubated in incubators at 4°C / 24°C for 30/45 days.

Trehalose was incorporated to reduce the damage to probiotics due to the high temperature during drying. Trehalose was prepared as the coating material then before and after the addition of trehalose, differences in output temperature, humidity, and viability rates were measured.

3.2.1. Spray Drying Process

The most appropriate coating material selection was targeted with the work to be carried out. The goal was to choose a material with a moisture level below 3.5-4 percent and with the highest recovery and liveliness.

Firstly, the coating materials were dried individually and 10 different trials were conducted via the spray drying process. Initial conditions were determined to achieve the targeted conditions, final temperature 68-70°C, moisture 3-4 percent: Inlet T 105°C, Pump 3ml/min, Aspirator 80 percent and Compressor 11L/min. It has been observed that when the inlet temperature is increased, the outlet temperature also increases. An outlet temperature higher than 70°C causes a decrease in the viability of the probiotic[24] . In addition, it was observed that the product yield decreased when the aspiration rate was reduced.

Although the targeted moisture content of less than 4 percent was achieved with all prepared coating materials, it was observed that the highest recovery belonged to WPI (Figure 3.1 (Figure 3.2)

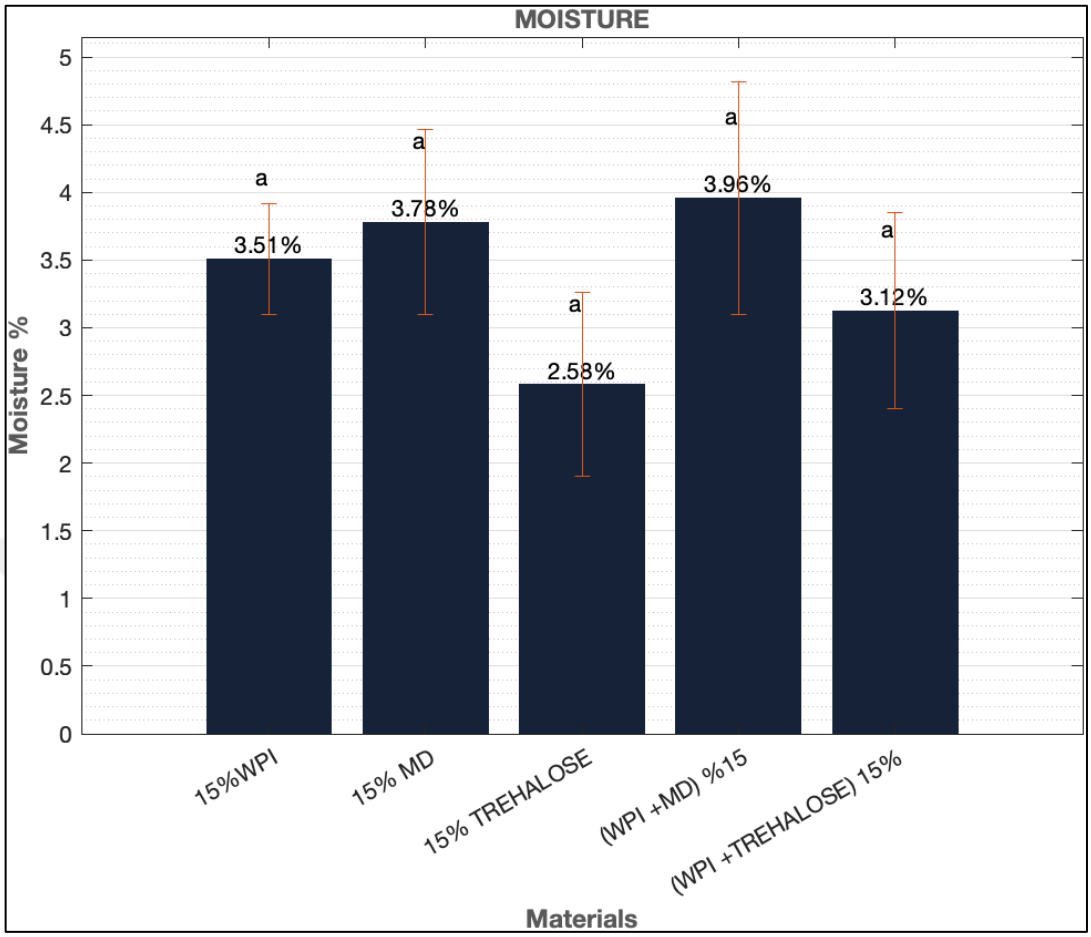


Figure 3.1. Variation of sample moisture the (spray drying conditions were not changed but starting materials were changed)

The mean values with the same letter are not significantly different ($p>0.05$)

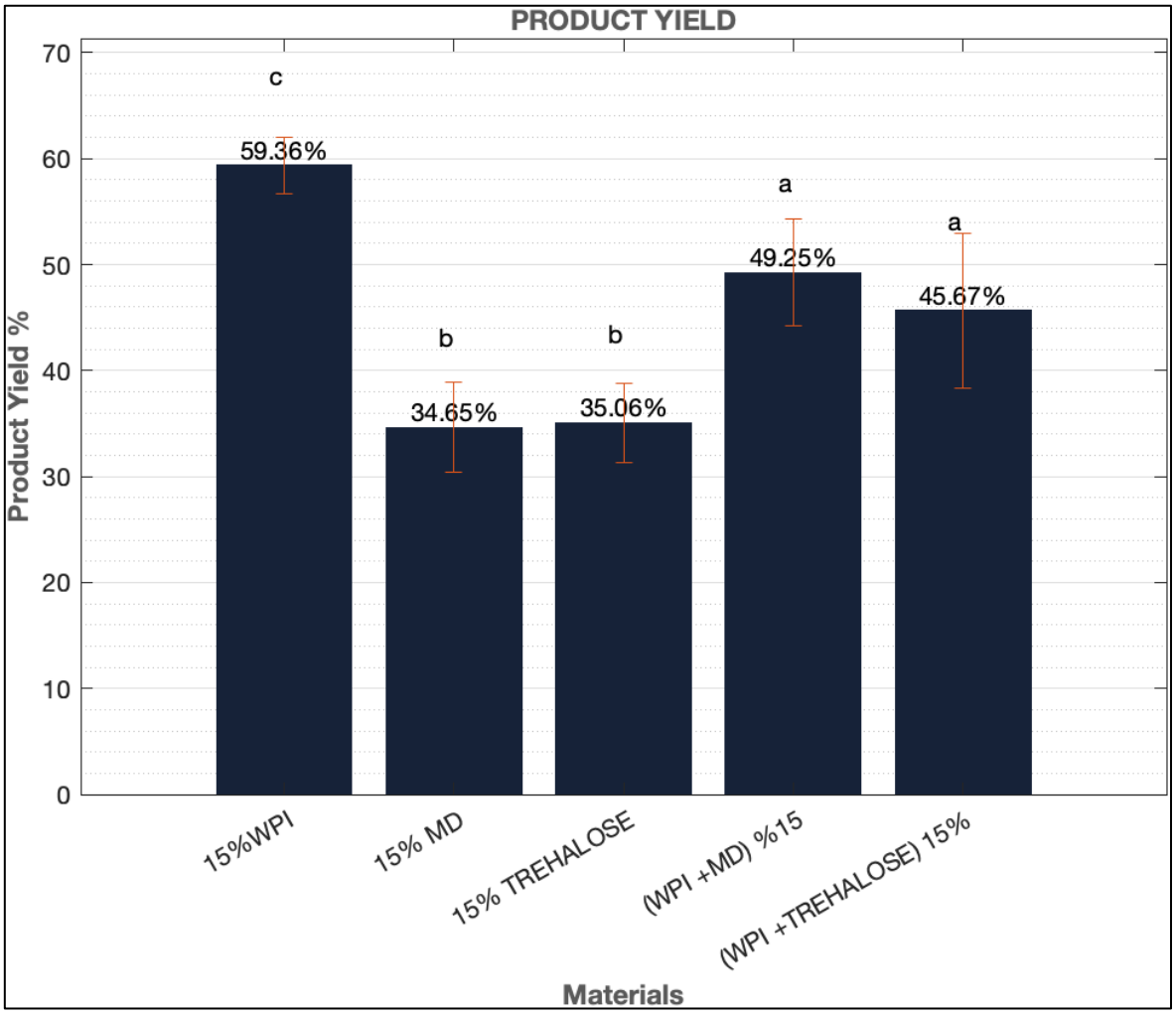


Figure 3.2. Variation of product yield (spray drying conditions are not changed but starting materials are changed)

The mean values with the same letter are not significantly different ($p>0.05$)

3.2.2. Viability of Cells After Spray Drying

Although the probiotic coated in the spray dryer is alive, it cannot show any signs of viability because it is under stress [25]. While the amount of viability of the stressed probiotic could not be determined by the direct culture method, it could be analyzed by the MTS method. The viability measurements of *L.rhamnosus GG* encapsulated in spray drying with different coating materials were measured by MTS and direct culture method (Figure 3.3).

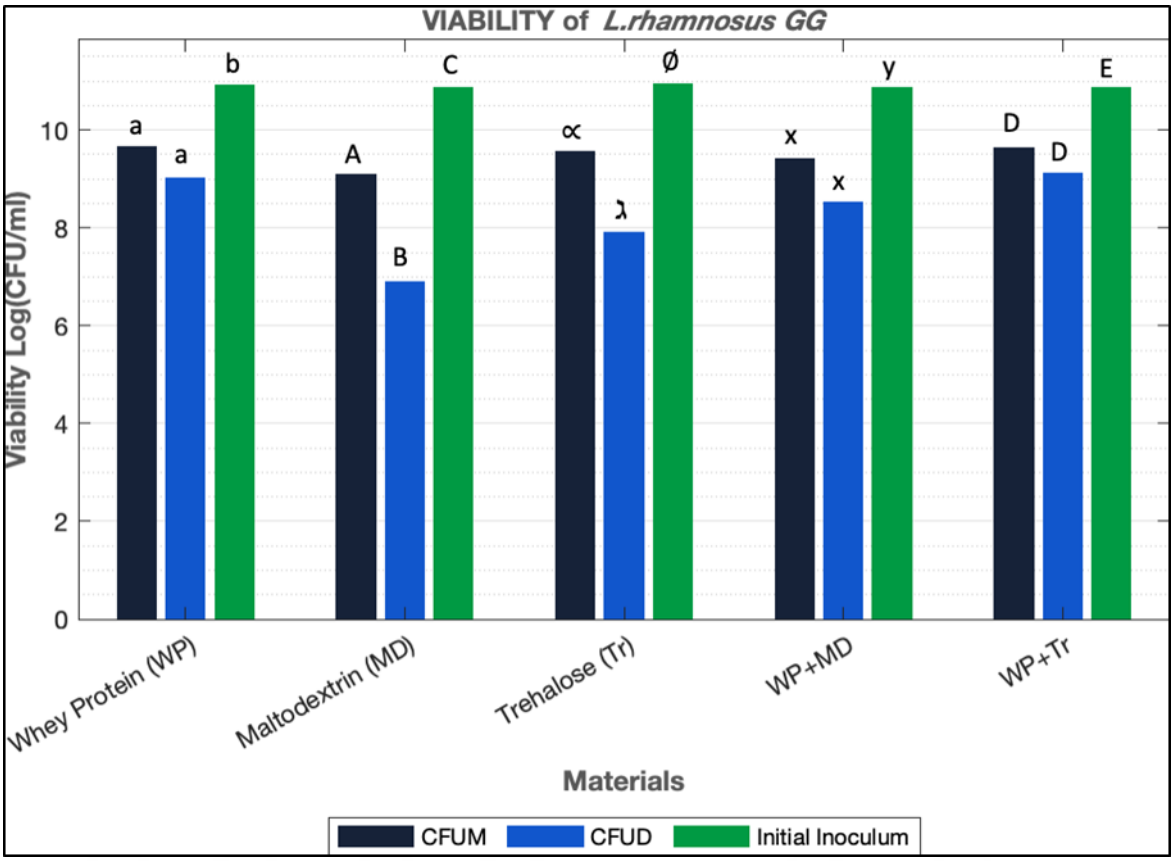


Figure 3.3. Assessing of *L.rhamnosus GG* viability and cultivability just after spray drying process with different coating materials: WP- whey protein, MD- maltodextrin, Tr- trehalose. CFUM = MTS method CFUD= Direct culture method

Statistical analysis was performed within each materials ($p < 0.01$) .

The difference between the two methods in the evaluation of bacterial viability is statistically significant. Since the direct culture method can detect viable but non-culturable (VBNC) bacteria, as in the spray drying process, MTS appears to be a more reliable choice for assessing bacterial viability. For a long time, the use of TS reduction was limited to eukaryotic cell research before it was widely applied for viability predictions of microbial cells. However, the bacterium's mechanism of reducing TS is still poorly understood [60], leading to risks of misinterpretation. Therefore, TS assays should be performed under suitably optimized conditions for an accurate absorbance value in direct proportion to the number of viable cells [57].

In the viability analyzes using these two methods, it was observed that the coating materials with the least viability differences were 15 percent WPI (w/v) and 15 percent (w/v) (WPI + trehalose). Trehalose seems to be suitable for heat protection but not as much as does whey protein. After spray drying, it was observed that the addition of trehalose did not increase the viability of *L.rhamnosus GG*' in whey protein-trehalose' and maltodextrin-trehalose mixtures 15 percent(w/v) prepared. However, it could enhance its viability during storage, as reported in many previous works [36]. Therefore, changes in viability during storage were observed. The percentage of VBNC state of spray-dried LAB strains were differentiated at storage (Figure 3.4) (Figure 3.5) (Figure 3.6) (Figure 3.7).

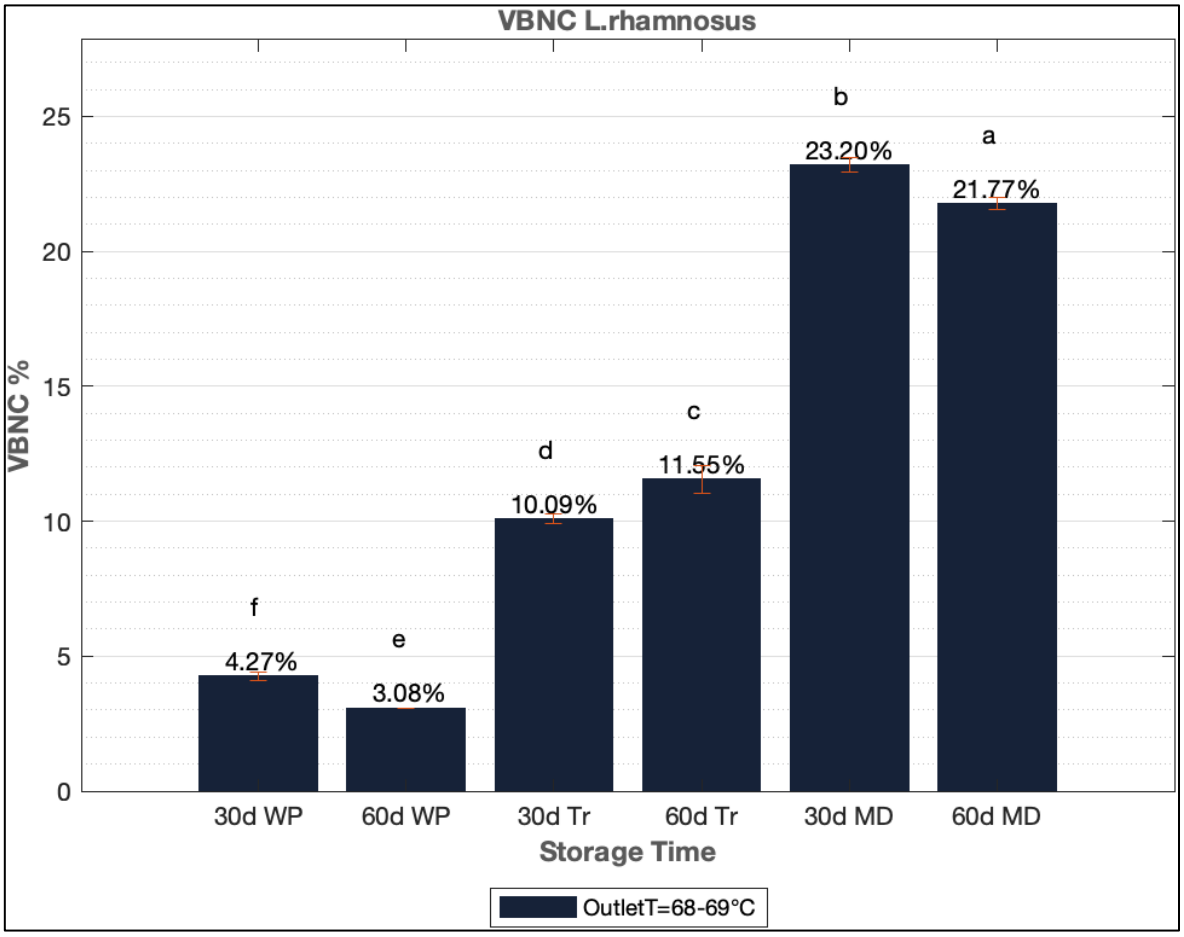


Figure 3.4. The evolution of VBNC state percentages of encapsulated *L.rhamnosus* strains according to spray drying conditions during storage (30 days and 60 days) at room temperature. The mean values with the same letter are not significantly different (p>0.01)

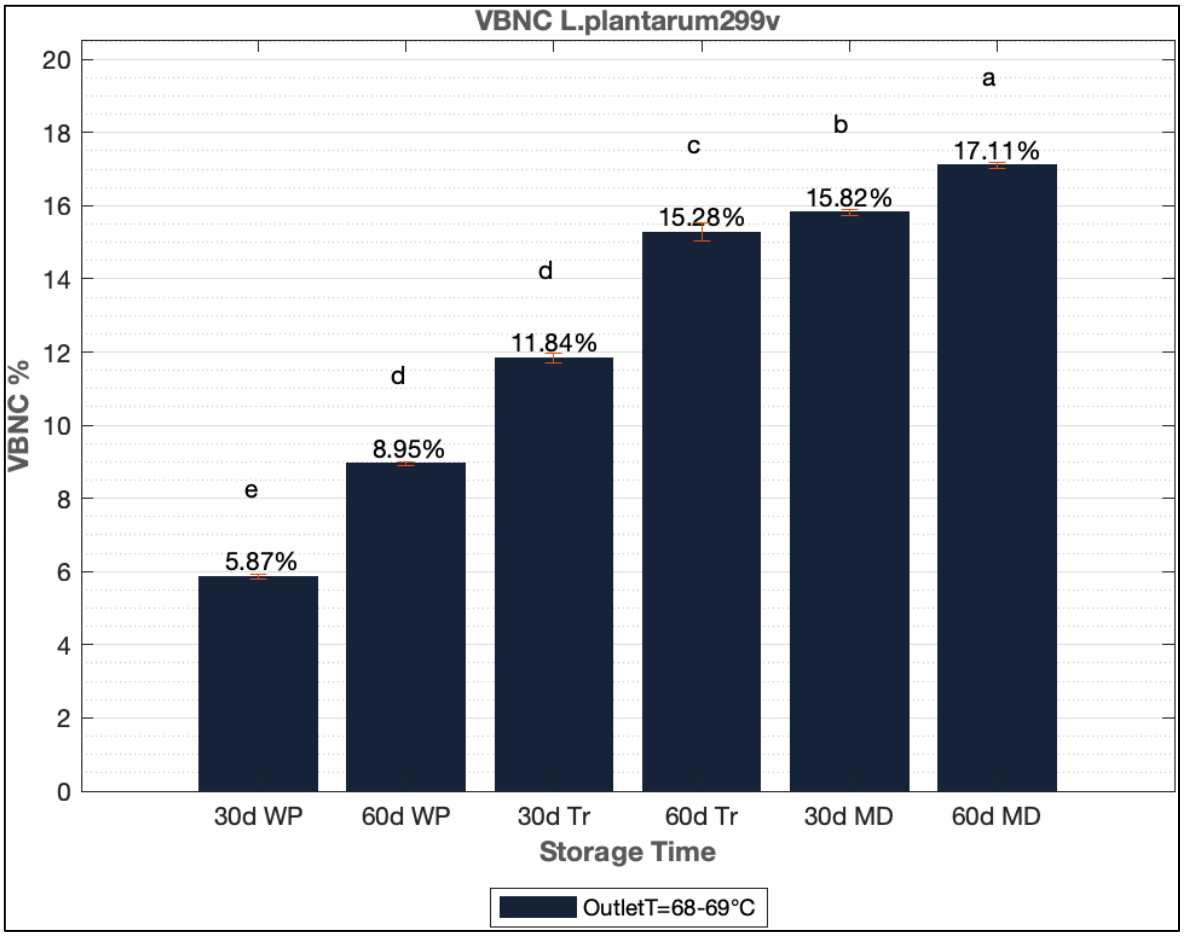


Figure 3.5. The evolution of VBNC state percentages of encapsulated *L.plantarum* strains according to spray drying conditions during storage (30 days and 60 days) at room temperature. The mean values with the same letter are not significantly different ($p>0.01$)

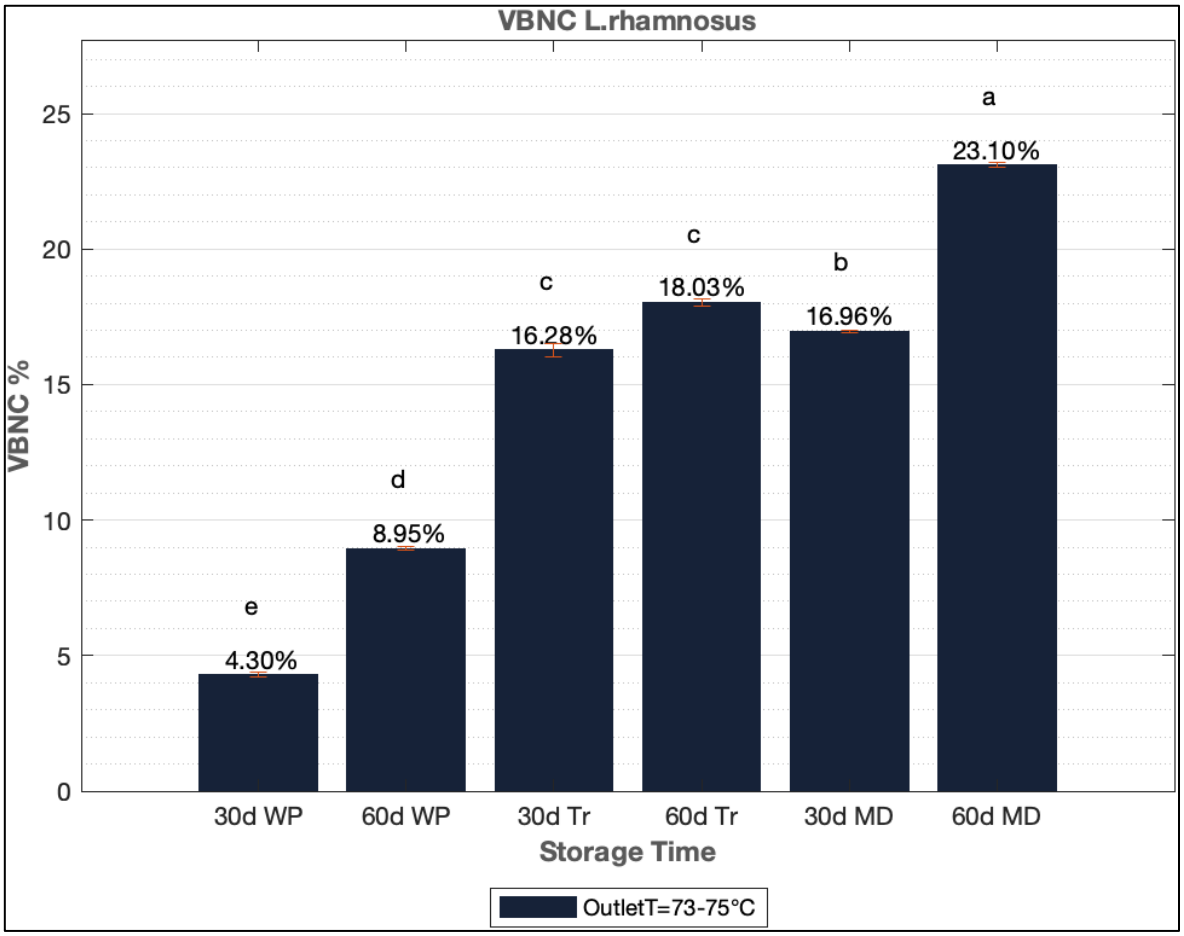


Figure 3.6. The evolution of VBNC state percentages of encapsulated *L.rhamnosus* strains according to spray drying conditions during storage (30 days and 60 days) at room temperature.

