DEVELOPMENT OF FUNCTIONAL CHOCOLATE USING GAMMA-AMINO BUTYRIC ACID PRODUCER PROBIOTIC: PROMINENT TO ALLEVIATE ANXIETY DISORDER

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ABSTRACT

DEVELOPMENT OF FUNCTIONAL CHOCOLATE USING GAMMA-AMINO BUTYRIC ACID PRODUCER PROBIOTIC: PROMINENT TO ALLEVIATE ANXIETY DISORDER

The purpose of this study was screening of four probiotics to investigate for their gamma-aminobutyric acid producing capabilities. *Lactobacillus rhamnosus* NRRL B-442 strain has been selected for its maximum gamma-aminobutyric acid concentration. In this study, a new chocolate product has been developed by incorporation of probiotic cells; stability of the probiotics was improved by microencapsulation by water-in-oil emulsion technique using whey-pullulan complex.

Survival rates of microencapsulated and free-cells of probiotic bacteria in chocolate have been compared. Probiotic counts were found as 6.75 log CFU/g and 7.20 log CFU/g in chocolate, for encapsulated and free-cells, respectively, at the end of 60 days. High cell counts and increased cell resistances were remained at 4°C when compared at 25°C, in chocolate samples. Furthermore, probiotics did not affect the physical, chemical and sensory properties of chocolate, whereas the texture analysis showed an increase in the hardness of free-cell in chocolate bacteria on the 0th and 60th day. Survival rate of microencapsulated bacteria in chocolate samples was observed at higher percentage 87% than free-cell bacteria e.g. 75% during simulated *in-vitro* analysis.

Consequently, *Lb. rhamnosus* with the highest GABA producing capability may provide insight for patients having an anxiety disorder, since this strain has been thought as having a therapeutic effect. A new functional food model was developed for “Gut-Brain Axis” phenomena since the chocolate could be accepted as a good carrier for probiotics.

ÖZET

KAYGI BOZUKLUĞUNU GİDERMEDE ÖNEMLİ OLAN: GAMA-AMİNO BÜTİRİK ASİT ÜRETİCİSİ PROBİYOTİK KULLANILARAK FONKSİYONEL ÇİKOLATA GELİŞTİRİLMESİ

Bu çalışmanın amacı dört probiyotik bakterilerinin taranmasıyla gama-amino bütirik asit üretim kapasitelerini araştırmaktır. *Lactobacillus rhamnosus* NRRL B-442 suşu en yüksek gama-amino bütirik asit konsantrasyonuna sahip olmasından dolayı seçilmiştir. Bu çalışmada, probiyotik ilaveli yeni bir çikolata geliştirilmiştir. Pobiyotik bakterinin stabilitesini arttırmak için peynir altı suyu proteini-pullulan birleşiği kullanılmıştır. Probiyotik bakterinin mikroenkapsülasyonu emülsiyon yöntemi ile gerçekleştirilmiştir.

Serbest ve enkapsüle probiyotik bakterilerinin çikolatadaki canlı kalma oranları karşılaştırılmıştır. 60 günlük depolama koşullarının sonunda, çikolatadaki enkapsüle probiyotik hücre sayısı 6.75 log KOB/ g ve serbest hücre sayısı 7.20 log KOB/ g olarak bulundu. Çikolata örneklerinde probiyotiklerin canlılık ve hücre dayanımları, 25°C'ye kıyasla 4°C'de daha yüksek bulundu ($P < 0.05$).

Ayrıca probiyotiklerin sütlü çikolataya eklenmesiyle, çikolatanın fiziksel, kimyasal ve duyusal özelliklerinin etkilemezken, 0. gün ve 60. gündeki tekstür analizinde, serbest hücreli çikolata örneklerinde sertlikte bir artış gözlemlenmiştir. Yapay *in-vitro* analizi boyunca, çikolata örneklerinde bulunan mikroenkapsül bakterinin yaşama oranı serbest hücreye göre yüksek oranda olduğu gözlemlenmiştir.

Sonuç olarak, en yüksek GABA üretim kabiliyetine sahip olduğu belirlenen *Lb. rhamnosus*'un anksiyete bozukluğu olan bireylere terapötik bir etkisi olabileceği düşünülmüştür. Bu çalışmada, "Bağırsak-Beyin Ekseni" kavramı açısından önemli bir yer tutabilecek olan yeni bir fonksiyonel gıda modeli olarak, probiyotikliler için iyi bir taşıyıcı olarak düşünülen çikolata ürünü geliştirildi.

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CHAPTER 1

INTRODUCTION

People start to be prone consuming functional foods that support a healthy lifestyle with changing concept of nutrition and diet in. Today, functional food is defined as foods having positive effects on biological functions, improved health status and/or reducing the risk of existing diseases. Recently, food products incorporated with probiotics have gained significant interest both in our country and worldwide. Therefore, in many scientific studies, the positive and improvement effects of probiotics have been revealed. Functional foods, which are supplemented with probiotics, have been confirmed their beneficial effects in supporting intestinal health, preventing diseases in a number of studies. These studies are related with effectiveness of intestinal microbiota on the immune system, brain development, as well as behavior; therefore it is envisaged that intestinal health is associated with brain and cognitive health. Furthermore, psychological disturbances such as stress and emotional changes have significant negative effects on the intestinal microbiota. Probiotics have positive effects on brain activity, brain development and behavior; some research on this area have shown significant findings for neurotransmitters having influences on anxiety disorder. There has been a lot of research carried out probiotics to date, probiotic chocolate studies are also present in the literature, but information on the gut-brain axis is lack.

The objective of this study is to develop a functional chocolate product, supplemented with GABA producing probiotics prominent to alleviate anxiety disorders. Therefore, the study covers information on GABA (so-called neurotransmitter) production by four strains of lactic acid bacteria having probiotic properties. Improvement of probiotic viability was achieved by microencapsulation with using emulsion technique. Chocolate samples incorporated with probiotics were examined for their shelf life, sensorial and structural characteristics, besides *in-vitro* digestion experiments were conducted. The chocolate samples were evaluated as control chocolate (C), chocolate with microencapsulation probiotic bacteria (M) and chocolate with free-cell bacteria (F).

CHAPTER 2

PROBIOTICS

Probiotics are identified "live microorganisms having beneficial effects on host's health when taken in sufficient quantities" as regards the World Health Organization and Food and Agriculture Organization of the United Nations (FAO/WAO, 2001). In addition, they are known as a microbial dietary supplement that are beneficial effects on animal and people (Douglas & Sanders, 2008). *Lactobacillus* and *Bifidobacterium* strains have been used most commonly probiotics because of seeing as dominant in the human intestine (Boyle & Tang, 2006; Douglas & Sanders, 2008). In Table 2.1. mentions from different probiotic species actively used.

