REPUBLIC OF TURKEY

YILDIZ TECHNICAL UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

PRODUCTION OF FUNCTIONAL YOGURT DRINK, APPLE AND ORANGE JUICE USING NANO-ENCAPSULATED L. BREVIS WITHIN SODIUM ALGINATE-BASED BIOPOLYMERS

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DOCTOR OF PHILOSOPHY THESIS

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Mohammed Jawad MOHAISEN

Signature

Dedicated to my family

and my friends

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Mohammed Jawad MOHAISEN

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LIST OF SYMBOLS

°C	Celsius
CFU	Colony Forming Unit
f	Frequency
Hz	Hertz
kHz	Kilo Hertz
min	Minute
P	Statistical Significance
pH	Power of Hydrogen
%	Percentage

LIST OF ABBREVIATIONS

E B	Encapsulated Bacteria
F B	Free Bacteria
FT-IR	Fourier-transform Infrared
g	Gram
L	Litter
LAB	Lactic Acid Bacteria
Log	Logarithm
mg	Milligram
mL	Millilitre
NMR	Nuclear Magnetic Resonance
SEM	Spectroscopy, Scanning Electron Microscopy
TGA	Thermogravimetric Analysis
XRD	X-ray Diffraction

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ABSTRACT

Production of Functional Yohgurt Drink, Apple and Orange Juice Using Nano-Encapsulated *L. Brevis* Within Sodium Alginate-Based Biopolymers

Mohammed Jawad MOHAISEN

Department of Food Engineering

Doctor of Philosophy Thesis

Advisor: Assoc Prof. Dr. Muhammed Zeki DURAK

Co-advisor: Prof. Dr. Mustafa Tahsin YILMAZ

This study aims to improve the functional properties of ayran, apple and orange juice, which were produced by the addition of free and encapsulated probiotics. Thus, probiotic *L. brevis* E25 bacterial cells were encapsulated in sodium alginate using the electrospinning method. In this study, fourier-transform infrared (FT-IR) spectroscopy, scanning electron microscopy (SEM), XRD, and Nuclear Magnetic Resonance (NMR) techniques were used to characterize the *L. brevis* encapsulated with nanofiber. The free and nano-encapsulated probiotic bacteria were inoculated into ayran, apple and orange juice, where bacterial viability, pH, Brix, and color were assessed every three days for the ayran and each week for the apple and orange juice for a six-week period. The encapsulated probiotic microorganisms showed an important survival ratio in the three products comparing to the free probiotic bacteria, where the pH and the Brix showed no decreasing leading to conclude that the nano-encapsulation of probiotic *L. brevis* cell using the electrospinning method lead to functional yet more stable food and have the potential of increased benefits.

Keywords: Nanoencapsulation; probiotic; *L. brevis*; electrospinning; ayran; fruit juice.

ÖZET

Sodyum Aljinet Biyopolimo ile Nano Encapsile Edilen *L. brevis* Biyopolimesi ile Kullanılarak Fonksiyonel Ayran , Elma ve Portokal Suyu Üretim

Mohammed Jawad MOHAISEN

Gıda Mühendisliği Bölümü

Doktora Tezi

Danışman: Doç. Dr. Muhammed Zeki DURAK

Eş-Danışman: Prof. Dr. Mustafa Tahsin YILMAZ

Bu çalışmanın amacı, serbest ve kapsüllenmiş probiyotiklerin eklenmesiyle üretilen yoğurtlu içecek, elma ve portakal suyunun fonksiyonel özelliklerini iyileştirmekti.Bu amaçla probiyotik *L. brevis* E25 bakteri hücreleri, elektrospinning yöntemi ile sodyum aljinatta kapsüllenmiştir. Bu çalışmada, fourier-transform kızılötesi (FT-IR) spektroskopisi, taramalı elektron mikroskobu (SEM), XRD ve Nükleer Manyetik Rezonans (NMR) teknikleri, nanofiber ile kaplanmış L. Brevis'i karakterize etmek için kullanılmıştır. Serbest ve nano kapsüllenmiş probiyotik bakteriler, ayran, elma ve portakal suyuna inoküle edilmiştir. Altı hafta boyunca ayran için her üç günde bir elma ve portakal suyu için her hafta örnek alınarak bakteri canlılığı, pH, Brix ve renk ölçümleri alınmıştır. Enkapsüle probiyotik organizmalar her üç üründe, serbest probiyotik bakterilere kıyasla önemli oranda hayatta kalma oranı göstermiştir. Bu sonuç ile pH ve Brix değerlerinin azalma

göstermemesi elektrospin metoduyla probiyotik *L.brevis'in* nanoenkapsülasyonunun fonksiyonel ürünün stabilitesini sağladığı ve ürünün potansiyel faydalarını arttırdığı sonucuna ulaşılmıştır.

Anahtar kelimeler: Nanoenkapsülasyon; probiyotik; *L. brevis*; elektrospinning; ayran; meyve suyu.

1
Introduction

1.1 Literature Review

All food and components need to be processed, stored and safely prepared to consumers either in mega packaging or packed and or encapsulated in proper materials. New developments in the preservation and packaging of food have led to a greater variety and range of safe and healthier products that proposed for people of all ages (Biji, Ravishankar et al. 2015, Augustin, Riley et al. 2016).

Utilization of proper preservation and packaging methods not only can help to prevent food deterioration and poisoning it even helps to improve the nutritional and functional values of food and additives. Sound knowledge of different packaging techniques for food and food additives would normally stabilize and improve the eating quality parameters and flavor of products and meals (Biji, Ravishankar et al. 2015, Augustin, Riley et al. 2016, Wyrwa and Barska 2017).

This module of research explains the micro- and the nanotechnology used in preserving probiotics that aimed to be incorporated into dairy and juice products, and how this technology affects the functionality, texture, color and flavor of Turkish ayran, apple and orange juice as a food model.

Encapsulation is known as a coating or covering a specific material using thin polymeric material to produce particles with a very small size to be known as microcapsules or a nano-capsules, depending on the size of the particles (Gharsallaoui, Roudaut et al. 2007).

While nano-technology is described as the ability to create, exploit and manipulate atoms and molecules (food components and menials) on the smallest of scales; the scale of nanometer (nm) which is a billion part of a meter that deals with anything measuring from 1 to 1000nm, such as probiotics, where the used polymers to cover and protect the main core which is the probiotic cell in this case from the extern medium that can easily stimulate the degradation of the covering material

so the cell can be released in the desired place and time (Silva, Fries et al. 2014, Suave, Dall et al. 2019).

Lately, the encapsulation has been widely used in the food industry to coat several cores and particles, such as microorganisms, antioxidant compounds, fatty acids and also flavor compounds, that are highly volatile all these using nano-encapsulation techniques (Gharsallaoui, Roudaut et al. 2007, Khan, Shinwari et al. 2011).

There are several products commercially available, such as dairy products, meat products, instant drinks (tea, coffee and skimmed milk), which are already benefiting from encapsulation and nanotechnology that both offer a wide range of opportunities in all the parts of the food industry.

In this direction, there are three main and precise aims reached using the microencapsulation and nanotechnology methods which can be split into food health approaches where nano-carrier used in the bio-delivery system of nutrients, ingredients, organic and inorganic additives and supplements, food safety and packaging applications, bio-plastic polymers, nano-surfactant, smart coatings, bio-degradable films and nanosensors are implemented to improve mechanical, physical, microbial and functional traits of foods and functional food approach, nano-sized bioactive components, such as peptides, antioxidants, functional lipids, and other nutraceuticals, were applied as therapeutic and functional materials (Sekhon 2010, Murugesan and Orsat 2011, Corbo, Bevilacqua et al. 2014, Contado 2015).

The other method of nano-encapsulation that is largely used is electro-spinning, in the recent years, electro-spinning, became one of the most important methods of nano-encapsulation, for being practical and very fast comparing to the traditional methods, producing a high amount of the fiber yet without applying heat to the main core which is the main advantage of this method where the structure and the nature of the bioactive substance is safe reaching by that all the nano-encapsulation goals (Paques 2015).

There are varied types of coating materials, and the most frequent coating material is alginate, especially in the encapsulation of probiotics; alginate is a water-soluble gelling polysaccharide recognized as safe (GRAS) for human consumption (Kailasapathy 2009, De Vos, Devroey et al. 2010).

Probiotic bacteria are widely used in functional food products for having many benefits to human health. However, probiotic bacteria can only show their actual effects when they reach the ideal environment inside the human body,

For this reason the encapsulation is needed to create a covering material that serve as a protective barrier from the outside medium of the product itself maintaining by that its shelf life and from the gastrointestinal tract, where the coating material degrades at the perfect spot which is usually the intestines (Bhushani and Chinnaswamy 2014, Başar, Castro et al. 2017).

More in-depth, the effects of nano-encapsulated probiotics on the eating quality parameters, including texture and rheological traits, have not yet deeply studied and remained under-researched. Thus, the present study aims to investigate the nano-encapsulated probiotics application in providing functional foods using electrospinning technology.

1.2 Objective of the Thesis

Probiotic bacteria, a microorganism that has a very important role in maintaining human being health and have been widely used in recent years in several domains especially in functional food production, yet the delivery of this microorganism to the inside human body so it can show it great benefits without been effected by the product proprieties or the human gastro-tract is the tricky task, there for the encapsulation of probiotic cells is currently gaining attention to increase the viability of this bacteria in the producing products without damaging the cell or causing any changes in the product properties and shelf life.

Thus, the research aimed to produce functional food items, one of the most consumed dairy products in Turkey “ayran” and two of the most common fruit juices “apple and orange” enriched with encapsulated probiotic *L. brevis* using the electrospinning method.

1.3 Hypothesis

The nano-encapsulation can be applied to encapsulate *L. brevis* as a probiotic microorganism to produce functional yogurt drink, apple and orange juice using sodium alginate as a biopolymer.

Therefore, it is hypothesized that the coating material can break down and the probiotic bacteria cannot survive since the three chosen food models are known by their high acidity and that are very hard to be enriched with probiotic cells, also in the other hand the nano-encapsulation using the electrospinning technique with sodium alginate biopolymer which is hard to be controlled as a coating material.

The results of this study will allow to clear the path on the possible industrial applications of *L. brevis* as a great probiotic cell applying the electrospinning technique as a nanotechnology method to deliver a very active probiotic bacteria to the human body and also to improve the functional, nutritional and sensorial properties of apple and orange juices and dairy products, namely Turkish ayran.

2
General Information

2.1 Probiotic bacteria

Probiotics, this term alludes to the live microorganisms that managed insufficient sums and cause no harms to the host on the contrary it gives it a many advantages a specially if the host is a human being, However, probiotic bacteria can only show their actual effects and benefits when they reach the medium where all the conditions are optimum for their growth, such as considerable increase in the drain, being steady and suitable amid capacity, so they can be controlled and consolidated in sustenance items without losing the practicality or viability (FAO/OMS, 2001).

All that will be in the intestines, a microorganism is perceived as probiotic in the event that it is a typical inhabitant of the gastrointestinal tract survives the section through the gut and keeps up the feasibility in the digestive system (Kechagia, Basoulis et al. 2013, Fijan 2014, Markowiak and Śliżewska 2017).

Lim et al. (Lim, Wang et al. 2004) consider the bacterial species that exist in the human intestines which tolerate acid and bile selected as probiotic strains, such as *Streptococcus, Lactobacillus* and *Bifidobacterium*, and that the good probiotics should present their antimicrobial actions particularly to the pathogens in the gastrointestinal system (Fijan 2014, Prabhurajeshwar and Chandrakanth 2019).

Probiotic bacteria have many benefits to the human health and confer a variety of medical advantages related with the ingestion of probiotic bacteria including the control of the magnification of unwanted microorganisms in the intestines by the anti-microbial substances they produce, such as acetic acid, bacteriocin, lactic acids and hydrogen peroxide (Dogan and Celik 2012, Fijan 2014, Piqué, Berlanga et al. 2019), which cause the inhibition of the adhesion of pathogenic genera including *Escherichia, Clostridium*, *Salmonella* and *Campylobacter* to the intestinal lumen.

Also, they destroy the carcinogens and eliminates the cancer-causing effects of these substances and thus have a cancer-preventing effect (Ozcan and Altun 2015).

The probiotics microorganisms increase the utilization of lactose and calcium, which have intrinsic thrombotic and cholesterol-lowering effects by stimulate it absorption, lowering of blood ammonia levels, synthesis of B vitamins and inhibition of tumor formation and strengthen the immune system ((Ashraf and Shah 2014, Liu, Lkhagva et al. 2018).

Probiotic microscopic organisms likewise delivered substances called bacteriocins, which goes about as characteristic anti-infection agents to execute nettlesome microorganisms (Oyetayo, Adetuyi et al. 2004).

Several factors are contributing to the control of probiotics bacteria in the digestive tract of the human being (Gilliland 1979). These factors include gastric juice, bile, fatty acids, organic acids, lysozyme, and antibiotics.

The primary barrier to the survival of salutary probiotic microorganisms in the gut is the gastric acid with the inhibitory action being cognate to pH and hydrochloric acid (Varankovich, Nickerson et al. 2015, Karami, Roayaei et al. 2017).

The majority of probiotics are strains of various types of *Bifidobacterium* and *Lactobacillus* (Vlasova, Kandasamy et al. 2016, Azad, Sarker et al. 2018). Lactobacilli are more resistant to the low pH, pancreatic juice and bile conditions and could successfully transit the human stomach conditions and functions effectively comparing to the *Bifidobacteria*. Nonetheless, most of the studies in this century are focused on *Lactobacillus* because of the reputation of *Bifidobacterium* as being difficult to work with and maintain (Lebeer, Vanderleyden et al. 2008, Ruiz, Ruas-Madiedo et al. 2011).

2.2 Lactic acid bacteria (LAB)

Lactic acid bacteria (Lactic acid bacterium) (LAB) is a group of bacteria that convert and transform sugar in to lactic acid is known to be one of the major group of probiotic, the most popular, widely available, the well-studied and reliable and very easy and to work with comparing to the author probiotics in general where some of LAB can survive even in very low pH of 1, such as *L. acidophilus*, *L. brevis*

(Axelsson 2004, Ozogul and Hamed 2015, Linares, Gómez et al. 2017, Zielińska and Kołożyn-Krajewska 2018).

LAB bacteria have a good antibacterial activity against several pathogenic strains, such as *E. coli, S. aureus* and, *S. Typhi*, *Serratia marcescens, Ps. aeruginosa*, and *Candida albicans* (Al-Bayati and Al-Mola 2008, Liu, Meng et al. 2017).

This group of bacteria gather in incorporated agents of the class *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Pediococcus*. The genus *Lactobacillus* was divided by Orla-Jensen (1943) into three main groups i.e. *Thermobacterium*, *Streptobacterium* and *Betabacterium* on the substructure of optimum magnification temperature and the end product of fermentation.

The species within these groups further can be classified using biochemical, physiological characteristics, where the *Lactobacillus* class is one of the most used LABs in the food production industry for having great functional properties (Cai, Kumai et al. 1999, Wirawati, Sudarwanto et al. 2019).

2.2.1. Lactobacillus

Lactobacillus is a heterogeneous group of Gram-positive bacteria, where the cells are nonmotile rods with a tendency to form chains and are non-spore-forming. *Lactobacillus* has a fermentative metabolism, are facultative anaerobes, their surface growth on solid media generally is enhanced by anaerobiosis, increased carbon dioxide and reduced oxygen pressure. Strictly aerobic conditions are commonly growth inhibitory.

Lactobacillus growth temperature is between 2 and 53°C, with optimal growth at 30-40°C, they grow at acidic conditions with optimal pH values between 5.5 and 6.2. Usually, growth occurs since pH 5.0 or even less and the growth rate is reduced in neutral or initially alkaline conditions (Tannock 2004, Hammes and Hertel 2006, 2015).

Lactobacillus shares with other lactic acid bacteria properties to inhibit the growth of competing for undesired microorganisms and thus to prevent food spoilage. Nowadays, there are 96 genus and species of *Lactobacillus* and 16 subspecies reported and their pathogenicity is absent in all of them. Infections arise from

Lactobacillus species are very rare and have been estimated to represent 0.05-0.48% of all cases of infective endocarditis and bacteraemia. In the vast majority of these cases an underlying disease indicated a predisposition of the patients (Bergey's Manual of Systematic Bacteriology, 2001; Rattanachaikunsopon and Phumkhachorn 2010, Markowiak and Śliżewska 2017).

Lactobacillus bacteria are widely found in nature and are easily isolated from milk, milk products, soil, water, meat, vegetables, beverages and dairy products (Sornplang and Piyadeatsoontorn 2016, Corsetti, Prete et al. 2018).

In the human body, Lactobacilli are found in the mouth, lower intestine and vagina (Könönen 2015, Bratcher 2018). In the last fifteen years, the genus *Lactobacillus* has contained more than 80 species, which are found in raw milk and dairy products (Coeuret et al., 2003).

All the *Lactobacillus* strains were reported as antibacterial activity against pathogens and identified as bacteriocin producing cultures were found to inhibit toxic bacterial human pathogens, such as against *Escherichia coli, Pseudomonas aeruginosa, Streptococcus sp, Staphylococcus aureus, Bacillus cereus, Enterococcus faecalis, Vibrio parahaemolyticus* and *Micrococcus luteus*. (Biswas, Upadhayay et al. 2017, Karami, Roayaei et al. 2017, Sharma, Singh et al. 2017).

Lactobacillus bacteria have the capacity to produce a bacteriocin that have a great antibacterial property where the activities of this bacteriocin is not affected by the treatment with lipase catalase and amylase yet it can lose its power after been treated with the proteolytic enzymes trypsin, chymotrypsin and pepsin (Todorov and Dicks 2006, Lim and Im 2007 , Topisirovic et al., (2006), where some strains of *Lactobacillus* can survive even when the host is under antibiotics treatment, such as *L. acidophilus* (Rodriguez-Ferri et al., 1979).

2.2.2. *Lactobacillus brevis*

Lactobacillus brevis one of the oldest discovered probiotic and the most common and widely found *Lactobacillus*, was the chosen cell for this study based on its great proprieties. As all microorganisms, this cell is classified as follows:

Table 2.1 Scientific classification of *Lactobacillus brevis*

Class	Bacilli
Order	Lactobacillales
Family	Lactobacillaceae
Genus	*Lactobacillus*
Specie	*brevis*

Since *Lactobacillus brevis* belong to the *Lactobacillus* species so it is automatically a gram-positive species with a rod-shaped cell structure with no spore-forming propriety; the main metabolic pathway which is hetero-fermentation includes transforming sugar into lactic acid-producing during this biochemical reaction carbon dioxide (Mayo, van Sinderen et al. 2008, Giraffa, Chanishvili et al. 2010).