The mean values with the same letter are not significantly different ($p>0.01$)

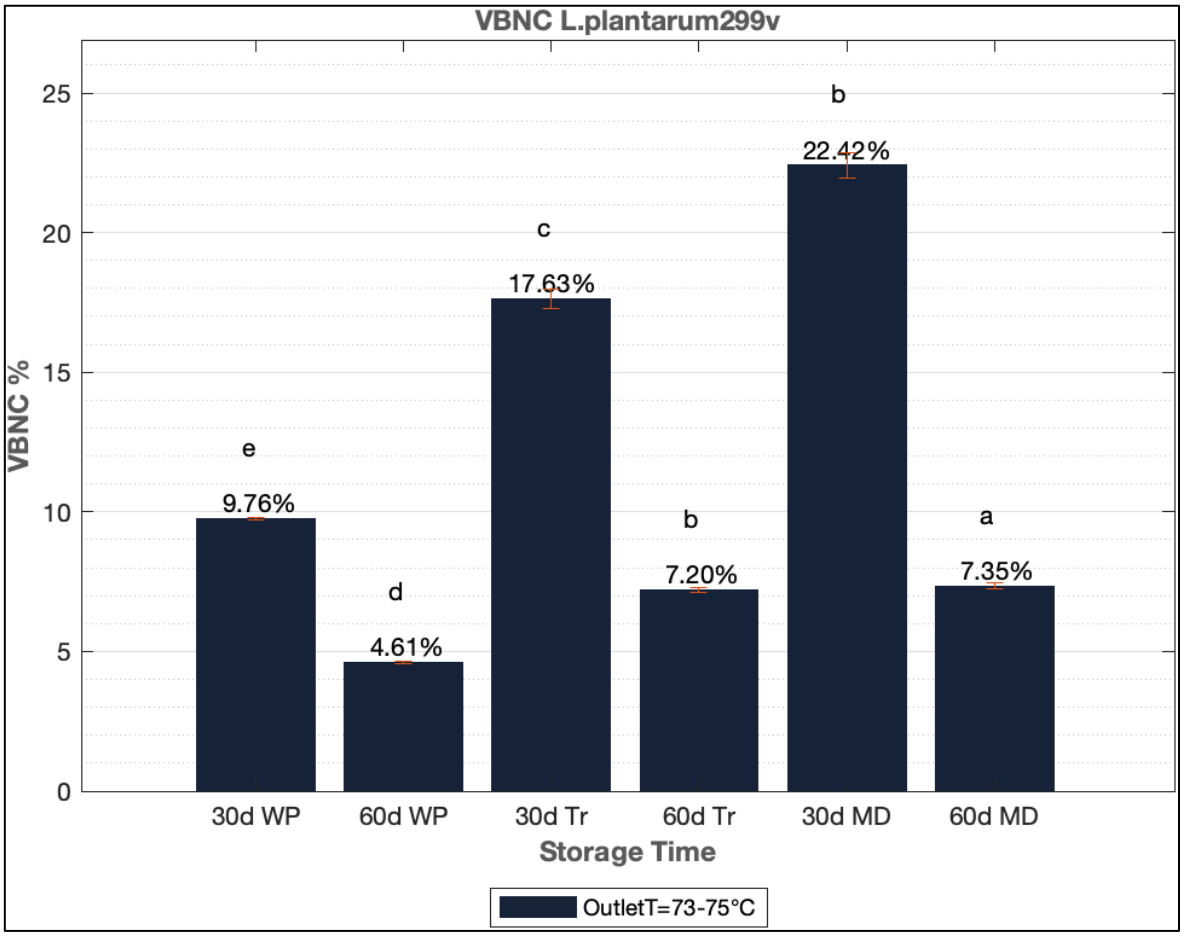


Figure 3.7. The evolution of VBNC state percentages of encapsulated *L.plantarum* strains according to spray drying conditions during storage (30 days and 60 days) at room temperature. WP- whey protein isolate, MD- Maltodextrin and Tr- Trehalose. Outlet temperature = 68-69°C and 73-75°C.

The mean values with the same letter are not significantly different (p>0.01)

The two LAB strains, *L.rhamnosus GG* and *L. plantarum 299v,* showed different evolution of their VBNC rate during the storage. While the VBNC state of encapsulated *L.rhamnosus GG* increased with time, under outlet T(73-75°C), encapsulated *L. plantarum 299v* showed a decreasing rate of VBNC state. The microencapsulation process was more responsible for the VBNC state inducing LAB strains than the starvation during storage. Although optimal conditions required to protect probiotics during spray drying and storage were well documented, it was still uncertain that cell damage can effectively predict survival to storage [61, 62].

However, the VBNC state of encapsulated LAB strains was significantly more stable ($p<0.01$), during the storage, under outlet T(68-69°C) for both strains encapsulated with different wall materials. Under higher outlet T(73-75°C), the VBNC state was more stable within bacteria encapsulated with only WP and Tr. The two LAB strains exhibited the lowest VBNC state in WP powders during storage for both outlet T's (less than 10 percent).

The cultivable encapsulated LAB strains significantly decreased after thirty days of storage regardless of the used wall material and outlet T. Bacteria encapsulated with MD and Tr collapsed drastically after thirty days of storage. In whey powders, LAB strains showed the highest cultivable percentage. Sugars and proteins containing formulations were reported to improve storage stability. Among the two used sugar powders, Tr was better than MD in maintaining bacterial viability during storage that served as a storage factor during carbon starvation in *Saccharomyces cerevisiae* [38].

The cultivability evolution of encapsulated LAB strains was not significant between thirty days and sixty days of storage except for *L.plantarum* strain in WP powders produced at outlet T=73-75°C. The difference of cultivable bacteria amount evolution between the two LAB species during the storage in different powders shows that this response is species-specific, as reported in many previous works [63, 64]. The impact of higher outlet T on the evolution of bacterial cultivability during storage was not always significant. The physicochemical properties of powders were measured under different drying temperatures. They were not enough to differentially affect storage stability. It was reported that at inlet and outlet temperatures above 100°C and 60°C respectively, the dry powder's moisture content was below 4 percent required for safe storage [62].

As a result, it was observed that the viability rate of the product encapsulated using WPI had the highest viability after 30 and 60 days of storage, as well as the viability determined after the spray dryer. In addition, when the viability values of *L.rhamnosus GG* during storage were measured by MTS and direct culture method, it was determined that the results were close.

Since WPI covers the probiotic surface better than others and provides a higher protective effect, it has been observed that it contributes to preventing the bacteria from being stressed during encapsulation[24, 25]. In addition, the test results of the maltodextrin coating material showed that; Maltodextrin, which is the best capsule material for many different core

materials, could not preserve the viability of *L.rhamnasus GG*, which was stressed after spray dryer[24, 25].Although there was a decrease in the viability of *L.rhamnosus GG*, which was coated with trehalose alone, the encapsule product with the lowest moisture content was obtained. When WPI and trehalose were used together, it was seen that the decrease in initial viability was less and also contributed to the moisture reduction.

According to the results of these experiments, it was seen that increasing the WPI concentration and using trehalose prepared in fewer proportions will provide low humidity, high product yield, and high viability. According to the articles, if 30 percent WPI were used, the efficiency will be increased, so the first studies were done by preparing 30 percent WPI [27, 34, 35, 65].

As the spray dryer's manuel, the nozzle of the spray dryer was clogged when the solution has high viscosity was used. For this reason, viscosity was measured by preparing different concentrations of WPI. The viscosities of 15 percent WPI and 20 percent WPI were compared. It was seen that the effect of increasing the concentration on viscosity increase was approximately five times. (Figure 3.8)

The high viscosity increase was measured by adding 1 percent instead of increasing the concentration by 5 percent. The viscosity of 16 percent WPI was measured. Even the viscosity of 16 percent WPI was almost four times greater than the 15 percent WPI. Since WPI is a useful coating material, but increasing the concentration causes viscosity problems, viscosity change was observed by adding trehalose to 15 percent WPI instead of increasing WPI by more than 15 percent. (Figure 3.8).

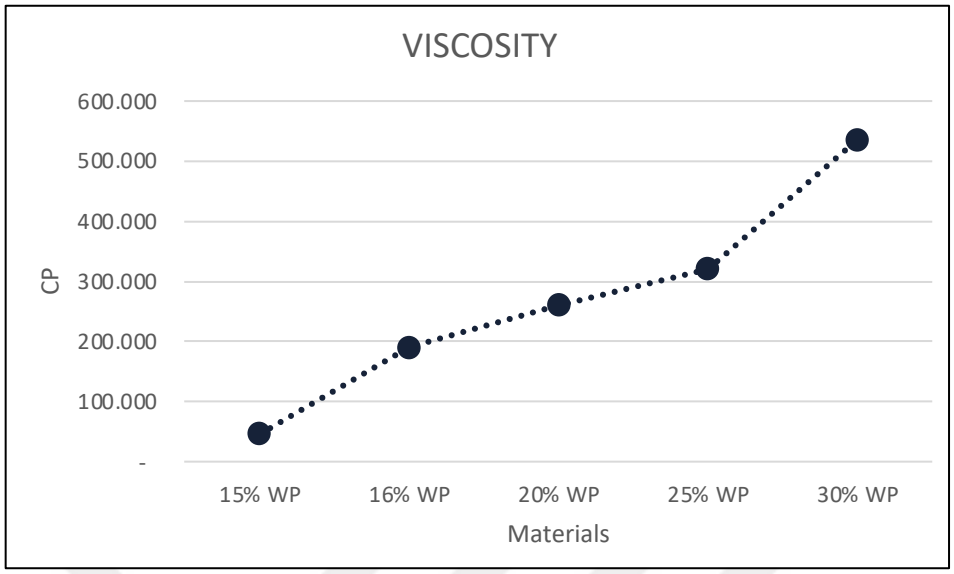


Figure 3.8. Comparison of viscosity of WPI mixture prepared 15-16-20-25-30 %

In the viscosity measurements made, it was observed that as the trehalose was added, the viscosity of the WP-trehalose mixture decreased (Figure 3.9). Trehalose was added to eliminate the high viscosity's risk, so it was decided to analyze the effects of trehalose on viability.

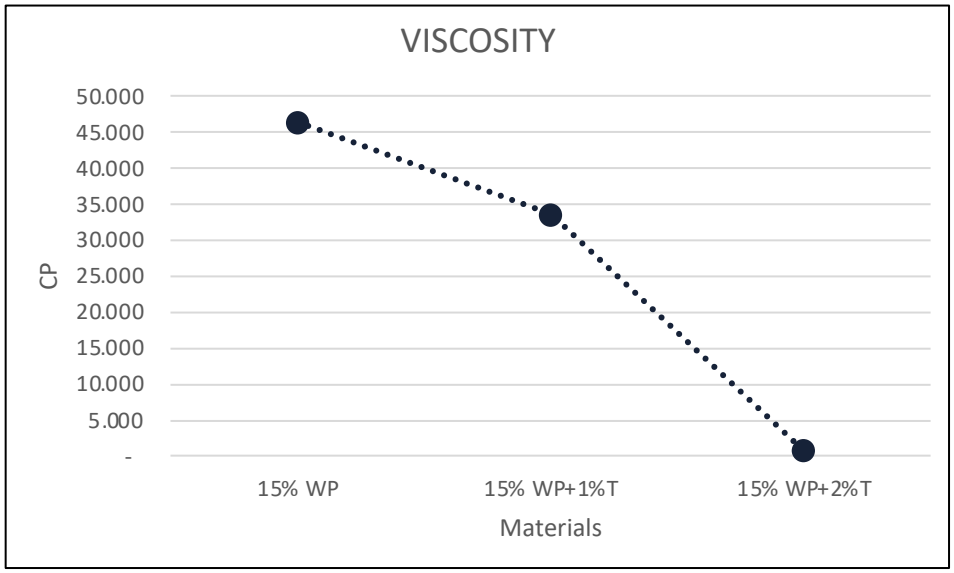


Figure 3.9. Viscosity with different coating material used with *L. rhamnosus* encapsulation (15% WPI/ 15%WPI + 1%T / 15%WPI + 2%T)

In literature studies, trehalose has been shown to increase the viability of bacteria. Therefore, the sample moisture, product yield and viability of *L.rhamnosus* in capsules prepared with WPI and trehalose mixtures, prepared in different proportions, were analyzed by working under the same working conditions as below (Figure 3.10) (Figure 3.11) (Figure 3.12).

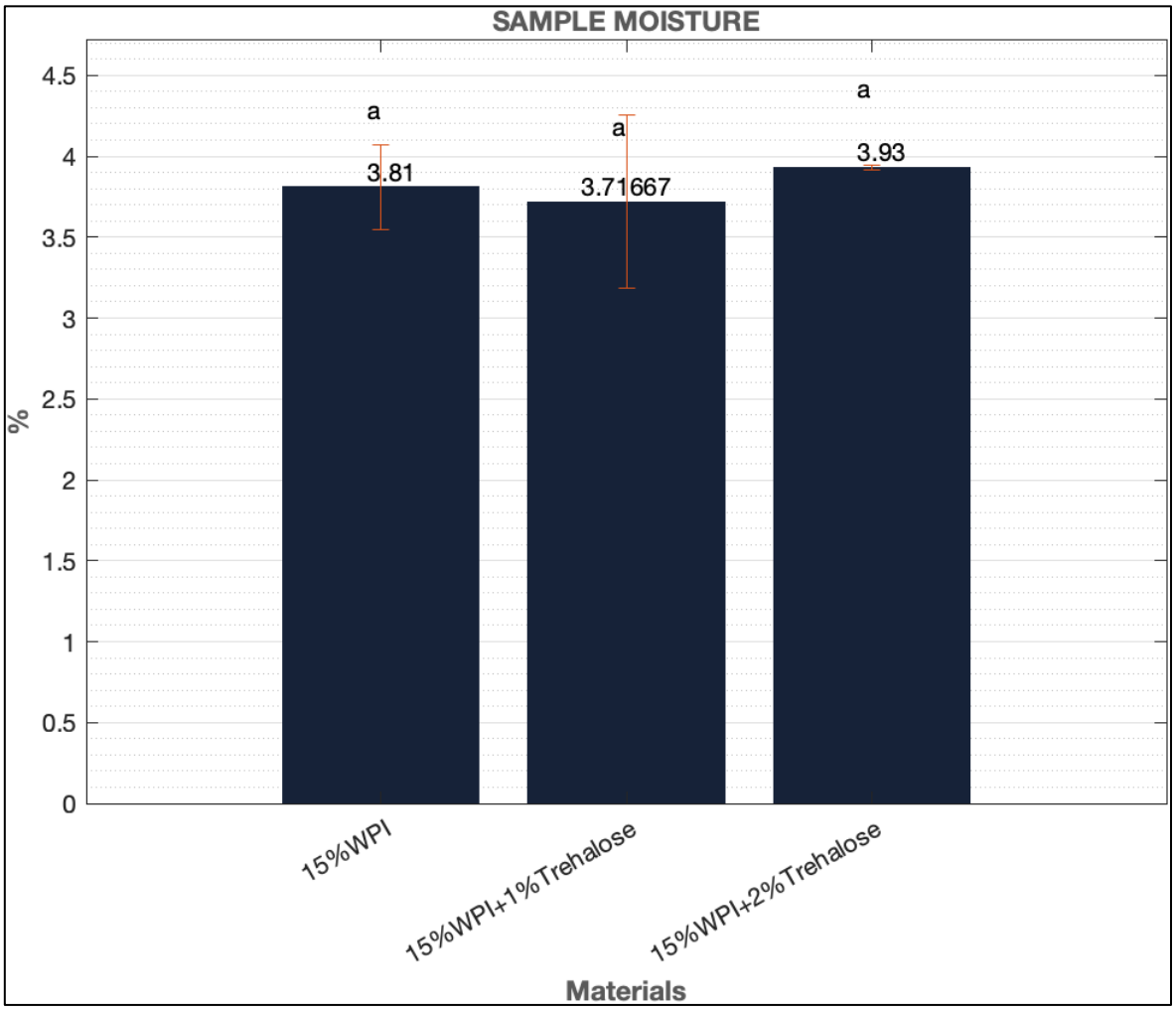


Figure 3.10. The moisture of *L.rhamnosus* in capsules prepared with WPI and trehalose mixtures, which will be prepared in different proportions (Working Conditions are Inlet temp. 105 C, outlet temp 68-70 C, pump 3 ml/min, aspirator 80%, compressor 11 lt/min, initial viability 10^{11} CFU/ml.).

The mean values with the same letter are not significantly different ($p>0.05$)

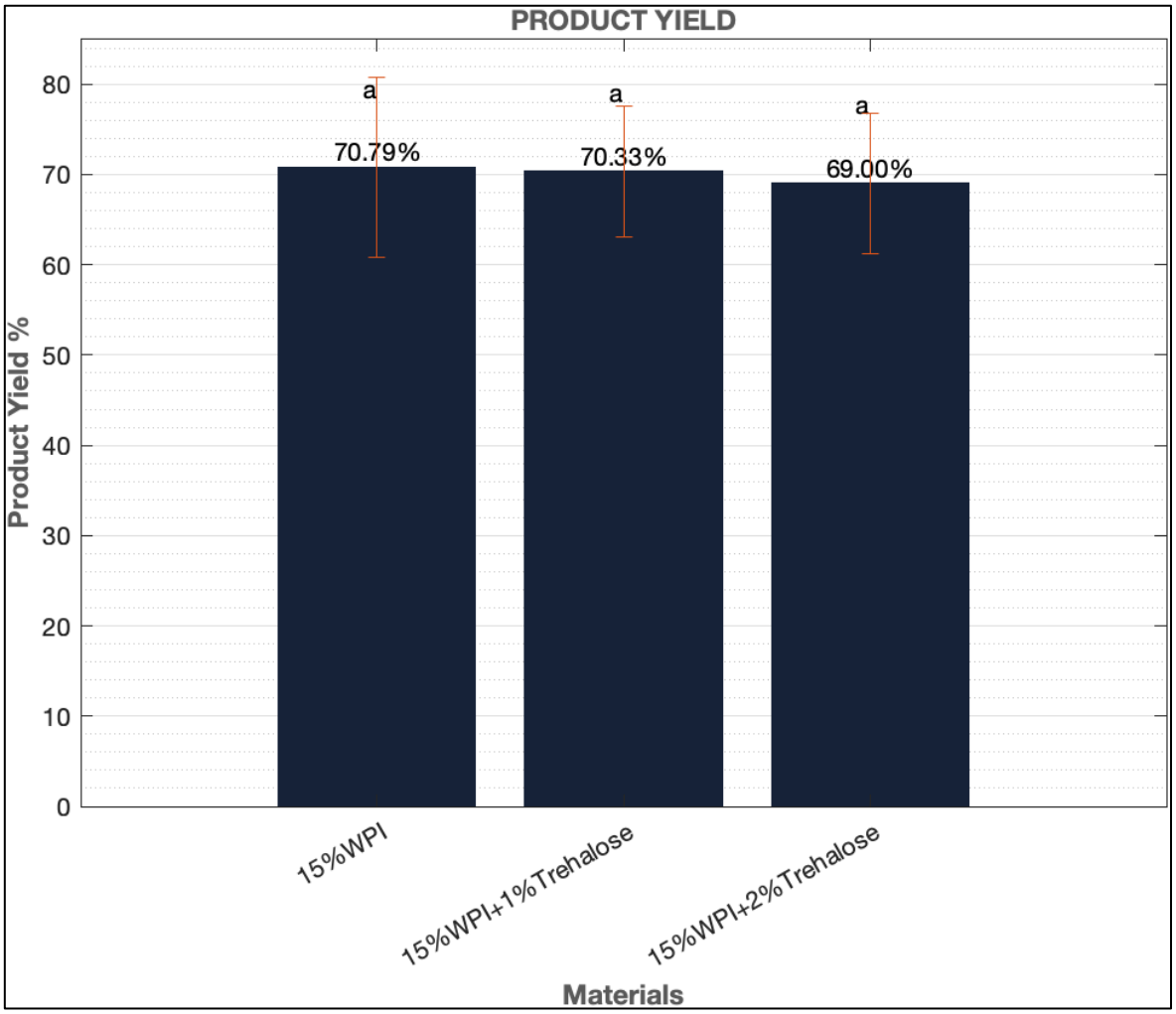


Figure 3.11. The product yield of *L.rhamnosus* in capsules prepared with WPI and trehalose mixtures, which will be prepared in different proportion (Working conditions are Inlet temp. 105 C, outlet temp 68-70 C, pump 3 ml/min, aspirator 80%,compressor 11 lt/min, initial viability 10^{11} CFU/ml.

The mean values with the same letter are not significantly different ($p>0.05$)

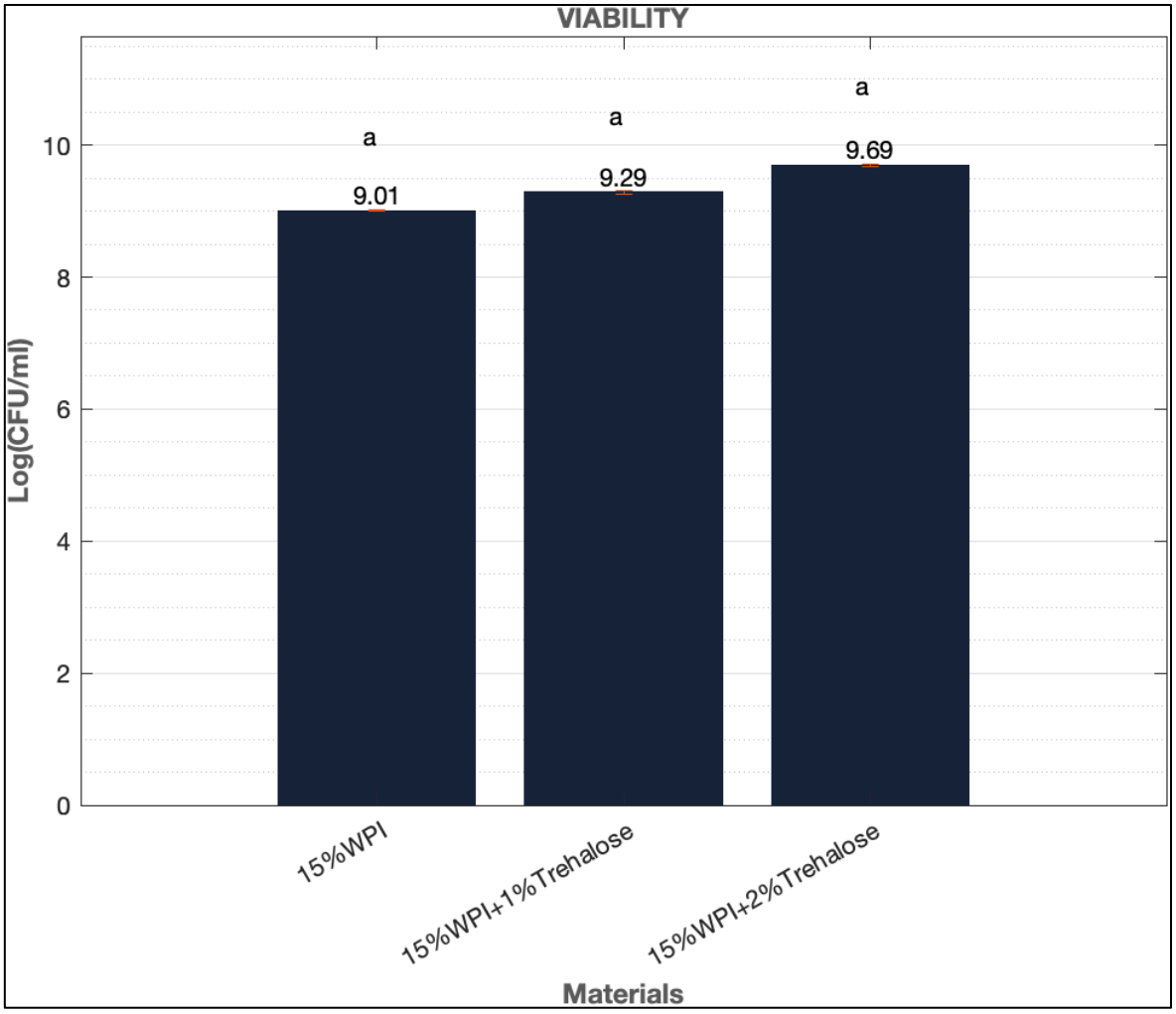


Figure 3.12. The viability of *L.rhamnosus* in capsules prepared with WPI and trehalose mixtures, which will be prepared in different proportions.