Table 2. 1. Types of probiotics

Types of Probiotics	**Probiotic species**	**Reference**
Lactobacillus	*Lb. rhamnosus, Lb. paracasei, Lb. reuteri, Lb. casei, Lb. bulgaricus, Lb. acidophilus and Lb. plantarum*	(Dixit, Wagle, & Vakil, 2016).
Bifidobacterium	*B. bifidum, B. longum, B. breve, B. catenulatum, and B. animals*	(Westermann, Gleinser, Corr , & Riedel, 2016).
Bacillus	*B. coagulans, B. laterosporus and B. subtilis*	(Nguyen, et al., 2016).
Lactococcus	*Lb. rhamnosus, Lb. reuteri, Lb. lactis, Lb. casei, Lb. acidophilus, Lb. plantarum and Lb. curvatus*	(Eid, et al., 2016).
Enterococcus	*E. facium*	(Onyenweaku, Obeagu, Ifediora, & Nwandikor, 2016).
Streptococcus	*S.thermophilus, S. sanguis,, S. salivarius, S. oralis, and S.mitis*	(Arora, Singh, & Sharma, 2013).
Saccharomyce	*S.boulardii*	(Chen, et al., 2013).

Probiotics have some properties;

- They are more resistant against gastric acid, bile salt than other bacteria. Thus, they have to be stable under these negative environmental conditions to be metabolized in gut systems.
- They must be safe. When humans or animals consume bacteria, they should not have an adverse side effect.
- They should be resistant against antibiotics because of the fact that they are effective on the gut flora.
- They should be held on the gut mucosa.
- When they are added to food, they should not decrease the food quality (Yılsay & Kurdal, 2000; Fernandez, Boris , & Barbes , 2003; Patterson & Burkholder, 2003).

Consumption of probiotic products has a beneficial effect on the strengthening of body health, immune system as well as they fight against other diseases and harmful bacteria in the gut system (Lourens-Hattingh & Viljoen, 2001). Probiotics also contribute to prevent from many diseases (Figure 2.1).

Many studies are reported that probiotic bacteria have a therapeutic effect on healthy subjects such as prevention of hypercholesterolemia, prevention of diarrhea, prevention of urogenital infection, protection against colon/bladder cancer, and prevention of osteoporosis. They have also beneficial effect such as protecting normal intestinal microflora toward pathogens, improving the immune system, and decreasing blood pressure (Ranadheera, Baines, & Adams, 2010; Douglas & Sanders, 2008; Lourens-Hattingh & Viljoen, 2001).

Lb. acidophilus and *Lb. casei* Shirota, both have been found to decrease *Helicobacter pylori* infection but not completely eradicate (Cats, et al., 2003). *Lb. plantarum* has been found effective decreasing inflammation in inflammatory bowel disease (Schultz, et al., 2002). It was demonstrated in a clinical study that the early atopic disease can be prevented by *Lactobacillus* GG (Kalliomrki, et al., 2001). It was observed that hypertensive patients who consumed fermented milk containing *Lactobacillus helveticus* experienced reductions in blood pressure (Hata, et al., 1996).

Probiotics may also be used therapeutically to reduce stress, anxiety disorder, depression, and other mental disorders (Logan & Katzman, 2005; Bruce-Keller, et al., 2015; Cryan & O'Mahony, 2011). For instance, it was found that *Lactobacillus*

rhamnosus has effective in reducing depressive behavior of mice (Bravo, et al., 2011) . In addition, multispecies probiotic bacteria may be used to reduce sad mood, depression as well (Steenbergen, Sellaro, van Hemert, Bosch, & Colzato, 2015). *Lactobacillus, Lactococcus* and *Bifidobacterium* genera have been found effective in depressive symptoms as well as anxious symptoms (Benton, Williams, & Brown, 2007; Yamamura, et al., 2009; Desbonnet, et al., 2010; Bravo, et al., 2011; Messaoudi, et al., 2011; Rao , et al., 2009).

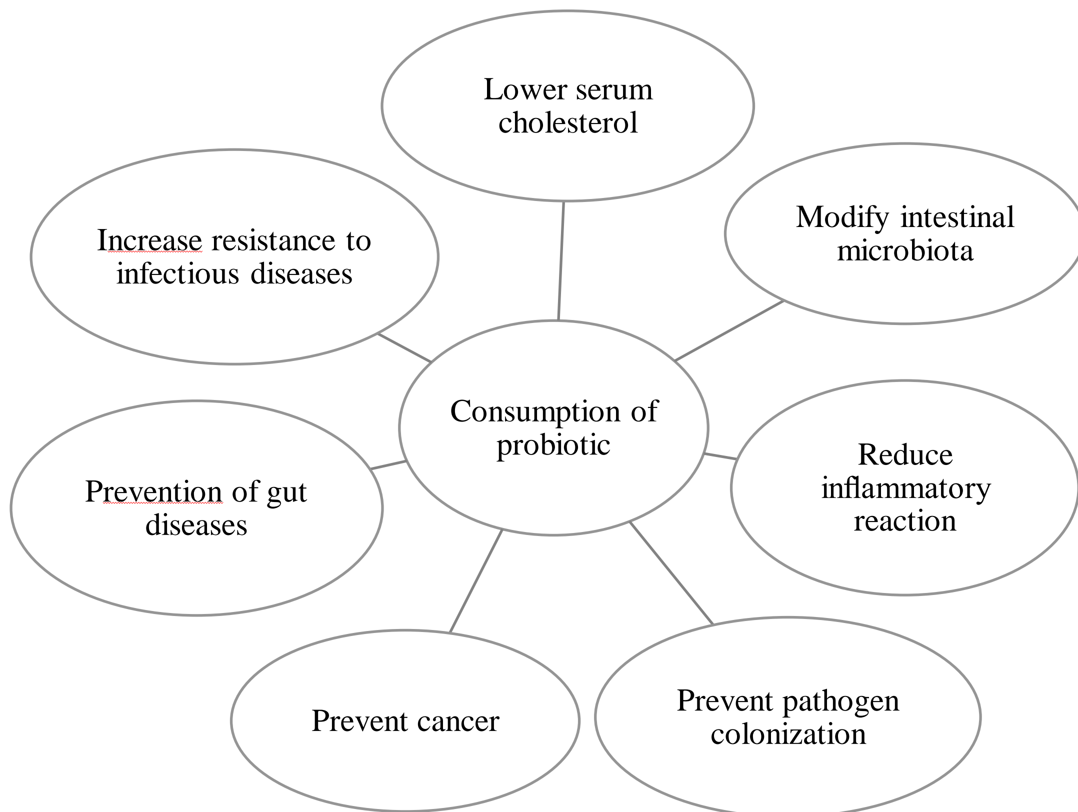

Figure 2. 1. Beneficial effects of consumption of probiotics.