Lactobacillus brevis has been widely studied in the recent years due to its major properties and benefits, this species is known with its great capacity to the low pH resistance where it can tolerate pH 1 for more than 4 hours (Utama, Hanim et al. 2018) and to survive even in a temperature that can reach 50°C, also it can tolerate high bile salt concentration (Ogunshe and Olasugba 2009).

So far, sixteen species of *L. brevis* have been identified; this probiotic bacterium can be found in several products, such as fermented foods as milk, yogurt, cheese, and inside the human body, especially and widely in the vaginal flora (Ghosh, Beniwal et al. 2019).

L. brevis offer a lot of benefits to human health, it is one of the strains with antibacterial capacity against *Ps. aeruginosa*, *K. pneumonia*, *E. coli*, and *Citrobacter aerogenes*. The most used strains as probiotics and the most reported in the literature belong to *Bifidobacterium* and *Lactobacillus* genera; it also helps in improving the human immune system (Fijan 2014, Prabhurajeshwar and Chandrakanth 2017).

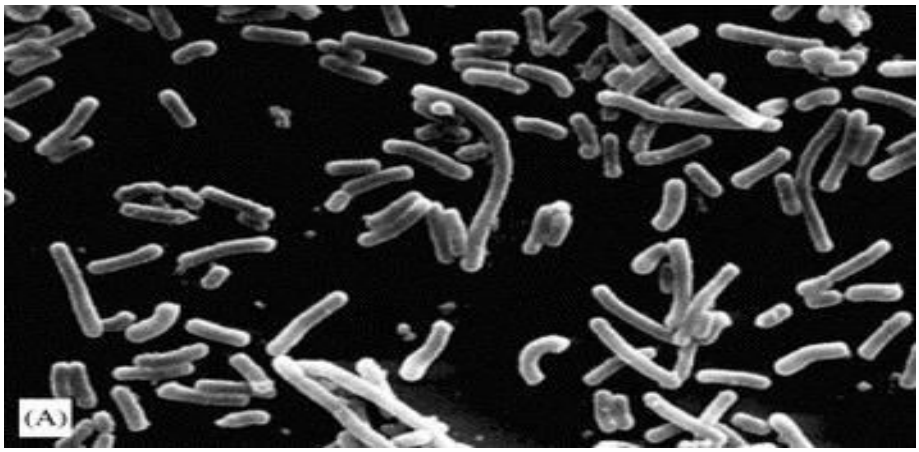

Figure 2.1. SEM of *L. brevis* (*2.000) (Elez-Martínez, Escolà-Hernández et al. 2005)

2.3 Encapsulation

In a broader sense, encapsulation can be defined as the technology of holding sensitive compounds in/onto functional matrices, as illustrated. This research focused mainly on the aspects of entrapment and containment procedures. In practice, the encapsulation step includes the complete envelopment of pre-selected core materials in either liquid, solid, or gaseous phase within a defined natural or synthetic porous or impermeable membrane using different techniques (Schleining 2007, Rathore, Desai et al. 2013, Engert, Balduini et al. 2016).

The encapsulation of microbial cells is a physicochemical or mechanical process to retain the microbes within other material to produce particles with a diameter between 1 to 1000nm.

The encapsulation process has several purposes in the food industry, such as controlling oxidative reactions, masking flavors, colors and odors, but the main purpose of probiotic encapsulation is to enhance their stability.

Probiotics are protected by encapsulation because capsules protect cells from unfavorable environmental conditions and also allow a controlled release in a viable and metabolically active state in the intestine (Nazzaro et al., 2011).

The encapsulation process can occur naturally in nature when bacterial cells grow and produce exo-polysaccharides.

The cells become entrapped in their secretions, which act as a protective structure/capsule, reducing permeability through the capsule and protecting the cells against adverse environmental conditions (Heidebach et al., 2010).

In the development of a successful encapsulation system for a target microorganism, it is necessary to know the stability of the encapsulated cells, the properties of the encapsulation material, and also if the delivery system is suitable for the final application.

The encapsulation process of probiotics usually has two main challenges; the first is the size of the produced capsules that should be under 100 μm, but it depends on the chosen encapsulation technique.

The other challenge is overcoming the low viability of probiotics in dairy products. Usually, the encapsulation process takes place in three stages (Favaro-Trindade 2011, Gbassi and Vandamme 2012, Terpou, Papadaki et al. 2019).

The first stage is the incorporation of the bioactive component, the second step is the micro or nano-capsules preparation, and the last phase is the capsules stabilization.

Therefore, probiotics encapsulation has the purpose of enhancing the survival of probiotic bacteria during processing, storage and in particular, in gastric transit encapsulation technology as previously mentioned, micro or nano-encapsulation technology evolved from the principle of cell immobilization (Priya, Vijayalakshmi et al. 2011, Iravani, Korbekandi et al. 2015, Malik, Sokolov et al. 2017).

Also, the procedure has been proved to enhance cell endurance against environment stress-related factors and thus prolonging cell viability, because of the versatility of immobilization, and later of the encapsulation, for the entrapment of many different microbial cells, this technology is still being explored in as many different applications.

Typical examples are as follows: the production of biofuels, environmental decontamination, novel food development and enzymes, vitamins, food and pharmaceutical products.

In the field of food science and technology, immobilization/nano-encapsulation technology gradually became a tool for the improvement of the performances of microorganisms in various areas of technological interest (Alonso 2016, Ahanger, Akram et al. 2017, Xie, He et al. 2019).

In addition, the technology is also being applied as a strategy for protection of microorganisms considered beneficial (i.e. probiotics) to human and/or animal health; the products that come from encapsulation procedure are the micro/nano-capsules.

Solid particles that can differ for their size and morphology depending on materials and techniques applied to produce them (López-Rubio, Sanchez et al. 2012, Martín, Lara-Villoslada et al. 2014, Markowiak and Śliżewska 2017). Capsule size varies and ranges from nano (>1000 nm), micro (1-1000 μm), as usually occurred in emulsification techniques (Whelehan and Marison, 2011). When capsules are not characterized by a well-defined and spherical morphology, they are defined as "irregular" shaped (Arpagaus, John et al. 2017).

A micro/nano-capsule consists of a semipermeable, spherical, thin and vigorous membrane circumventing a solid or liquid core, with a diameter varying from a few microns to 1mm (Anal and Singh, 2007). Coating bulwarks the active component from environmental stresses, such as acidity, oxygen, and gastric conditions, and can be utilized, for example, to avail the content pass through the stomach (Kim, Xiong et al. 2008).

Cell encapsulation has its roots in the primordial microbial immobilization technique, referred to as the procedure for physical confinement of cells to a certain defined space with the main purpose of preserving their viability. Through the tailoring of innovative techniques and the research on new suitable encapsulating materials, micro and nano-encapsulation have become a sophisticated technology for the entrapment of living cells targeting a variety of applications (Stormo and Crawford 1992, Zhang and He 2009).

Encapsulation of bacterial cells has been described by some authors (O'Riordan et al., 2001; Anal and Singh, 2007; Sohail et al., 2012), and the main purpose, or at

least, the most investigated aspect until now, is the protection of the cells under gastrointestinal conditions. Encapsulation, separated to the previously mentioned impacts, can offer numerous focal points in improving the treatment of probiotic societies and also the veiling of taste and smell given by generation of various metabolic mixes (e.g. acidic corrosive). Delivered amid maturation in nourishments where they are not required. As recently reviewed by De Prisco and Mauriello (2016), the application of encapsulation to probiotic cells contributed to the research development of conventional and non-conventional probiotic products. Some applications of encapsulated probiotics are present in bakery, meats, fruits and vegetables and dairy products (Anselmo, McHugh et al. 2016, Silva, Balthazar et al. 2017).

The microencapsulation of probiotics is not conventionally practiced for developing of probiotic foods, it is additionally utilized in other fields, more spreading of scientific results towards industry should be performed to offer for example incipient business chances in the engenderment's of desirable and currently unavailable probiotic foods, such as bakery products or instant sultry beverages (Heidebach, Forst et al. 2012, Kechagia, Basoulis et al. 2013). There are three main and precise aims reached using the microencapsulation and nanotechnology methods which can be split into:

- Food health approaches, nano-carrier used in the bio-delivery system of nutrients, ingredients, organic and inorganic additives, and supplements.
- Food safety and packaging applications, bio-plastic polymers, nano-surfactant, smart coatings, bio-degradable films, and nano-sensors are implemented to improve physical, microbial and or functional traits of foods.
- Functional food approach, nano-sized bioactive components, such as peptides, antioxidants, functional lipids and other nutraceuticals were applied as therapeutic, and functional materials.

The application of nanotechnology in food and nutrition area is of great importance where many applications and products that some of them are commercially available (Table 2).

Table 2.2. The application of encapsulation technology in the food and nutrition area

Food processing and products	Nutrition	Source
Flavours and aromas	Neutraceuticals	Plants and animal tissues
Gelation agents	Nutrient delivery	Animals tissue
Antioxidant agents	Bioactive peptides	Animal and plants proteins
Nano emulsions	Mineral/vitamin fortification	Natural and plant mass
Anti-caking	Potable water purification	Plants tissue
Antimicrobials	Bioactive compounds	Animal and plants tissue
Flavors and aromas	Sensory properties of supplements	Plant tissues
Contaminant sensors	Supplementation	Microorganisms

2.3.1 Electrospinning

Electrospinning or spinning with electrostatic is the technique the most used to fabricate a very thin polymeric fiber with a perfect efficiency to be used in different domains and areas.

This method is mainly based on using a high electric force and power to produce fibers in diameters of the order of nano-meters (Mamun 2019).

This technique became the first choice for all the domain where fibers in general and nanofibers in specific are used, for been easy to work with, very practical and fast and at the same level the high fabrication quality.

Using the electrospinning method helps to produce a well establish fiber with the wanted size, high surface areas and a very reduced pores size in limited time which makes the produced fiber the ideal tool in the industrial application and employment of fibers (Li and Tuan 2009, Wang, Wang et al. 2019).

The fibers produced using the electrospinning technique can be used in several applications, such as filtration, especially that the produced fiber is characterized by their very small pores, textile and weaving areas regarding the high quality yet fast of the operation. It can be applied in the medical pharmaceutical and cosmetical domain where the tissues made by the spinning with electrostatic method are taken the place of the old and unwell made fibers (Schreuder-Gibson, Gibson et al. 2002, Bhardwaj and Kundu 2010, Mao 2016, Mamun 2019). Easily, this is how the electrospinning is processed; the polymers solution that takes place in an injector usually syringe is stamp to the deep on the metallic needle, an electric file is created between the end of the needle and the collector plate by applying high voltage power in the system.

A taylor cone will start to be formed after the first drops been evacuated progressively by the needle and it will move in the direction of the collector stating to formed a fiber and a line fiber polymer is generated as it is shown in figure 2 (Bhardwaj and Kundu 2010, Zhang and Yu 2014).

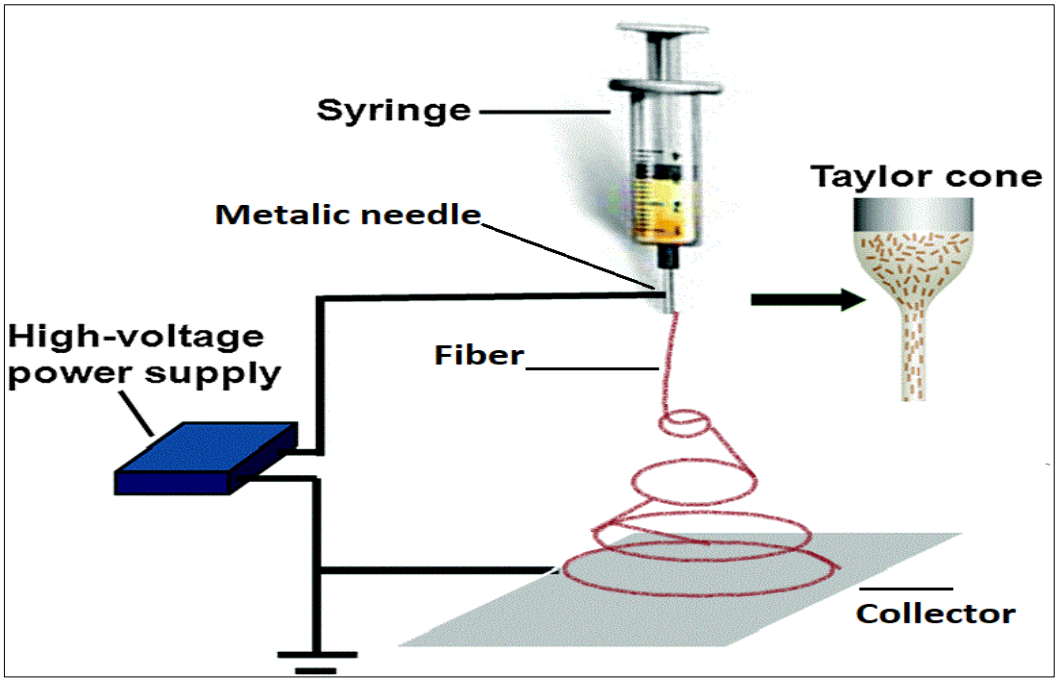

Figure 2.2 Illustration on the electrospinning process (Zhang and Yu 2014)

2.3.2 Sodium alginate

Sodium alginate is nature extracted polysaccharides that took a big place lately for been used as a biopolymer in the electrospinning technique to produce nanofibers; this sugar is characterized by (Table 2.3):

Table 2.3. General proprieties of sodium alginate

Molecular formula	$(C_6H_7NaO_6)_n$
Structure	Liner with (1–4) linked β-d-mannuronic acid (M units) and α-l-guluronic acid (G units).
Molecular weight	216.12 g/mol.
Solubility	Water-soluble.
Origin	marine brown algae.

The main utilization reason of this sugar is its capacity to behave as liquid-gel in the aqueous solutions where this changing from a low viscous to a gel is immediately processed after the exchange between monovalent ions with divalent ions is exhibited. It has been widely investigated as being biodegradable (Mukopadhyay 2010, Loureiro dos Santos 2017, Sawant 2017).

Sodium alginate is used in many applications as in food for producing biodegradable films, which is also applied in pharmaceutical and cosmetic products, in the other hand the texture that alginate add to the products is the major reason that make it main ingredient in it, more over this sugar took a new way of application where it is used in the medical side to help the human body tissues regeneration (Loureiro dos Santos 2017).

Alginate became one of the most utilized polymers in fibers production, which is usually made by injection the sodium alginate solution with the presence of salt as calcium or acidic solution to be used as a textile in different employment, especially coating probiotic microorganisms (Mukopadhyay 2010).

2.4. Functional food

A functional food, universally used concept in the recent years that includes a food enriched with bioactive ingredients that offer a major health therapeutic benefits to the consumer's body this is how a functional food being identified from the National Academy of Sciences and The International Life Sciences Institute (Hasler 2002).

"Healing the human body with food" became the main topic for the new treatment paths; where a normal food is consumed mainly to ensure the main and essential compounds to the consumer body, such as proteins, lipids and carbohydrates, to reach the main goals like growth, maintenance energy -in a general way for the main body metabolism satisfaction. The role of the functional food is more important, where the consumption of this food allows the penetration of bioactive ingredients and compounds with medicinal properties called nutraceuticals, such as fatty acids, amino acids, peptides, vitamins, enzymes and antioxidants, and probiotics that are known with their high capacity and activity in the body

regulation and cure at deferent levels, this nutraceuticals can be delivered from either animal sources and called zoo-chemicals or from the plants and being called phytochemicals sources (Rincón-León 2003, Prakash and van Boekel 2010, Utama, Hanim et al. 2018).

The consumption of the functional food beside that it will ensure the needed basic element to the body it will also help to treat several health disorder and disease, such as diabetes, blood pressure, oxidative stress, heart disorder inflammation, infection and even cancer and tumoral cases without fearing a toxicity or sides effects (Mao 2016, Dudeja and Gupta 2017, Loureiro dos Santos 2017, Vukasović 2017).

A lot of commercially available product are considered to be a functional aliment, where the dairy product takes the first order as active food items the other aliments takes second places based on the richness of the product on the bioactive compounds and microorganisms, such as juices types and canned food (Pessione and Cirrincione 2016, Iriondo-DeHond, Miguel et al. 2018).

2.4.1 Turkish ayran

Ayran, the most consumed drink in Turkey, is a traditional called drink made of mixing milk water and salt, whose milk comes from cow, sheep or goat, where the production of ayran was the first started in Iraq and Turkey who tamed and domesticated the cows and used their milk as a food substance for human being.

This drink is consumed at any time of the day, especially during meals. Ayran is a salty drink with a pH 5 that it is reported to have two weeks maximum as a storage period at 4°C (Baruzzi, Quintieri et al. 2016, Nergiz-Unal, Akal Yildiz et al. 2017).

Probiotic microorganisms found in fermented yogurt are known as the ability to prevent the growth of pathogenic bacteria in the intestine and prevent the formation and absorption of toxic products, which causes diarrhea, dyspepsia and constipation (Metchnikoff, 1908).

The other desirable functions carried out by those probiotics in the intestinal tract are the production of the antimicrobial substances, organic acids, bacteriocins and

hydrogen peroxide, etc. Also aid in maintaining lower redox potential (Bested, Logan et al. 2013, Markowiak and Śliżewska 2017).

Table 2.4. Nutrition values of ayran (Freitas, 2017, Lokumcu Altay et al., 2013)

Nutritional Elements	Unit	Amount*	Nutritional Elements	Unit	Amount *
General			**Vitamin**		
Water	%	75	Retinol	mg	0.01
Energy	kcal	34	Thiamine	mg	0.02
Energy	kJ	143	Riboflavin	mg	0.09
Protein	g	1.7	Vitamin	mg	0.04
Total Lipid (fat)	g	1.9	Vitamin B-6	mg	0.02
Carbohydrate	g	2.6			
Minerals					
Calcium, Ca	mg	63	Copper, Cu	mg	0.045
Magnesium, Mg	mg	7	Potassium, K	mg	77
Phosphorus, P	mg	46	Chlorine	mg	35
Zinc, Zn	mg	0.2	Sulphur	mg	27

*** Chart values are for 100ml of**

2.4.2 Orange juice

Orange juice the most popular juice in the world, a drink made from pressed oranges that are coloured with orange and characterized with a pH value that

ranges from 3.3 to 4.2 and that and stand for a three days period at 4°C (Serpen 2012, 2016).