The mean values with the same letter are not significantly different (p>0.01)

In the mixtures added with trehalose, the edible film's moisture , product yield and viability didnt changed. When all the results obtained were evaluated; there isn't advantage to using trehalose. A decision was taken considering that as the cost of trehalose is high, the use of trehalose causes no significant logarithmic increase in the number of viability in the studies, the studies were continued with the encapsulated product prepared with only 15 percent WPI as the coating material.

The selection of the coating materials was summarized with (Figure 3.13).

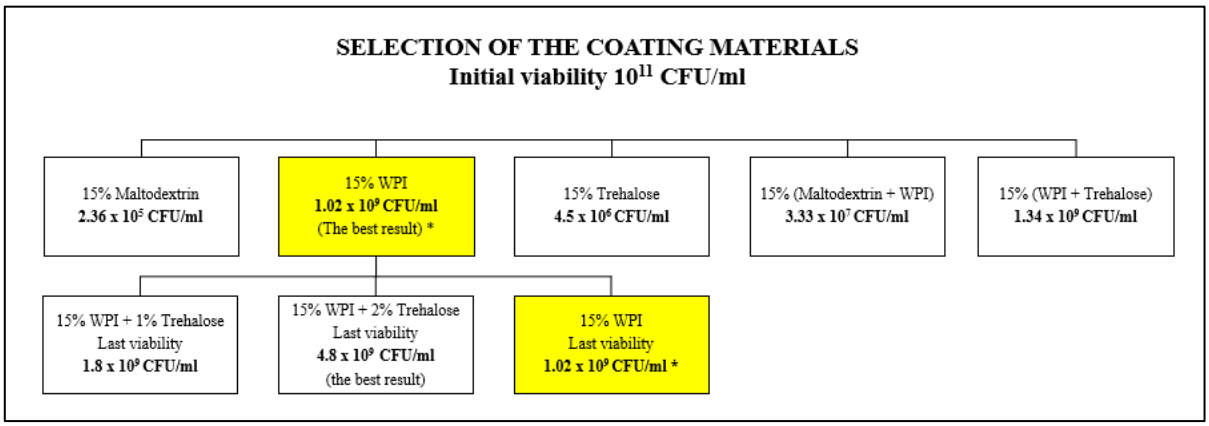


Figure 3.13. Selection of the coating materials

SEM images of raw WPI(a), spray-dried WPI(b), probiotic products encapsulated with WPI(c-d) were taken. Capsule formation was observed when compared to the SEM image of the raw WPI(a). (Figure 3.14). Particle morphology showed smooth spherical powders.

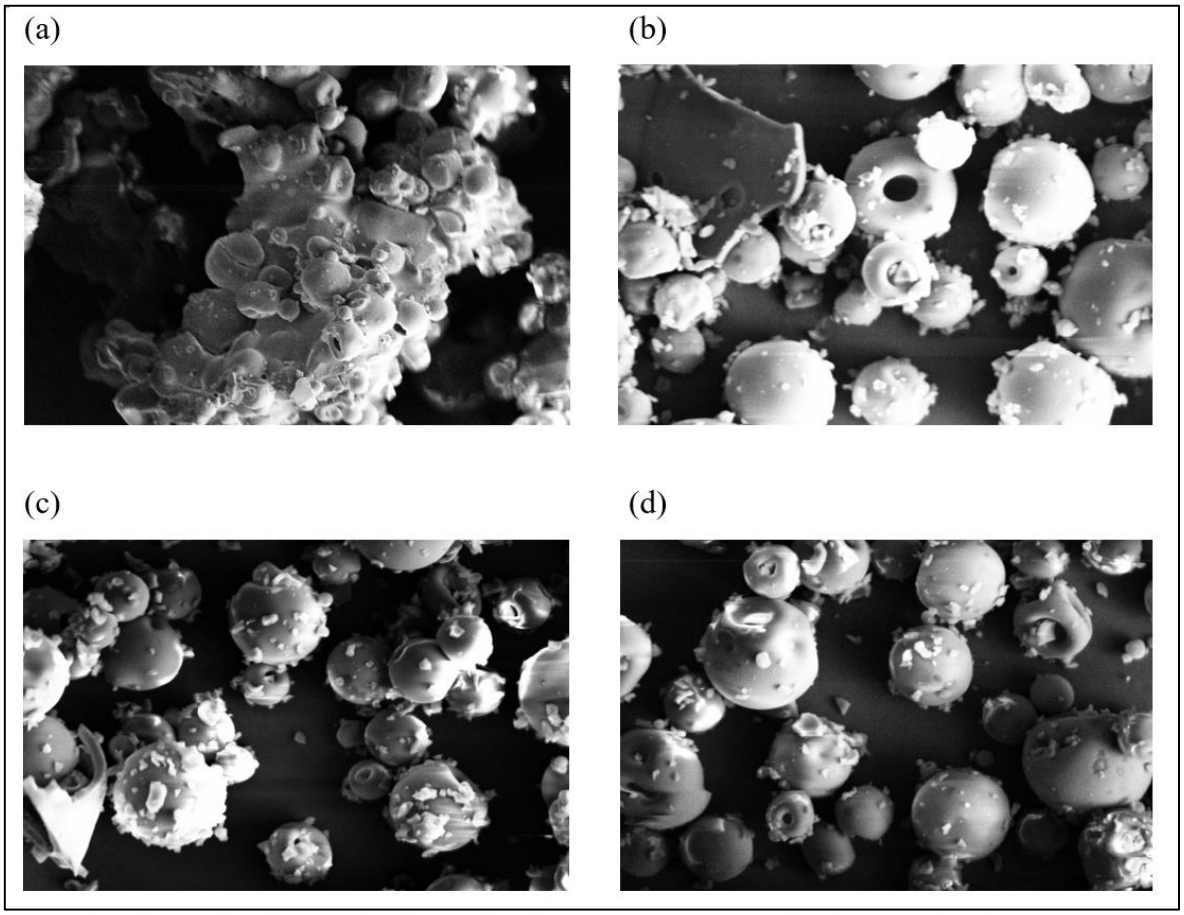


Figure 3.14. Scanning electron microscopy of whey protein isolate microparticles (x3000 magnification). Bar scale = 20 μm. Different outlet air temperatures and compressor values were used during spray-drying. Final products were observed via SEM(Scanning electron microscopy): (a)- Raw whey protein. (b)- Whey protein spray-dried at 58-59°C and 11L/min. (c)- Whey protein with *L.rhamnosus GG* spray-dried at 70-71°C and 12L/min. (d)- Whey protein with *L.rhamnosus GG* spray-dried at 66-70°C and 10L/min.

No bacteria was observed on the microparticles' surface, even at x5000 magnification through many examined microparticles. The same phenomenon was already observed in other studies [47, 66, 67]. It may be suggested that bacterial cells were embedded inside the microparticles.

Dried WPI microcapsules (with and without lactic acid bacteria) were analyzed using FT-IR THERMO Brand Spectrometer, too. In the spectrum of dried WPI, the diagnostic signals show that the significant absorption region is between 1633 cm-1 and 1238 cm-1. The wave

number at 1633 cm-1, 1515 cm-1, and 1391 cm-1 are associated with amide I (C─N) bonds, amide II (C=O), and amide III of proteins, respectively [68]. The wavenumber 1237 cm-1 is related to the vibration of carbohydrate rings, 2359 cm-1 is related to NH component.

In the spectrum of encapsulated bacteria with WPI, the wave number 2359 disappeared. This phenomenon can be attributed to the electrostatic interaction between the fatty acids of WPI and *L.rhamnosus* . According to these observations, our next analyzes should be interested in the FT-IR window between 2500 cm-1 and 500 cm-1, knowing that the primary characteristic absorption in bacteria was observed in the fatty acid composition (3000–2800 cm−1 region), carbohydrates (1200–900 cm−1 region) and fingerprint region (900–700 cm−1) reported that for lactic acid bacterial starter cultures, classification models showed significant discrimination in the 1200–900 cm−1, supporting their use as a potentially useful tool to identify and monitor the activity of dairy starters [69, 70]. Thus, it has to be proceed with the FT-IR analyses of *L.rhamnosus* only to choose the best absorption region for the FT-IR analyses during the storage (Figure 3.15).

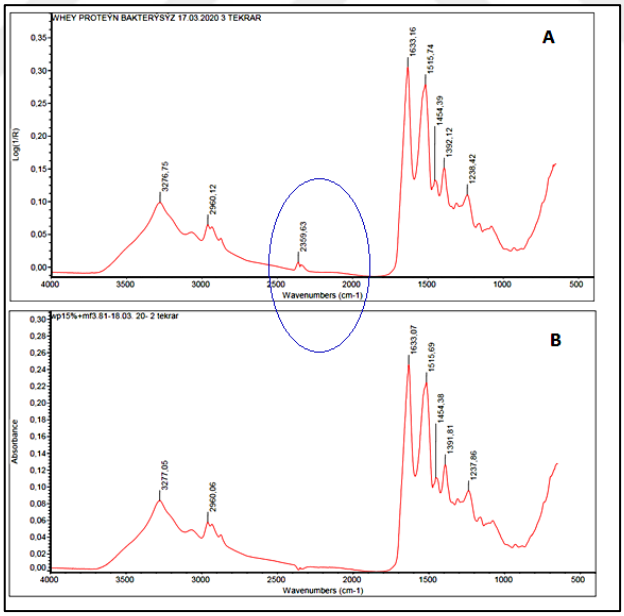


Figure 3.15. The FT-IR spectra of WPI (a), and encapsulated *L.rhamnosus* in WPI (b)

Determining the glass transition temperature is also important, as it is essential for the stability of the encapsulated materials during storage; The glass transition temperature (Tg) of the encapsulated product used in the production of edible films is measured using differential scanning calorimetry, [59]. The encapsulated product exhibits a long shelf life

when stored below the glass transition temperature since deterioration due to bacterial proliferation and chemical reactions are minimal. The low permeability of the carrier material at temperatures below the glass transition temperature helps prevent oxygen and preserve core materials.

T_g of the *L.rhamnossus GG* encapsulated with 15 percent WPI was determined as 35°C by DSC (at a heating rate of 5°C/min) (Figure 3.16). Since the storage temperature, which is accepted as room temperature, is lower than T_g of the coating material, bacterial proliferation and chemical reactions will be minimal and oxygen diffusion will not increase. Therefore, it has been verified that room temperature is a suitable storage temperature.

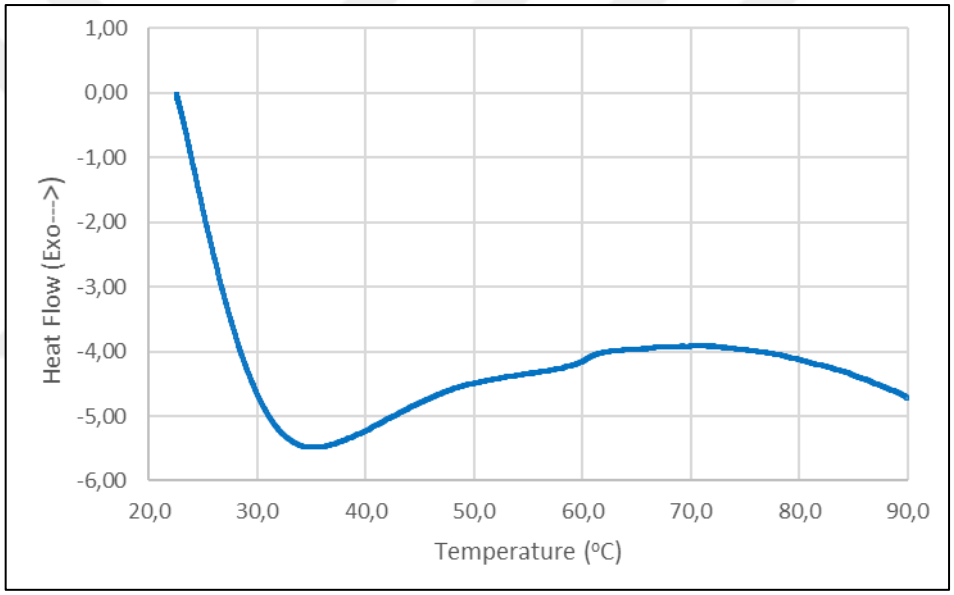


Figure 3.16. DSC thermogram of the *L.rhamnossus GG* encapsulated with 15 percent WPI

As a result; Whey protein isolate was found to be a suitable coating material to protect *L.rhamnosus* from the high temperature of the spray drying process. WPI has been shown to be more effective in preserving the viability of the probiotic because it covers the surface of the probiotic better than the others, forming a protective layer or having slow drying kinetics. Thus, it was analyzed that *L.rhamnosus* coated with WPI had high viability[25]. MTS method and direct culture method were applied in the viability analyzes and in the viability analysis of the encapsulated product obtained by using WPI, only 1 log level difference was observed compared to the other coating materials. Since the encapsulated product produced

with WPI had the highest viability and there was no significant difference between MTS and CFU methods, the direct culture method continued to be used.

4. PRODUCTION OF THE EDIBLE FILMS WITH ENCAPSULATED PRODUCT

4.1.MATERIALS AND METHODS

4.1.1. Materials

Pectin (76282, Sigma, Germany), alginate (CR8223, FMC, USA), agar (E406 CasNo 9002-18-0, Tito, Turkey), sucrose (477180, Carlo Erba, Italy), glycerol (346160, Carlo Erba, Italy) were used to produce the edible films. Glycerol and sucrose were used to reduce the dissolution time of the films.

The edible film was dissolved in the dissolution apparatus (PTWS 120D, Pharmatest, Germany) to perform viability analysis. NaCl (106404, Merck, Germany) potassium phosphate monobasic (P5655, Sigma, Germany), sodium phosphate (71642, Sigma, Germany) dibasic were used to simulated the saliva solution in the dissolution apparatus.

The bacteria was incubated in incubators (IFA 110, ESCO, USA).

4.1.2. Production of the Edible Films

A 10 ml mixture was prepared using these polysaccharides in 3 different ways to make an edible film.

The three different mixtures were prepared with different contents. The first one was pectin-sucrose-glycerol; the second one was alginate-sucrose-glycerol, and the third one was pectin-agar-sucrose-glycerol.

6 ml of 2 percent pectin (w/v) solution, 2 ml of 20 percent sucrose (w/v) solution, and 2 ml of 10 percentglycerol(w/w) were mixed at 1000rpm 45°C using magnetic mixer to prepare the solution. After 10 minutes, the mixer was stopped, and 2 percent w/v (0.2g for 10mL solution) encapsulated probiotic was added, and mixing was started gradually increasing. Stirring was continued until the encapsulated product was completely dissolved. 2 percent$CaCl_2$ solution(w/v)) in different amounts (2-1-0.5ml) were added into the film

mixture to observe the effect of $CaCl_2$ on gelling by keeping the solution's total volume of pectin-sucrose-glycerol mixture prepared was always 10 ml. The prepared film solution was divided into two and poured into two petri dishes in equal amounts and left to dry.

While preparing the film with alginate, firstly, the mixing ratios of the best film prepared using pectin were used. The first film was produced by using alginate instead of pectin in the mixture, then the mixing ratios were changed to produce the best film.

Another film preparation experiment was continued by preparing a pectin-agar mixture. In this solution, a pectin agar mixture was used instead of just pectin. Again, the content of the 10 ml mixture was prepared in the same way. 60 percent of the 10 ml solution was formed with pectin - agar. 3 ml 2 percent pectin solution (w/v), 3 ml 1.5 percent agar(w/v), 2 ml 20 percent sucrose solution (w/v), 2 ml 20 percentglycerol were used. In mixture, there were 30 percent pectin, 30 percent agar mixture, 20 percent sucrose, 20 percent glycerol, and 2 g of encapsulated product.

Two methods were used to ensure crosslinking. In the first; $CaCl_2$ solution was added to the edible film mixture without waiting for it to dry. It was called old method. In the second, the mixture was kept for one day and then $CaCl_2$ solution was added after one day. It was called new method. In order to increase viability in the film, two different methods were tried. First method; the encapsulated product was added to the film solution and mixed. It was called mixing method. In the second method, the encapsulated product was poured onto the film mixture crosslinked in the petri dish using a funnel. It was called pour-on method.

4.1.3. Dissolution Test and Viabilities

The edible film composed of gelatin, pectin, glucose, the encapsulated probiotics was poured into the petri dish. Analysis of which ratio and which edible film material has the highest viability was conducted.

The edible film was cut into 2.5x1.5cm pieces then the films viability was analyzed in each piece. The cut piece was dissolved to perform viability analysis in the dissolution apparatus. The mouth fluid was simulated as a solution in the device (Table 4.1). To produce the saliva solution, 8 gr/lt NaCl, 0.19 g/lt potassium phosphate monobasic, 2.38 g/lt sodium phosphate dibasic were mixed. This solution was autoclaved.(Table 4.1.) [46].

Table 4.1. Simulated saliva (SS) i.e. biological fluids

COMPOSITON	SS 1 (g/l)	SS 3 (g/l)	SS 4 (g/l)	SS 5 (g/l)
Potassium chloride	0.72	-	0.149	-
Calcium chloride dihydrate	0.22	0.228	-	-
Sodium chloride	0.6	1.017	0.117	8
Potassium phosphate monobasic	0.68	-	-	0.19
Sodium phosphate dibasic	0.866 (dodecahydrate)	0.204 (dodecahydrate)	-	2.38
Potassium bicarbonate	1.5	-	-	-
Potassium thiocyanate	0.06	-	-	-
Citric acid	0.03	-	-	-
Magnesium chloride hexahydrate	-	0.061	-	-
Potassium carbonate hemihydrate	-	0.603	-	-
Sodium phosphate monobasic monohydrate	-	0.273	-	-
Sodium bicarbonate	-	-	2.1	-
Submaxillary mucin	-	1	-	-
Alpha-amylase	-	2	2	-
Mucin gastric	-	-	1	-
Properties				
pH	6.5	-	-	6.8

The dissolution time of the edible film forms that were prepared was measured in the saliva solution, too. The solution's pH is 6.8.

The edible film's viability rate was analyzed immediately after production and incubated in incubators at 4°C / 24°C temperature for 30/45 days.

4.2.RESULTS AND DISCUSSION

4.2.1. Development of the Edible Film

The first edible film trials were started with pectin-sucrose-glycerol ingredients. $CaCl_2$ was added to the edible film mixture because of its crosslink feature. The prepared mixture was

poured into a petri dish to make an edible film, and two different studies were done. In the first edible film prepared, the $CaCl_2$ solution was immediately dropped. In the other, the mixture was allowed to dry for one day, and then the $CaCl_2$ solution was added dropwise. It was observed that when the amount of $CaCl_2$ was increased, added to the mixture quickly or before the mixture dries, the film produced had a heterogeneous structure. Because of the rapid crosslink of the film mixture with the Ca^{+2} ion in $CaCl_2$, rapid swelling was occurred at the drip site[71]. For this reason, $CaCl_2$ was added to the edible film mixture after keeping dry for 1 day. Thus, a more homogeneous film was obtained. The film prepared to dry for one day, was more homogeneous and could be removed more easily from the petri dish. It was also analyzed whether there was a difference in viability between adding $CaCl_2$ solution immediately to the prepared mixture and adding it after 24 hours. In addition, effect on viability was also analyzed adding $CaCl_2$ 1 day later was dried or removed, and there was no effect on the amount of viability. It was preferred not to be removed for ease of operation. As a result; the best film feature and viability was seen in the film that was encapsulated with WPI, prepared with a mixture of pectin, and $CaCl_2$ solution was added after 24 hours.

Trials were made. First of all, the effect of reducing the amount of $CaCl_2$ used was investigated. It was added into the 10 ml solution, and mixed at 45 C at 1000 rpm. But high bubble film formation was occurred inside the film. To decrease of the bubble's amount, the mixer rate was decreased from 1000 rpm to 750 rpm. Moreover, it could not be removed from the petri dish. Therefore, the mixture was prepared again by reducing the amount of $CaCl_2$. Instead of 2 ml $CaCl_2$, the amount of $CaCl_2$ was decreased and 1ml 2 percent $CaCl_2$ was prepared and added to the solution prepared under the same conditions. It was observed that the hardening was excessive and the amount of film decreased slightly. When 0.5 ml of $CaCl_2$ was added to the same solution this time, a homogeneous, bubble-free film that could be removed from the petri was obtained (Table 4.2).

Table 4.2. The effect of the amount of $CaCl_2$ on film formation (Pectin)

Pectin			Sucrose			Gylcerol			$CaCl_2$		Encapsulated Product	Film Solution	Film Prepared		
$C_{solution}$ (w/v)	film %	Amount (mL)	$C_{solution}$ (w/v)	film %	Amount (mL)	$C_{solution}$ (w/v)	film %	Amount (mL)	$C_{solution}$ (w/v)	Amount (mL)	Consantration (w/v)	Amount (mL)	Film Formation	The Amount of Bubbles	Homogeneity
2%	60	6	1:1	20	2	-	20	2	2%	2	2%	10	can not remove	many	-
2%	60	6	1:1	20	2	-	20	2	2%	1	2%	10	Couldn't pour because it hardened		
2%	60	6	1:1	20	2	-	20	2	2%	0.5	2%	10	+	-	+

According to this result; As seen in the studies, reducing $CaCl_2$, that is, reducing the Ca^{+2} ion, reduces the swelling rate [71]. Thus, a more homogeneous film was obtained (Figure 4.1).

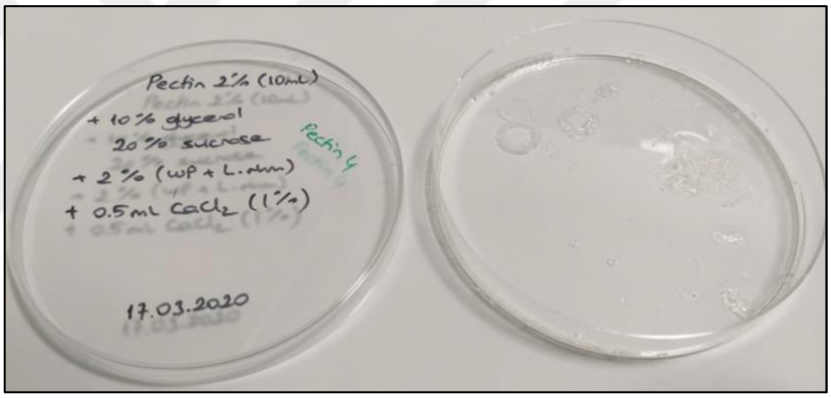


Figure 4.1. Representative image of the edible film composed of 6 ml of 2% pectin (w/v) solution, 2 ml of 20% sucrose (w/v) solution, and 2 ml of 10%glycerol(w/w), 0,5ml 2%$CaCl_2$ (w/v), 2 g encapsulated product(w/v)

In a previous study, when 2 ml of glycerol was added to the mixture, the film could be removed from the petri dish, but this was not easy. When 1 ml was used instead of 2 ml glycerol in the mixture, the homogeneity of the film decreased, but it became easier to remove from the Petri dish. (Table 4.3).

Table 4.3. The effect of the amount of glycerol on film formation (Pectin)

Pectin			Sucrose			Gylcerol			$CaCl_2$		Encapsulated Product	Film Solution	Film Prepared		
$C_{solution}$ (w/v)	film %	Amount (mL)	$C_{solution}$ (w/v)	film %	Amount (mL)	$C_{solution}$ (w/v)	film %	Amount (mL)	$C_{solution}$ (w/v)	Amount (mL)	Consantration (w/v)	Amount (mL)	Film Formation	The Amount of Bubbles	Homogeneity
2%	60	6	1:1	20	2	-	20	2	2%	1	2%	10	Couldn't pour because it hardened		
2%	60	6	1:1	20	2	-	10	1	1%	1	2%	10	+	-	-

According to previous studies, the decreasing in the amount of glycerol was caused deterioration in the homogeneity of the film[72] (Figure 4.2).