Attention to functional foods with probiotics has been increased because of their overall contribution beneficial effect on human health. Therefore, there are a lot of new commercial functional food products with probiotic bacteria and continue to be produced (Burgain, Gaiani, Linder, & Scher, 2011). Fermented foods such as yogurt, ice cream, kefir, and cheese are known as probiotic products for ancient times (Cross, Stevenson, & Gill, 2001; Kopp-Hoolihan, 2001; Şengün, 2011; Desmond, et al., 2005; Stanton, Desmond, Fitzgerald, & Ross, 2003). Recently, various probiotic strains have been used as commercial sources in industrial products (Table 2.2) (Kerry, et al., 2018).

Table 2. 2. Used probiotic bacteria in commercial products

Sold as ingredient		Probiotics supplement		Dairy products	
Probiotics strain	**Source**	**Probiotics strain**	**Source**	**Probiotics strain**	**Source**
Lb. acidophilus, *B. lactis*, *Lb. rhamnosus*	Danisco	*S. cerevisiae boulardii*	Biocodex	*Lb. casei* Shirota *B. breve* strain Yakult	Yakult
Lb.rhamnosus, *Lb. acidophilus*	Institut Rosell	*B.infantis* 35,264	Procter and Gamble	*B. animals*	Dannon
Lb. paracasei, *Lb. rhamnosus*	GenMont Biotech	*Streptococcus oralis*, *Streptococcus uberis*	Oragenics Inc.	*Lb. rhamnosus*	Valio Dairy
Lb. acidophilus, *Lb. paracasei*, *B. lactis*	Chr. Hansen	*Lb. rhamnosus*, *Lb. gasseri*	Bifodan	*Lb. casei*, *Lb. rhamnosus*	Danone
Lb. rhamnosus, Lb. lactis	Essum AB	*Bacillus coagulans* BC30	Ganeden Biotech Inc.	*Lb. johnsonii* and *Lb. acidophilus*	Nestlé
Lb. salivarius	University College Cork	*Lb. reuteri*, *Lb. rhamnosus*	Jarrow Formulas		
B. longum	Morinaga Milk Industry Co. Ltd.				
Lb. acidophilus	Lacteol Laboratory				
Lactobacillus paracasei	Medipharm				
Lb. fermentum	Probiomics				
Lb. rhamnosus	Probi AB				

(Source: Kerry et al., 2018).

2.1. Encapsulation

Survival of bacteria is essential in the human gut due to their beneficial effects (Anal & Singh, 2007). Therefore, probiotics content must include minimum 10^6-10^7 CFU per ml/per gram of viable probiotic bacteria (FAO/WAO, 2001) as the standard. However, there are many concrete factors affecting the viability of probiotics, for example, pH, dissolved oxygen ingredients, hydrogen peroxide, species, storage condition and concentration of bacteria (Dave & Shah, 1997; Lankaputhra, Shah, & Britz, 1996). Encapsulation methods used for probiotics may overcome these problems (Gismodo, Drago, & Lombardi, 1999). Microencapsulation is also effective for their survival under gastrointestinal conditions and used to improve the viability of bacteria during storage to resume their health-promoting effects. There are different types of microencapsulation techniques shown in Figure 2.2. However, microencapsulation techniques such as emulsion, spray drying, and extrusion are commonly preferred than many other techniques since their effectiveness (Dubey , Shami , & Bhasker Rao , 2009; Ünal & Erginkaya, 2010).

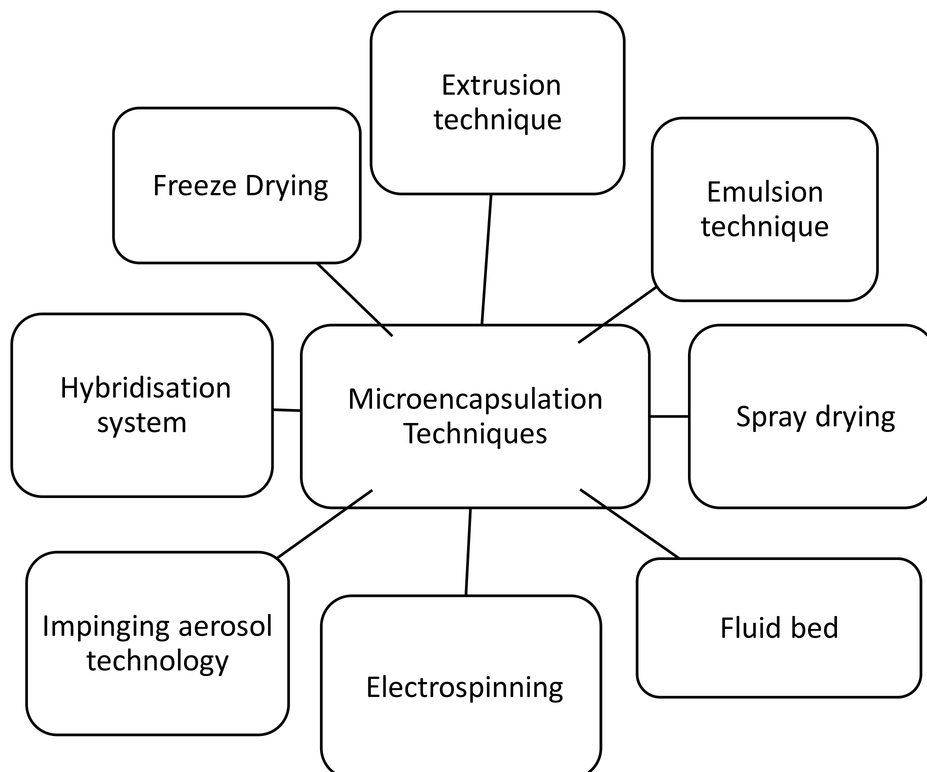

Figure 2. 2. Techniques for microencapsulation of probiotics (Source: Martin, Lara-Villoslada, Ruiz, & Morales, 2015).