Drinking orange juice offer many benefits to human health and this juice is one of the main sources of vitamin C, which is a very powerful antioxidant that contributes to some diseases, such as the influenza; consuming this juice will also help the immune system and the body metabolism regulation.

In addition, orange juice can prevent many diseases and health disorders, including cancer, cardiovascular disease by contributing to red cell production and blood circulation regulation and inflammation.

Table. 2.5 Nutrition values of orange juice (Lanza, 2003)

Nutritional Elements	Unit	Amount*	Nutritional Elements	Unit	Amount*
General			**Vitamin**		
Water	G	86.7	Vitamin C	Mg	50
Energy	Kcal	45	Thiamine	Mg	0.2
Energy	kJ		Riboflavin	Mg	3
Protein	G	0.1	Niacin	Mg	0.5
Total Lipid (fat)	G	0.2	Vitamin B-6	Mg	0.4
Carbohydrate	G	11			
Fiber	G	2.4			
Minerals					

Table 2.5 (continued)

Calcium, Ca	Mg	0.42			

Iron, Fe	Mg	3	Manganese, Mn	Mg	0.4
Magnesium, Mg	Mg	0.4	Potassium, K	Mg	0.2
Phosphorus, P	Mg	0.55	Sodium, Na	Mg	0.4

* Chart values are for 100g of edible orange

2.4.3 Apple juice

Apple juice, the most consumed juice by adults and children as reported by the FAO in 2014, a drink made from apples that it can be yellow, light brown, dark brown or green color depending on the type of the apples used in manufacturing this juice which is characterized with a pH values that ranges from 3.35 to 4 (Varming, Amigo et al. 2014) .

Besides the high energetic and nutritional value of apple juice, it contains a very high number of phenolic compounds that have a lot of benefits to the consumer health. Consumption of this juice ensures many advantages to the human body, such as preventing some diseases as inflammation, atherosclerotic, cardiovascular problems and cancer; also it can play a role as a neuroprotective and antiaging activity (Altunakar, Gurram et al. 2007, Sahar, Rahman et al. 2019, Sikorska, Khmelinskii et al. 2019).

Table 2.6. Nutrition values of apple juice (Lee and Wrolstad, 1988, Medina et al., 2019, Pilando and Wrolstad, 1992)

Nutritional Elements	Unit	Amount*	Nutritional Elements	Unit	Amount*
General			**Vitamin**		
Water	g	86.7	Vitamin C	Mg	0.9
Energy	kcal	46	Vitamin A	Mg	0.2

Energy	kJ		Riboflavin	Mg	3
Protein	g	0.1	Niacin	Mg	0.5
Total Lipid (fat)	g	0.1	Vitamin B-6	Mg	0.4
Carbohydrate	g	11			
Fiber	g	0.2			
Minerals					
Calcium, Ca	mg	8	Sodium, Na	Mg	4
Iron, Fe	mg	0.1	Potassium, K	Mg	101
Magnesium, Mg	mg	5			

* Chart values are for 100g of edible orange

3
Experimental Studies

3.1 Bacterial strain preparation

In this study, *Lactobacillus brevis* E25 bacteria strain was provided and identified from previous work (Castro-Mayorga, Fabra et al. 2016) from Istanbul, Turkey, these cells were revived and counted applying the standard plate method, where the bacteria strain was incubated using MRS agar for two days under anaerobic conditions at 37°C, the probiotic organism was collected and divided into two parts:

• The first part was directly used without encapsulation as free bacteria in all the assays.

• The second part was subjected to the nano-encapsulation within sodium alginate-based biopolymers using the electrospinning method to be used as encapsulated cells.

3.2 Fabrication of nanofiber mats and their characterization

3.2.1 Materials and solutions

An aqueous of 100 µl of the stored probiotic cells (-80°C) was restored for one day where it was submerged in 10ml of MRS broth solution after that 2.5ml of the previous solution was mixed and incubated in 250ml of MRS broth solution.

The final bacterial solution was divided into six tubes and centrifuged at 8000 rpm for 15min at 25°C; then, the precipitate was washed twice with 30ml of sterile phosphate buffer saline (PBS). Eventually, 5ml of PBS solution was used which precisely contain the main probiotic stock. The Encapsulation buffer containing the probiotic was made up of mixing 70% of PVA solution (20% w: v), 20% of alginate solution (SA) with a concentration of 3% (w: v) and 10% of the bacterial stock buffer.

3.2.2 Electrospinning process

Sodium alginate was used as the main coating polymers in this study, where those compounds were dissolved in distilled water and sterilized. Afterwards, the probiotic solution was added into the polymer solution with the optimized concentrations and the mixture was homogenized, then this solution was filled in the injector which was placed in the electrospinning equipment to start the processes that was carried according to the methods published by Librán, Castro and Lagaronb (Addison, M Osborn Popp et al. 2014, Qi, Jiang et al. 2015).

Briefly as stated in a previous article reported by Dong and others (2010) (De Vos, Devroey et al. 2010), where four parameters had to be adjusted before starting the electrospinning to make the solution spun which were the flow rate that was 0.4ml/hour, voltage 18-20, electric current 6-7 and distance from the injector the surface of collecting the yield was 12-13cm.

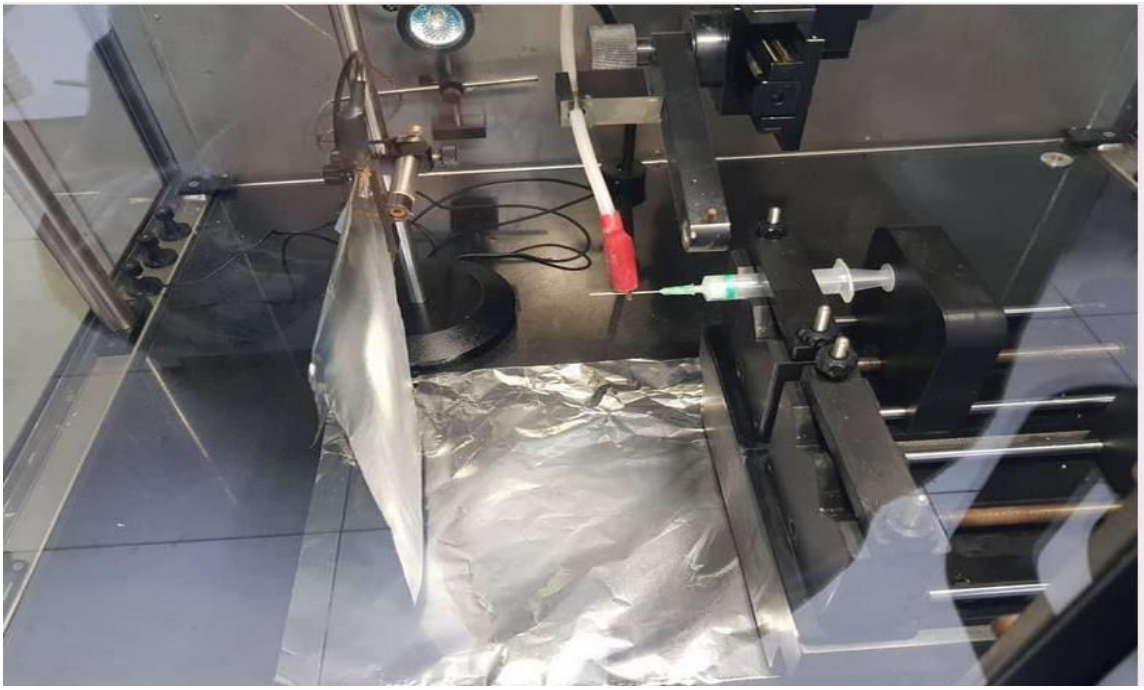

Figure 3.3. Electrospinning processing

3.2 Viability analysis and encapsulation efficiency

Petri dishes were prepared for both of the non-covered and encapsulated bacteria (both subject to different dilutions), where 100 µl of the diluted bacterial solution was added to 900 µl of Peptone to prepare the final bacterial solution. After 48hours of incubation in Petri dishes, the harvest of the bacteria was counted and collected (bacterial colony).

The encapsulation efficiency was determined by the number of bacteria present in the nanofibers, where the ratio of the number of bacteria in the solution were determined in CFU/ml.

The viability was calculated using the following formula:

$$\text{Viability \%} = \text{encapsulated (log)} / \text{free (log)} * 100 \quad (3.1)$$

3.4 Nanofiber characterization

To have a global and a more in-depth view of the encapsulation technique efficiency and the covering nanofiber characterization, all the following tests were performed on nanofibers and the encapsulated bacteria, which means the coating material with and without the probiotic organism.

3.4.1 Fourier-transform infrared spectroscopy (FTIR) characterization

Fourier transformation infrared spectroscopy is a technique used to obtain the absorption and emission spectrum for molecular and atomic analysis, it is principally qualitative method that study and detect the interaction between matter and infrared radiations that can trigger and give rise to the vibration of specific molecular bands, this technique can be used as a fingerprint for chemical compounds which mean a good method for materials characterization and identification (Thomson 2015).

ATR FTIR spectroscopy is a technique with many advantages. First of all, it is a fast way that saves a lot of time and requires only a small amount of sample, also, largely used in product development (Qian, Qi et al. 2016, Akbar, Zahoor et al. 2018); more than that FTIR has no negative effect on the biological studied sample

(which was a probiotic organism in this research), plus this method requires no preparation steps.

In the present study, this technique was employed to determine the efficiency of the encapsulation process and to have a more information on the possible interaction between the functional groups of the nanofiber and the probiotic cell an FTIR scanning was performed at a band interval of 4000-600 s^{-1}, where distilled water was used as a background where the samples were directly compressed on the ATR crystal surface. The Bruker Tensor 27 spectrometer equipped with a DLA TGS detector (Bremen - Germany) was the equipment used for this analysis, and the data acquisition was accomplished using OPUS program version 7.2 for Windows from Bruker GMBH.

3.4.2 Characterization by Nuclear Magnetic Resonance (NMR)

Each living organism, such as probiotic bacteria, consists of matters that are made up of atoms, nuclear magnetic resonance can zoom inside the matter to observe the molecules, their interaction, behaviour, position, and movement.

In a brief way, the molecules that are mainly belt up from atoms behave like small magnet when they are placed in strong magnet filed and by that the absorption of radiation of those molecules can be measured giving a spectrum that consist of several peaks, the high of each peak represents the number of nuclides and their position and the structure of the molecules. Nuclear magnetic resonance non-destructive, quantitative, reproducible, untargeted and unbiased method that requires no or minimal sample preparation, this assay was managed on a 400 MHz Varian wide-bore instrument equipped with a 1.6 mm triple resonance MAS probe, where samples were spun at the magic angle at 30 kHz (Pitino, Randazzo et al. 2012).

3.4.3 Characterization by X-Ray Diffraction (XRD)

X-ray diffraction (XRD) is used to measure the crystal structure of organic materials; based on the fact that is most of the materials are made up from many small crystals that are composed of number of atoms, the XRD instrument applies the X-rays to the sample (that are placed in to the sample holder) that produces

signals, those signals are detected by the equipment detector which gives graphs related to the atomic structure of the tested sample. XRD is a powerful method for the study of nanomaterials (materials with structural features of at least one dimension in the range of 1-1000 nm).

The wavelength of X-rays is on the atomic scale, so X-ray diffraction (XRD) is a primary tool for probing the structure of nanomaterials. The synthesized nanofiber particles and the encapsulated probiotic were characterized under X-ray diffractometer (XRD) using CuKα (λ = 1.54 Å) as a radiation source (Bharti, Bhardwaj et al. 2016).

Table 3.7. Measurement conditions of XRD

Measurement conditions	
Scan Axis	Gonio
Start Position [°2θ]	10.0250
End Position [°2θ]	79.9750
Step Size [°2θ]	0.0500
Scan Step Time [s]	2.0000
Scan Type	Continuous
Offset [°2θ]	0.0000
Divergence Slit Type	Fixed
Divergence Slit Size [°]	0.4354
Specimen Length [mm]	10.00
Receiving Slit Size [mm]	0.5000
Measurement Temperature [°C]	25.00

Table 2.5 (continued)

Anode Material	Cu
K-Alpha1 [Å]	1.54060
K-Alpha2 [Å]	1.54443
K-A2 / K-A1 Ratio	0.50000
Generator Settings	40 mA, 45 Kv
Diffractometer Type	0000000080920314
Goniometer Radius [mm]	240.00
Dist. Focus-Diverg. Slit [mm]	100. 00

3.4.4 Characterization by Optical Microscope and Scanning (SEM) Microscopes

The optical photographs of the free and encapsulated used bacteria in this research was captured by a microscope attached with a digital camera with magnification and also using Scanning Electron Microscopy (SEM).

The microscope works on the electrons that exist on the samples surface to gives photons images applying electrons optics and signal detection system, the SEM is known by being very specific because it gives a full-size result and quite clear images of the observed samples.

3.4.5 Thermal characterization

Thermogravimetric analysis (TGA) was analysed using the DSC tool (DSC Q20, TA Instruments, Inc., USA). Each of the nanofiber and encapsulated bacteria was placed into an aluminium-coated DSC pan where the heat was applied with a ratio of 10°C/ min from a range of 20 to 250°C, and an empty aluminium pan was used as a negative control (Jana, Trivedi et al. 2015).

3.5 Viability of free and encapsulated strains under simulated gastrointestinal conditions

The free and encapsulated bacteria were subjected to the in vitro digestion process using pepsin and liver salt bile (alkaline buffer containing trypsin) treatment to determine the resistance of the encapsulated probiotic to the environment of the stomach and intestines (pH and enzymes).

In brief way, the pH of the medium was adjusted to 1.5 (with HCl) for the pepsin treatment, where the bacteria (free and encapsulated) was added and left for two hours at 37°C, after that the pH was adjusted to 7.5 using NaOH for the trypsin treatment (intestines conditions) and left for another two hours at 37°C.

After finishing the digestion treatment, the tested bacteria were then inoculated and incubated for survival purposes, where the living colonies were counted to calculate the stability and growth rate, which means the resistance of the probiotic cells to the gastronomic conditions (pH and enzymes).

This was proposed for the possible application of the encapsulated probiotics for the food industry and bio-delivery system in the human body (Shalumon, Anulekha et al. 2011, Jana, Trivedi et al. 2015, Palama, Canard et al. 2016).

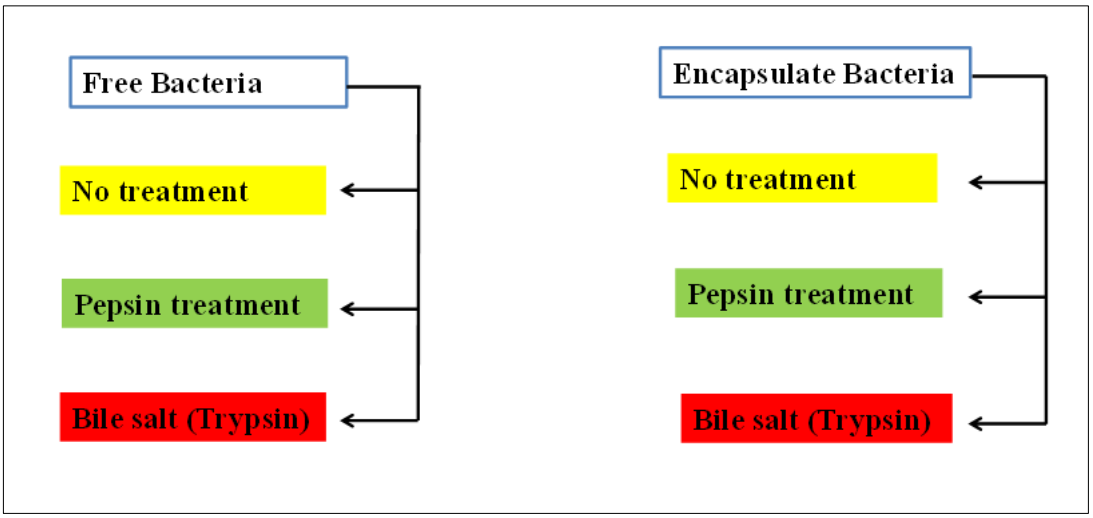

Figure 3.4 Scheme plan of the in vitro analysis.

3.6 In situ viability in selected food products

Commercially available ayran, orange and apple juice with no added preservation were used for all experiments (Istanbul, Turkey).

Where those products were treated with Ultra-high temperature processing (UHT) to ensure a total absence of any microorganisms and stored at 4°C for 24h before inoculation.

Table 3.8. Brands of the used food items

The product	Brand
Ayran	Sutas
Apple juice	SADE
Orange juice	Exotic

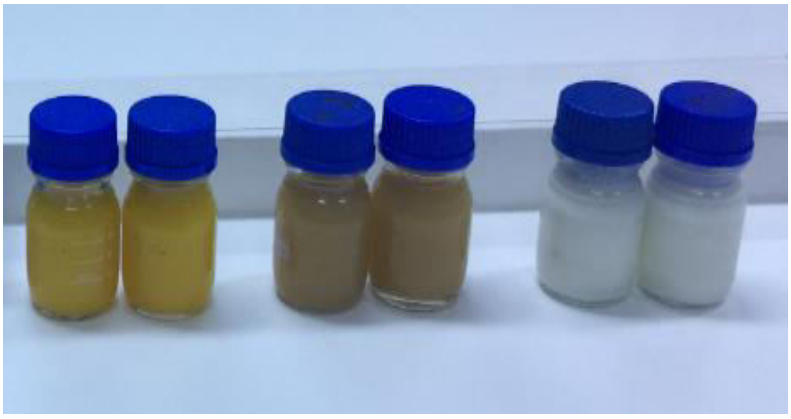

Figure 3.5 Samples prepared for storage

3.7 Enumeration of Bacteria

The enumeration of the probiotic bacteria in the ayran was performed on a three days basis over a storage period of 12 days, while the orange and the apple juice enumeration was carried out for a period of six weeks on a weekly basis using MRS agar and incubation at 37°C for 48 h under anaerobic conditions (Süle, Kõrösi et al. 2014).