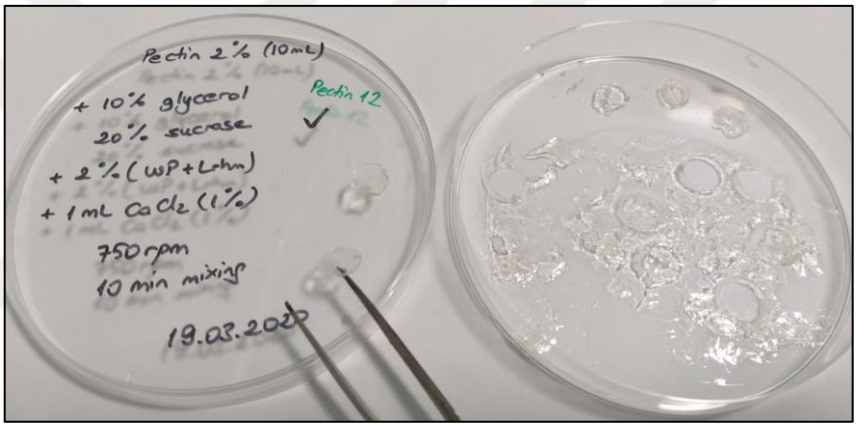


Figure 4.2. Representative image of the edible film composed of 6 ml 2% pectin (w/v) solution, 2 ml 20% sucrose (w/v) solution, and 1 ml 10%glycerol(w/w), 1ml 2%$CaCl_2$ (w/v), 2 g encapsulated product(w/v)

Film preparation trials were continued by preparing an alginate mixture. The effect of $CaCl_2$ on gelling was observed by adding 0.5 ml and 1 ml $CaCl_2$. While the edible film mixture containing 1 ml of $CaCl_2$ could not be removed from the Petri dish, the edible film mixture containing 0.5 ml of $CaCl_2$ could be easily removed from the Petri dish. In addition, no bubble formation was observed in this mixture, in which 0.5 ml of $CaCl_2$ was added, unlike other mixtures (Table 4.4) (Figure 4.3).

Table 4.4. The effect of the amount of $CaCl_2$ on film formation (Alginate)

Alginate			Sucrose			Gylcerol			$CaCl_2$		Encapsulated Product	Film Solution	Film Prepared		
$C_{solution}$ (w/v)	film %	Amount (mL)	$C_{solution}$ (w/v)	film %	Amount (mL)	$C_{solution}$ (w/v)	film %	Amount (mL)	$C_{solution}$ (w/v)	Amount (mL)	Consantration (w/v)	Amount (mL)	Film Formation	The Amount of Bubbles	Homogeneity
1%	60	6	1:1	20	2	-	20	2	2%	1	2%	10	The film formed crumbles when being removed	Little	-
1%	60	6	1:1	20	2	-	20	2	2%	0.5	2%	10	+	-	-

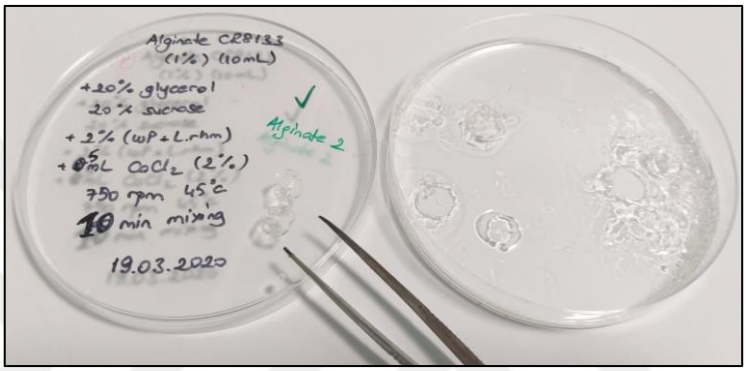


Figure 4.3. Representative image of the edible film composed of 6 ml 1% alginate (w/v) solution, 2 ml 20% sucrose (w/v) solution, and 2 ml 20%glycerol(w/w), 0.5ml 2%$CaCl_2$ (w/v), 2 g encapsulated product(w/v)

Another film preparation experiment was continued by preparing a pectin-agar mixture. The effect of $CaCl_2$ was observed by adding 1 ml and 0.5 ml of $CaCl_2$ to two separate mixtures prepared in this way (Table 4.5).

Table 4.5. The effect of the amount of $CaCl_2$ on film formation (Pectin-agar)

Pectin			Agar			Sucrose			Gylcerol			$CaCl_2$		Encapsulated Product	Film Solution	Film Prepared		
$C_{solution}$ (w/v)	film %	Amount (mL)	$C_{solution}$ (w/v)	film %	Amount (mL)	$C_{solution}$ (w/v)	film %	Amount (mL)	$C_{solution}$ (w/v)	film %	Amount (mL)	$C_{solution}$ (w/v)	Amount (mL)	Consantration (w/v)	Amount (mL)	Film Formation	The Amount of Bubbles	Homogeneity
2%	30	3	1.5%	30	3	1:1	20	2	-	20	2	2%	1	2%	10	can remove	little	-
2%	30	3	1.5%	30	3	1:1	20	2	-	20	2	2%	0.5	2%	10	+	little	-

It was observed that a better film was obtained from the mixture with 0.5 ml of $CaCl_2$ compared to the other. However, it was observed that both had bubbles inside (Figure 4.4).

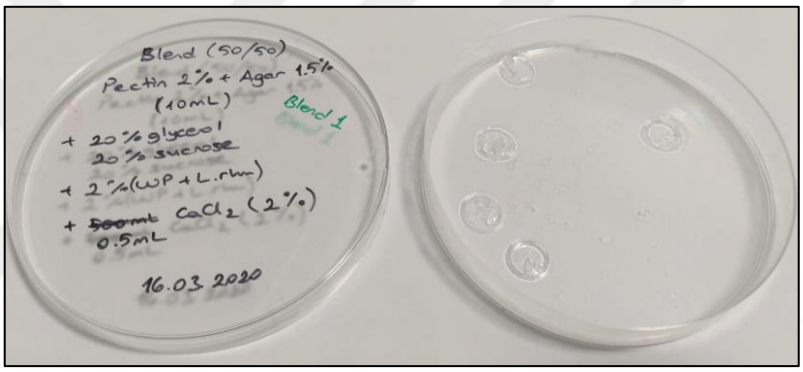


Figure 4.4. Representative image of the edible film composed of 3 ml 2% pectin (w/v) solution, 3 ml 1.5% agar(w/v), 2 ml 20% sucrose (w/v) solution, and 2 ml 20%glycerol(w/w), 0.5ml 2%$CaCl_2$ (w/v), 2 g encapsulated product(w/v)

As a result, homogeneity is low and the problem of not being easily removed was observed in all films obtained by the old(mixing) method. ($CaCl_2$ solution was added to the edible film mixture without waiting for it to dry) . The trials were summarised (Figure 4.5).

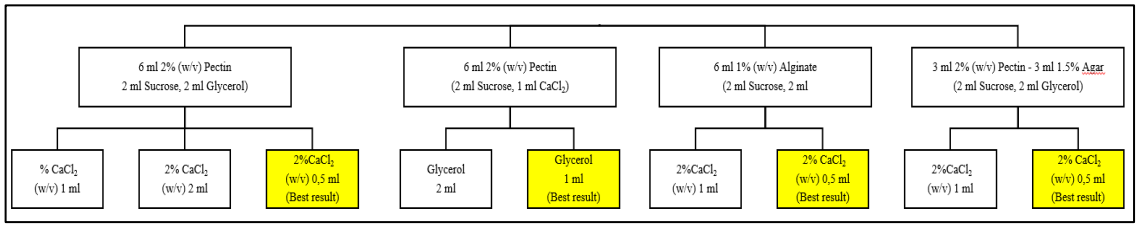


Figure 4.5. Development of the edible film prepared old (mixing) method

When $CaCl_2$ was added to the prepared edible film mixture, it was expected to form a homogeneous film with the crosslink effect, but generally a heterogeneous film was formed instead of homogeneous. A different method was developed to obtain a homogeneous film, and the film mixture prepared without adding $CaCl_2$ was poured into the petri dish, left to dry for 24 hours, then $CaCl_2$ was added and after 10 minutes, the $CaCl_2$ solution was removed from the petri dish. [41, 73]. Therefore, this new(immersion) method was used to add $CaCl_2$ to the edible film mixture after the film had dried, in order to obtain a more homogeneous and easily removable film.

Before starting all trials, initial viability was checked, again.

All edible film mixtures were prepared with the new method, again. The pectin mixture was prepared under the same conditions and poured into the petri dish. Unlike the other study, it was left to dry for 24 hours in this study. After 24 hours, 5 ml of $CaCl_2$ was added dropwise to the prepared petri dish, cross-linking was achieved (Table 4.6).

Table 4.6. The effect of $CaCl_2$ was added 24 hours later on film formation (Pectin)

Pectin			Sucrose			Gylcerol			$CaCl_2$		Encapsulated Product	Film Solution	Film Prepared		
$C_{solution}$ (w/v)	film %	Amount (mL)	$C_{solution}$ (w/v)	film %	Amount (mL)	$C_{solution}$ (w/v)	film %	Amount (mL)	$C_{solution}$ (w/v)	Amount (mL)	Consantration (w/v)	Amount (mL)	Film Formation	The Amount of Bubbles	Homogeneity
2%	60	6	1:1	20	2	-	20	2	2%	5	2%	10	+	-	-

A homogeneous non-bubble film was obtained (Figure 4.6).

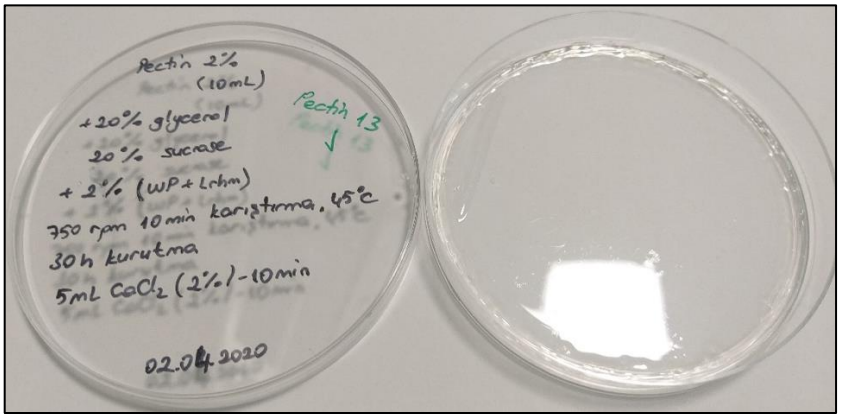


Figure 4.6. Representative image of the edible film composed of 6 ml 2% pectin (w/v) solution, 2 ml 20% sucrose (w/v) solution, and 2 ml 10%glycerol(w/w), 5ml 2%$CaCl_2$ (w/v), 2 g encapsulated product(w/v)

Similar work was repeated for the alginate. However, the film formed from the solution prepared with 6 ml of alginate was broken, experiments were done by increasing the alginate amount (Table 4.7).

Table 4.7. The effect of $CaCl_2$ was added 24 hours later on film formation (Alginate)

Alginate			Sucrose			Gylcerol			$CaCl_2$		Encapsulated Product	Film Solution	Film Prepared		
$C_{solution}$ (w/v)	film %	Amount (mL)	$C_{solution}$ (w/v)	film %	Amount (mL)	$C_{solution}$ (w/v)	film %	Amount (mL)	$C_{solution}$ (w/v)	Amount (mL)	Consantration (w/v)	Amount (mL)	Film Formation	The Amount of Bubbles	Homogeneity
1%	60	6	1:1	20	2	-	20	2	2%	5	2%	10	The film formed crumbles when being removed	-	+
1%	70	7	1:1	20	2	-	10	1	2%	5	2%	10	The film formed crumbles when being removed	-	+
1%	70	7	1:1	10	1	-	20	2	2%	5	2%	10	+	-	+

A homogeneous non-bubble film was obtained (Figure 4.7).

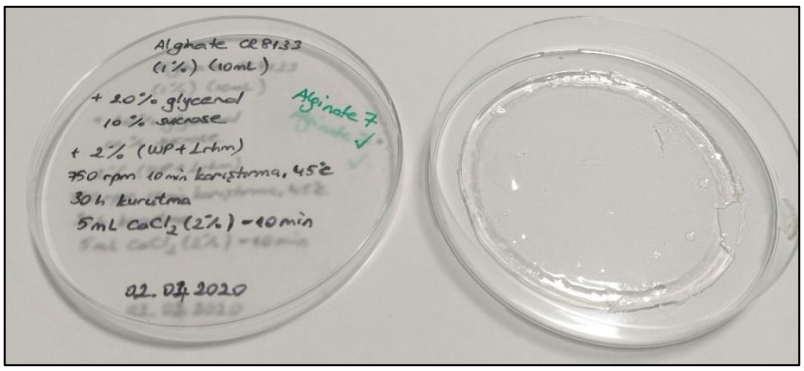


Figure 4.7. Representative image of the edible film composed of 7 ml 1% alginate (w/v) solution, 1 ml 20% sucrose (w/v) solution, and 2 ml 20%glycerol(w/w), 5ml 2%$CaCl_2$ (w/v), 2 g encapsulated product(w/v)

A similar procedure was repeated for the pectin-agar mixture (Table 4.8).

Table 4.8. The effect of $CaCl_2$ was added 24 hours later on film formation (Pectin-agar)

Pectin			Agar			Sucrose			Gylcerol			$CaCl_2$		Encapsulated Product	Film Solution	Film Prepared		
$C_{solution}$ (w/v)	film %	Amount (mL)	$C_{solution}$ (w/v)	film %	Amount (mL)	$C_{solution}$ (w/v)	film %	Amount (mL)	$C_{solution}$ (w/v)	film %	Amount (mL)	$C_{solution}$ (w/v)	Amount (mL)	Consantration (w/v)	Amount (mL)	Film Formation	The Amount of Bubbles	Homogeneity
2%	30	3	1.5%	30	3	1:1	20	2	-	20	2	2%	5	2%	10	+	-	+

A homogeneous non-bubble film was obtained (Figure 4.8).

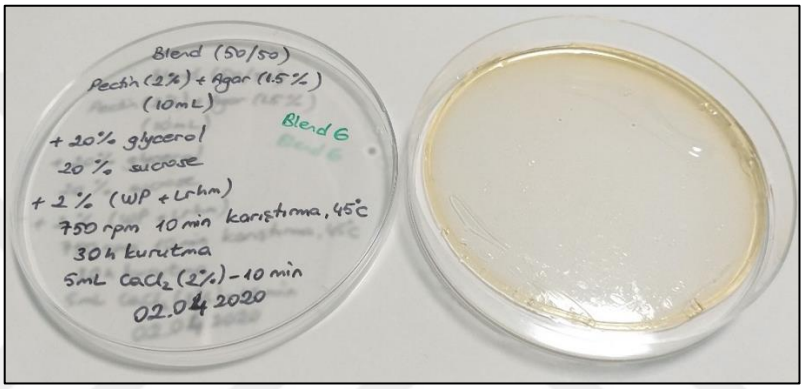


Figure 4.8. Representative image of the edible film composed of 3 ml of 2% w/v pectin solution, 3 ml of 1.5% w/v agar, 2 ml sucrose solution

Summarly, the appropriate inputs were determined to obtain a homogeneous, easily removable, bubble-free edible film (Figure 4.9).

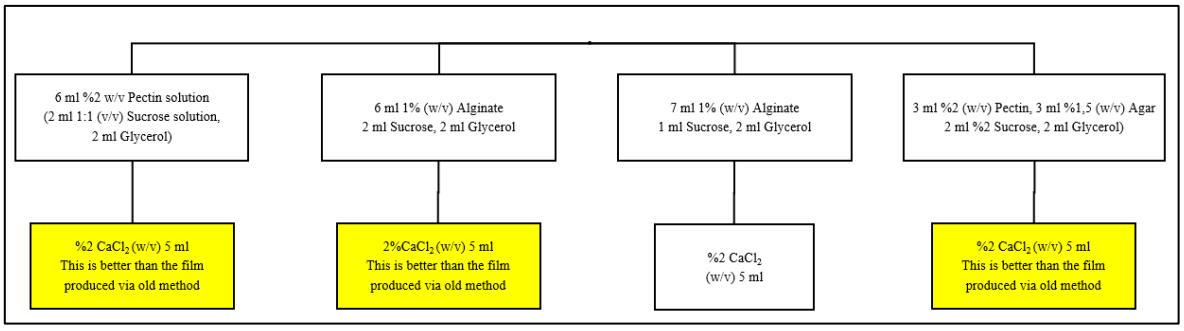


Figure 4.9. Development of the edible film prepared with new (immersion) method

4.2.2. Comparison of the New and Old Method's Viabilities

The viability measurement of bacteria was made in the film prepared with the old and new method. A high decline had been detected. It was observed that there was no significant difference in viability between the old method and the new method (Figure 4.10).

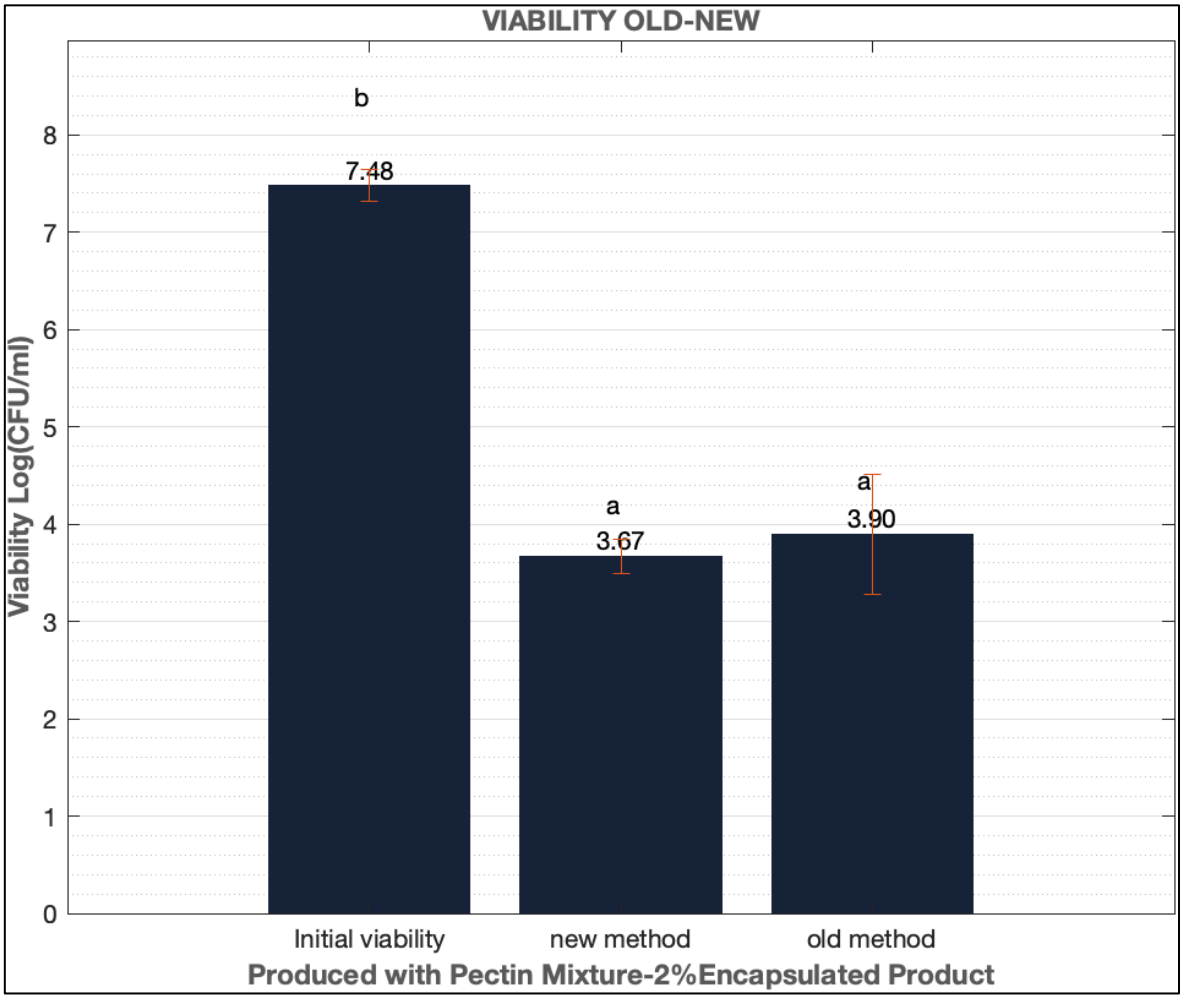


Figure 4.10. The viability measurement of bacteria with the old and new method. The mean values with the same letter are not significantly different ($p>0.01$)

The aim was to analyze the difference in viabilities and homogeneity between edible films produced with the old and new methods. Unfortunately, there was no significant difference according to the results. However, since a more homogeneous film was produced with the new method, it was decided to continue working with the new method.

With the new method, homogeneous removable films were obtained. As a next step, it was necessary to demonstrate that the bacteria was alive by performing a viability analysis. As is known, bacteria dissolve in the water phase. For this reason, it was thought that if $CaCl_2$ were kept the added for 10 minutes and remove it, the bacteria may decrease away from the film. Consequently, the viability must be analyzed using two different methods. $CaCl_2$ solution was added 1 day later to 2 films prepared according to the new method. $CaCl_2$, which was placed in one petri dish, was removed after 10 minutes, while $CaCl_2$ was left to dry completely in the other. Removal or complete drying of $CaCl_2$ added to the film mixture after 1 day had no effect on viability. For this reason, in recent studies, it was preferred to not-remove $CaCl_2$ from the petri dish (Figure 4.12).

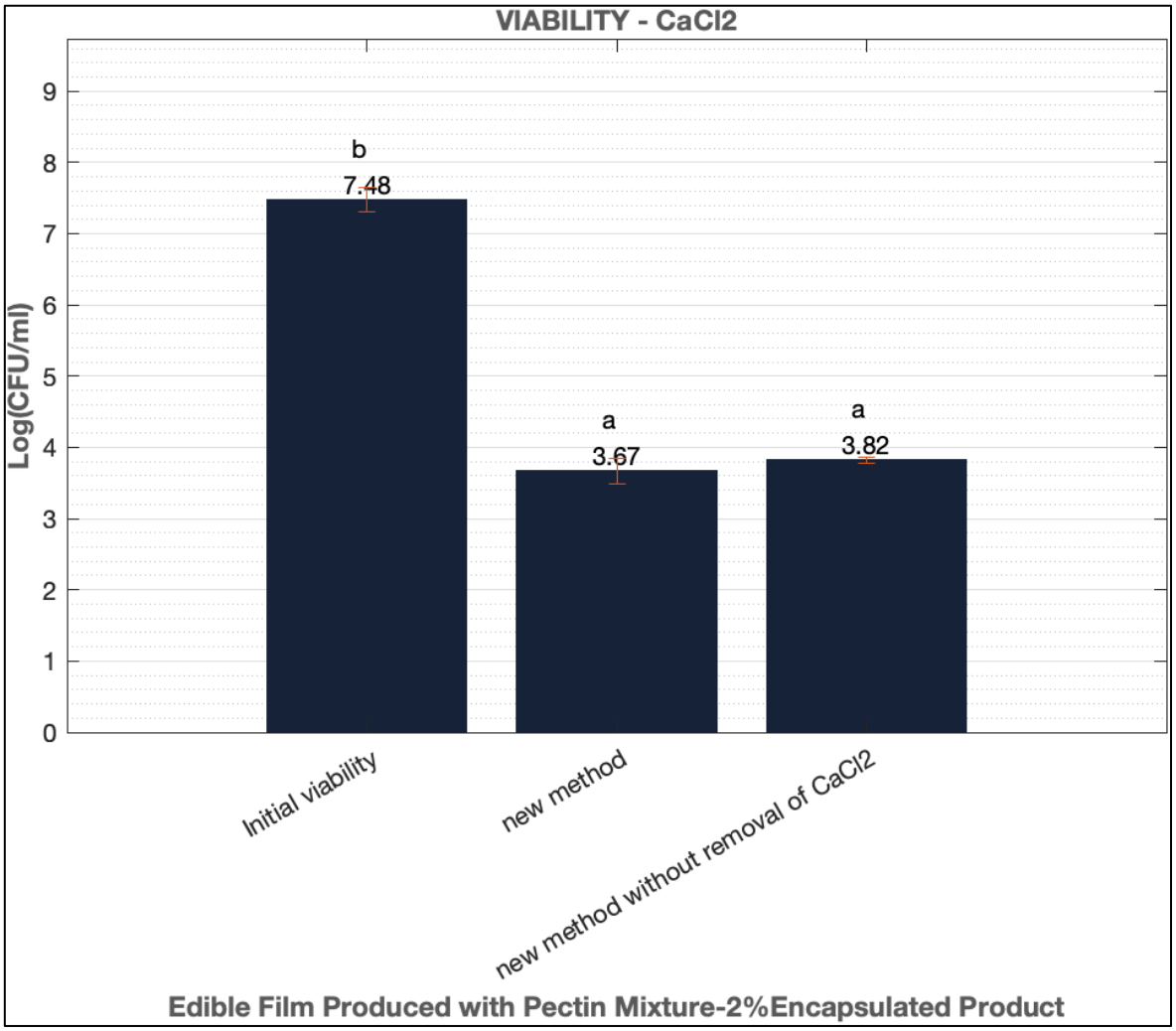


Figure 4.11. Effect of $CaCl_2$ removal or not removal on viability . The mean values with the same letter are not significantly different ($p>0.01$)

The viability quantities were measured and compared (Figure 4.12). Since, there was no significant difference between the results, it was seen that there was no need to removal of $CaCl_2$ solution after it was dropped into the edible film mixture by using the new method.