2.1.1. Emulsion Technique

This method depends on the cell-polymer suspension as the discontinuous phase and a large volume of oil as a continuous phase. After the water-in-oil emulsion is formed, hardening solution is added to the emulsion and the soluble polymer is insolubilized to form microcapsules including bacteria into the oil phase (Heidebach, Först, & Kulozik, 2012). Then, the beads are harvested by filtration. While this technique has high microencapsulation efficiencies, it possesses poor survival rates through gastrointestinal transit because of reduced microcapsule size (Winder , et al., 2003). There are various polymers used such as carrageenan, sodium carboxymethyl cellulose, alginate, chitosan, gelatin and pullulan for the method.

2.1.2. Spray Drying Technique

This method is the most widely used microencapsulation technique because it is flexible and economical to make powders. Microencapsulation has been commonly used not only for probiotics but also vitamins, fish oil, and flavor (Desai & Jin Park, 2005). This method depends on mixing the probiotic bacteria with a polymer solution. This mixing is homogenized with the oil phase to form emulsions and later this mixture is directly spray-dried to obtain microcapsules. Formed emulsion/polymer mixture is sprayed *via* a nozzle into a drying chamber circulating hot air.

The functionality of encapsulated materials and microcapsule size depends on nozzle size, viscosity, inlet, and outlet temperatures, flow rate of solution and solid concentration. The cell survival is based on outlet temperature of process. Using high temperatures during spray drying leads to allow intracellular substances leak into the cellular pores (Anekella & Orsat, 2013).

2.1.3. Extrusion Technique

This technique is the most widely used microencapsulation method because of its simplicity, low-cost and it provides high cell viability (Krasaekoopt, Bhandari, & Deeth,

2006). This method depends on preparing a solution using different wall material such as alginate, whey protein, pectin and milk, adding bacteria, and extruding the cell mixing through a syringe needle at high pressure into a hardening solution (Heidebach, Först, & Kulozik, 2012) (Table 2.3). However, different microencapsulation techniques were used to improve the viability of *B. longum*. The viability of bacteria microencapsulated with spray-drying technique decreased 2 log CFU/g, whereas in extrusion technique viable bacteria count was reduced 2.9 log CFU/g (Khan, Korber, Low, & Nickerson, 2013).

There are various cryoprotectants such as whey protein, maltodextrin, skim milk powder, glucose, etc. in order to protect the viability of bacteria. There are different types of encapsulating wall material such as pectin, gelatin, alginate, carrageenan, chitosan, maltodextrins, whey protein and chickpea proteins (Martin, Lara-Villoslada, Ruiz, & Morales, 2015).

Proteins are preferred for microencapsulation because of well emulsifying characteristics, heat-sensitive bioactive materials. Besides, polysaccharides indicate good solubility in water, good oxygen and, moisture barrier properties compared to the proteins (Kagami, et al., 2003; Murano, 1998).

It was found that whey protein was more effective than pullulan in the stabilization of bacteria. Whey protein is a commonly used protein for probiotic bacteria microencapsulation since it is a good encapsulating agent (Table 2.3) (Heelan & Corrigan, 1998; Rosenberg & Lee, 2004). Moreover, many studies have indicated that a combination of proteins with polysaccharides enhance the barrier properties of protein-based encapsulate and emulsifying properties (Ducel, Richard, Saulnier, Popineau, & Boury, 2004; Mendanha, et al., 2009; Pereira, et al., 2009; Yu, Wang, Yao, & Liu, 2007).

Aureobasidium pullulans produce pullulan, which is an extracellular polysaccharide. In addition, various industrial wastes such as olive oil and potato peel wastes can be used as a carbon source for the synthesis of pullulan (Barnett, Smith, Scanlon, & Israilides, 1999; Singh, Saini, & Kennedy, 2008). Pullulan is used in the food industry due to its properties e.g. water-soluble, tasteless, colorless, heat stable, odorless and non-toxic (Wu & Imai, 2011). Based on the functional properties of protein-polysaccharide complex, it is decided to explore both the potential of whey protein and pullulan for microencapsulation of GABA-producer probiotic bacteria.

Table 2. 3. Microencapsulation techniques with wall materials

Microencapsulation techniques	Probiotics	Microencapsulation matrix	References
Emulsification	*Lb. acidophilus* CGMCC1.2686	Alginate, $CaCO_3$ and Ca-EDTA	(Cai, Zhao, Fang, Nishinan, & Phillips, 2014).
Extrusion	*Lb acidophilus* 5, *Lb. casei* 01	Inulin, , chitosan, alginate, galactooligosaccharide	(Krasaekoopt & Watcharapoka, 2014).
Spray chiller	*B. animalis and Lb. acidophilus*	Cocoa butter	(Pedroso, Dogenski , Thomazini, Heinemann, & Favaro-Trindade, 2013).
Emulsification /Spray-drying, freeze-drying	*Lb. acidophilus*	Whey protein and pullulan, Pectin and whey protein	(Çabuk, 2014; Gebara, et al., 2013).
Emulsification	*Lb. delbrueckii spp lactis Lb. helveticus*	Alginate and gellan gum	(Rosas-Flores , Ramos-Ramírez , & Salazar-Montoya, 2013).
Spray drying	*Lb. reuteri*	Alginate and calcium chloride	(Malmo, La Storia, & Mauriello, 2013).
Spray drying	*Lb. rhamnosus, Lb. acidophilus*	Maltodextrin	(Anekella & Orsat, 2013).
Spray drying, freeze-dried, emulsification method	*Lb. rhamnosus* GG	Polydextrose, skim milk, inulin, whey protein isolate and maltodextrin, whey protein isolate and inulin	(Ananta, Volkert, & Knorr, 2005; Ying , et al., 2010; Burgain, Gaiani, Cailliez-Grimal, Jeandel, & Scher, 2013).

(cont.on next page)

Table 2.3. (cont.)