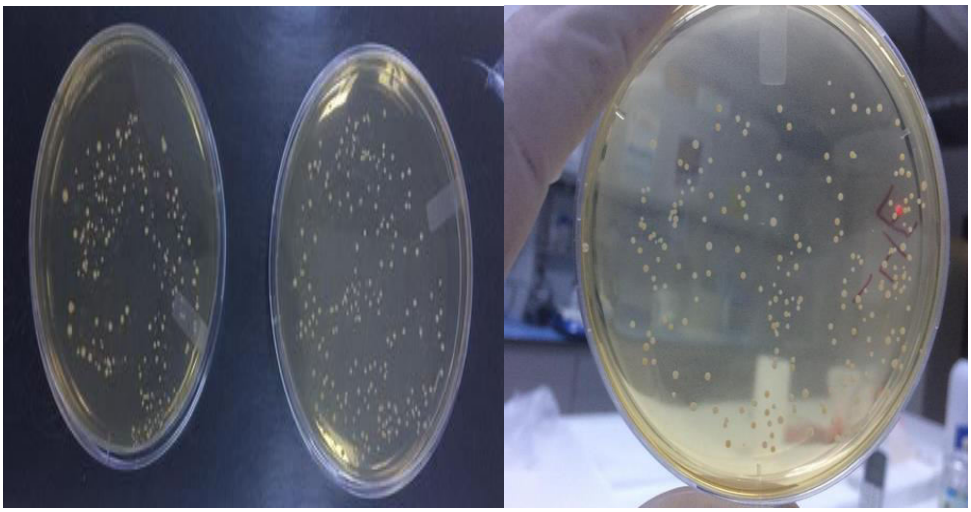

Figure 3.6 Encapsulated and non-encapsulated *L. brevis* counts in Ayran, apple and orange juices

3.8 pH and Brix determination

The potential of hydrogen (pH) and Water-soluble dry matter contents (Brix) values of the ayran samples were measured every 72 hours of intervals for 12 days, while those values of the orange and apple juice samples were established weakly for one and half month period.

The measurement of the Brix was conducted using a Abbe refractometer at the room temperature where the pH of the samples was determined using a pH meter (Thermo Scientific Orion Star A111, Indonesia), where it was calibrated before starting the measurement with a standard buffer at 25 °C (Cen, He et al. 2006, Kaddumukasa, Imathiu et al. 2017).

Figure 3.7 Determination of the pH of ayran

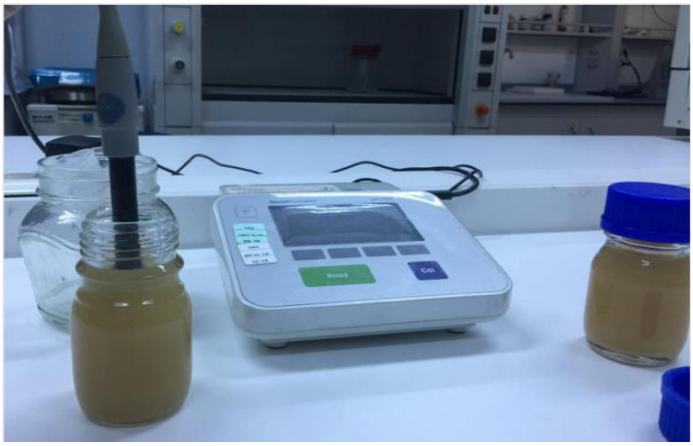

Figure 3.8 Determination of the pH of orange juice

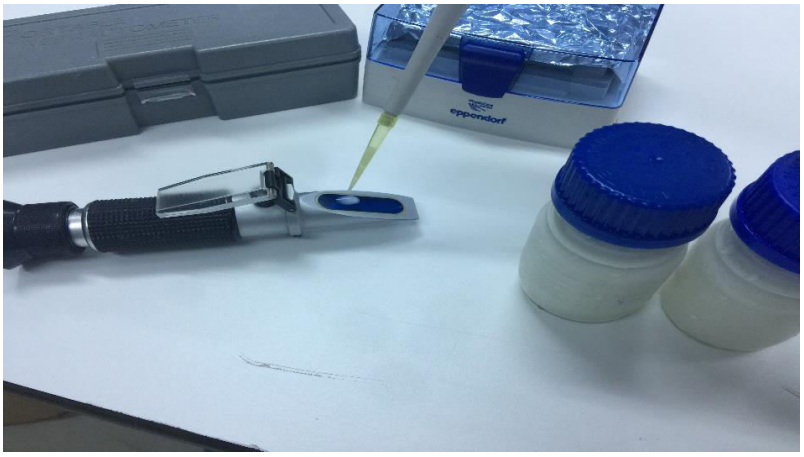

Figure 3.9 Determination of the total soluble solids in ayran

Figure 3.10 Determination of the total soluble solids in orange juice

3.9 Color properties

Konica Minolta Chroma Meter (CR-400, Minolta Co. Ltd., Osaka, Japan) was used to determine the color proprieties of the ayran, orange and apple juice samples, at first the used device was first calibrated with white ceramic calibration plate properties, three parameters were taken as following the L* which signified the brightness (whiteness-blackness), the a* referring to the redness and greenness and the b* for the blueness-yellowness.

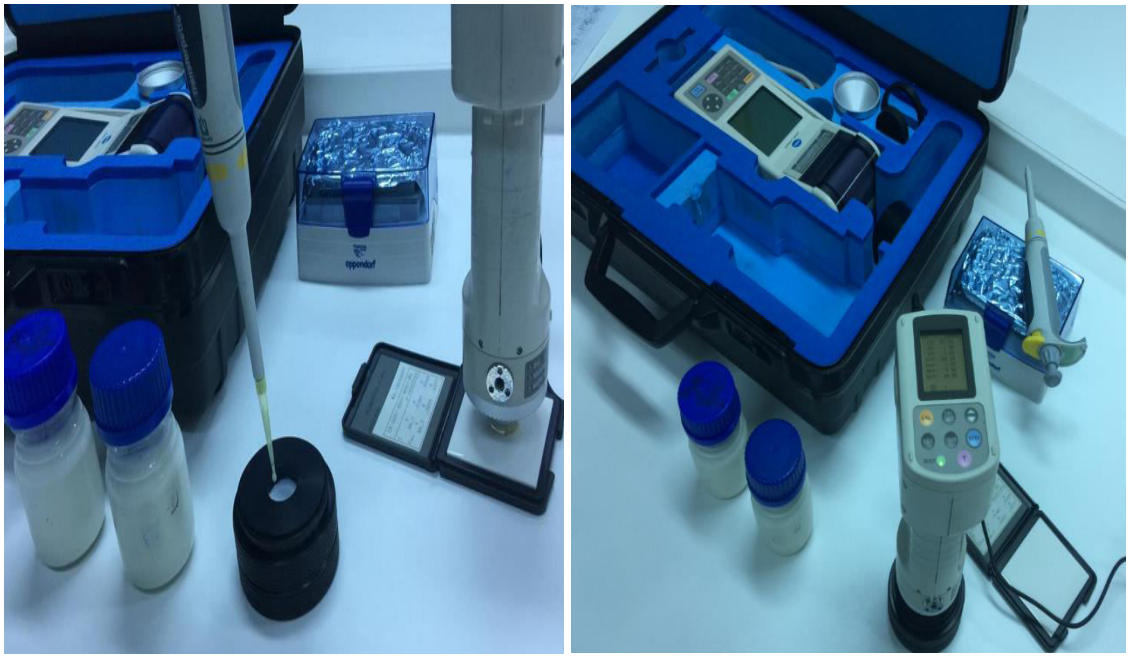

Figure 3.11 Color measurements of ayran

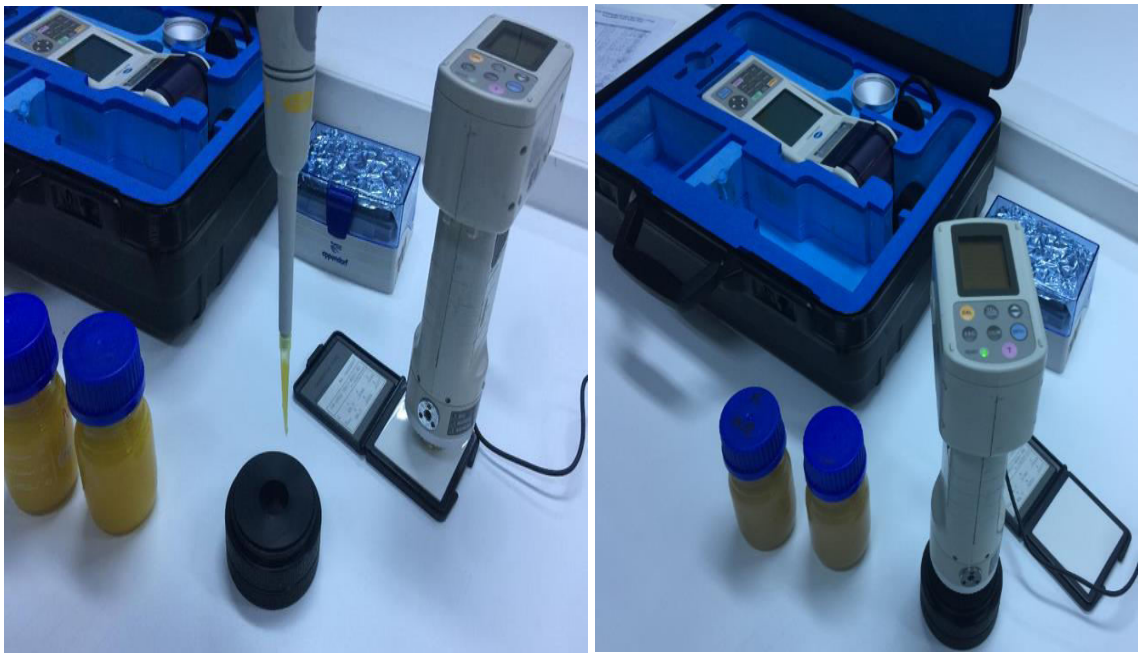

Figure 3.12 Color measurements of apple and orange juices.

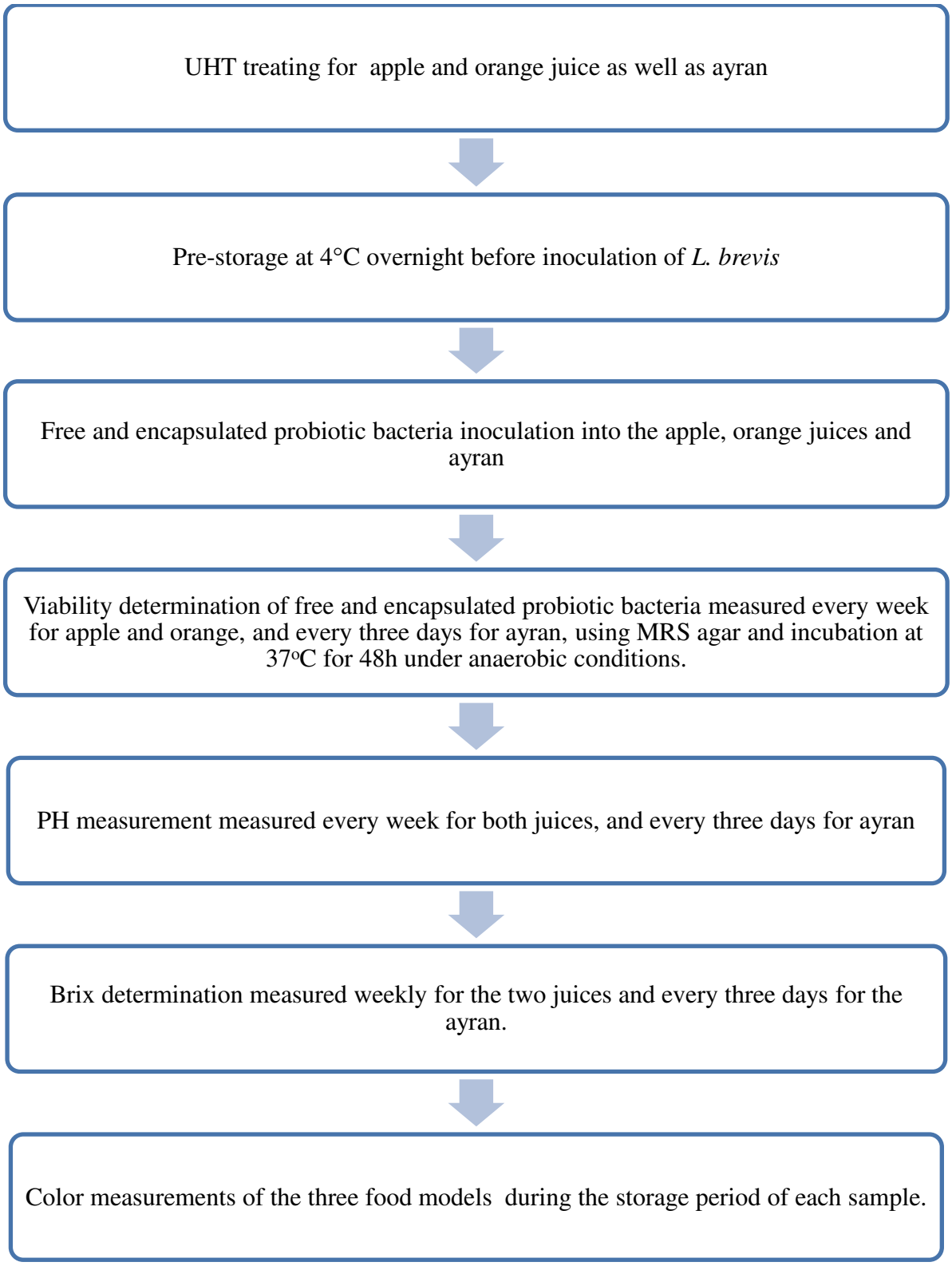

Figure 3.13 Scheme plan of the microbial and physical analysis.

3.10 Statistical analysis

Using the JMP package program (JMP 5.0.1) statistical analysis was carried out performing a two-way analysis of variance (ANOVA) and the statistical differences between the means were evaluated at the significance level of 0.05. The experimental analyses were performed in triplicate.

4
Results and Discussion

4.1 Viability Analysis

In this research, the probiotic bacteria *L. brevis* E 25 was encapsulated within PVA/SA based nanofibers produced using electrohydrodynamic method (electrospinning).

To detect the possible effects on the tested bacterial, a viability analysis was established, where a serial dilution was prepared from washed bacterial cells and encapsulated bacterial cells using the MRS agar spot method and Petri plates were inoculated and incubated at 37°C for 48 hours. The growth and survival ratio of free probiotic bacteria was compared to its counterparts, the nano-encapsulated probiotics.

The results of the viability ratio of the non-coated and the coated bacteria are shown in Table 4.9. As illustrated, the free bacteria cells showed a growth of 11.5 CFU/ml, while the encapsulated cells had a survival ratio of 9.3 CFU/ml. However, the viability ratio of the covered bacteria using electrospinning was overall a percentage of 81, this was regarded as an outstanding ratio since the electrospinning process appeared to lead almost no inactivation of the probiotic microorganism.

The results showed that the encapsulation process is efficient and present no harm or damages to the *L. brevis* E 25 probiotic bacteria.

L. brevis bacteria showed an ability to be encapsulated for a further application that would improve the economic value of the food and nutrition sector.

Plainly, the electrospinning technique and the chosen biopolymer have no negative effects on this stain, the obtained data is similar to a previous work established by Ding and Sahah (Ding and Shah 2008).

Where eight strains from LAB group showed a great ability to be encapsulated using electrospinning method, which disapproves the theory that electrospinning would inactivate bacteria, in contrast, the results provide evidence that the method is efficient and even protective rather than being viewed as destructive.

Table 4.9. Comparison of the viability ratio of the non- and encapsulation probiotics

Type of Bacteria *L. brevis*	**Free Bacterium** **Log CFU/ml**	**Encapsulated Bacterium** **Log CFU/ml**	**Survival (%)**
Value	11.5 ± 0.1^{A}	9.3 ± 0.2^{B}	81.1

Different superscript uppercase letters indicate significant differences between the free and encapsulated bacterium according to the viability ($P<0.05$).

4.2 Nanofibers characterization

In this research, the encapsulation of probiotic bacteria (*L. brevis*) was carried out using electrohydrodynamic method (electrospinning).

The probiotic was electrically coated in the nano-size with PVA and sodium alginate polymer mixtures in the study.

The electrospinning process of sodium alginate polymer mixtures was optimized without bacteria and the collected, dried products were investigated by SEM. Bacteria containing polymer solutions were prepared at the determined optimal concentration.

The effect of the polymers on the viability of the bacteria was examined as a function of time through 4 h at 25°C. After that, bacteria containing polymer solutions were electro-spun.

To have a deeper and a better vision about the fabricated of nanofibers with and without the main probiotic using the nano-encapsulation method with the electrospinning technique, several testes were effectuated to characterize the

made fibers in terms of their molecular, crystallographic and morphological properties by analyses of FTIR, XRD, Optical Microscopy and SEM.

To detect the functional groups and the possible interactions between the made-up polymer and the extracellular matrix of the probiotic cells and to confirm the combination and bonds between the two materials that lead to the immobilization of the *L. brevis*.

FTIR spectroscopy was carried out to reveal the molecular structure of the fabricated nanofibers, understand if the used strain could be successfully encapsulated in the nanofiber mats, and to observe any possible interaction between the functional groups of the nanofiber and the probiotic strain.

As seen in figure 14, the obtained spectrums of the nanofiber and the encapsulated cells present almost the same peaks pattern, yet with a higher concentration of the functional groups, which are mainly related to the coating material and the extra-cell matrix compounds of the probiotic bacteria.

From the obtained graph the following bands and peaks are observed, where the bands at 1545 cm^{-1} and at 1642 represents the N–H bond of amino group (amide I and amide II) with an amid III peak at 1042 cm^{-1} (Jana, Trivedi et al. 2015), this is mainly related to the proteins groups of the extra-cell matrix of the *L. brevis* such as peptidoglycan, stretching vibrations of C–N bond is observed at 1417 cm^{-1} and C–H appeared at 2900 cm^{-1}.