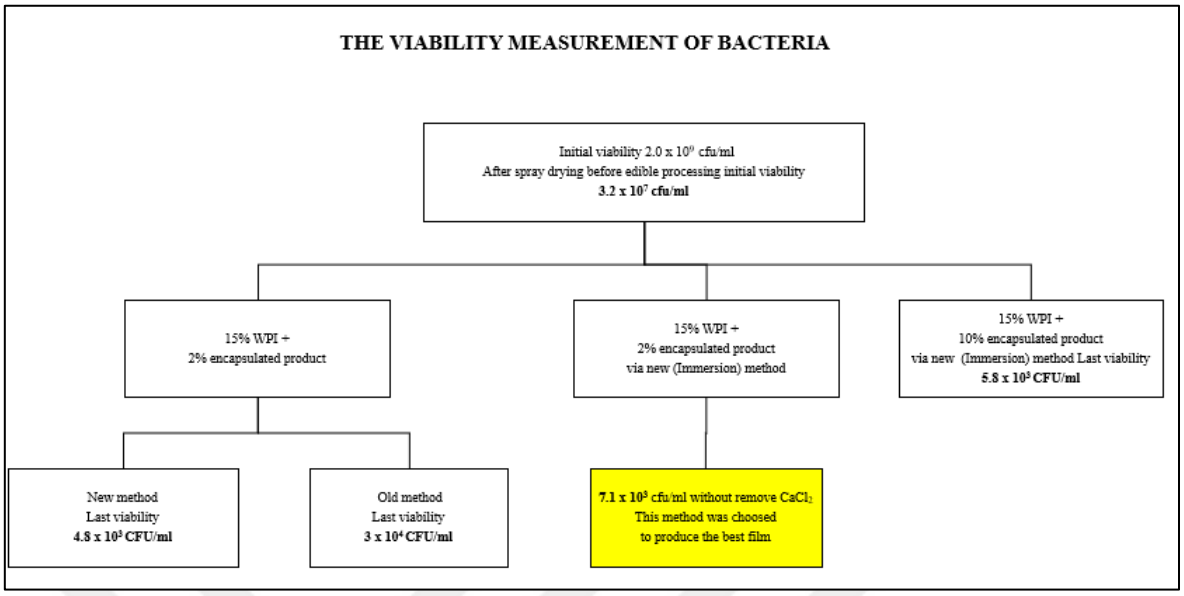


Figure 4.12. Viability measurement of bacteria

4.2.3. Increasing the Viability in the Edible Film

Although a homogeneous film was obtained by the new method, the amount of viability could not reach the recommended daily level of 10^6 CFU/ml[43] The amount of encapsulated product added to the edible film was increased from 2 percent to 10 percent to increase the viability level. According to the results, increasing the amount of encapsulated product in the film did not contribute to an increase in the viability of the probiotic (Figure 4.13).

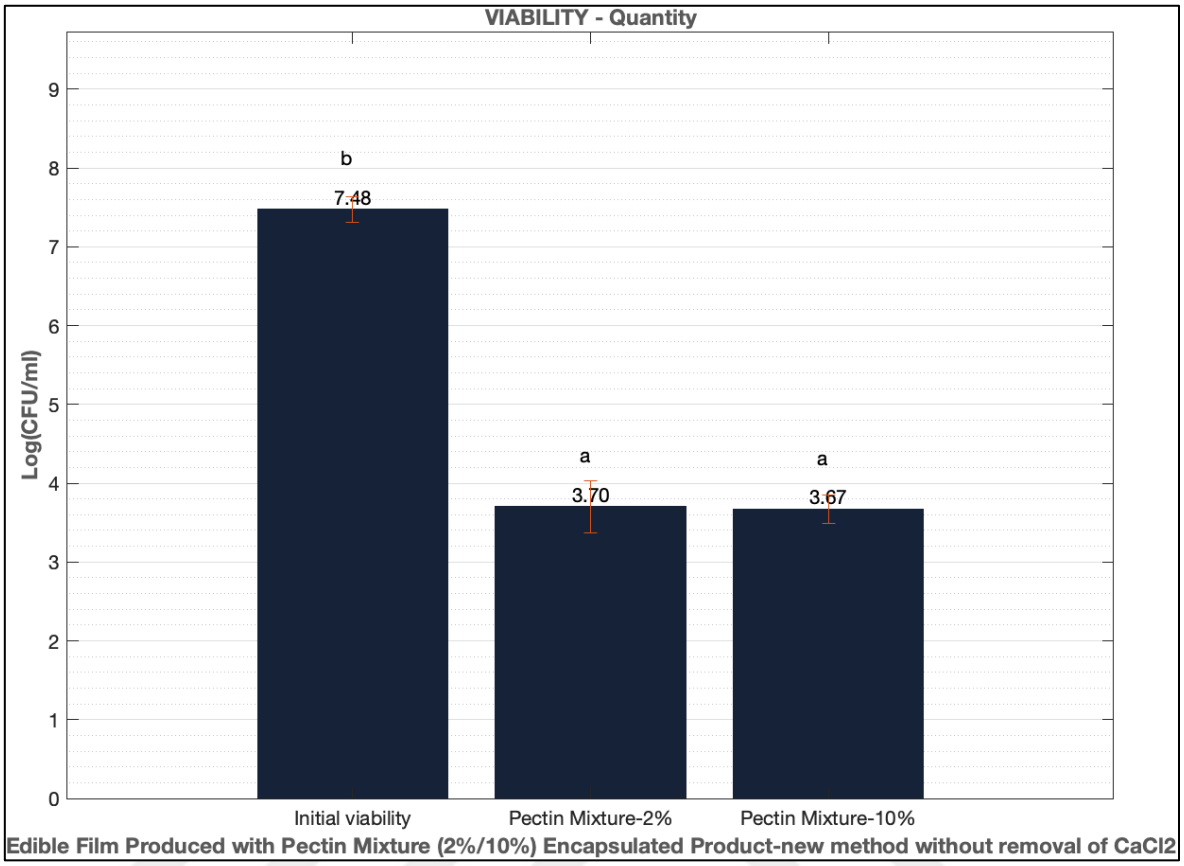


Figure 4.13. The effect of the increase the amount of encapsulated product in the mixture on the viability. The mean values with the same letter are not significantly different ($p>0.01$)

When the encapsulated products were mixed with the edible film mixture, other trials were carried out to prevent the decrease in the amount of viability after mixing. Firstly; Instead of mixing the encapsulated probiotics with the film mixture, the edible film, which does not contain the encapsulated product, was dipped into a solution containing 10 percent encapsulated product, to ensure that the encapsulated product was absorbed into the film. However, no significant difference was found in viability values.

In another trials to increase the amount of viability, 10 percent encapsulated product was sprayed onto the edible film that did not contain the encapsulated product. The film solution prepared pectin mixture was poured into the petri dish, then 10 percent (w/v) of the encapsulated product was added with a filter, then the film was left to dry. After 24 hours, 5 mL 2 percent (w/v) $CaCl_2$ solution was added and dried in the cabinet for 24 hours at room temperature (Figure 4.14).

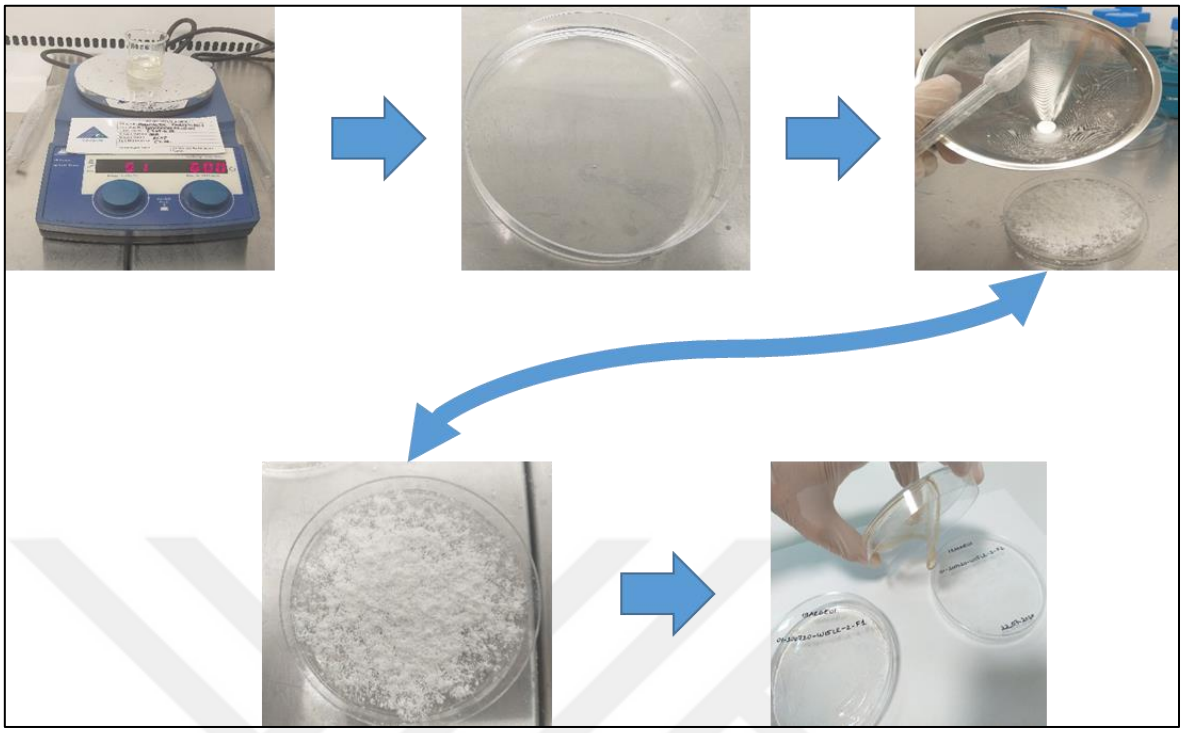


Figure 4.14. Preparing o the edible film with pouring powder method

From this point, this method was referred to as the "powder pouring method on edible film - pour on method".It was recognised with this method, the viability was increased. (Figure 4.15).

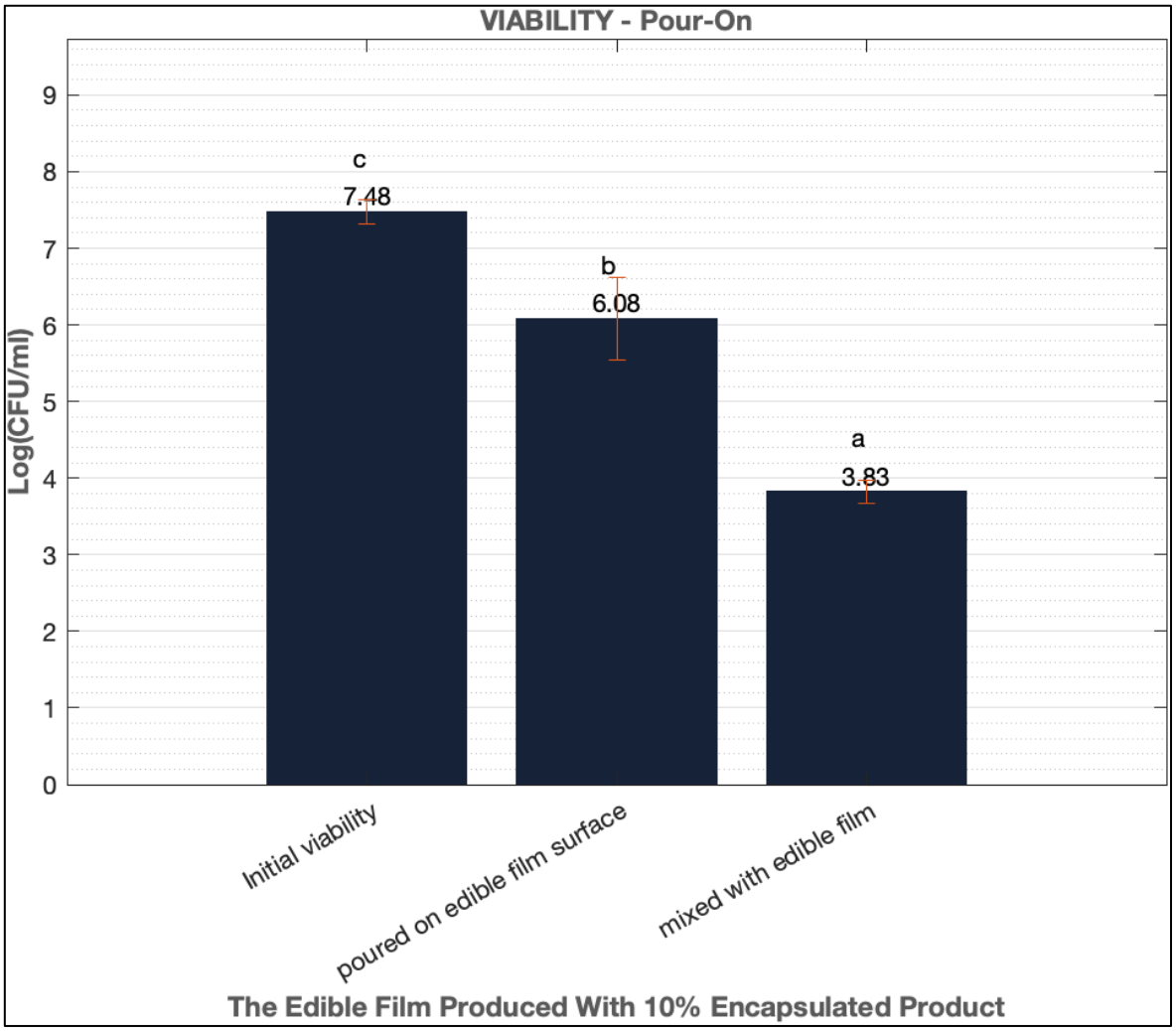


Figure 4.15. The effect of the pour-on method on viability.

The mean values with the same letter are not significantly different ($p>0.01$)

After reaching the desired values of 10 percent, the next step was to measure viability changes during storage. It was observed that the decrease in the number of bacteria was at expected levels in our film, which was stored at incubators under room conditions for 90 days (Figure 4.16).

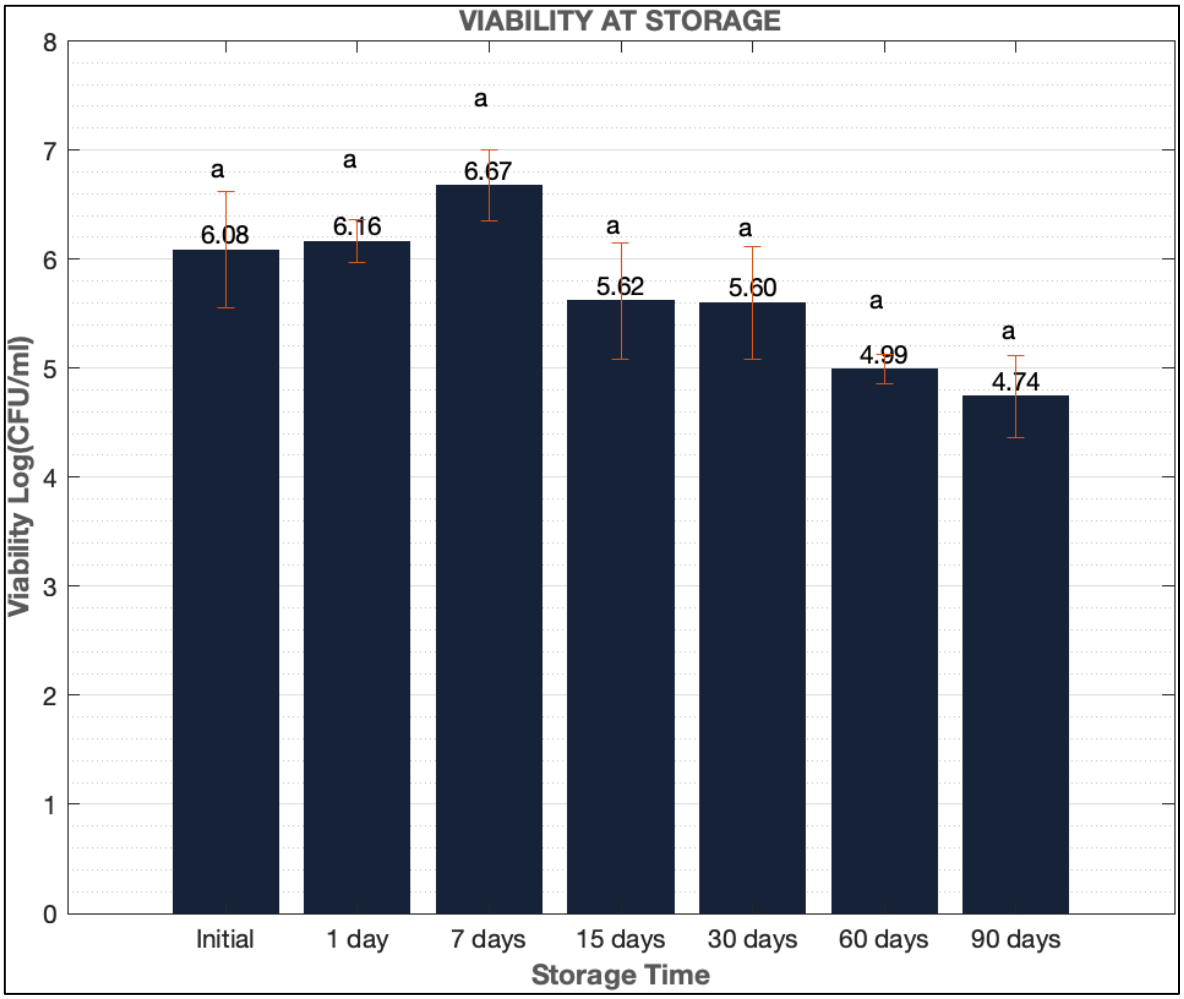


Figure 4.16. Bacteria viability changes during storage in the final product obtained by pouring the encapsulated product onto edible film with pectin.

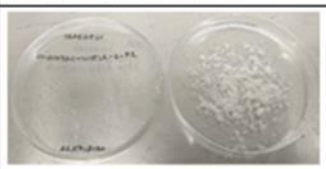
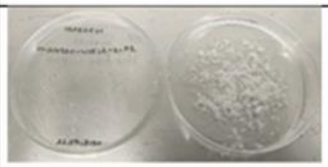
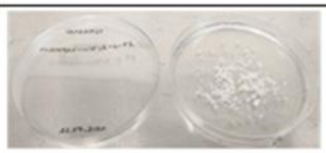
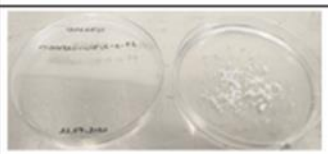
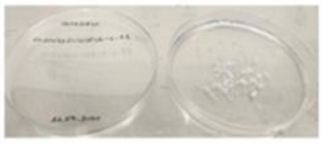
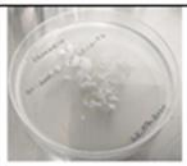
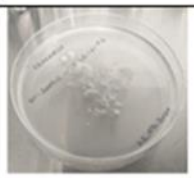
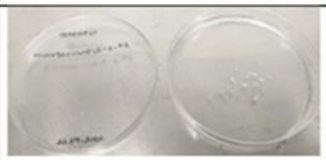
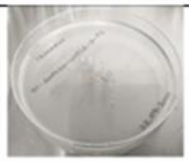
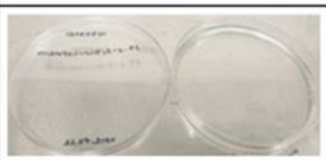
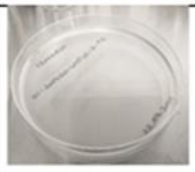
The mean values with the same letter are not significantly different ($p>0.01$)

In the shelf life studies, the film with the highest vitality was used. The viability of the probiotics in the edible film produced by the pour on method, 1st, 7^{th}, 15^{th},30^{th}, 60^{th}, 90^{th}, measured in days. At the end of 90 days, two log level decrease in viability was detected.

It was observed that the viability rates of the product attached to the surface by pouring the powdered encapsulated powder probiotic on the uncross linked edible film, which did not contain probiotics, were higher than other studies. While the film was produced using the pour on method, the new method ($CaCl_2$ was added to the prepared film after 1 day) was continued to be used. Then the encapsulated product was poured into the film.

The dissolution time of the added encapsulated product on the film surface varies according to the amount of added product. For example, F1 contains 2 percent encapsulated product, F2 contains 10 percent encapsulated product. Therefore, the absorption of the encapsulated powder on the film was observed with a time-dependent change (Table 4.9).

Table 4.9. The disappearance of the encapsulated powder on the edible film – time dependent change

Time (min)	2% encapsulated product-poured on the edible film	Time (min)	10% encapsulated product-poured on the edible film
0		10	
5		15	
10		25	
15		35	
20		45	
25		55	
30		65	
35		75	

Viabilities of bacteria in the films prepared by pour-on and old (mixing) method were compared and summarized (Figure 4.17). It was determined that the edible film produced by pour-on method, was not damaged due to not mixing the probiotics, and the viability values

were three logs cycle higher than the other method (89). With this method, the decrease in the viability of the probiotic during production was partially reduced.