Microencapsulation techniques	**Probiotics**	**Microencapsulation matrix**	**References**
Spray drying or freeze-drying	*Lb. acidophilus* *Lc. lactis ssp. cremoris*	Vegetable oil, sodium caseinate, mannitol, fructooligasaccharides and glucose	(Dianawati, Mishra, & Shah, 2013).
Electrospinning	*Bifidobacterium animalis subsp. lactis*	Whey protein and pullulan	(López-Rubio, Sanchez, Wilkanowicz, Sanz, & Lagaron, 2012).
Freeze drying/ spray drying	*Lb. plantarum*	Whey protein and alginate	(Rajam, Karthik, Parthasarathi, Joseph, & Anandharamakrishnan, 2012).
Spray drying	*Lb. casei, Lb. paracasei, Lb. acidophilus, Lb. plantarum*	Skim milk	(Paez, et al., 2012).
Spray drying	*Lb. rhamnosus* *Lb. rhamnosus* GG *Lb. salivairs*	Skim milk and polydextrose, inulin, skim milk.	(Corcoran, Ross, Fitzgerald, & Stanton , 2004).
Spray drying	*B. longum* *B.infantis*	Gelatin, starch, skim milk and arabic gum	(Hsiao , Lian, & Chou, 2004).
Emulsification/Spray-drying	*B. breve* *B.longum*	Whey protein isolate and milk fat	(Picot & Lacroix, 2003).
Spray drying	*Lb. acidophilus* *B.lactis*	Cellulose Acetate pathalate	(Fávaro-Tindale & Grosso, 2002).

Recently, encapsulation technique has been used to preserve product taste and to maintain the stability of microorganisms in the products. Thus, the probiotics used in the products can survive for a long time under environmental stress conditions. Lipid fractions of cocoa butter in chocolate have also been shown to protect probiotics (Burgain, Gaiani, Linder, & Scher, 2011; Lahtinen, Ouwehand, Salminen , Forssell, & Myllarinen, 2007). Nevertheless, the encapsulated probiotic content of foods may represent a negative impact on the quality as well as sensory qualities of the products (Succi , et al., 2017). Therefore, the encapsulation technique selected is quite important.

2.2. The Relationship between Nutrients, Diet and Mood

Nutrition is thought important for prevention and treatment not only obesity, diabetes, cardiovascular disease, and cancer but also mental illness and neuropsychiatric disorders (Jacquelyn & Flaskerud , 2015). Brain function and hormones such as serotonin (5-HT), dopamine (DA) and gamma-aminobutyric acid (GABA) can be affected by diet. Serotonin hormone plays an essential role in increased positive mood. When many people feel stressful (serotonin level is decreased), they generally want to eat the food containing carbohydrate, because carbohydrate-rich food increases serotonin synthesis of the brain (Prasad, 1998). The result of related studies appeared that individuals who live depressive problems such as premenstrual tension and mood disorder associated with season were changed their mood as a positive way with consuming some foods (Prasad, 1998; Özenoğlu, 2018).

Consumption of whole-grain, vegetable and fruits in women were diagnosed with less anxiety disorder, depression and bipolar disorder (Gold , 2015). It was revealed that undergraduate students increased their sense of energy and happiness by consuming more fruits in one of the research (White, Horwath, & Conner, 2013). *Lycium barbarum L.* (goji berry) fruits and juice reduce the level of anxiety disorder and depression in rats (Karabaş, Coşkun, Sağlam, & Bozat, 2016; Amagase & Nance, 2008). Presence of omega-3 fatty acids at a low ratio in the body is able to cause mental illnesses such as bipolar disorder and depressive disorder (Grosso, et al., 2014; Hennebelle , Champeil-Potokar, Lavialle , Vancassel , & Denis , 2014; Sublette, Ellis, Geant, & Mann, 2011). Nutrition elements like vitamin D, calcium, magnesium, zinc, iron, vitamin B12, and

unsaturated fatty acid are able to use as add-on-therapy to an antidepressant (Lakhan & Vieira , 2008; Miki , et al., 2015). Moreover, two studies showed that consuming zinc supplement by women decrease anger and can be used as an antidepressant for men and women (Sawada & Yokoi , 2010; Vashum , et al., 2014). Prebiotics like apple, legume, banana, jerusalem artichoke, oat, wheat-unrefined and persimmon fruit have been supporting intestinal bacteria. The diet with low Mg content reduce bacterial variety, anxiety and similar behaviors have been appearing, so nutrition can use as therapeutic for psychiatric disorders (Oriach , Robertson , Stanton , Cryan , & Dinan , 2016; Cani & Delzenne , 2011). A functional food can be identified as fortified nutritive values by usage of special ingredients. Furthermore, it can be used to decrease the risk of some diseases (Konar, et al., 2018). There are many functional foods that probiotics and prebiotics are incorporated. There has been an increasing trend of that type of functional foods for well-being and health concern (Özer, Özyurt, & Tellioğlu, 2019).

Choosing food materials to be consumed are affected by our psychological structure, which has an important influence on emotional interactions. For example, exposing on stress causes excessive consumption of food because of increasing release of cortisol in the body (Greeno & Wing, 1994; Gonzalez-Bono, Rohleder, Hellhammer, Salvador, & Kirschbaum, 2002). In addition to this situation, stress leads to a lack of some materials in the body. This results in lack of dopamine and serotonin such as iron, magnesium, and copper (Benton & Donohoe, 1999; Greeno & Wing, 1994; Robbins & Fray, 1980). Thus, foods can be important to prevent various psychiatric disorders.

2.3. Relationship of Gut-Brain

The intestine and the brain are closely related through the gut-brain axis, which comprises bidirectional communication immune, endocrine, and neural pathways (Dinan & Cryan, 2017; Mayer, Knight, Mazmanian, Cryan, & Tillisch, 2014). Many studies reveal that intestinal health is directly related to the brain and cognitive functions (Mayer, 2011). The intestinal microbiota is able to produce short-chain fatty acids (SCFAs) such as acetate, butyrate and propionate with the contribution of prebiotics and various polysaccharides. SCFAs are also involved in regulatory effects on food-energy intake and inflammatory processes (Petra, et al., 2015). As a result of this, SCFAs increase the

feeling of satiety (Everard & Cani, 2013). The metabolites of probiotics such as SCFAs, bile acids, bioactive lipids in the intestinal microbial have influences on different hormones like dopamine (DA), gamma-aminobutyric acid (GABA), serotonin (5-HT) and noradrenaline (NA) monoamines; therefore these are related closely with gut and brain health (Cani, Everard, & Duparc, 2013).

Vagus nerve is directly connected with the brain and the gastrointestinal tract. The neuronal and hormonal changing in the gastrointestinal tract are transmitted to the brain vagus nerve (Borovikova , et al., 2000; Perez-Burgos , et al., 2013; Wang , et al., 2002). The intestinal microbiota stimulates the central nervous system (CNS) and the immune system; endocrine, immune (chemokines, cytokines), autonomic nervous system and enteric nervous system with the sequence of microbiota-intestine-brain (Mayer, 2011).

Emotional changes and psychological stress adversely affect the intestinal microbiota (Dinan & Cryan, 2012). The gut microbiota in the digestive tract may affect the brain function in different ways (Figure 2.3). For instance, neurotransmitters produced by microbiota have an important role in the nervous system and cognitive functions (Gill, et al., 2006; Qin, et al., 2010; Yatsunenko, et al., 2012).