Besides, the band present at 1088 cm-1 which correspond to CO and C-O-C groups (Shalumon, Anulekha et al. 2011). A large band observed at 3285 cm^{-1} can refer to the elongation of the O-H group. All the obtained bands correspond to the functional groups of the proteins, lipids, and carbohydrates.

The obtained spectrum from FTIR technique that mainly study and detect the interaction between the matter's molecules, which mean a good method for materials characterization and identification, showed no clear evidence of a remarkable bonding between the functional groups of the coated material and the molecules of the biological material where no new peaks were observed and no absence or changing in the profiles are noted.

To have a deeper vision and more clear image of the interaction between the coating and the coated materials a nuclear magnetic resonance (NMR) spectroscopy was used for further investigation especially that it's the quickest yet reliable method (Palama, Canard et al. 2016) and a very powerful tool for characterizing biopolymers and to elucidate molecular-level (Addison, M Osborn Popp et al. 2014), the NMR spectrum of the nanofibers fabricated using sodium alginate applying the electrospinning technique was compared with the spectrum of the coated *L. brevis* (Fig. 15), the interpretation of the graphs that investigate mainly the proton environment especially that NMR analyses the hydrogen types inside the molecules their position and movements and by that detecting the possible bonding between all sorts of molecule's atoms.

Slight changes were observed in the NMR spectrum which is due to the changing in the hydrogen (H) environment due to the deferent interaction between the coating material and the microorganism membrane extra molecules.

Changing in H environment means changing in the proton position and by that we can conclude that a new bond between the fiber molecule's and the probiotic membrane compounds are created, this is considered as a good result because the more the interaction appears, the more the encapsulation is butter making the microorganism trapped in the fiber, so the bacterial surface is more protected yet without causing any damage to the cell which already confirmed by the results obtained in the viability testes.

The synthesized nanofiber particles with and without the probiotic bacteria were characterized under X-Ray Diffractometer (XRD) using CuKα (λ = 1.54Å) as a radiation source. In the present research encapsulated fiber showed diffraction peaks at 2θ of 8.5º, 10.4º, and 20.1º the diffraction peaks in PVA at 2θ= 8.5º and 20.1º that showed inter and intramolecular hydrogen bonding between the coating polymer and the probiotic cell, the XRD diffractogram (Fig. 15).

Also showed less crystalline nature of the nanofiber with large peak at 2θ equals to 20.1º comparing to the covered bacteria with the fiber which showed the same peak area yet more intense and sharp which mean more crystalline nature (Fontes,

Calado et al. 2013, Jana, Trivedi et al. 2015) and the formation of new covalent bonds between the SA polymer and the cell (Shalumon, Anulekha et al. 2011).

All pattern and graphs of the XRD and NMR confirm the existence of molecular bonding between the fiber mats that contains mainly from sodium alginate and the extracellular matrix of the probiotic microorganism that consist of varied group of dynamic macromolecules (proteins, carbohydrate and lipids), where the changing in the hydrogen position means the formation of new covalent bonds between the two materials.

The obtained results mean a successful coating and bacteria immobilization which was the one of the research goals, so the fiber will dissolve in the desired place and release the bacterial core to start its functionality.

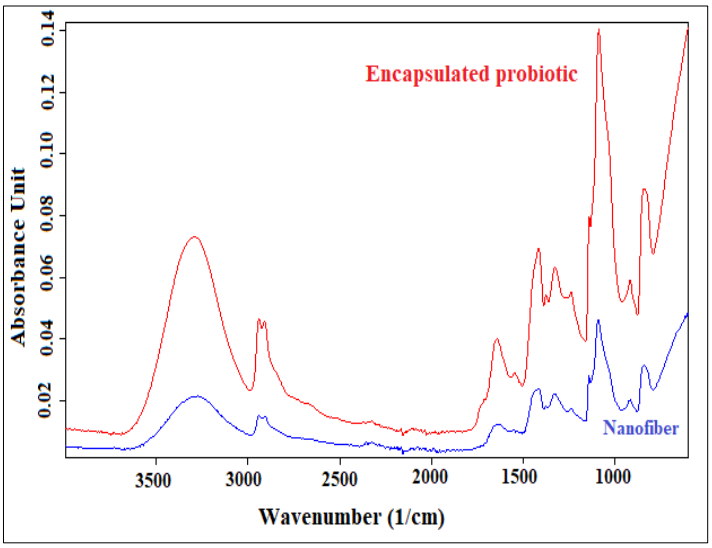

Figure 4.14 FTIR spectrum of encapsulated probiotic and of nanofiber.

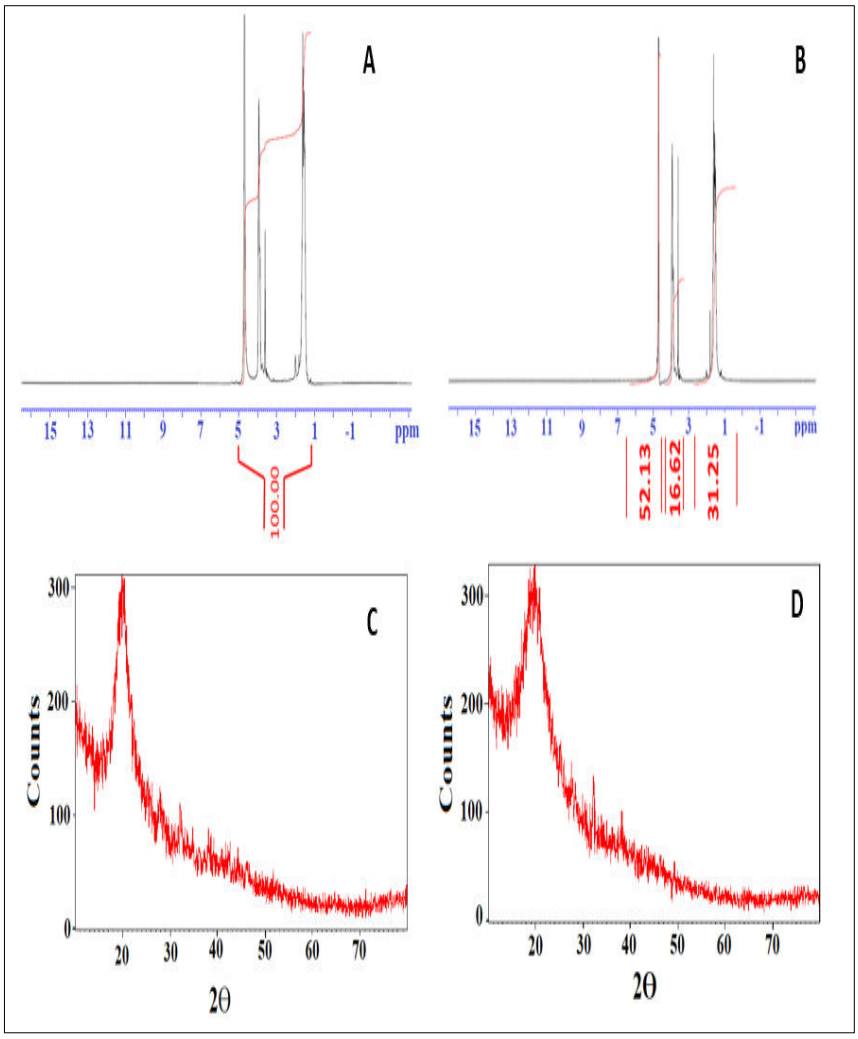

Figure 4.15 NMR and XRD spectrum: A, NMR spectrum of encapsulated probiotic; B, NMR spectrum of nano fiber; C, XRD graph of encapsulated probiotic; D, XRD graph of nanofiber

To evaluate and determine the morphological properties of the nanofiber an optical photograph of the nanofiber with and without the probiotic cell was captured by a microscope attached with a digital camera. Figures 16 and 17 illustrate the general shape and the tissue spreading in the encapsulated samples, plainly showed that the fibers are well made up, which resulted in forming a homogenized texture (Fig. 18), the obtained images also demonstrate that the bacteria are trapped inside the web forming by the fibers.

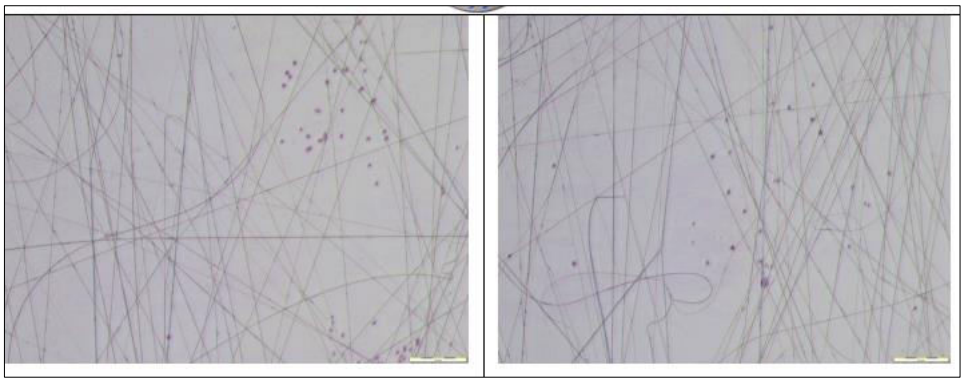

Figure 16 Image by optical microscopy of encapsulated bacteria

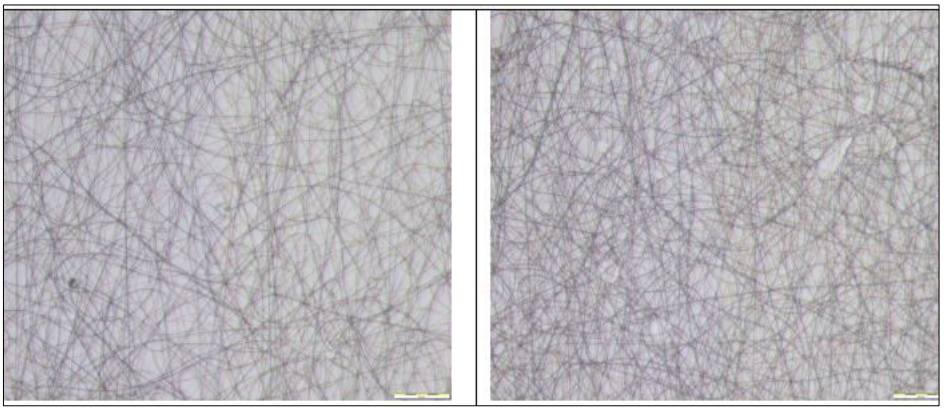

Figure 4.17 Image by optical microscopy of the nano_fiber without bacteria.

To have more specific details concerning the diameter and the size of the nanofiber and the nanofiber bonding with the probiotic cells a scanning electron microscopy (SEM) was effectuated. The obtained images are shown in figures 18 and 19, where it can be noticed that the fiber diameter was 200 nm, the same size was reported by a previous work of (Shalumon, Anulekha et al. 2011).

The SEM data confirm that the fabricated fiber is acceptable since the dimeters is in the naon average, which means that the combination and the concertation used are optimum, also the electrospinning was successful. It is also showing the uniformity and homogenization of nanoparticle distribution leading to good encapsulation and covering of the probiotic bacteria that was perfectly entrapped in the fabricated mats.

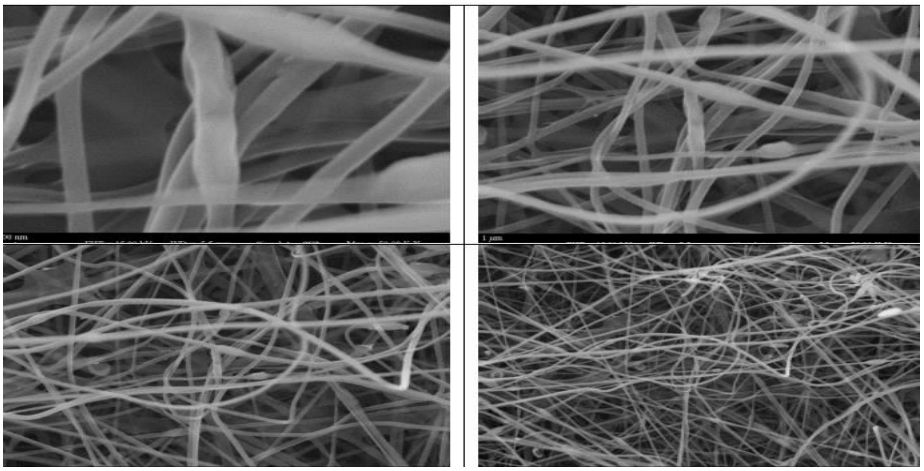

Figure 4.18 Fiber diameter distribution of encapsulated bacteria by SEM.

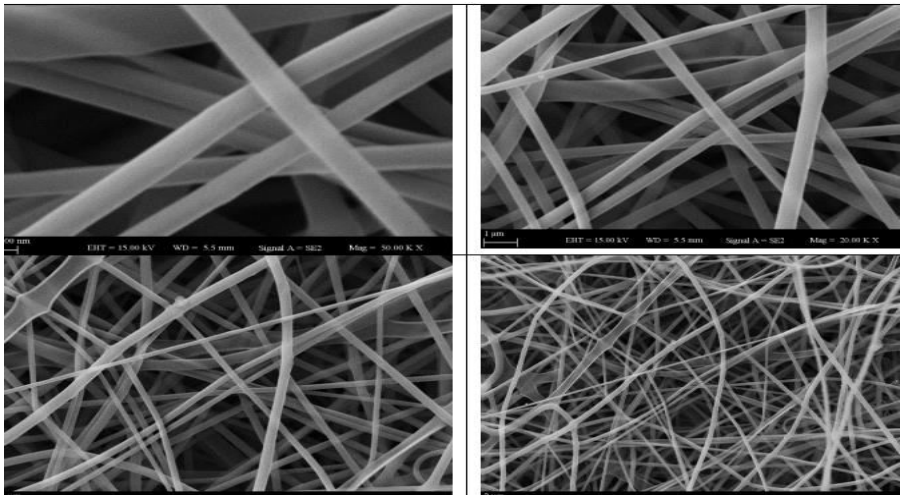

Figure 4.19 Fiber diameter by SEM

FTIR, NMR, and XRD analysis highlighted that a clear interaction between the nanofiber material and the extra molecules of the probiotic organism is present, yet without causing any membrane damage, these results confirming good trapping and immobilization to the bacteria that was later confirmed using the SEM.

The sodium alginate made up fiber was a perfect biopolymer for coating this probiotic bacteria, where a good adhesion was illustrated after the bonds bacteria-fiber was characterized, the obtained results shed light also at the technique used to coat the *L. brevis* which was the electrospinning, so as a characterization side the chosen technique and biopolymer was a great achievement to be further employed in several domains.

The thermogravimetric analysis profile is shown in figure 21. The weight loss started around 50°C to 600°C was observed for the free fiber samples, which were caused by hemadsorption of physically adsorbed material, such as moisture, which was around 15–18% for PAC and CNF, respectively.

The mass of both samples slightly decreased around 7%, between 200 and 400°C, this value is usually related to water loss. Also, the curve decreasing illustrated in figure 21. indicates a small weight and structure loss linked to the degradation of the sodium alginate (Qi, Jiang et al. 2015).

In the temperature range between 400 and 440°C, the loss of the bacterial cell mass was 2%, and of alginate anthocyanins 18%, which is associated with the first endothermic peak observed by DSC (120 °C) the total weight loss was with an 86.36% which was 7.1 mg for the encapsulated bacteria, while it was 6.74 mg (91.74%) for the coating material (figure. 20).

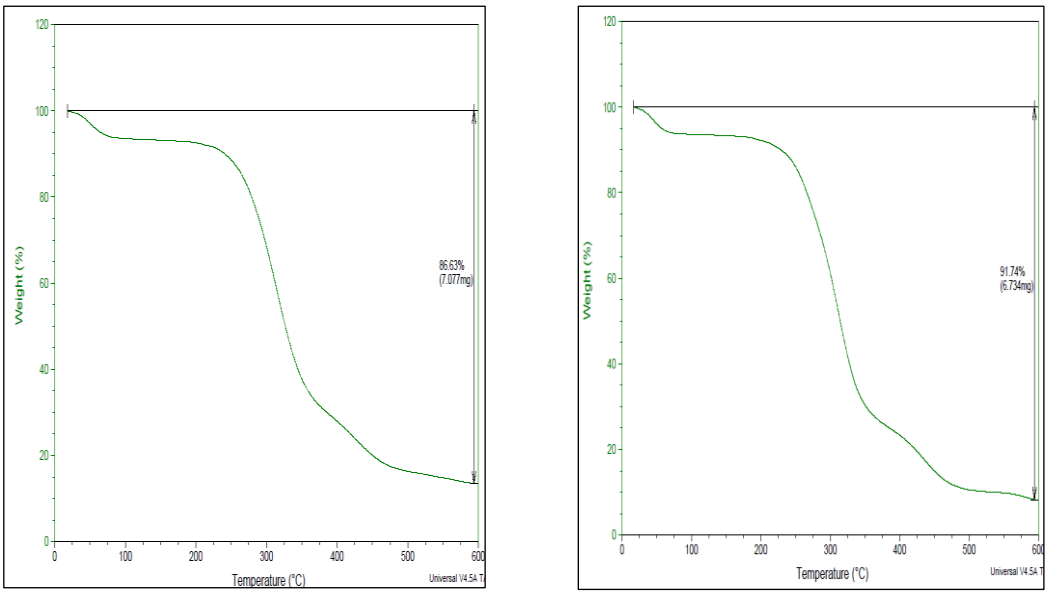

Figure 4.20. Nanofiber thermograms of differential scanning calorimetry graph for the total weight loss for the free and encapsulated bacteria