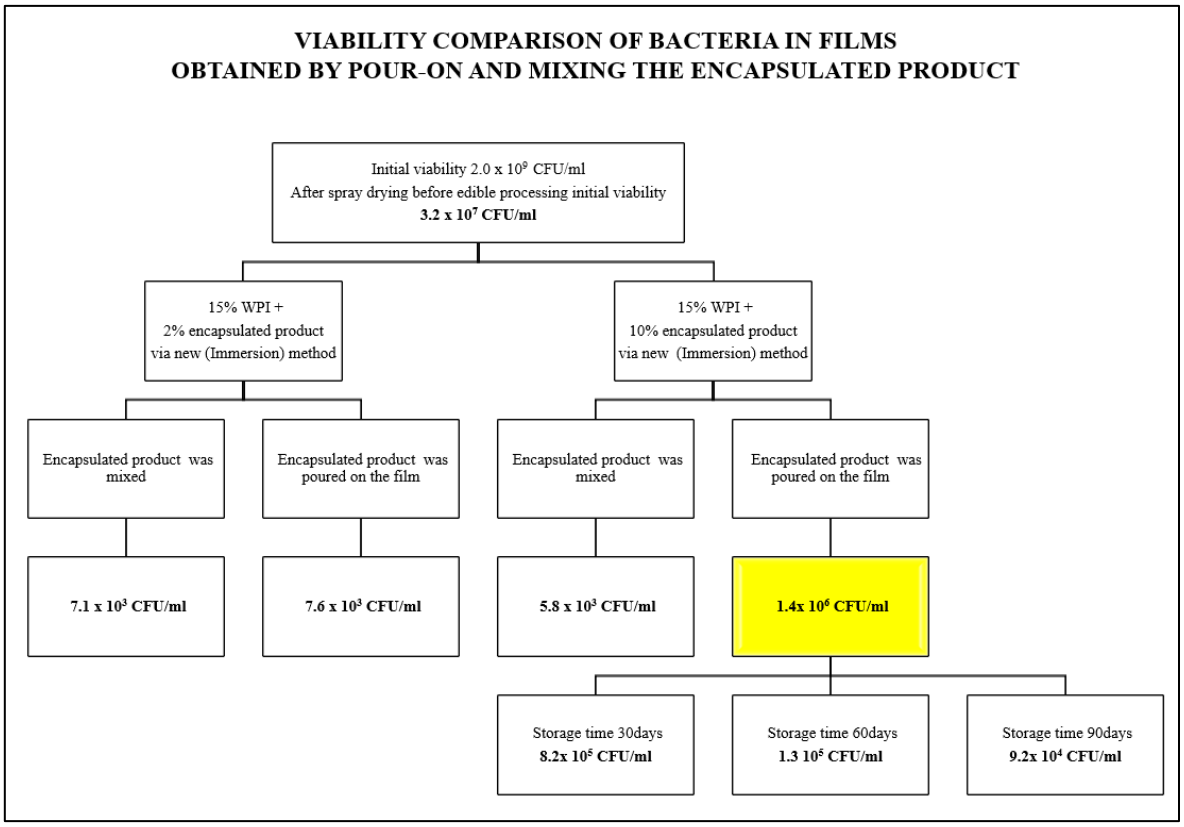


Figure 4.17. Viability comparison

4.2.4. Preperation of Saliva Solution

The mouth fluid was simulated as a solution in the device [46]. 40 mL of simulated oral fluid was prepared using the SS5 solution given in (Table 4.1)

4.2.5. Dissolution of the Edible Film in Saliva

The solubility of the films was examined by heating to 37°C body temperature. Since it was obtained the best results amongst the films made, 2x3 cm size cuts were tried in 3 different ways. The results were written in Table 4.10.

(i) Without mixing

(ii) Using the mixer with low mixing speed (100 rpm)

(iii) Using an ultrasonic bath

Table 4.10. Simulated Biological Fluids with Possible Application in Dissolution Testing

Process	Pectin Mixture		Pectin-Agar Mixture		Alginate Mixture	
	Initial Weight (g)	Time (min)	Initial Weight (g)	Time (min)	Initial Weight (g)	Time (min)
No Mixing	0.086	60	0.0877	120	0.0884	150
Stirrer (100 rpm)	0.0824	35	0.0814	75	0.0846	90
Ultrasonic Bath	0.084	7	0.0819	11	0.081	15

According to the distribution studies done in the mouth, it has been documented with the patent [74] that the product reaches every point in the mouth in 3-6 hours. According to this patent, in order to ensure the effectiveness of the product, the distribution in the mouth must be at least 1 hour. For this reason, 1 hour was taken as a reference for the contents of the prepared films to spread well in the mouth. In addition, this period was considered to be an acceptable period for the consumer.

In this study, it was tried to evaluate the rate of absorption in the mouth. It was decided that without mixing at all or mixing at 100 rpm is the closest method to the absorption in the mouth. It was compared the melting times of the prepared mixtures in the oral fluid under three different conditions. It was achieved the most successful result with the mixture with pectin (Figure 4.18, Figure 4.19).

It was also determined the number of bacteria spread in the mouth after the edible film was swallowed. Bacteria count determination study was also carried out depending on time.

In this dissolution test, 10 percent probiotic film prepared by the pour-on method was used. In order to see the difference of edible films prepared with sodium alginate and pectin, the study was conducted on two different samples in 3 repetitions. Viability results were showed (Figure 4.18, Figure 4.19).

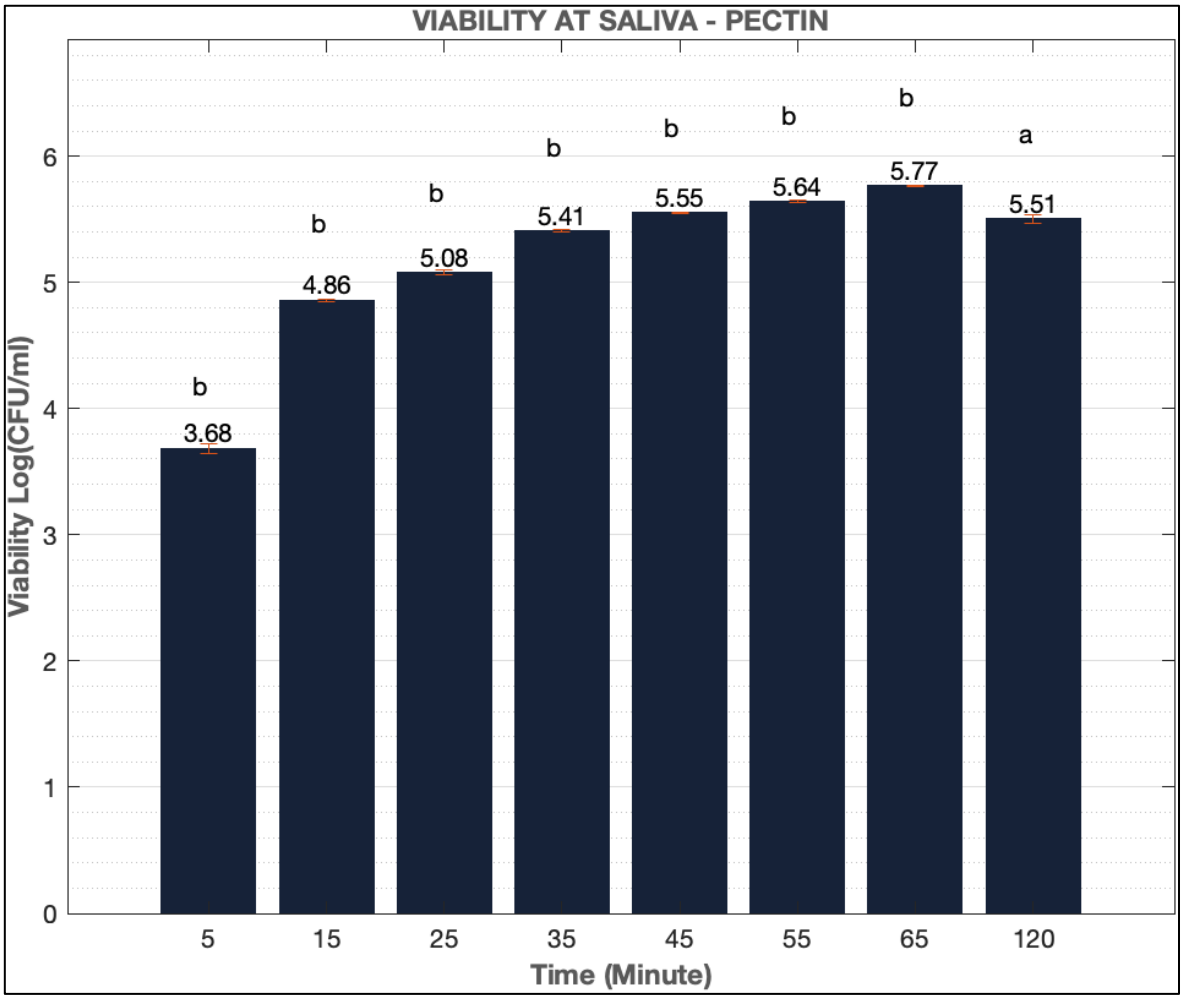


Figure 4.18. Viability of *L.rhamnosus* of the edible film prepared pectin with the pour-on method in Saliva.

The mean values with the same letter are not significantly different ($p>0.01$)

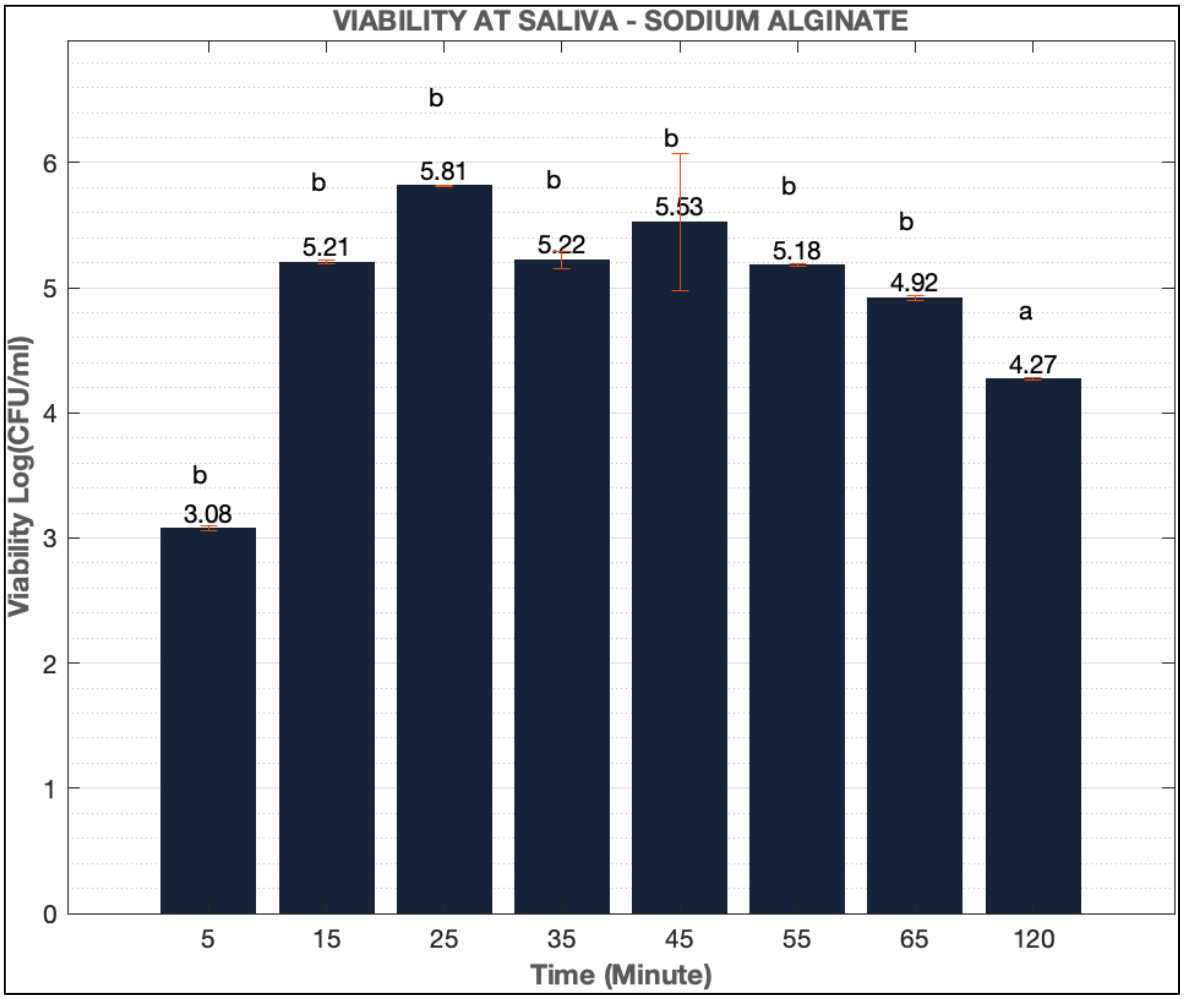


Figure 4.19. Viability of *L.rhamnosus* of the edible film prepared alginate with the pour-on method in saliva.

The mean values with the same letter are not significantly different ($p>0.01$)

According to the time-dependent dissolution test, a logarithmic increase in viability was observed after 15 and 25 minutes. As stated in previous studies, it is recommended that the daily dose should be 10^6 and above for beneficial effects from probiotics [75–77]. Thus, the daily recommended probiotic dose has been almost approached.

5. EFFECTIVENESS OF THE EDIBLE FILM

Efficiency studies were conducted against oral pathogens (*E.coli, S.aureus, C.albicans, P. aeruginosa*) on 5 films prepared. Probiotic-free film was used as the control group. The study was performed in triplicate for each pathogen. The initial viability of the pathogens was compared with the changes in the viability after the edible film was added.

5.1.MATERIALS AND METHODS

5.1.1. Materials

E.coli (25922, ATCC, Germany), *S.aureus* (25923, ATCC, Germany), *C.albicans* (10231, ATCC, Germany), *P. aeruginosa* (27853, ATCC, Germany) were used as pathogen. TBX (Tryptone Bile X-Glucuronide) (116122, Merck, Germany), BPW (Buffered Peptone Water) (107228, Merck, Germany), BPA (Baird Parker Agar) (105406, Merck, Germany), SDA (Sabouraud Dextrose Agar) (105438, Merck, Germany), CFC agar (107620, Merck, Germany) were used for growth and cultivation of pathogens. The strains present at -80°C were dissolved at room temperature.

5.1.2. Preperation of Pathogens

Freeze-dry pathogens were reactivated in appropriate 2 ml medium and 0.3 ml suspension was taken and cultured in the medium via incubators at require temperature and time and kept (Table 5.1). After that, conservation was made from an overnight culture in suitable broth, and mixed with glycerol 50 percent (v/v) and kept at -80°C. Finally, for short-term preservation, the pathogen was inoculated with medium into a 15 ml falcon tube and incubated for 48 hours, then kept at 4°C for daily use.

Table 5.1. Growth conditions suitable for pathogens

Pathogen	Agar Medium			Broth Medium		
	Medium	Incubation Temprature	Incubation time	Medium	Incubation Temprature	Incubation time
E. coli	TBX (Tryptone Bile X-Glucuronide)	44°C	24 h	BPW (Buffered Peptone Water)	37C	24 h
S. aureus	BPA (Baird Parker Agar)	37°C		BPW (Buffered Peptone Water)		
C. albicans	SDA (Sabouraud Dextrose Agar)	25°C		BPW (Buffered Peptone Water)		
P. aeruginosa	CFC (Pseudomonas CFC/CN Agar)	37°C		BPW (Buffered Peptone Water)		

E.coli was planted on TBX (Tryptone Bile X-Glucuronide) agar by line planting. It was kept in the incubator at 44°C for 18-24 hours. At the end of the incubation period, green colonies were observed on TBX agar. The pathogen seen in the (a) figure was *E.coli* (Figure 5.1) .

Taking a loopful of *S.aureus* strain dissolved in medium BPA. Moreover, the temperature was reduced to 37°C, which is the appropriate incubation temperature. It was left in incubator 24 hours. At the end of the incubation period, colonies with black zones and transparent opaques were observed in BPA. *S.aureus* was seen in the (b) (Figure 5.1).

C.albicans were grown in SDA. It was kept at a suitable incubation temperature 24-25°C for 24-36 hours. For growth, a loopful of *C.albicans* grown in a petri dish was mixed with approximately 30 ml of BPW . Then, it was kept at 37°C for 24 hours. At the end of the incubation period, beige-white, cream-colored zoned colonies were observed in SDA. The colonies was *C.albicans* in the (c) (Figure 5.1).

A loop of *P.aeruginosa* strain dissolved in Eppendorf was taken and streaked in a petri dish with CFC media spilled. It was left in the incubator at 37°C, the appropriate incubation temperature 37°C, for 18-24 hours. At the end of the incubation, *P.aeruginosa* , yellow-green pigmented colonies with UV fluorescence, were seen on the CFC agar in the (d) (Figure 5.1).

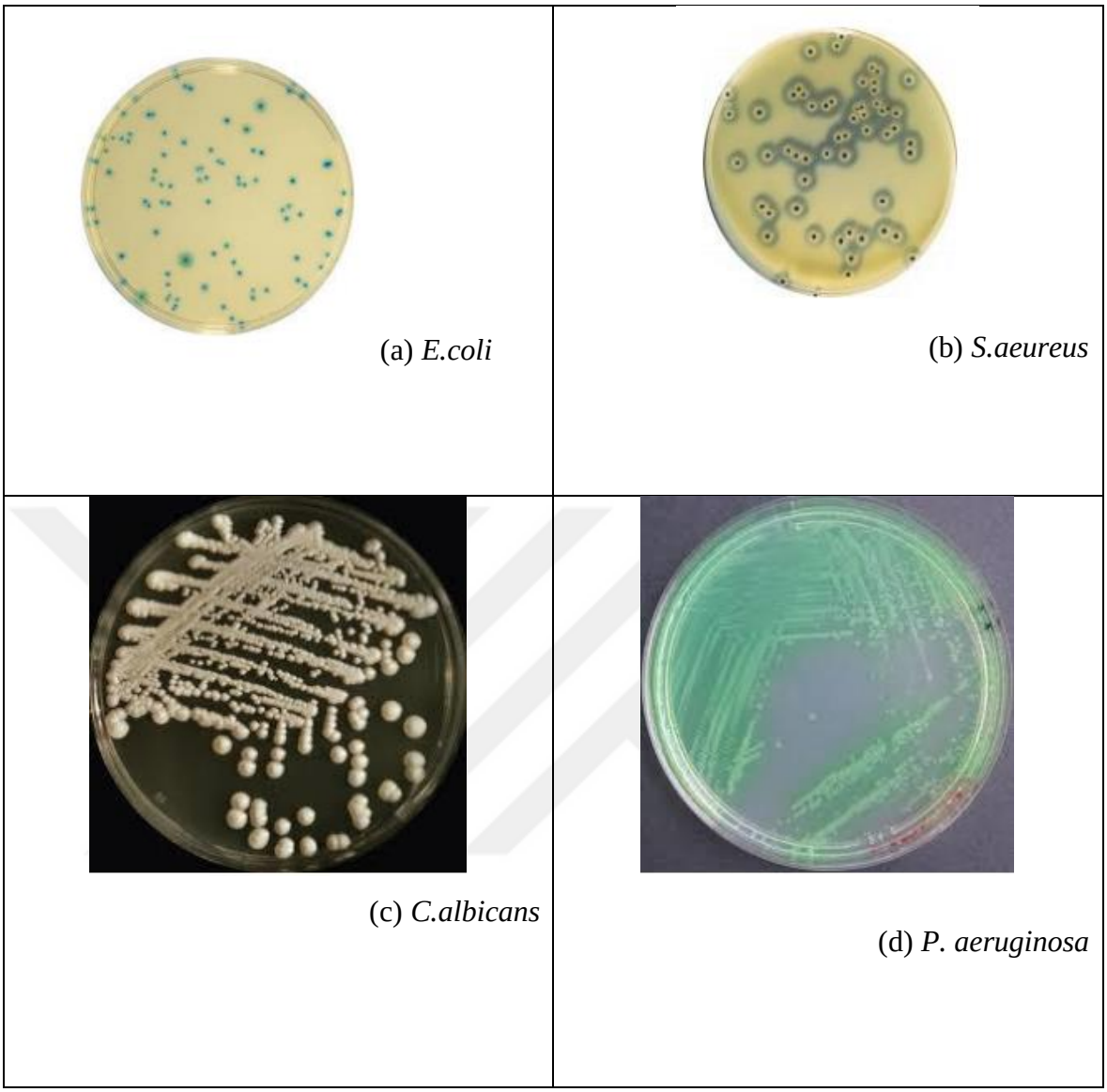


Figure 5.1. Figures of pathogens (a) *E.coli*, (b) *S.aeureus*, (c) *C. albicans*, (d) *P.aeruginosa*

5.1.3. The Efficiency Test of The Edible Film on Pathogen

In the previous dissolution test at 4.chapter, the disappearance time of the edible film in saliva was controlled. This study was aimed to determine the number of probiotics released into saliva from the edible film dissolved in oral fluid over time.

A loopful of selected pathogen bacteria was grown overnight at 37°C and 100 rpm in a shaker-water bath on the respective medium. The whole mixture in the prepared falcon was centrifuged, and the medium was removed by washing with two times 10 ml of distilled

water. It was dissolved with 30 mL of MRD. 1 ml solution was taken into two falcon tubes. It was dissolved by adding 10 mL of saliva and then vortexed. Edible film prepared by the pour-on method was added to one of the falcons. It was kept in a shaker water bath for 24 hours at 37°C and 100 rpm. At the end of the specified hour, samples were taken from both falcons and planted with dilution. Pathogens were grown under standard operating conditions, changing agar and medium according to the pathogen. Four bacteria causing disease in the mouth; halitosis, tooth decay, gingivitis; were separately added into saliva solution. The encapsulated probiotic film prepared by the pour-on method was placed in saliva containing the pathogen, and the viability of the bacteria and probiotic in the liquid was observed. In each study, a control group without edible film was prepared. The viability levels of this control group were compared with the viability results of saliva in which pathogen and edible film were added. All studies were repeated three times.

Then, 100 µL of the sample was taken from the mixture at certain time intervals and inoculated on MRS agar medium. Finally, it was left to incubate for 48 hours in an incubator with 5 percent CO_2 at 37°C. Later; viability counts were made.

5.2.RESULT AND DISCUSSION

The effectiveness of the produced edible film containing *L.rhamnosus* against pathogens causing oral diseases was tested. The viability levels of the control group were compared with the viability results of saliva in which a pathogen and edible film were added. All studies were repeated three times.

In the control group containing the *E.coli* pathogen, viability was determined to be $3.7x10^7$ cfu/g. After the film prepared by the pour-on method was kept in a saliva solution containing *e-coli* for 24 hours, the viability of the pathogen decreased by two logs to $3.37x10^5$ cfu/g. The change in the viability of the probiotic decreased from $1.70x10^6$ to $5.83x10^5$ cfu/g. While the viability number of the pathogen decreased by two digits logarithmically, that of the probiotic dropped by one (Figure 5.2)

In a similar study with the *S.aeurus* pathogen; while the viability of the pathogen decreased by 1 step logarithmically, it was observed that there was no decrease in the viability of the probiotic (Figure 5.2)

The study was conducted with the *C.albicans* pathogen; while the viability of the pathogen decreased by 1 step logarithmically, it was observed that there was almost no decrease in the viability of the probiotic (Figure 5.2)

In a similar study with *P. aeruginosa* pathogen; a significant decrease such as three logarithmics was detected in the viability number of the pathogen. *P.aeruginosa* pathogen was decreased three log from the initial level of $1.2x10^7$ to $5.73x10^4$ cfu / g after being kept in the edible film-containing medium for 24 hours. On the other hand, the viability of the probiotic was decreased only one log cycle (Figure 5.2.)

As a result of the study, it was observed that the viability of pathogens in saliva was reduced using the edible film containing probiotics prepared by the pour-on method. The best result was obtained for *P. aeruginosa* with four logs cycle reduction in viability and the second best result was obtained with *E. coli* with a two logs cycle reduction in viability. The decline of viability in *S. aureus* and *C. albic*ans was remained at a one log cycle (Figure 5.2.)