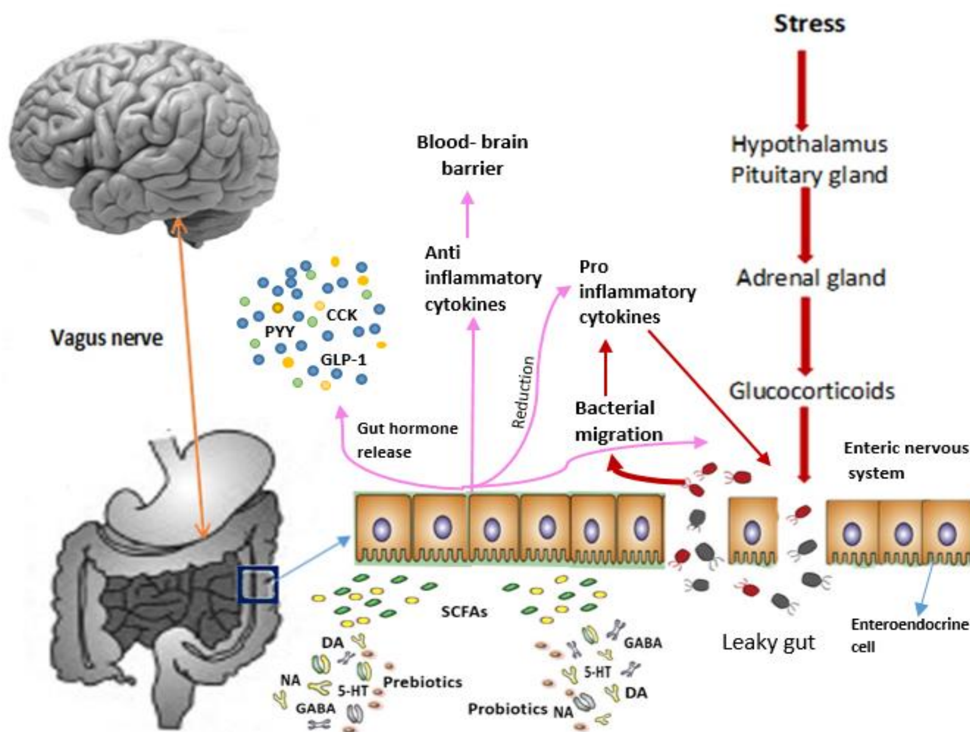

Figure 2. 3. Effect of probiotics to the gut-brain axis (Source: Sarkar, et al., 2016).

Pink arrows show probiotic and prebiotic processes and effects, whereas red arrows demonstrate processes associated with inflammation, leaky gut. It is known that probiotics are introduced as beneficial bacteria into the gut and prebiotics affect the growth of bacteria especially probiotics. In addition, not only prebiotics but also probiotics can increase the production of SCFAs. They interact with enteroendocrine cells and catalyze the gut hormones such as glucagon-like peptide 1 (GLP1), cholecystokinin (CCK) and peptide tyrosine tyrosine (PYY). Both probiotics and prebiotics increase neurotransmitter production in the gut such as 5-HT, DA, GABA and NA. The neurotransmitters can modulate neurotransmission in the enteric nervous system. Under stress, they stimulate the hypothalamus, pituitary gland, adrenal gland, and glucocorticoids respectively and glucocorticoid level changes. It causes the migration of bacteria with pro-inflammatory, which enhance inflammation as well as an increase in pro-inflammatory cytokines. These cytokines permit access to inflammatory elements or pathogenic elements blood-brain barrier. In addition, pro-inflammatory cytokines have decreased the integrity of the gut barrier. However, consumption of probiotics and/or prebiotics are effective on regulating the gut barrier, reduce concentrations of pro-inflammatory cytokines glucocorticoids level. On the other hand, they increase concentrations of the anti-inflammatory cytokines, increasing the integrity of the gut barrier, the blood-brain barrier decreases inflammation (Sarkar, et al., 2016).

Social, environmental and psychological factors can change nutritional behavior. In addition, changes in diet may result in the changes in microbiota composition and gut hormones that are affected by stress level and mood (Özenoğlu, 2018). Consumption of antibiotics, drug and experiencing behavior depends on stress, affect microbiota negatively whereas consumption of probiotic, prebiotic, various beneficial diet, and nutrition affect microbiota positively.

Probiotic is known as modulating neurotransmission in the synapses of the enteric nervous system and increase the production of the neurotransmitters 5-HT, NA and GABA. Thus, probiotics may be effective on anxiety disorder because they were tried on a range of psychiatric disorder such as insomnia, and stress and shown to be effective. For example, *Lb. rhamnosus* and *Lb. helveticus* consumption was found protective in memory disorder of mice, having *Citrobacter rodentium* infection, when they were exposured to stress (Mckernan, Fitzgerald, Dinan, & Cryan, 2010). While *Lb. helveticus NS8* has shown antidepressant effect, *Lb. rhamnosus* was found to reduce the risk of

ADHD since they regulate 5-HT (Liang, et al., 2015; Pärtty, Kalliomäki, Wacklin, Salminen, & Isolauri, 2015; Pessi, Sütas, Hurme, & Isolauri, 2000). Furthermore, fermented milk contains *Lb. helveticus* provided a healthy sleep to individuals, according to randomized, double-blinded study (Benton, Williams, & Brown, 2007; Yamamura, et al., 2009).

Researches conducted with *B. longum* and *B. breve* showed a therapeutic effect in psychological disorders related to stress (Savignac, Tramullas, Kiely, Dinan, & Cryan, 2015). In addition, *B. infantis* was effective for preventing depression (Desbonnet, Garrett, Clarke, Bienenstock, & Dinan, The probiotic Bifidobacteria infantis: An assessment of potential antidepressant properties in the rat., 2008). Consumption of *B. bifidum, Lb. brevis, Lb. acidophilus, Lc. lactis, Lb. casei* and *B. lactis* by healthy individuals, depression were decreased (Steenbergen, Sellaro, van Hemert, Bosch, & Colzato, 2015). When *B. animalis* subsp *lactis, Lc. lactis* and *Streptococcus thermophilus* were consumed by healthy women, their mood was changed positively by probiotic usage, especially during menstruation (Tillisch, et al., 2013). *Lb. acidophilus* and *B. longum* have treated nausea caused by stress (Diop, Guillou, & Durand, 2008). Consumption of *B. lactis, Lb. fermentum* and *Lb. acidophilus* was demonstrated to affect brain functions positively (Davari, Talaei, Alaei, & Salamı, 2013).