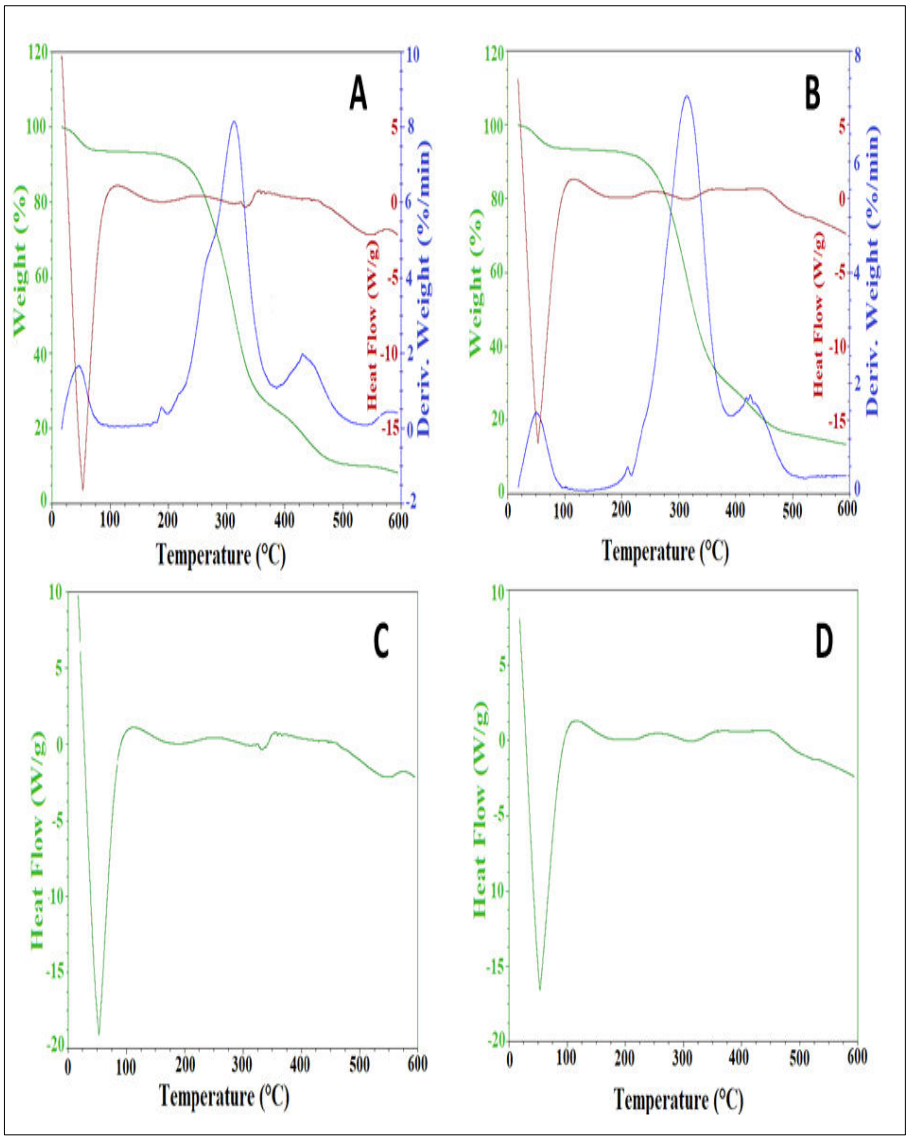

Figure 4.21. Thermograms of differential scanning calorimetry graph: A and B illustrate the encapsulated bacteria graph, where B and D graphs are for the nanofiber

4.3 In vitro analyses

The health benefits provided by probiotic bacteria have led to their increasing use in fermented and other types of food products. Encapsulation has been investigated to protect the probiotic bacteria in the product's environment and improve their survival in food products and from the gastronomic condition by protecting those microorganisms against digestive stomach juice.

Table 10 shows the survival ratio of the free and encapsulated strains after the treatment with pepsin and trypsin under in vitro gastrointestinal conditions.

The survival ratio of the free bacteria was very high were it was 79.9% after been treated with pepsin in an acid medium for two hours of incubation. The survival ratio has dropped down to the half where it was 39.1% after being treated with trypsin in a pH of 7.5 for two hours with a continuous rotation (table 10).

The obtained data confirm a previous study where it was mentioned that *L. brevis* has the ability to resist to a very low pH (pH of 1) for more than four hours (Utama, Hanim et al. 2018).

After been treated pepsin in acid medium the survival ratio of the encapsulated bacteria was 57.53%, a close ratio of 55.85% was showed with the same bacteria after being treated with trypsin in alkaline medium (table 10).

The encapsulated type showed an almost compatible survival ratio; this leads to suggest that the encapsulated type was more stable than the free type after both digestion processes. On the other hand, the increasing of the survival ratio after the trypsin treatment is a good since the desirable place of the *L. brevis* growth is the intestines where the bacteria can show its functionality.

Furthermore, this would evidence that the encapsulation of beneficial bacteria using alginate as a coating material and electrospinning as a technique so the probiotics can be utilized in the functional foods industry. It also protects the bacteria so it can reach the small intestine in a safe condition that may enhance the micro-flora of the human digestive tract.

Table 4.10. The survival performance and Survival ratio of the free and encapsulated bacterium after two treatments with pepsin and bile salt

	Free Bacterium		Encapsulated Bacterium	
Treatment	**(Log CFU/ml)**	**Survival ratio (%)**	**(Log CFU/ml)**	**Survival ratio (%)**
No treatment	11.3 ±0.1^{Aa}	100	9.2 ±0.07^{Ab}	81.5
After pepsin	9.1 ±0.12^{Ba}	79.9	6.5 ±0.14^{Bb}	57.5
After trypsin	4.5 ±0.17^{Cb}	39.1	6.3 ±0.08^{Ba}	55.9

Different superscript uppercase letters in the same column indicate significant differences between the treatment, different superscript lowercase letters in the same line indicate significant differences between free bacterium and encapsulated bacterium ($p<0.05$).

4.4.1 Free and Encapsulated bacteria survival in ayran

The survival ratio of the encapsulated and non-encapsulated probiotic bacteria is shown in figure 22. After a period of 12 days which was the storage period a progressive loss of the free probiotic bacteria was noted where it started with 6.27 log CFU/ml to get down and be 4.53 log CFU/ml in the last reading (12 days).

On the other hand the nano-encapsulated bacteria with the nanofiber showed a remarkable increase in their viability where it started with a log/ml of 5.29 to be at the last reading 7.34 log CFU/ml, this is mainly due to the coated material that cover the probiotic organisms causing by that as a protection wall from the medium conditions, such as the acidity and the high salinity of the product (Köksoy and Kılıç 2003), which are considerate as factors that effect in major way the growth of the tested probiotics.

On the other hand, the case of the free probiotic organisms was deferent where it had no protection barrier from the inoculation medium conditions and by that no

ability to resist to the acidity and salinity of the ayran despite the richness of the ayran in lactose and the necessary elements needed for the *L. brevis* bacterial growth (Akkaya, Kara et al. 2015).

The important viability index showed by the Nano-encapsulation probiotic coated by the sodium alginate and PVA fiber using the electrospinning technology confirm that the bio-polymer used in this study count as a great protection barrier where even that the sodium alginate polysaccharide is a water-soluble sugar that forms gel in the acid medium (Lee and Mooney 2012), yet it did not break down and it has a very good resistance to the acid medium and the high salinity.

Moreover, it caused no harm to the viability of the tested microorganism which would contribute to furnish and promote a commercial application of probiotics designed for the improvement of novel functional ayran enriched with *L. brevis* probiotic.

It is found as a conclusion of this test that this probiotic bacterial cell supports the encapsulation with sodium alginate where it had great viability index in the ayran, which is the most consumed drink in Turkey so that the society will get dairy products with excellent quality.

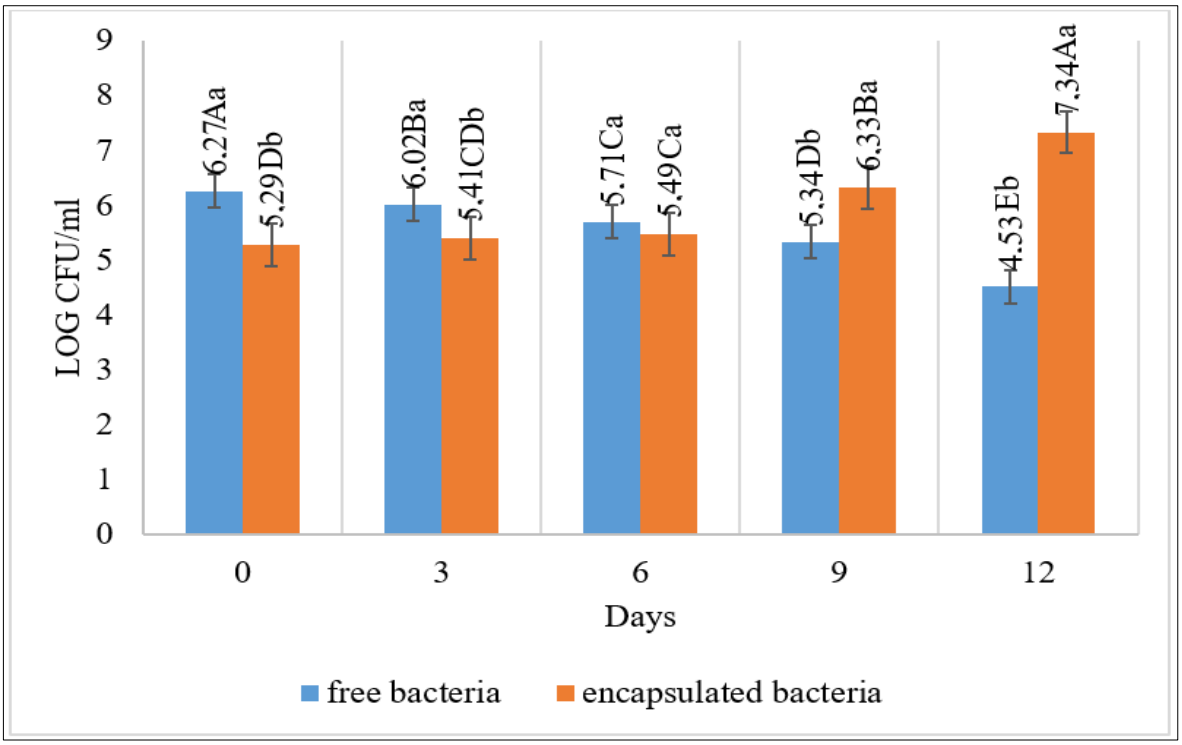

Figure 4.22. Free and encapsulated bacteria survival in ayran during the storage period (Different uppercase letters indicate significant differences between the storage time, different lowercase letters indicate significant differences between free and encapsulated bacterium in the same storage time $p<0.05$)

4.4.2 Free and encapsulated bacteria survival in orange and apple juice

Changes in the total viability ratio load throughout the storage period of orange and apple juice inoculated with a non-covered probiotic cell and a nano-covered probiotic cells are given in figure 23 and 24.

4.4.3 Free and encapsulated bacteria survival in apple juice

The survival ratio of the free cells in the apple juice for six weeks of a storage period was decreasing where it started with a 6.36 CFU /ml to be after four weeks 3.6 CFU /ml (figure. 23).

Where in the fifth and the sixth week no remaining bacteria were present in the apple juice despite the presence of all the nutrient elements in this juice, such as carbohydrate, proteins and vitamins, the free cells could not tolerate the medium

conditions, such as acidity or the high amount of antioxidants, that are known with their slow antibacterial activity (Naqvi, Nadeem et al. 2019).

The obtained results are similar to a previous study established by of Ding and Shah (Ding and Shah 2008), that was effectuated on eight LAB strains, where the maximal survival period was four weeks for the *L. brevis* in the apple juice in the free form.

Unlike the results demonstrated by the coated *L. brevis* bacteria with the sodium alginate polymer where the ratio was increasing after each reading until the fifth week when it started to decrease yet without despairing.

As the figure 23 present, the first reading gave a survival ratio of 5.22 CFU/ml this amount of bacteria kept getting higher until the 5^{th} week when it reached the maximum counting number of 7.44 CFU/ml, in the last week (6^{th} week) the loss of the viability started to be 6.95 CFU/ml and this could be explained by the solubility of the coating material, since one of the sodium alginate major properties is that its viscosity increase as the pH decrease (Lee and Mooney 2012) where the probiotic cells started to be released in the medium and effected by the low pH. However, the nano-encapsulated bacteria showed a greater survival ratio in the apple juice comparing to the free *L. brevis* calls that kept following down until total despairing.

At this point, the role of the covering nanofiber shine where it was like an armor protector that separate the microorganism from the medium yet it kept it safe from any antibacterial activity of the bioactive compounds of the apple juice, allowing by that the production of a functional juice enriched with probiotic *L. brevis* .

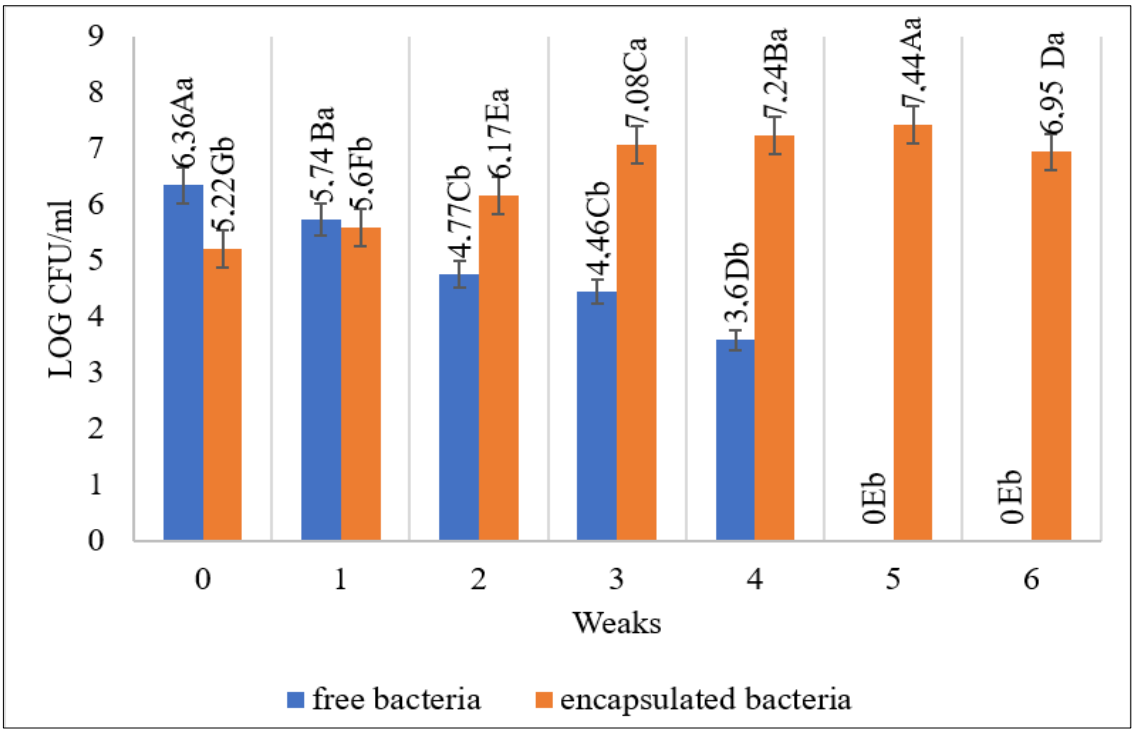

Figure 4. 23 Free and encapsulated bacteria survival in apple juice during the storage period (Different uppercase letters indicate significant differences between the storage time, different lowercase letters indicate significant differences between free and encapsulated bacterium in the same storage time $p<0.05$)

4.6.2 Free and encapsulated bacteria survival in orange Juice

Changing in the *L. brevis* viability count of orange juice samples during the storage period is seen in figure 24.

The non-coated probiotic bacteria were found to be in the first reading 6.04 CFU/ml in the orange juice this amount starts to drops every week to be 3.6 CFU /ml in the third week, the rest of the three weeks no prebiotic cells was found in the tested product and it was the most rapid viability loss of the free bacteria comparing to the other products.

On the other hand the high acidity of the medium and the richness of the product in the bioactive compounds which can have an antimicrobial role (Okeke, Okoli et al. 2015) this affected the growth of *L. brevis* probiotic, which was not the case for the nano-encapsulation bacteria where it started with CFU/ml of 5.15 to reach a

maximum growing of 7.17 CFU/ml in the fourth week to start to decrease to be 6.47 CFU/ml (figure.24), the covering polymers helped to maintain the bacteria from the acidity and any antimicrobial compounds of the product allowing it to survive and grow.

The obtained results indicate all the three tested products are very acid for the *L. brevis* growth which showed no tolerance, this confirm that only encapsulated probiotic can grow and survive under high acidity and salinity condition due to the coating material that serves as a protection barrier (Ding and Shah 2008, Gbassi and Vandamme 2012, Das, Roy et al. 2014), it also appears that the nanofiber protect the probiotic cells from any compound that could have an antimicrobial capacity.

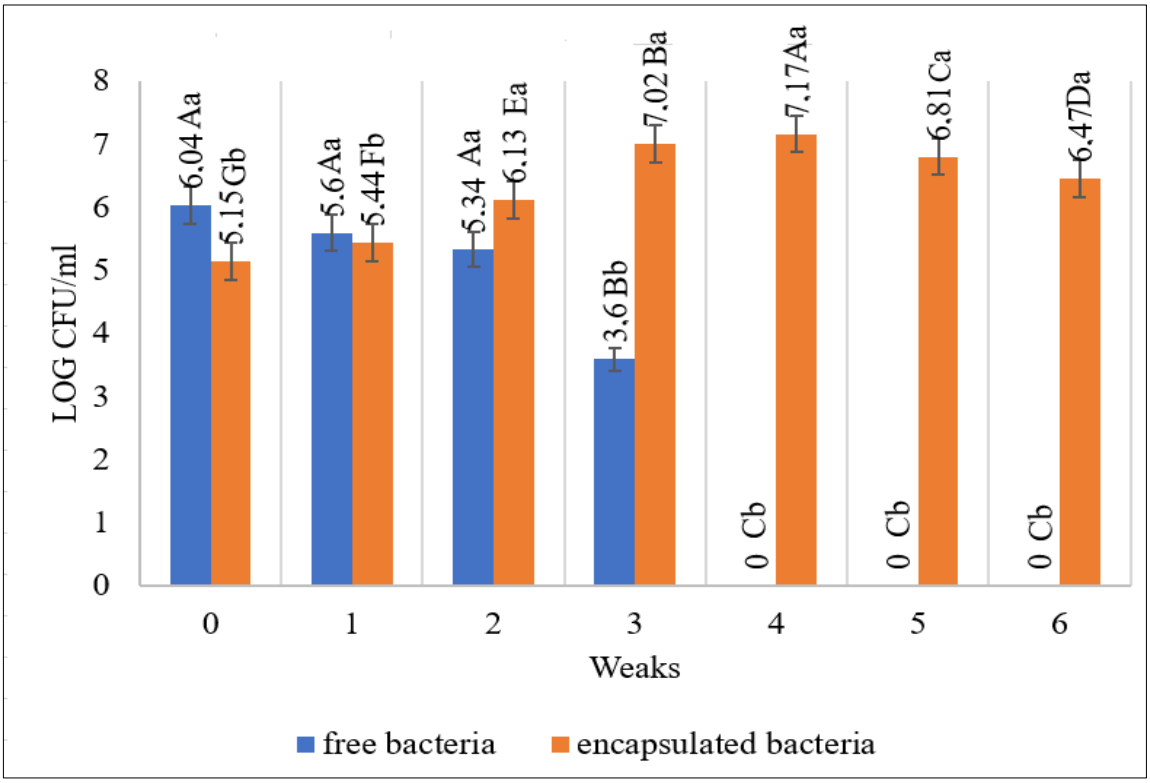

Figure 4.24 Free and Encapsulated bacteria survival in orange juice during the storage period (Different upper letters indicate significant differences between the storage time, different lowercase letters indicate significant differences between free and encapsulated bacterium in the same storage time $p<0.05$)

For the both cases of apple and orange juice, the obtained results found to support the inoculation of encapsulated of the *L. brevis* that in normal cases (free state) this probiotic has no capacity to tolerate the activities of the bio-actives compounds that existed in the orange and apple juice so the probiotic loss its viability and the inoculation process fails.