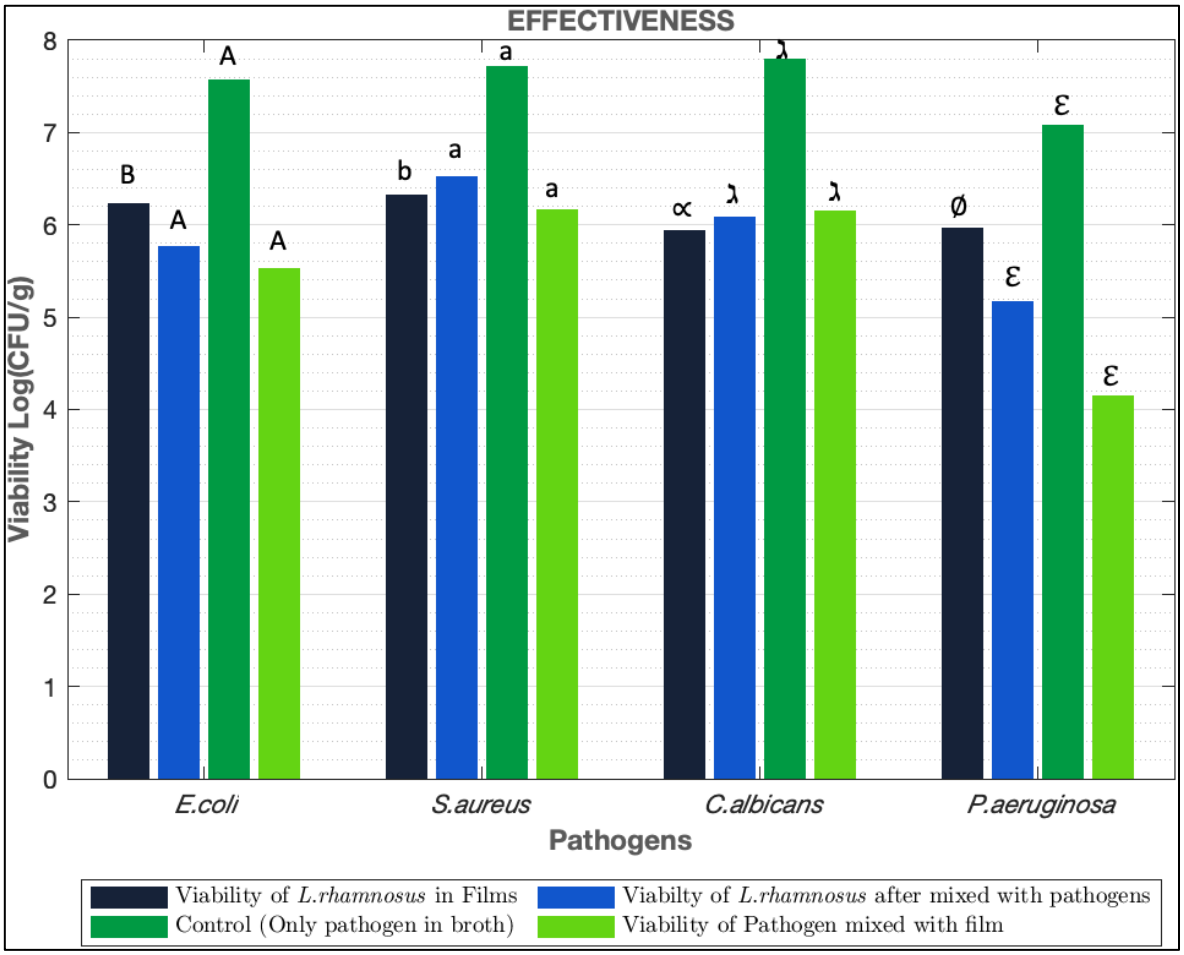


Figure 5.2. Efficacy of edible film containing *L.rhamnosus* in pathogen-containing saliva)

Statistical analysis was performed within each microorganism (*E.coli, S.aereus, C.albicans, P.aeruginosa*)

6. CONCLUSION

In this project, the aim is to develop edible films that will release encapsulated probiotics to the oral cavity in a short time, and contribute to general oral health, once placed on the tongue. In order to protect the oral health, the spray dryer method was used to preserve the viability of the *L.rhamnosus* bacteria to be used. Experimental studies were carried out to select the most suitable coating material for bacteria to be used in the spray dryer. An edible film form was created to ensure easy use of the encapsulated probiotic in maintaining oral health. Experimental studies were carried out to make this film a bubble-free, easily removable form that melts in the mouth in a maximum of 1 hour. Methods were developed to minimize the decrease in the viability of the probiotic during the addition of the encapsulated probiotic to this film and the storage of the obtained edible film. in the end; The effectiveness of edible film containing encapsulated probiotics against pathogens that cause deterioration of oral health was tested.

Whey protein isolate was found to be a suitable coating material to protect *L.rhamnosus* from the high temperature of the spray drying process. Thus, it was analyzed that *L.rhamnosus* coated with WPI had high viability.

The effect of trehalose using on increasing vitality, which was mentioned in other articles, was not seen in studies. This is thought to be because WPI covers the *L.rhamnosus* surface better than trehalose and provides higher protection.

The pectin-containing mixture was seen as the perfect edible film. because, with pectin mixture; a film with the desired melting time of 1 hour was produced, which is much more homogeneous, easier to remove from the petri dish and disappears in the mouth faster than other mixtures. It was seen that the saccharide/glucose combinations played a synergistic role in achieving a longer shelf life by providing the necessary energy source to maintain the high population of bacteria during storage.

The film prepared to dry for one day, was more homogeneous and could be removed more easily from the petri dish. It was also analyzed whether there was a difference in viability between adding $CaCl_2$ solution immediately to the prepared mixture and adding it after 24 hours. In addition, effect on viability was also analyzed adding $CaCl_2$ 1 day later was dried or removed, and there was no effect on the amount of viability. It was preferred not to be

removed for ease of operation. As a result; the best film feature and viability was seen in the film that was encapsulated with WPI, prepared with a mixture of pectin, and $CaCl_2$ solution was added after 24 hours.

In the experiments; the viability in the edible films was less than the recommended daily dose of 10^6. According to this result, the consumer could be recommended to use twice a day. In order to increase the viability and reach the recommended daily consumption amounts, instead of using 2 percent encapsule product, 10 percent encapsule product was used, but this was not enough and a new method was developed to produce the film. It was determined that the edible film produced by this method, called pour-on, was not damaged due to not mixing the probiotics, and the viability values were three logs cycle higher than the other method. With this method, the decrease in the viability of the probiotic during production was partially reduced. According to this result; almost the recommended daily dose level has been reached.

In addition, the effect of this edible film on pathogens that harm oral health was investigated. In dissolution testing with this product, viability of pathogens and *L.rhamnosus* was observed for a minimum recommended period of 1 hour as effective time to spread in the mouth. It was analyzed that while maintaining the number of probiotics in the edible film, it was also effective in reducing oral pathogens.

7. FUTURE STUDIES

In order to reach a daily dose of 10^6 probiotics, the initial viability should be increased. In the first studies, bacteria were produced as 10^{11} CFU/ml, but since it was not easy to achieve this, the first viability was continued with 10^9 CFU/ml. According to the logarithmic reduction in these studies, if these experiments are repeated with an initial viability of 10^{11} CFU/ml, the viability of the final product will be achieved at levels of 10^7 CFU/ml. Thus, it will be ensured that the daily recommended dose is obtained.

As a coating material, a slight increase in vitality was observed when trehalose was used together with WPI. This mixture; The effect on the viability of the probiotic in the stock product can be investigated. If there is an increase in viability, even if it increases the cost, experimental studies can be continued on the use of WPI-trehalose mixture as a coating material.

In the studies carried out, it was determined that WPI was a suitable coating material, but the concentration could not be increased above 15 percent due to its high viscosity. In the viscosity studies, it was determined that the viscosity of the mixture started to decrease as trehalose was added to the WPI-trehalose mixture. According to these data, WPI concentration can be increased up to 30 percent by using trehalose and higher protection can be achieved.

In addition, better protection of the encapsulated product from external conditions, multi-layer edible film can be formed with more than one film.

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APPENDIX A: MATLAB CODES FOR FIGURES

Algorithm A.1. Matlab Code for figure moisture (Figure 3.10):

```
clear all
data = [  3.10     3.51     3.92 ;
   3.50   3.28     4.56 ;
   1.90   2.58     3.26 ;
   3.10   3.96     4.82 ;
   2.40   3.12     3.85 ];
lbl=["15%WPI" "15% MD" "15% TREHALOSE" "(WPI +MD) %15" "(WPI +TREHALOSE)
15%"];
p=anova2(data, 1, 'off');
disp(p);
[p,t,stats]=anova1(data',lbl,'off');
c = multcompare(stats,'Display','off');
disp(c);
data_mean=mean(data,2);
data_mean_sorted=sort(data_mean);
p=1;
val=97;
data_label(1)=char(val);
for i=1:length(data_mean)-1
   indx(i)=find(data_mean==data_mean_sorted(i));
   if abs(data_mean_sorted(i)-data_mean_sorted(i+1))<p
      data_label(i+1)=data_label(i);
   else
      data_label(i+1)=char(data_label(i)+1);
      val=val+1;
   end

end
indx(i+1)=find(data_mean==data_mean_sorted(i+1));
figure
b= bar(mean(data,2));
xtips1 = b(1).XEndPoints;
ytips1 = b(1).YEndPoints+0.5;
labels1 = char(sort(data_label,'descend'));
C = textscan(labels1,'%c');
text(xtips1,ytips1,C{1},'HorizontalAlignment','right',...
   'VerticalAlignment','bottom')
title('MOISTURE','Color', [0.349019607843137 0.349019607843137 0.349019607843137],...
   'FontWeight', 'bold');
ylabel('Moisture %', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
  'FontWeight', 'bold');
xlabel('Materials', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');
b.FaceColor = '#162238';
b.EdgeColor = '#162238';
ylim([0 max(mean(data,2))*1.3]);
set(gca, 'xticklabel', lbl, 'YGrid','on', 'YMinorGrid','on');
xt = get(gca, 'XTick');
text(xt, mean(data,2), num2str(mean(data,2),'%0.2f%%'),...
   'HorizontalAlignment','center', 'VerticalAlignment','bottom');
hold on
```

```
er = errorbar(mean(data,2),std(data,0,2));
er.LineStyle = 'none';
hold off
```

Algorithm A.2. Matlab Code for figure product yield (Figure 3.2).

```
clear all
data=[ 62.00    59.36    56.73 ; 30.43    34.65    38.87 ; 31.31    35.06    38.80 ; 44.21
49.25    54.30 ; 38.33    45.67    53.00 ];
lbl=["15%WPI" "15% MD" "15% TREHALOSE" "(WPI +MD) %15" "(WPI +TREHALOSE)
15%" ];
p=anova2(data, 1, 'off');
disp(p);
[p,t,stats]=anova1(data',lbl,'off');
c = multcompare(stats,'Display','off');
disp(c);
data_mean=mean(data,2);
data_mean_sorted=sort(data_mean);
p=5;
val=97;
data_label(1)=char(val);
for i=1:length(data_mean)-1
   indx(i)=find(data_mean==data_mean_sorted(i));

   if abs(data_mean_sorted(i)-data_mean_sorted(i+1))<p
      data_label(i+1)=data_label(i);
   else
      data_label(i+1)=char(data_label(i)+1);
      val=val+1;
   end

end
indx(i+1)=find(data_mean==data_mean_sorted(i+1));
figure
b= bar(mean(data,2));
xtips1 = b(1).XEndPoints;
ytips1 = b(1).YEndPoints+7;
labels1 = char(sort(data_label,'descend'));
C = textscan(labels1,'%c');
text(xtips1,ytips1,C{1},'HorizontalAlignment','right',...
   'VerticalAlignment','bottom')
title('PRODUCT        YIELD','Color',        [0.349019607843137        0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');
ylabel('Product    Yield    %',    'Color',    [0.349019607843137    0.349019607843137
0.349019607843137],...
  'FontWeight', 'bold');
xlabel('Materials',          'Color',          [0.349019607843137          0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');
b.FaceColor = '#162238';
b.EdgeColor = '#162238';
ylim([0 max(mean(data,2))*1.2]);
set(gca, 'xticklabel', lbl, 'YGrid','on', 'YMinorGrid','on');
xt = get(gca, 'XTick');
text(xt, mean(data,2), num2str(mean(data,2),'%0.2f%%'),...
   'HorizontalAlignment','center', 'VerticalAlignment','bottom');
hold on
er = errorbar(mean(data,2),std(data,0,2));
er.LineStyle = 'none';
hold off
```

Algorithm A.3. Matlab Code for figure viability l (Figure 3.3).

```
clear all

data=[ log10(4482145767) log10(1021000000) log10(82833333333);
      log10(1254479100) log10(8000000)              log10(74666666667);
      log10(3619979100) log10(81000000)   log10(88166666667);
      log10(2625812433) log10(333333333)  log10(74666666667);
      log10(4206479100) log10(1333333333) log10(72166666667)];

lbl=["Whey Protein (WP)" "Maltodextrin (MD)" "Trehalose (Tr)" "WP+MD" "WP+Tr"];

cfum = [log10(4482145767) log10(4382145767) log10(4582145767);
       log10(1224479100) log10(1254479100) log10(1284479100);
       log10(3119979100) log10(3619979100) log10(3919979100);
       log10(2125812433) log10(2625812433) log10(2925812433);
       log10(4201479100) log10(4206479100) log10(4209479100)];


cfud = [log10(1011000000) log10(1021000000)  log10(1091000000);
       log10(5000000)       log10(8000000)            log10(9000000)          ;
       log10(21000000)     log10(81000000)          log10(51000000)        ;
       log10(313333333)  log10(333333333)   log10(339333333) ;
       log10(1233333333) log10(1333333333)  log10(1833333333) ];



initial = [log10(82233333333) log10(82833333333) log10(82433333333);
         log10(72666666667) log10(74666666667) log10(79666666667);
         log10(82166666667) log10(88166666667) log10(88126666667);
         log10(72666666667) log10(74666666667) log10(74266666667);
         log10(71166666667) log10(72166666667) log10(79166666667)];

data2=[cfum cfud initial];

p=anova2(data, 1, 'off');
disp(p);

lbl_2=["CFUM" "CFUD" "INITIAL"];

for i=1:5
   data_gen = [cfum(i, :); cfud(i, :); initial(i, :)];
   [~,~,stats]=anova1(data_gen',lbl_2,'off');
   c = multcompare(stats,'Display','off');
   disp("block" + i);
   disp(c);
end

[~,~,stats]=anova1(data',lbl,'off');
c = multcompare(stats,'Display','off');
disp("MultiCompare");
disp(c);

figure
b= bar(data);

title('VIABILITY OF L.RHAMNOSUS GG','Color', [0.349019607843137
0.349019607843137 0.349019607843137],...
   'FontWeight', 'bold');
```

```
ylabel('Viability Log(CFU/ml)', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
  'FontWeight', 'bold');
xlabel('Materials', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');

b(1).FaceColor = '#162238';
b(1).EdgeColor = '#162238';
b(2).FaceColor = '#1156cc';
b(2).EdgeColor = '#1156cc';
b(3).FaceColor = '#009a43';
b(3).EdgeColor = '#009a43';

ylim([0 max(mean(data,2))*1.2]);

set(gca, 'xticklabel', lbl, 'YGrid','on', 'YMinorGrid','on');
legend('CFUM', 'CFUD', 'Initial Inoculum',
'Orientation','horizontal','Location','southoutside',...
   'Interpreter','none');
```

Algorithm A.4. Matlab Code for figure VBNC 68-69 *L.rhamnosus*: (Figure 3.4)

```
clear all

data =[
 4.30    4.10    4.40
 3.08    3.08    3.08
10.16    9.90   10.20
11.66   11.00   12.00
23.29   22.90   23.40
21.70   22.00   21.60];

lbl =["30d WP" "60d WP" "30d Tr" "60d Tr" "30d MD" "60d MD"];

data_mean=mean(data,2);
data_mean_sorted=sort(data_mean);

[~,~,stats]=anova1(data',lbl,'off');
c = multcompare(stats,'Display','off');
disp(c);

p=1;
val=97;
data_label(1)=char(val);
for i=1:length(data_mean)-1
   indx(i)=find(data_mean==data_mean_sorted(i));

   if abs(data_mean_sorted(i)-data_mean_sorted(i+1))<p
      data_label(i+1)=data_label(i);
   else
      data_label(i+1)=char(data_label(i)+1);
      val=val+1;
   end

end
indx(i+1)=find(data_mean==data_mean_sorted(i+1));

figure
b= bar(mean(data,2));
xtips1 = b(1).XEndPoints;
ytips1 = b(1).YEndPoints+2;
labels1 = char(sort(data_label,'descend'));
C = textscan(labels1,'%c');
text(xtips1,ytips1,C{1},'HorizontalAlignment','right',...
   'VerticalAlignment','bottom')

title('VBNC L.rhamnosus','Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');
ylabel('VBNC %', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
  'FontWeight', 'bold');
xlabel('Storage Time', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');

hold on
legend('OutletT=68-69°C', 'Orientation','horizontal','Location','southoutside',
'Interpreter','none');
```

```
b.FaceColor = '#162238';
b.EdgeColor = '#162238';

ylim([0 max(mean(data,2))*1.2]);
set(gca, 'xticklabel', lbl, 'YGrid','on', 'YMinorGrid','on');
xt = get(gca, 'XTick');
text(xt, mean(data,2), num2str(mean(data,2),'%0.2f%%'),...
    'HorizontalAlignment','center', 'VerticalAlignment','bottom');

hold on
er = errorbar(mean(data,2),std(data,0,2));
er.LineStyle = 'none';
er.HandleVisibility = 'off';
hold off
```

Algorithm A.5. Matlab Code for figure VBNC 68-69 L.plantarium: (Figure 3.5)

```
clear all

data =[
5.82    5.85    5.95;
8.96    9.00    8.90;
11.87   11.70   11.95;
15.35   15.00   15.50;
15.82   15.90   15.75;
17.04   17.20   17.10];

lbl =["30d WP" "60d WP" "30d Tr" "60d Tr" "30d MD" "60d MD"];

data_mean=mean(data,2);
data_mean_sorted=sort(data_mean);

[~,~,stats]=anova1(data',lbl,'off');
c = multcompare(stats,'Display','off');
disp(c);

p=1;
val=97;
data_label(1)=char(val);
for i=1:length(data_mean)-1
   indx(i)=find(data_mean==data_mean_sorted(i));

   if abs(data_mean_sorted(i)-data_mean_sorted(i+1))<p
      data_label(i+1)=data_label(i);
   else
      data_label(i+1)=char(data_label(i)+1);
      val=val+1;
   end

end
indx(i+1)=find(data_mean==data_mean_sorted(i+1));

figure
b= bar(mean(data,2));
xtips1 = b(1).XEndPoints;
ytips1 = b(1).YEndPoints+2;
labels1 = char(sort(data_label,'descend'));
C = textscan(labels1,'%c');
text(xtips1,ytips1,C{1},'HorizontalAlignment','right',...
   'VerticalAlignment','bottom')

title('VBNC L.plantarum299v','Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');
ylabel('VBNC %', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
  'FontWeight', 'bold');
xlabel('Storage Time', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');

hold on
legend('OutletT=68-69°C', 'Orientation','horizontal','Location','southoutside',
'Interpreter','none');
```

```
b.FaceColor = '#162238';
b.EdgeColor = '#162238';

ylim([0 max(mean(data,2))*1.2]);
set(gca, 'xticklabel', lbl, 'YGrid','on', 'YMinorGrid','on');
xt = get(gca, 'XTick');
text(xt, mean(data,2), num2str(mean(data,2),'%0.2f%%'),...
    'HorizontalAlignment','center', 'VerticalAlignment','bottom');

hold on
er = errorbar(mean(data,2),std(data,0,2));
er.LineStyle = 'none';
er.HandleVisibility = 'off';
hold off
```

Algorithm A.6. Matlab Code for figure VBNC 73-75 L.rhamnosus: (Figure 3.6)

```
clear all

data =[
4.30    4.20    4.40;
8.96    9.00    8.90;
16.33   16.00   16.50;
18.19   18.00   17.90;
16.99   17.00   16.90;
23.10   23.00   23.20];

lbl =["30d WP" "60d WP" "30d Tr" "60d Tr" "30d MD" "60d MD"];

data_mean=mean(data,2);
data_mean_sorted=sort(data_mean);

[~,~,stats]=anova1(data',lbl,'off');
c = multcompare(stats,'Display','off');
disp(c);

p=1;
val=97;
data_label(1)=char(val);
for i=1:length(data_mean)-1
   indx(i)=find(data_mean==data_mean_sorted(i));

   if abs(data_mean_sorted(i)-data_mean_sorted(i+1))<p
      data_label(i+1)=data_label(i);
   else
      data_label(i+1)=char(data_label(i)+1);
      val=val+1;
   end

end
indx(i+1)=find(data_mean==data_mean_sorted(i+1));

figure
b= bar(mean(data,2));
xtips1 = b(1).XEndPoints;
ytips1 = b(1).YEndPoints+2;
labels1 = char(sort(data_label,'descend'));
C = textscan(labels1,'%c');
text(xtips1,ytips1,C{1},'HorizontalAlignment','right',...
   'VerticalAlignment','bottom')

title('VBNC L.rhamnosus','Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');
ylabel('VBNC %', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
  'FontWeight', 'bold');
xlabel('Storage Time', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');

hold on
legend('OutletT=73-75°C', 'Orientation','horizontal','Location','southoutside',
'Interpreter','none');
```

```
b.FaceColor = '#162238';
b.EdgeColor = '#162238';

ylim([0 max(mean(data,2))*1.2]);
set(gca, 'xticklabel', lbl, 'YGrid','on', 'YMinorGrid','on');
xt = get(gca, 'XTick');
text(xt, mean(data,2), num2str(mean(data,2),'%0.2f%%'),...
    'HorizontalAlignment','center', 'VerticalAlignment','bottom');

hold on
er = errorbar(mean(data,2),std(data,0,2));
er.LineStyle = 'none';
er.HandleVisibility = 'off';
hold off
```

Algorithm A.7. Matlab Code for figure VBNC 73-75 L.plantarium (Figure 3.7)

```
clear all

data =[
9.78    9.70    9.80;
4.64    4.60    4.59;
17.59   18.00   17.30;
7.21    7.10    7.30;
22.36   22.90   22.00;
7.35    7.25    7.45];

lbl =["30d WP" "60d WP" "30d Tr" "60d Tr" "30d MD" "60d MD"];

data_mean=mean(data,2);
data_mean_sorted=sort(data_mean);

[~,~,stats]=anova1(data',lbl,'off');
c = multcompare(stats,'Display','off');
disp(c);

p=1;
val=97;
data_label(1)=char(val);
for i=1:length(data_mean)-1
   indx(i)=find(data_mean==data_mean_sorted(i));

   if abs(data_mean_sorted(i)-data_mean_sorted(i+1))<p
      data_label(i+1)=data_label(i);
   else
      data_label(i+1)=char(data_label(i)+1);
      val=val+1;
   end

end
indx(i+1)=find(data_mean==data_mean_sorted(i+1));

figure
b= bar(mean(data,2));
xtips1 = b(1).XEndPoints;
ytips1 = b(1).YEndPoints+2;
labels1 = char(sort(data_label,'descend'));
C = textscan(labels1,'%c');
text(xtips1,ytips1,C{1},'HorizontalAlignment','right',...
   'VerticalAlignment','bottom')

title('VBNC L.plantarum299v','Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');
ylabel('VBNC %', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
  'FontWeight', 'bold');
xlabel('Storage Time', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');

hold on
legend('OutletT=73-75°C', 'Orientation','horizontal','Location','southoutside',
'Interpreter','none');
```

```
b.FaceColor = '#162238';
b.EdgeColor = '#162238';

ylim([0 max(mean(data,2))*1.2]);
set(gca, 'xticklabel', lbl, 'YGrid','on', 'YMinorGrid','on');
xt = get(gca, 'XTick');
text(xt, mean(data,2), num2str(mean(data,2),'%0.2f%%'),...
    'HorizontalAlignment','center', 'VerticalAlignment','bottom');
 hold on
er = errorbar(mean(data,2),std(data,0,2));
er.LineStyle = 'none';
er.HandleVisibility = 'off';
hold off
```

Algorithm A.8. Matlab Code for figure viability moisture (Figure 3.10)

```
clear all

data=[  3.51     3.98     3.94 ; 3.10      4.05     4; 3.92  3.95     3.92 ];
lbl=["15%WPI" "15%WPI+1%Trehalose" "15%WPI+2%Trehalose" ];

p=anova2(data, 1, 'off');
disp(p);

[p,t,stats]=anova1(data',lbl,'off');
c = multcompare(stats,'Display','off');
disp(c);

data_mean=mean(data,2);
data_mean_sorted=sort(data_mean);

sapma=5;
val=97;
data_label(1)=char(val);
for i=1:length(data_mean)-1
   indx(i)=find(data_mean==data_mean_sorted(i));

   if abs(data_mean_sorted(i)-data_mean_sorted(i+1))<sapma
      data_label(i+1)=data_label(i);
   else
      data_label(i+1)=char(data_label(i)+1);
      val=val+1;
   end

end
indx(i+1)=find(data_mean==data_mean_sorted(i+1));

figure
b= bar(mean(data,2));
xtips1 = b(1).XEndPoints;
ytips1 = b(1).YEndPoints*1.10;
labels1 = char(sort(data_label,'descend'));
C = textscan(labels1,'%c');
text(xtips1,ytips1,C{1},'HorizontalAlignment','right',...
   'VerticalAlignment','bottom')

title('SAMPLE      MOISTURE','Color',      [0.349019607843137      0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');
ylabel('%', 'Color', [0.349019607843137 0.349019607843137 0.349019607843137],...
   'FontWeight', 'bold');
xlabel('Materials',          'Color',          [0.349019607843137          0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');

b.FaceColor = '#162238'; %yeşil
b.EdgeColor = '#162238'; %yeşil

ylim([0 max(mean(data,2))*1.2]);
set(gca, 'xticklabel', lbl, 'XTickLabelRotation', 30,...
   'YGrid','on', 'YMinorGrid','on');

xt = get(gca, 'XTick');
```

```
text(xt, mean(data,2), num2str(mean(data,2),'%g'),...
    'HorizontalAlignment','center', 'VerticalAlignment','bottom');

hold on
er = errorbar(mean(data,2),std(data,0,2));
er.LineStyle = 'none';
hold off
```