2.4. Anxiety Disorder

Anxiety disorder is common mental health problem. There are many anxiety disorder types such as generalized anxiety disorder, separation anxiety disorder, obsessive-compulsive disorder, social anxiety disorder and posttraumatic stress disorder (Brand, Wilhelm, Kossowsky, Holsboer-Trachsler, & Schneider, 2011; Rapp, Dodds, Walkup, & Rynn, 2013; Hill, Waite, & Creswell, 2016). Anxiety disorder is common during childhood, which occurs at age of seven (Herren, In-Albon, & Schneider, 2013; Özyurt, 2013). This disease is seen between 9% and 32% in young and adolescent children. Having anxiety disorder in children and young people have a significant effect in school performances, social, family functions, and in their daily lives, such as sleep patterns (Hill, Waite, & Creswell, 2016). Anxiety disorders are thought to cause serious mental health problems such as substance abuse and suicide risk (Lavallee , et al., 2011).

Main origination of anxiety disorder is the central nervous system. A subcortical structure in the brain includes limbic system which is an essential zone in the brain responsible for a change of mood and memory and it consists of neuroanatomic such as the thalamus, hypothalamus, amygdala, and pituitary (Uzbay, 2002). In addition, Amygdala plays a fundamental role in our emotional responses such as anxiety, pleasure, and fear (Davis, Rainnie, & Casell, 1994). Amygdala and neuronal communicating with amygdala such as lateral hypothalamus, ventral tegmental area, and locus seruleus are important neuroanatomic structures in the anxiety formation (Carvey, 1998; Ninan, 1999). Many types of research indicate that there are anxiety disorder is caused by the three main central neurotransmitters, which includes less amount of GABA, 5-HT and NA (Cryan & Kaupmann , 2005; Leonardo & Hen, 2008; Hill, Waite, & Creswell, 2016; Stein, Seedat, & Gelernter, 2006).

The absence of GABA receptors especially $GABA_A$ receptors is correlated with anxiety symptoms (Malizia , et al., 1998; Hasler , et al., 2008; Cryan & Kaupmann , 2005). Since these receptors are anti-anxiety agents, they are significant in pharmacological evaluations (Bravo, et al., 2011).

There are two ways of treatment for persons having anxiety disorder; which are cognitive behavioral therapy and pharmacotherapy. Educated professionals select cognitive behavioral therapy as the first treatment stage, especially on children. Selective serotonin reuptake inhibitors and glutamatergic drugs like those that riluzole and ketamine generally used, being on the first-line as pharmacotherapy (Hill, Waite, & Creswell, 2016; Möhler, 2012). However, pharmacotherapy has an adverse effect such as insomnia, sexual dysfunctions and nausea (Baldwin, et al., 2005; Nutt, 2000). In addition, there are different antidepressants, which affect anxiety disorder and depression such as Tricylic antidepressant, but all antidepressants show the similar side effects (Baldwin, et al., 2005; Anderson, et al., 2008).

The relationship between diet and psychiatric disorder could be related not only because of depression risk but also anxiety disorder (Logan & Jacka, 2014). Thus, probiotics may be effective on anxiety disorder *via* neurochemicals they produce (Lyte, 2011). Probiotic bacteria, especially *Lactobacillus* and *Bifidobacterium* play a significant role in depression and anxiety disorder (Barrett, Ross, O'Toole, Fitzgerald, & Stanton, 2012). In addition, consumption of probiotics can provide more 5-HT and NA synthesized

in the body (Collins & Bercik, 2009). There are several studies on the effect of using probiotic on anxiety disorder (Table 2.4).

Table 2. 4. Effect of probiotics on anxiety disorder

Probiotic	**Group**	**Clinical Study Type**	**The conclusion**	**Reference**
Lb. helveticus NS8	In rats with given water	Morris water maze, Elevated plus maze	shows effect therapeutic	(Luo, et al., 2014).
Lb. rhamnosus (JB-1)	In mice	Elevated plus maze and stress-induced hyperthermia	Reduced depression and anxiety-related behavior, can modulate the GABAergic system	(Bravo, et al., 2011)
Lb. helveticus ROO52	In mice	Barnes maze forced swim test	Can be modulated mouse physiological function and decreased anxiety	(Ohland, et al., 2013).
Lb. helveticus ROO52 and *B. longum* R0175	In rats and human	Randomize double-blind Hopkins Symptom Checklist, Hospital Depression and Anxiety Scale, anxiety testing for rats	Taken in combination can prevent anxiety disorder in rat and can be a beneficial effect as psychological for human	(Messaoudi, et al., 2011).
Bifidobacterium longum NCC30001	In mice	Step-down test	It can be used as a treatment for anxiety-like behavior	(Bercik, et al., 2011)
Lb. casei strain Shirota	In human	Beck Anxiety Inventory and the Beck Depression Inventory	Increased in fecal *Lb.* spp. and *B.* spp. And also decrease in anxiety symptoms	(Rao , et al., 2009).

Consequently, probiotics have been revealed to have reducing the effect on anxiety disorders.

CHAPTER 3

CHOCOLATE

Chocolate; it has an aroma, unique taste, structure, bioactive compounds like polyphenols. Cocoa has a very bitter taste since it contains high amounts of polyphenols. Therefore, cocoa beans cannot be consumed directly, they are exposed to different process to produce chocolate. Even though 90% of its polyphenol content is lost during the process, phenolic compounds are still present in the final chocolate product (Andújar, Recio , Giner, & Ríos, 2012). Cocoa includes a significant amount of catechin, methylxanthines, proanthocyanidins, phenolic acids, and epicatechin (Meng, Mhd Jalil, & Ismail, 2009). While dark chocolate illustrated the highest phenolic content, white and milk chocolates exhibited lower total phenolic content (Grassi, Lippi, Necozione, Desideri, & Ferri, 2005; Meng, Mhd Jalil, & Ismail, 2009; Andújar, Recio , Giner, & Ríos, 2012). The polyphenol content in chocolates is altered by various processing techniques, such as alkalinization of cocoa and fermentation of cocoa beans. It is known that chocolate has a positive impact on human health, especially on the cardiovascular system thanks to the polyphenols it contains (Richelle , Tavazzi , & Offord , 2001; Steinberg, Bearden, & Keen, 2003).

There are different types of chocolate such as white, milk and dark chocolate, which depend on the raw material (Table 3.1).