At this point the efficient of the coating utilization is illustrated where using the electrospinning technique within sodium alginate stand to be the needed solution, so juices (orange and apple) can be easily enriched with probiotic *L. brevis* despite their high number of bio-active compounds. The obtained results are similar of deferent *Lactobacillus* stains where all the encapsulated bacteria showed a batter survival ratio comparing to the free cells, and also the tested products containing the encapsulated microorganisms where more stable, this encourage more and more the utilisation and the production of functional food items using the encapsulated microorganism (table 11).

Table 4.11 Free and encapsulated deferent lactobacillus species in yogurt, apple and orange juice.

Product	Bacteria species	Free Log CFU/ml		Encapsulated Log CFU/ml		Survival ratio %		References
		Initial rate	Final rate	Initial rate	Final rate	Free	Encapsulated	
Apple juice	*L. rhamosus GG*	/	/	/	/	13.6	61.2	(Gandomi et al., 2016)
	L. rhamosus	8.5	1.5 (4 W)	8.6	5.2 (6W)	/	/	(Ding and Shah, 2008)
	L. sahvarius	8.2	1.9 (4 W)	8.5	5.5 (6W)	/	/	(Ding and Shah, 2008)
	L. plantanun	8.3	1.9 (4 W)	8.4	5.2(6 W)	/	/	(Ding and Shah, 2008)
	L. acidophilus	8.3	1.8 (4 W)	8.5	5.6(6 W)	/	/	(Ding and Shah, 2008)
	L. paracasei	8.4	1.7 (4 W)	8.6	5.3(6 W)	/	/	(Ding and Shah, 2008)

Table 4.11	**Table 4.11** Free and encapsulated deferent lactobacillus species in yogurt, apple and orange juice (continued)							
Orange juice	*L. paraacasei*	9.2	3.5 (12 W)	9.2	5 (12 W)			(Rawee et al, 2015)
	L. rhamosus	8.2	6.1 (3 W)	8.5	5.2(6 W)	/	/	(Ding and Shah, 2008)
	L. sahvarius	8.3	5.3 (3 W)	8.4	5.5(6 W)	/	/	(Ding and Shah, 2008)
	L. plantanun	8.4	5.3 (3 W)	8.5	5.2(6 W)	/	/	(Ding and Shah, 2008)
	L. acidophilus	8.1	5.4 (3 W)	8.5	5.6(6 W)	/	/	(Ding and Shah, 2008)
	L. paracasei	8.2	5.4 (3 W)	8.5	5.3(6 W)	/	/	(Ding and Shah, 2008)
	L. delbrueckii	9	1.5	9	8.8	/	/	(L Hruyia et al. 2018)
	L. bulgarius							
	L. plantarum							

Table 4.11	**Table 4.11** Free and encapsulated deferent lactobacillus species in yogurt, apple and orange juice							(continued)
yogurt drink	*L. acidophilus*	7.8	6.5 (45D)	8	8.4(45 D)	/	/	(Shahin zomorodi, 2019)
	L. casei	8.6	8.79 (6 W)	8.8	8.65 (6 W)	/	/	(Ines maria, 2013)
	L. plantarum TISTR1465	9.62	00	9.51	7.48	/	/	(Phoem, Mayiding et al. 2019)
	L. acidophillus (ATCC 314)	10.9	00 (4 W)	11	10.2 (4 W)	/	/	(Urbanska, Bhathena et al. 2007)

4.5.1 pH and Brix changes during the storage of probiotic ayran (yogurt drink), apple, and orange juice

Changes in the pH of ayran, apple and orange juice samples during the storage are provided in figures 25, 62 and 27. After twelve days of a storage period which is the standard storage time for the ayran, both non-encapsulated and encapsulated probiotics *L. brevis* showed a similar pH values which varied between 4.27 and 4.22, so it decreased with a value of 0.1 that is considerate as no significant change ($p \leq 0.05$), where the obtained results of the both studied probiotic were very close indicating that the probiotic organism in both cases had a very week use of the ayran compound especially lactose that is considerate to be the most consumed element by the lactobacillus (Teixeira 2014) producing lactic acid that causes the increase of the acidity of the product and by that decreasing the pH which almost maintains stable.

Likewise, no author reactions showed to have a place inside the product causing by the inoculated bacteria which mean that this probiotic organism bring no negative effect on the product proprieties and shelf life.

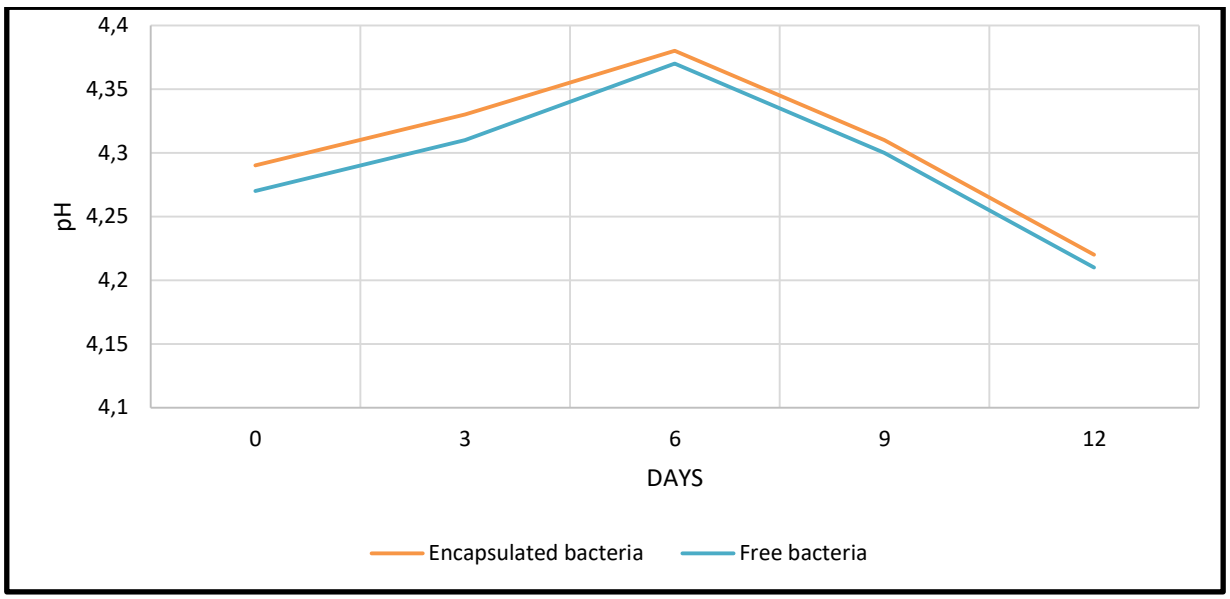

Figure 4.25 pH changing of ayran during the storage period (P<0.05 indicates statistical significance according to the ANOVA tukey test)

The same results were illustrated in the orange and apple juice where no remarkable change ($p \leq 0.05$) in the average pH value was noticed, wherein the apple juice case the pH started with a value of 4.13 to drop in the last reading and with a value of 0.1 and became 4 after one month

and half as a storage period, while in the orange the hydrogen potential was in the first reading 3.4 to decrease with a value of 0.1 and be 3.3 after six weeks of conservation period.

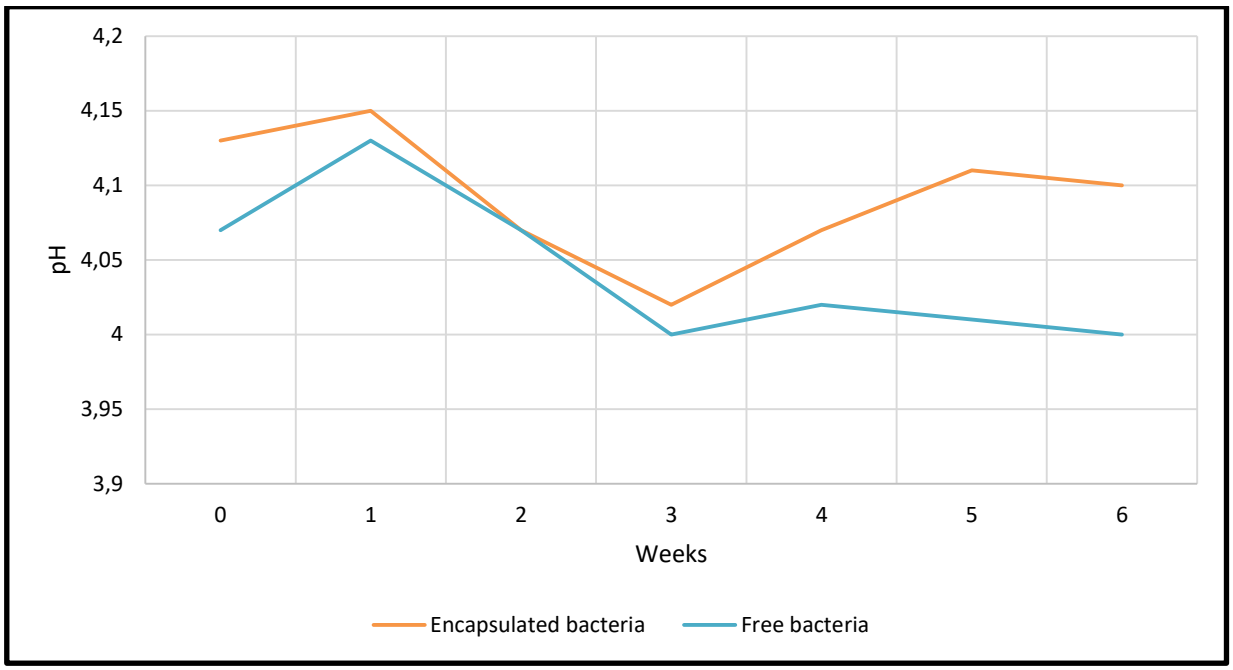

Figure 4.26 pH changing of apple juice during the storage period (P<0.05 indicates statistical significance according to the ANOVA tukey test)

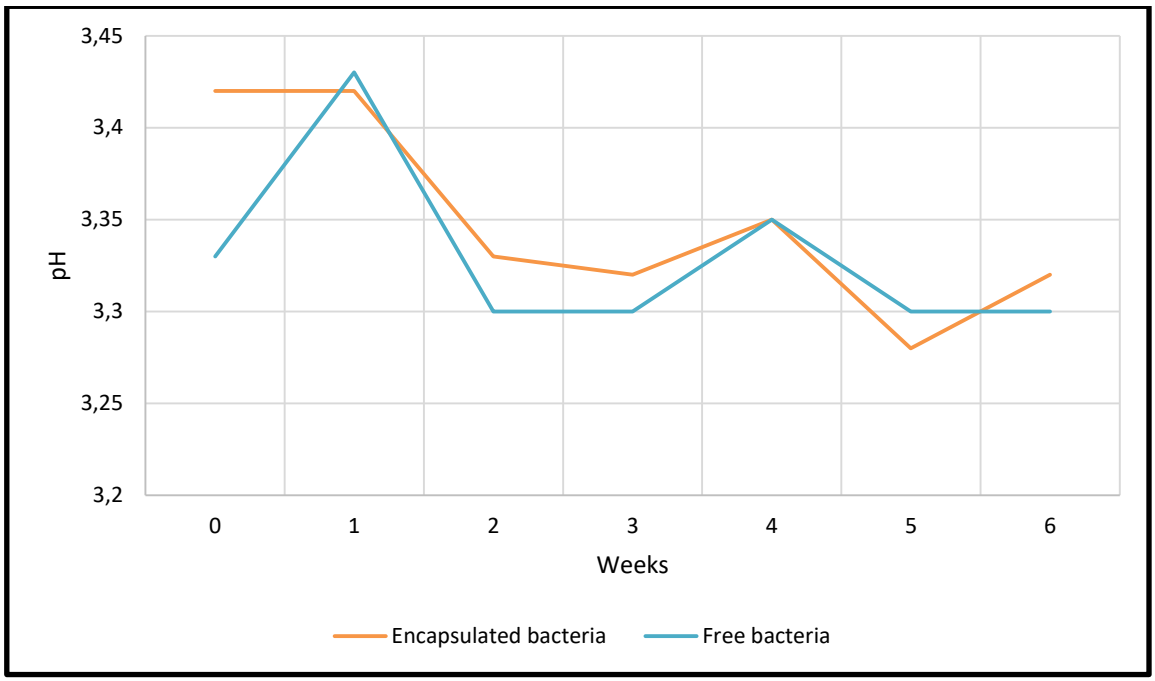

Figure 4.27 pH changing of orange juice during the storage period (P<0.05 indicates statistical significance according to the ANOVA tukey test)

The obtained results showed in both apple and orange juices are probably due to the non-consumption of the carbohydrate of or any other compounds of those products by the incorporated probiotic in both cases.

The total soluble solids in all the products ayran, orange, and apple juice maintain stable were no remarkable decrease have been noticed where especially for the ayran case where it was 5.

For both the apple and orange juices the dropping in the Brix value was very miner comparing to the storage period, where it starts with 12 to finish and be 10 at the last reading for the apple juice, while for the orange juice the first Brix reading gave a value of 13 to be decreased to 11 after one and half month.

The results were very close for both coated and non-coated bacteria the lost amount of the total soluble solids in the apple and orange juices is not very important comparing to the storage period in the presence of a living microorganism, this can be easily explained by the rapid viability loss which was noted by the free cells and by that the probiotic had no or very few consumption of organic compounds of the products.

The encapsulated was immobilized by the coating material that helped to reduce the contact between the soluble elements of the ayran and the juices and the probiotic microorganism. These results demonstrated that the encapsulation of probiotic bacteria would make a more stable product over a longer storage period (Ding and Shah 2008).

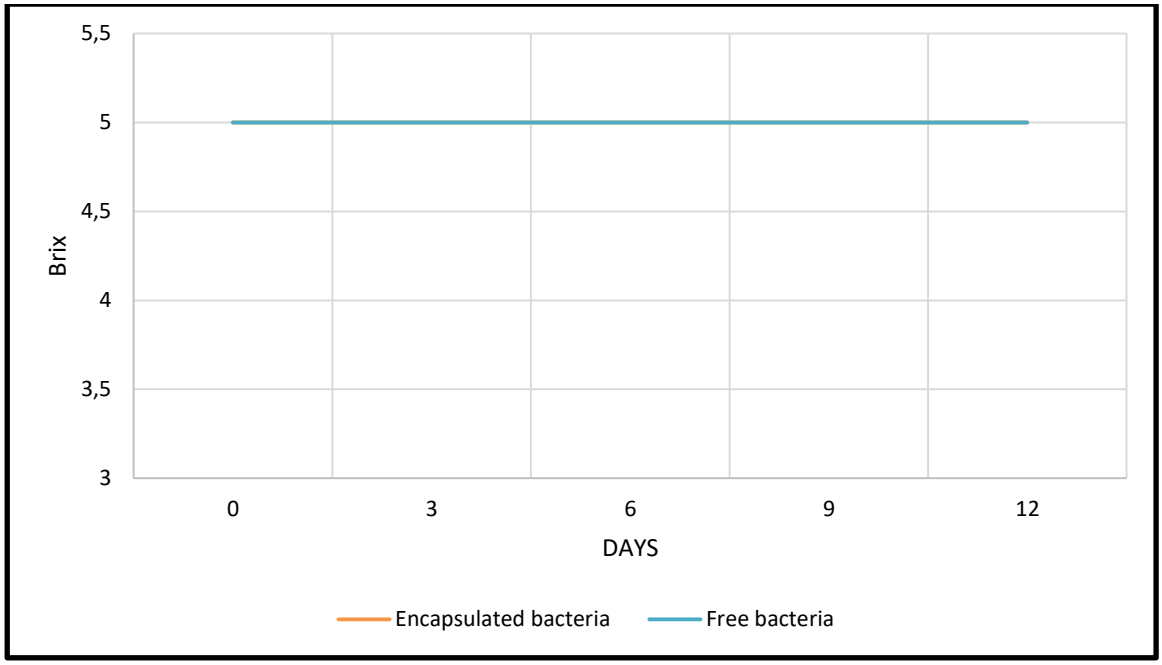

Figure 4.28 Brix changing of ayran during the storage period (P<0.05 indicates statistical significance according to the ANOVA tukey test)

The no statistical difference in terms of changes in the total soluble solids and the pH value between the storage periods in the ayran for both of the coated and non-coated bacteria, the high salinity of this medium is the main cause for the non-viability of the free bacteria which was mentioned previously were after 12 days of a storage period the free bacteria did not survive which explain exactly the no consumption of the soluble solids of this product by the inoculated microorganism. In the other hand the encapsulated *L. brevis* survived the high salinity of the ayran yet with no utilization of any nutritional element of this product where no changing was mentioned after each Brix measurement, this can be explained by the state of the bacteria, where the non-fiber acted as a barrier between the cell and the ayran compounds and by that the bacteria had no ability to consume those elements.