Algorithm A.9. Matlab Code for figure viability productyield (Figure 3.11)

```
clear all

data=[ 59.36     76        77;62    74        75; 60   73        74 ];
lbl=["15%WPI" "15%WPI+1%Trehalose" "15%WPI+2%Trehalose"];

p=anova2(data, 1, 'off');
disp(p);

[p,t,stats]=anova1(data',lbl,'off');
c = multcompare(stats,'Display','off');
disp(c);


data_mean=mean(data,2);
data_mean_sorted=sort(data_mean);

p=5;
val=97;
data_label(1)=char(val);
for i=1:length(data_mean)-1
   indx(i)=find(data_mean==data_mean_sorted(i));

   if abs(data_mean_sorted(i)-data_mean_sorted(i+1))<p
      data_label(i+1)=data_label(i);
   else
      data_label(i+1)=char(data_label(i)+1);
      val=val+1;
   end

end
indx(i+1)=find(data_mean==data_mean_sorted(i+1));

figure
b= bar(mean(data,2));
xtips1 = b(1).XEndPoints;
ytips1 = b(1).YEndPoints+7;
labels1 = char(sort(data_label,'descend'));
C = textscan(labels1,'%c');
text(xtips1,ytips1,C{1},'HorizontalAlignment','right',...
   'VerticalAlignment','bottom')

title('PRODUCT YIELD','Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');
ylabel('Product Yield %', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
  'FontWeight', 'bold');
xlabel('Materials', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');

b.FaceColor = '#162238';
b.EdgeColor = '#162238';

ylim([0 max(mean(data,2))*1.2]);
set(gca, 'xticklabel', lbl, 'XTickLabelRotation', 30,...
   'YGrid','on', 'YMinorGrid','on');
```

```
xt = get(gca, 'XTick');
text(xt, mean(data,2), num2str(mean(data,2),'%0.2f%%'),...
    'HorizontalAlignment','center', 'VerticalAlignment','bottom');

hold on
er = errorbar(mean(data,2),std(data,0,2));
er.LineStyle = 'none';
hold off
```

Algorithm A.10. Matlab Code for figure viability (Figure 3.12)

```
clear all

data=[ log10(1020000000)        log10(1020000000)       log10(1030000000);
   log10(1800000000)   log10(2010000000)       log10(2010000000);
   log10(4800000000)   log10(5100000000)       log10(4900000000)];
lbl=["15%WPI" "15%WPI+1%Trehalose" "15%WPI+2%Trehalose" ];

p=anova2(data, 1, 'off');
disp(p);

[p,t,stats]=anova1(data',lbl,'off');
c = multcompare(stats,'Display','off');
disp(c);

data_mean=mean(data,2);
data_mean_sorted=sort(data_mean);

sapma=1;
val=97;
data_label(1)=char(val);
for i=1:length(data_mean)-1
   indx(i)=find(data_mean==data_mean_sorted(i));

   if abs(data_mean_sorted(i)-data_mean_sorted(i+1))<sapma
      data_label(i+1)=data_label(i);
   else
      data_label(i+1)=char(data_label(i)+1);
      val=val+1;
   end

end
indx(i+1)=find(data_mean==data_mean_sorted(i+1));

figure
b= bar(mean(data,2));
xtips1 = b(1).XEndPoints;
ytips1 = b(1).YEndPoints*1.1;
labels1 = char(sort(data_label,'descend'));
C = textscan(labels1,'%c');
text(xtips1,ytips1,C{1},'HorizontalAlignment','right',...
   'VerticalAlignment','bottom')

title('VIABILITY','Color',              [0.349019607843137              0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');
ylabel('Log(CFU/ml)',        'Color',        [0.349019607843137        0.349019607843137
0.349019607843137],...
  'FontWeight', 'bold');
xlabel('Materials',          'Color',          [0.349019607843137          0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');

b.FaceColor = '#162238';
b.EdgeColor = '#162238';

ylim([0 max(mean(data,2))*1.2]);
set(gca, 'xticklabel', lbl, 'XTickLabelRotation', 30,...
```

```
    'YGrid','on', 'YMinorGrid','on');

xt = get(gca, 'XTick');
text(xt, mean(data,2), num2str(mean(data,2), '%.2f'),...
    'HorizontalAlignment','center', 'VerticalAlignment','bottom');

hold on
er = errorbar(mean(data,2),std(data,0,2));
er.LineStyle = 'none';
hold off
```

Algorithm A.11. Matlab Code for figure viability old-new (Figure 4.10)

```
clear all

data=[ log10(32000000) log10(20000000)          log10(42000000);log10(4800)
          log10(3100)      log10(7000);log10(30000)          log10(1800)      log10(9000) ];
lbl=["Initial viability" "new method" "old method" ];

p=anova2(data, 1, 'off');
disp(p);

[~,~,stats]=anova1(data,lbl,"off");
c=multcompare(stats, Display="off");
disp(c);


data_mean=mean(data,2);
data_mean_sorted=sort(data_mean);


sapma=1;
val=97;
data_label(1)=char(val);
for i=1:length(data_mean)-1
   indx(i)=find(data_mean==data_mean_sorted(i));

   if abs(data_mean_sorted(i)-data_mean_sorted(i+1))<sapma
      data_label(i+1)=data_label(i);
   else
      data_label(i+1)=char(data_label(i)+1);
      val=val+1;
   end

end
indx(i+1)=find(data_mean==data_mean_sorted(i+1));

figure
b= bar(mean(data,2));
xtips1 = b(1).XEndPoints;
ytips1 = b(1).YEndPoints*1.1;
labels1 = char(sort(data_label,'descend'));
C = textscan(labels1,'%c');
text(xtips1,ytips1,C{1},'HorizontalAlignment','right',...
   'VerticalAlignment','bottom')

title('VIABILITY      OLD-NEW','Color',      [0.349019607843137      0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');
ylabel('Viability    Log(CFU/ml)',    'Color',    [0.349019607843137    0.349019607843137
0.349019607843137],...
  'FontWeight', 'bold');
xlabel('Produced      with      Pectin      Mixture-2%Encapsulated      Product',      'Color',
[0.349019607843137 0.349019607843137 0.349019607843137],...
   'FontWeight', 'bold');

b.FaceColor = '#162238';
b.EdgeColor = '#162238';

ylim([0 max(mean(data,2))*1.2]);
```

```
set(gca, 'xticklabel', lbl, 'YGrid','on', 'YMinorGrid','on');
xt = get(gca, 'XTick');
text(xt, mean(data,2), num2str(mean(data,2), '%.2f'),...
    'HorizontalAlignment','center', 'VerticalAlignment','bottom');

hold on
er = errorbar(mean(data,2),std(data,0,2));
er.LineStyle = 'none';
hold off
```

Algorithm A.12. Matlab Code for figure viability $CaCl_2$ (Figure 4.11)

```
clear all

data = [  log10(32000000)          log10(20000000)          log10(42000000);
log10(4800)          log10(3100)      log10(7000);
log10(7100)          log10(6000)      log10(6900) ];
lbl=["Initial viability" "new method" "new method without removal of CaCl2" ];

p=anova2(data, 1, 'off');
disp(p);

[p,t,stats]=anova1(data',lbl,'off');
c = multcompare(stats,'Display','off');
disp(c);

data_mean=mean(data,2);
data_mean_sorted=sort(data_mean);

sapma=1;
val=97;
data_label(1)=char(val);
for i=1:length(data_mean)-1
   indx(i)=find(data_mean==data_mean_sorted(i));

   if abs(data_mean_sorted(i)-data_mean_sorted(i+1))<sapma
      data_label(i+1)=data_label(i);
   else
      data_label(i+1)=char(data_label(i)+1);
      val=val+1;
   end

end
indx(i+1)=find(data_mean==data_mean_sorted(i+1));

figure
b= bar(mean(data,2));
xtips1 = b(1).XEndPoints;
ytips1 = b(1).YEndPoints+0.5;
labels1 = char(sort(data_label,'descend'));
C = textscan(labels1,'%c');
text(xtips1,ytips1,C{1},'HorizontalAlignment','right',...
   'VerticalAlignment','bottom')

title('VIABILITY - CaCl2','Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');
ylabel('Log(CFU/ml)', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
  'FontWeight', 'bold');
xlabel('Edible Film Produced with Pectin Mixture-2%Encapsulated Product', 'Color',
[0.349019607843137 0.349019607843137 0.349019607843137],...
   'FontWeight', 'bold');

b.FaceColor = '#162238';
b.EdgeColor = '#162238';

ylim([0 max(mean(data,2))*1.3]);
set(gca, 'xticklabel', lbl, 'YGrid','on', 'YMinorGrid','on');
```

```
xt = get(gca, 'XTick');
text(xt, mean(data,2), num2str(mean(data,2),'%.2f'),...
    'HorizontalAlignment','center', 'VerticalAlignment','bottom');

hold on
er = errorbar(mean(data,2),std(data,0,2));
er.LineStyle = 'none';
hold off
```

Algorithm A.13. Matlab Code for figure viability quantity (Figure 4.13)

```
clear all

data = [  log10(32000000)          log10(20000000)          log10(42000000);
log10(5800)          log10(2200)      log10(10000) ;
log10(4800)          log10(3100)      log10(7000)];
lbl=["Initial viability" "Pectin Mixture-2%" "Pectin Mixture-10%" ];

p=anova2(data, 1, 'off');
disp(p);

[p,t,stats]=anova1(data',lbl,'off');
c = multcompare(stats,'Display','off');
disp(c);

data_mean=mean(data,2);
data_mean_sorted=sort(data_mean);

sapma=1;
val=97;
data_label(1)=char(val);
for i=1:length(data_mean)-1
   indx(i)=find(data_mean==data_mean_sorted(i));

   if abs(data_mean_sorted(i)-data_mean_sorted(i+1))<sapma
      data_label(i+1)=data_label(i);
   else
      data_label(i+1)=char(data_label(i)+1);
      val=val+1;
   end

end
indx(i+1)=find(data_mean==data_mean_sorted(i+1));

figure
b= bar(mean(data,2));
xtips1 = b(1).XEndPoints;
ytips1 = b(1).YEndPoints+0.5;
labels1 = char(sort(data_label,'descend'));
C = textscan(labels1,'%c');
text(xtips1,ytips1,C{1},'HorizontalAlignment','right',...
   'VerticalAlignment','bottom')

title('VIABILITY - Quantity','Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');
ylabel('Log(CFU/ml)', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
  'FontWeight', 'bold');
xlabel('Edible Film Produced with Pectin Mixture (2%/10%) Encapsulated Product-new
method without removal of CaCl2', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');

b.FaceColor = '#162238';
b.EdgeColor = '#162238';

ylim([0 max(mean(data,2))*1.3]);
```

```
set(gca, 'xticklabel', lbl, 'YGrid','on', 'YMinorGrid','on');
xt = get(gca, 'XTick');
text(xt, mean(data,2), num2str(mean(data,2), '%.2f'),...
    'HorizontalAlignment','center', 'VerticalAlignment','bottom');

hold on
er = errorbar(mean(data,2),std(data,0,2));
er.LineStyle = 'none';
hold off
```

Algorithm A.14. Matlab Code for figure viability pour-on (Figure 4.15)

```
clear all

data = [  log10(32000000)          log10(20000000)          log10(42000000);
log10(330000)       log10(1400000)            log10(3800000) ;
log10(5800)          log10(5200)       log10(10000)];
lbl=["Initial viability" "poured on edible film surface" "mixed with edible film" ];

p=anova2(data, 1, 'off');
disp(p);

[p,t,stats]=anova1(data',lbl,'off');
c = multcompare(stats,'Display','off');
disp(c);

data_mean=mean(data,2);
data_mean_sorted=sort(data_mean);

sapma=1;
val=97;
data_label(1)=char(val);
for i=1:length(data_mean)-1
   indx(i)=find(data_mean==data_mean_sorted(i));

   if abs(data_mean_sorted(i)-data_mean_sorted(i+1))<sapma
      data_label(i+1)=data_label(i);
   else
      data_label(i+1)=char(data_label(i)+1);
      val=val+1;
   end

end
indx(i+1)=find(data_mean==data_mean_sorted(i+1));

figure
b= bar(mean(data,2));
xtips1 = b(1).XEndPoints;
ytips1 = b(1).YEndPoints+0.5;
labels1 = char(sort(data_label,'descend'));
C = textscan(labels1,'%c');
text(xtips1,ytips1,C{1},'HorizontalAlignment','right',...
   'VerticalAlignment','bottom')


title('VIABILITY - Pour-On','Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');
ylabel('Log(CFU/ml)', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
  'FontWeight', 'bold');
xlabel('The Edible Film Produced With 10% Encapsulated Product', 'Color',
[0.349019607843137 0.349019607843137 0.349019607843137],...
   'FontWeight', 'bold');

b.FaceColor = '#162238';
b.EdgeColor = '#162238';

ylim([0 max(mean(data,2))*1.3]);
```

```
set(gca, 'xticklabel', lbl, 'XTickLabelRotation', 30,...
    'YGrid','on', 'YMinorGrid','on');
xt = get(gca, 'XTick');
text(xt, mean(data,2), num2str(mean(data,2), '%.2f'),...
    'HorizontalAlignment','center', 'VerticalAlignment','bottom');

hold on
er = errorbar(mean(data,2),std(data,0,2));
er.LineStyle = 'none';
hold off
```

Algorithm A.15. Matlab Code for figure viability storage (Figure 4.16)

```
clear all

data=[  log10(330000)  log10(1400000) log10(3800000);
log10(1700000) log10(2100000) log10(870000);
log10(6300000) log10(2000000) log10(8200000);
log10(753000)  log10(940000)  log10(100000);
log10(100000)  log10(820000)  log10(760000);
log10(71000)   log10(130000)  log10(100000);
log10(20000)   log10(92000)   log10(90000) ];

lbl=["Initial" "1 day" "7 days" "15 days" "30 days" "60 days" "90 days"];

p=anova2(data, 1, 'off');
disp(p);

[p,t,stats]=anova1(data',lbl,'off');
c = multcompare(stats,'Display','off');
disp(c);

data_mean=mean(data,2);
data_mean_sorted=sort(data_mean);

sapma=1;
val=97;
data_label(1)=char(val);
for i=1:length(data_mean)-1
   indx(i)=find(data_mean==data_mean_sorted(i));

   if abs(data_mean_sorted(i)-data_mean_sorted(i+1))<sapma
      data_label(i+1)=data_label(i);
   else
      data_label(i+1)=char(data_label(i)+1);
      val=val+1;
   end

end
indx(i+1)=find(data_mean==data_mean_sorted(i+1));

figure
b= bar(mean(data,2));
xtips1 = b(1).XEndPoints;
ytips1 = b(1).YEndPoints*1.1;
labels1 = char(sort(data_label,'descend'));
C = textscan(labels1,'%c');
text(xtips1,ytips1,C{1},'HorizontalAlignment','right',...
   'VerticalAlignment','bottom')

title('VIABILITY AT STORAGE','Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');
ylabel('Viability Log(CFU/ml)', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
  'FontWeight', 'bold');
xlabel('Storage Time', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');
```

```
b.FaceColor = '#162238';
b.EdgeColor = '#162238';

ylim([0 max(mean(data,2))*1.2]);
set(gca, 'xticklabel', lbl,    'YGrid','on', 'YMinorGrid','on');

xt = get(gca, 'XTick');
text(xt, mean(data,2), num2str(mean(data,2),'%.2f'),...
   'HorizontalAlignment','center', 'VerticalAlignment','bottom');

hold on
er = errorbar(mean(data,2),std(data,0,2));
er.LineStyle = 'none';
hold off
```

Algorithm A.16. Matlab Code for figure viability saliva pectin (Figure 4.18)

```
clear all

data=[  log10(5200)     log10(4400)     log10(4900);
log10(72000)    log10(71000)    log10(73000);
log10(125000)  log10(115000)  log10(120000);
log10(260000)  log10(250000)  log10(255000);
log10(357000)  log10(350000)  log10(360000);
log10(443000)  log10(430000)  log10(440000);
log10(578000)  log10(580000)  log10(590000);
log10(302000)  log10(310000)  log10(350000)
 ];

lbl=["5" "15" "25" "35" "45" "55" "65" "120"];

p=anova2(data, 1, 'off');
disp(p);

[p,t,stats]=anova1(data',lbl,'off');
c = multcompare(stats,'Display','off');
disp(c);

data_mean=mean(data,2);
data_mean_sorted=sort(data_mean);

sapma=1;
val=97;
data_label(1)=char(val);
for i=1:length(data_mean)-1
   indx(i)=find(data_mean==data_mean_sorted(i));

   if abs(data_mean_sorted(i)-data_mean_sorted(i+1))<sapma
      data_label(i+1)=data_label(i);
   else
      data_label(i+1)=char(data_label(i)+1);
      val=val+1;
   end

end
indx(i+1)=find(data_mean==data_mean_sorted(i+1));

figure
b= bar(mean(data,2));
xtips1 = b(1).XEndPoints;
ytips1 = b(1).YEndPoints*1.1;
labels1 = char(sort(data_label,'descend'));
C = textscan(labels1,'%c');
text(xtips1,ytips1,C{1},'HorizontalAlignment','right',...
   'VerticalAlignment','bottom')


title('VIABILITY AT SALIVA - PECTIN','Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');
ylabel('Viability  Log(CFU/ml)',  'Color',  [0.349019607843137  0.349019607843137
0.349019607843137],...
  'FontWeight', 'bold');
```

```
xlabel('Time (Minute)', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
    'FontWeight', 'bold');

b.FaceColor = '#162238';
b.EdgeColor = '#162238';

ylim([0 max(mean(data,2))*1.2]);
set(gca, 'xticklabel', lbl,    'YGrid','on', 'YMinorGrid','on');

xt = get(gca, 'XTick');
text(xt, mean(data,2), num2str(mean(data,2),'%.2f'),...
    'HorizontalAlignment','center', 'VerticalAlignment','bottom');

hold on
er = errorbar(mean(data,2),std(data,0,2));
er.LineStyle = 'none';
hold off
```

Algorithm A.17. Matlab Code for figure viability saliva alginate (Figure 4.19)

```
clear all

data=[  log10(1200)     log10(1150)     log10(1240);
log10(157000)  log10(160000)  log10(165000);
log10(643000)  log10(650000)  log10(665000);
log10(144000)  log10(200000)  log10(160000);
log10(168000)  log10(155000)  log10(1450000);
log10(155000)  log10(150000)  log10(148000);
log10(86400)   log10(85000)   log10(79000);
log10(18400)   log10(19000)   log10(18400)
 ];

lbl=["5" "15" "25" "35" "45" "55" "65" "120"];

p=anova2(data, 1, 'off');
disp(p);

[p,t,stats]=anova1(data',lbl,'off');
c = multcompare(stats,'Display','off');
disp(c);

data_mean=mean(data,2);
data_mean_sorted=sort(data_mean);


sapma=1;
val=97;
data_label(1)=char(val);
for i=1:length(data_mean)-1
   indx(i)=find(data_mean==data_mean_sorted(i));

   if abs(data_mean_sorted(i)-data_mean_sorted(i+1))<sapma
      data_label(i+1)=data_label(i);
   else
      data_label(i+1)=char(data_label(i)+1);
      val=val+1;
   end

end
indx(i+1)=find(data_mean==data_mean_sorted(i+1));


figure
b= bar(mean(data,2));
xtips1 = b(1).XEndPoints;
ytips1 = b(1).YEndPoints*1.1;
labels1 = char(sort(data_label,'descend'));
C = textscan(labels1,'%c');
text(xtips1,ytips1,C{1},'HorizontalAlignment','right',...
   'VerticalAlignment','bottom')


title('VIABILITY AT SALIVA - SODIUM ALGINATE','Color', [0.349019607843137
0.349019607843137 0.349019607843137],...
   'FontWeight', 'bold');
ylabel('Viability Log(CFU/ml)', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
```

```
   'FontWeight', 'bold');
xlabel('Time (Minute)', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');

b.FaceColor = '#162238';
b.EdgeColor = '#162238';

ylim([0 max(mean(data,2))*1.2]);
set(gca, 'xticklabel', lbl,    'YGrid','on', 'YMinorGrid','on');

xt = get(gca, 'XTick');
text(xt, mean(data,2), num2str(mean(data,2),'%.2f'),...
   'HorizontalAlignment','center', 'VerticalAlignment','bottom');
 hold on
er = errorbar(mean(data,2),std(data,0,2));
er.LineStyle = 'none';
hold off
```

Algorithm A.18. Matlab Code for figure viability effectiveness (Figure 5.2)

```
clear all

data=[ 6.23      5.76      7.57      5.52;6.32          6.52      7.72      6.16;5.94          6.08
          7.79 6.15;5.97   5.17      7.08      4.15 ];
lbl=["E.Coli" "S. Aureus " "C. Albicans" "P. aeruginosa"];

p=anova2(data, 1, 'off');
disp(p);

data_ecoli =[5.23 6.23 7.23; 5.76 5.67 5.85; 6.57 7.57 8.57; 5.63 5.49 5.43];
lbl_ecoli =["initial probiotic" "final probiotic" "initial pathogen" "final pathogen"];

[~,~,stats]=anova1(data_ecoli',lbl_ecoli,'off');
c = multcompare(stats,'Display','off');
disp("e-coli");
disp(c);


data_saures =[5.32 6.32 7.32; 6.51 6.61 6.43; 6.72 7.72 8.72; 6.00 6.36 6.11];

[~,~,stats]=anova1(data_saures',lbl_ecoli,'off');
c = multcompare(stats,'Display','off');
disp("s.aureus");
disp(c);


data_calbicans =[4.94 5.94 6.94; 6.04 6.30 5.90; 6.79 7.79 8.79; 6.43 6.23 5.80];

[~,~,stats]=anova1(data_calbicans',lbl_ecoli,'off');
c = multcompare(stats,'Display','off');
disp("C.albicans");
disp(c);

data_aeruginosa =[4.97 5.97 6.97; 4.94 5.08 5.49; 6.08 7.08 8.08; 4.72 4.83 4.72];

[~,~,stats]=anova1(data_aeruginosa',lbl_ecoli,'off');
c = multcompare(stats,'Display','off');
disp("P.aeruginosa");
disp(c);

[~,~,stats]=anova1(data',lbl,'off');
c = multcompare(stats,'Display','off');
disp("MultiCompare");
disp(c);


figure
b= bar(data);
xtips1 = b(1).XEndPoints;
ytips1 = b(1).YEndPoints+( 100000000);


b(1).FaceColor = '#162238';
b(1).EdgeColor = '#162238';
b(2).FaceColor = '#1156cc';
b(2).EdgeColor = '#1156cc';
b(3).FaceColor = '#009a43';
```

```
b(3).EdgeColor = '#009a43';
b(4).FaceColor = '#64d413';
b(4).EdgeColor = '#64d413';
set(gca, 'xticklabel', lbl, 'YGrid','on', 'YMinorGrid','on');

legend('Viability of L. rhamnosus in Films', 'Viabilty of L. rhamnosus after mixed with
pathogens', ...
    'Control (Only pathogen in broth)', 'Viability of Pathogen mixed with film', ...
    'Orientation','horizontal','Location','southoutside', 'Interpreter','none', 'NumColumns', 2);
title('EFFECTIVENESS','Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
    'FontWeight', 'bold');
ylabel('Viability Log(CFU/g)', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
   'FontWeight', 'bold');
xlabel('Pathogens', 'Color', [0.349019607843137 0.349019607843137
0.349019607843137],...
    'FontWeight', 'bold');
xt = get(gca, 'XTick');
```