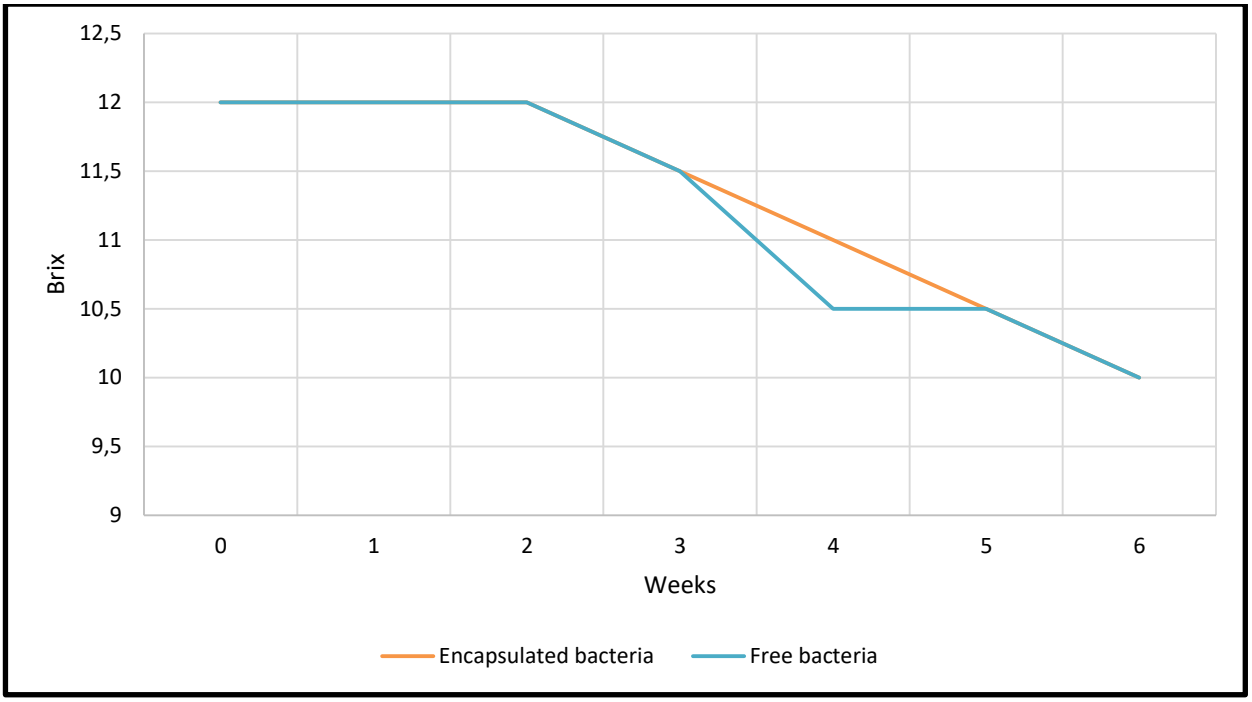

Figure 4.29 Brix changing of apple juice during the storage period (P<0.05 indicates statistical significance according to the ANOVA tukey test)

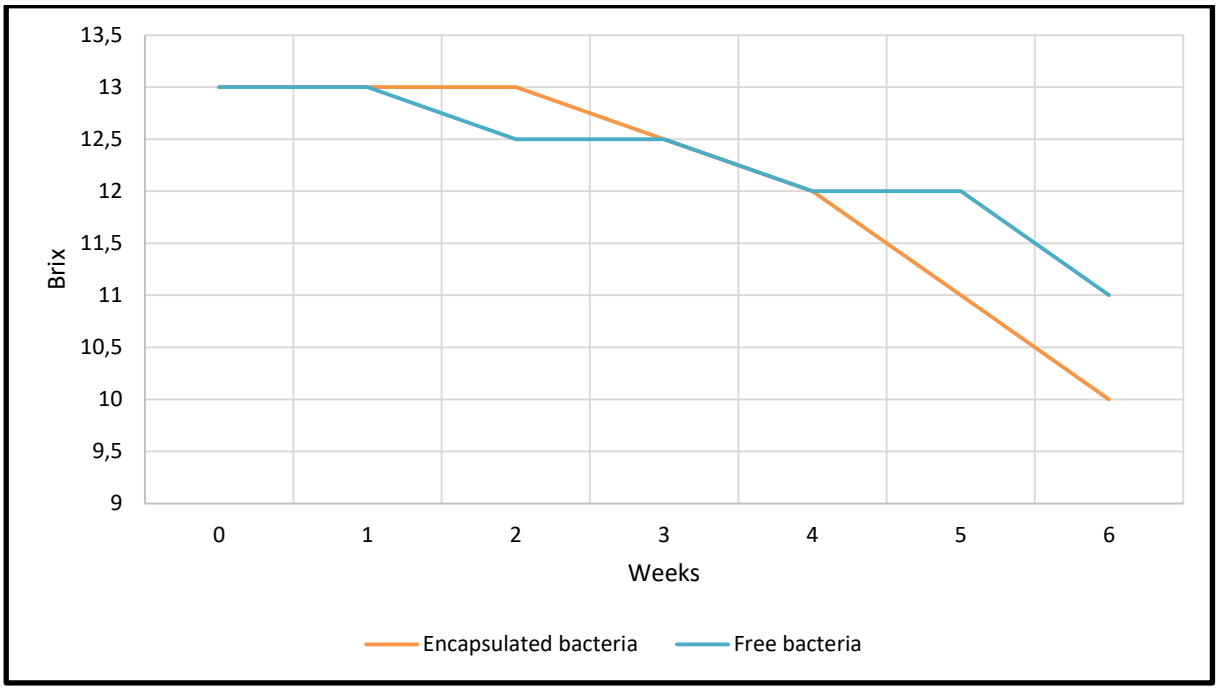

Figure 4.30 Brix changing of orange juice during the storage period (P<0.05 indicates statistical significance according to the ANOVA tukey test)

According to the obtained results that are present in the three figures (28, 29, and 30), it is observed that the inoculation of the free bacteria causes no chaining in the Brix values this is mainly due to the non-survive and viability capacity of the free *L. brevis* where it couldn't tolerate the medium condition acidity from one side.

On the other hand, the high number of bioactive compounds as in the case of orange and apple juices so the probiotic cell started to die and by that it had no ability to consume a high amount of the soluble solids that exist in those food products.

In the encapsulated bacteria *L. brevis*, no significant ($p \leq 0.05$) changes were noted in the Brix values during all the storage period of both juices' apple and orange, despite the viability of this inoculated bacteria for one and half month in the two products.

Yet no noticeable consumption of soluble solids was shown which is mainly due to the coating nanofiber that separated the probiotic microorganism from the nutritional elements of the products.

So, the cell was trapped inside the fiber with no ability to use the soluble solids this was confirmed previously by all the profiles and scans of the characterization part, where the adhesion between the biopolymer that was sodium alginate and the bacteria was very strong in a global view.

The electrospinning technique within sodium alginate as a coating material for the *L. brevis* is very efficient where the bacteria survive in both apple and orange juice yet without affecting the elements of the constitution of those products (no changing accrues to the propriety of the product).

4.5.2 Color changes

Tables 11, 12 and 13 illustrate the color changes in the three products, where the *L**, *a** and *b** values were taken, few differences were showed between the product inoculated with encapsulated bacteria and free bacteria may be due to the covering material (SA/PVA) that cause slight reducing in the product lightness and by that the *L** value changed with a slight difference in a non-stable way in the three food items.

The obtained results of the pH and Brix in the three chosen food items illustrated during the storage period confirm that the inoculated bacteria had no interference in the lightness changing, where the observed changes was caused by the browning causing either by the

enzymatic activity as in the case of apple and orange juices (Joslyn and Marsh 1935, Özoğlu and Bayındırlı 2002); or non-enzymatic browning (Selen Burdurlu and Karadeniz 2003, Bharate and Bharate 2014), which could be caused by either the autooxidation of the products organic elements oxidation as indicated by the *a** and *b** values (Pathare et al., 2013), or the storage periods or the light all those factors play a role in changing the product color.

Table 4.11 Color properties of yogurt drink sample with free and encapsulated bacteria

Color		T0	3 Days	6 Days	9 Days	12 Days
L	E-B	57.80 ±0.13^{Aa}	55.40 ±0.17^{Ba}	48.55 ±0.22^{Eb}	53.23 ±0.19^{Cb}	51.33±0.29^{Db}
	F-B	51.53 ±0.03^{Db}	53.39 ±0.3^{Cb}	56.55 ±0.42^{Aa}	55.47 ±0.44^{Ba}	53.44±0.36^{Ca}
b*	E-B	-2.81 ±0.06^{Da}	-3.16 ±0.12^{Ea}	-1.55 ±0.14^{Ca}	-1.19 ±0.15^{Ba}	-0.39 ±0.27^{Aa}
	F-B	-2.83 ±0.16^{Ca}	-3.13 ±0.1^{Ca}	-1.53 ±0.27^{Ba}	-1.18 ±0.08^{Ba}	-0.48 ±0.28^{Aa}
a*	E-B	-0.83 ±0.08^{Bb}	-0.24 ±0.12^{Aa}	-3.48 ±0.41^{Ca}	-3.28 ±0.28^{Ca}	-3.63 ±0.2^{Ca}
	F-B	-0.51 ±0.14^{Aa}	-0.43 ±0.24^{Aa}	-4.22 ±0.07^{Bb}	-4.36 ±0.47^{Bb}	-4.59 ±0.44^{Bb}

E-B: Encapsulated Bacteria, F-B: Free Bacteria (Different superscript uppercase letters in the same line indicate significant differences according to storage time, different superscript lowercase letters in the same column indicate significant differences between free and encapsulated bacterium in the same storage time $p<0.05$).

Table 4.12 Colour properties of apple juice sample with free and encapsulated bacteria

Color		T0	1 Week	2 Week	3 Week	4 Week	5 Week	6 Week
L	E-B	34.50 ± 0.12^{Bb}	28.60 ± 0.16^{Ea}	31.52 ± 0.27^{Db}	27.45 ± 0.27^{Fb}	28.47 ± 0.29^{Ea}	33.57 ± 0.38^{Ca}	36.39 ± 0.25^{Aa}
	F-B	36.49 ± 0.25^{Aa}	27.25 ± 0.06^{Db}	33.20 ± 0.13^{Ba}	29.45 ± 0.40^{Ca}	27.56 ± 0.47^{Db}	26.51 ± 0.09^{Eb}	26.38 ± 0.41^{Eb}
b*	E-B	-1.54 ± 0.27^{Ea}	0.69 ± 0.15^{Ab}	-0.84 ± 0.10^{Db}	-0.06 ± 0.04^{Ba}	0.69 ± 0.24^{Aa}	-0.38 ± 0.15^{BCa}	-1.61 ± 0.21^{CDb}
	F-B	-4.14 ± 0.09^{Gb}	2.47 ± 0.14^{Aa}	0.51 ± 0.24^{Ca}	-0.79 ± 0.18^{Eb}	0.08 ± 0.22^{Db}	-1.84 ± 0.11^{Fb}	1.73 ± 0.09^{Ba}
a*	E-B	-1.36 ± 0.12^{Ca}	-0.62 ± 0.26^{Ba}	-0.15 ± 0.07^{Aa}	-0.12 ± 0.06^{Aa}	$-0.44\pm 0.32A^{Ba}$	-0.48 ± 0.32^{ABa}	-0.40 ± 0.14^{ABa}
	F-B	-2.23 ± 0.08^{Db}	-1.27 ± 0.04^{Cb}	-0.12 ± 0.04^{Aa}	-0.15 ± 0.1^{Aa}	-0.21 ± 0.12^{ABa}	-0.39 ± 0.22^{Ba}	-1.10 ± 0.07^{Cb}

E-B: Encapsulated Bacteria, F-B: Free Bacteria (Different superscript uppercase letters in the same line indicate significant differences according to storage time, different superscript lowercase letters in the same column indicate significant differences between free and encapsulated bacterium in the same storage time $p<0.05$).

Table 4.13 Colour properties of Orange juice sample with free and encapsulated bacteria

Color		T0	1 Week	2 Week	3 Week	4 Week	5 Week	6 Week
L	**E-B**	31.19 ±0.02^{Aa}	28.20 ±0.01^{Cb}	27.01 ±0.02^{Eb}	29.91 ±0.01^{Ba}	26.43 ±0.01^{Fb}	27.98 ±0.01^{Da}	26.24 ±0.01^{Gb}
	F-B	30.04 ±0.04^{Db}	29.13 ±0.01^{Ea}	33.19 ±1.01^{Aa}	26.80 ±0.10^{Fb}	31.41 ±0.01^{Ca}	25.77 ±0.01^{Gb}	31.93 ±0.02^{Ba}
b*	**E-B**	-2.23 ±0.01^{Gb}	4.53 ±0.01^{Ba}	2.10 ±0.02^{Da}	5.25 ±0.00^{Aa}	4.04 ±0.01^{Ca}	1.99 ±0.01^{Eb}	0.43±0.00^{Fa}
	F-B	-1.52 ±0.01^{Fa}	3.65 ±0.02^{Ab}	0.16 ±0.01^{Eb}	1.56 ±0.02^{Cb}	0.66 ±0.01^{Db}	2.27 ±0.00^{Ba}	-4.53 ±0.01^{Gb}
a*	**E-B**	0.87 ±0.02^{Ab}	-1.92±0.01^{Fb}	-0.67 ±0.02^{Cb}	-2.32 ±0.02^{Gb}	-1.67 ±0.02^{Eb}	-1.40 ±0.01^{Db}	0.27 ±0.01^{Bb}
	F-B	1.10 ±0.04^{Aa}	-1.82 ±0.02^{Ga}	-0.53 ±0.02^{Ca}	-0.91 ±0.01^{Da}	-1.14 ±0.01^{Ea}	-1.24 ±0.02^{Fa}	0.88 ±0.01^{Ba}

E-B: Encapsulated Bacteria, F-B: Free Bacteria (Different superscript uppercase letters in the same line indicate significant differences according to storage time, different superscript lowercase letters in the same column indicate significant differences between free and encapsulated bacterium in the same storage time $p<0.05$).

4.6 Conclusion

In this study, the production of three functional food models, Turkish ayran apple juice and orange juice using nano-encapsulated *L. brevis* within sodium alginate-based biopolymers was the main goal. To promote the functionality of those three-food item using a very good prebiotic microorganism, the ability of encapsulation this probiotic using the nanotechnology utilizing sodium alginate as a biopolymer coating nanofiber was investigated.

1. Applying the nanotechnology method more specifically the electrospinning technique using sodium alginate fiber as a coating material for the *L. brevis* cell was a very successful operation where this bacterium found with a remarkable viability ratio that reached 81%.
2. The encapsulated probiotic *L. brevis* showed a very high resistance to the in vitro gastric conditions with a viability ratio more than 50% after both treatment with pepsin and trypsin, this results confirm that the sodium alginate nanofiber manufactured by the electrospinning technique serve as a good coating material to protect the probiotic cell under the gastro-tract conditions.
3. Fourier-transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR), characterization by X-ray diffraction (XRD) and characterization by optical microscope and scanning (SEM) microscopes scans, pictures and profiles illustrate the good adhesion between the extra-cellular-matric of the tasted probiotic microorganism and the used biopolymer as coating fiber where this will serve for the immobilization of the cell inside the products which was one of the ultimate aims.
4. The sodium alginate biopolymer that was used as an encapsulation material for the *L. brevis* shows a good protection capacity of this cell from the high treatment temperature, which was presented by the DSC test result.
5. The surviving ratio of the *L. brevis* under encapsulation stadium in the ayran was very good, no loss of viability was noticed until the last reading, which made the used encapsulation technique and the covering material very efficient to produce a functional ayran with probiotic bacteria despite its high salinity.
6. Apple juice can be successfully enriched with nano-encapsulated probiotic *L. brevis* with sodium alginate as covering barrier, wherein normal cases free bacteria cannot survive for more than four weeks inside this product, the coated probiotic cell stands to survive

for six weeks which is a major period for a probiotic microorganism inside an acid product.

7. Based on the obtained result, encapsulated *L. brevis* have the ability to survive for a period of one and half month in the orange juice while a non-coated *L. brevis* disappears after three weeks as maximum, this concludes to efficiency of the nanotechnology used technique which was electrospinning in the production of functional orange juice inoculated with *L. brevis* within sodium alginate-based biopolymers.
8. As a further physicochemical property, there was no changess in pH values as a result after 12 days of storage for the ayran and six weeks of storage for the orange and apple juices.
9. It was found that the inoculation of encapsulated *L. brevis* in the three food products ayran, apple, and orange juice, did not cause any difference in the total soluble solids amount (Brix) during the storage periods.
10. The effects of the inoculation of encapsulated *L. brevis* probiotic on the color proprieties of the three food items were analyzed by color analysis during storage time while there was no significant difference in *L* value during storage time.

This research aimed to coat probiotic cells using the nano-encapsulation technique with an environment-friendly coating material for the application of the bio-delivery system. The present study suggests that the viability of the encapsulated bacteria was observed at a considerable rate, which was as excellent as in the survival and growth rate in the peptic treatment.

Bio-encapsulation using the electrospinning technology would contribute to furnish and promote the industrial and commercial application of probiotics designed for the improvement of many properties, which are microbial and nutritional. However, further analysis of encapsulated probiotics bacterial cells supports the encapsulation of *L. brevis.*

Yogurt drink produced has also shown a very excellent viability index. Thus, society will get several types of products with excellent quality. For wise consumers, this is economical and also reduces food wastage, improves food quality research.

The encapsulated probiotic microorganism showed high survival ratio in the ayran, apple and orange juice despite the acidity and the salinity of the products comparing to the free probiotic microorganism, no significant decreasing ($p \leq 0.05$) was noticed in the pH and Brix of the three

products and by that causing no reducing to the products shelf life and by that maintaining the food stability.

Moreover, the three food products are considered to be suitable as nano-encapsulated probiotic vehicles, which leads to producing a functional yet stable ayran, apple and orange juice that is tailored to the consumers' needs.

5

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Publications from the thesis

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Papers

1. Mohammed Jawad Mohaisen, Rusen Metin Yildirim, Mustafa Tahsin Yilmaz, Muhammed Zeki Durak, "Production of functional yogurt drink, apple and orange juice using nano-encapsulated *L. brevis* within sodium alginate-based biopolymers". Science of Advanced Material Journal SAM, Dec,2019.