Table 3. 1. Chocolate composition (Turkish food codex: Communication on cocoa and chocolate products, No: 29)

Chocolate types	Total dry cocoa solid	Fat-free cocoa dry solid	Cacao fatty	Total fatty	Dry milk solids	Milk fat
White chocolate			≥ 20 %		≥ 14 %	≥ 3.5 %
Milk chocolate	≥ 25 %	≥ 2.5 %		≥ 25 %	≥ 14 %	≥ 3.5 %
Dark chocolate	≥ 35 %	≥ 14 %	≥ 18 %			

3.1. Chocolate Manufacturing

There are 4 steps to produce chocolate process.

3.1.1. Mixing

Cocoa butter, sugar, cocoa liquor, skimmed milk powder and milk fat are the components depending on the chocolate types. These ingredients are mixed for 12-15 min at 40-50 °C to provide a constant density (Minifie, 1989).

3.1.2. Refining

Refining is an essential process so that the chocolate can gain a smooth structure as required. In the refining process, two and five roll refiners are common to provide particles size of fewer than 30 μm (Beckett, 2000) (Beckett, 1999). The particular size of the end product affects sensory properties and rheology structure.

3.1.3. Conching

Conching is the final phase in producing chocolate and it is an important stage to develop final texture, flavor and viscosity of chocolate. The process contributes to volatile acid, moisture removal, and flavor development. Furthermore, conching also aims to decrease particle size, to remove big sized particle and to reduce viscosity from the previous processes (Minifie, 1989).Conching temperature and time show alteration according to chocolate types. For example, chocolate including milk powder is made conching maximum for 16-24 hours at 60°C, whereas in dark chocolate is generally made conching at 70°C and maximum 82°C (Awua, 2002). Figure 3.1 shows the chocolate manufacture process.

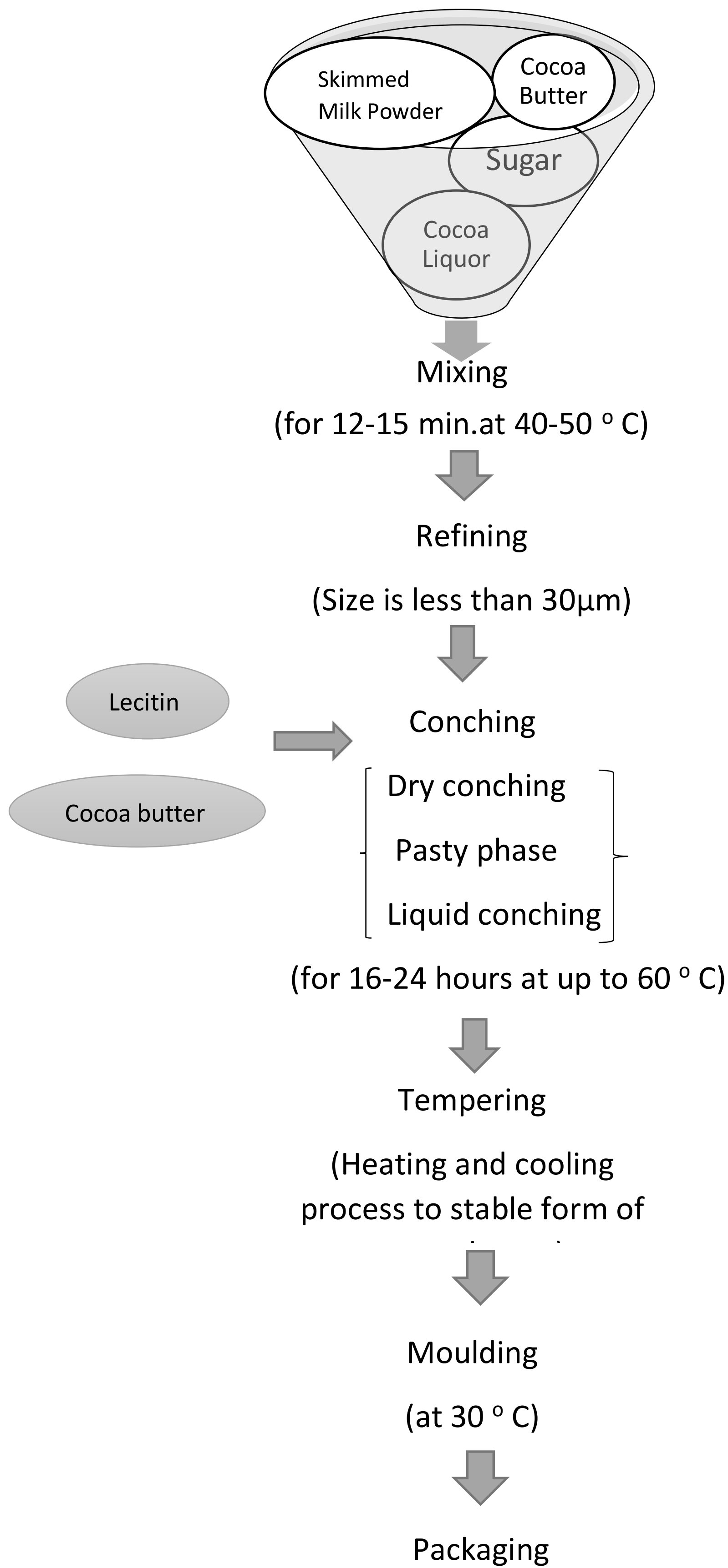

Figure 3. 1. Milk chocolate manufacture process (Source: Afoakwa et al., 2007).

3.1.4. Tempering

Chocolate fat has six polymorphic forms (Figure 3.2). Form V is the most requested form in well-tempered chocolate because of providing good snap, glossy appearance, and resistance to bloom (Beckett, 2000). Form I is an unstable form, which has a melting point of 17 °C and is rapidly transformed into Form II. Furthermore, form II converts more slowly into III and IV. Form V and VI of cocoa butter are the most stable.

Polymorphic forms of cocoa butter		Melting point (°C)	Chain packing
Form I	β'_2	16–18	Double
Form II	α	21–22	Double
Form III	Mixed	25.5	Double
Form IV	β_1	27–29	Double
Form V	β_2	32–34	Triple
Form VI	β'_1	34–36	Triple

Figure 3. 2. Polymorphic forms of chocolate butter (Source: Afoakwa., 2010).

Tempering is essential for acceptable polymorphic form in the chocolate and this step influences texture, color, shelf life and handling of chocolate. Tempering includes pre-crystallization of small quantities of triglycerides. The tempering process has four steps (Figure 3.3).

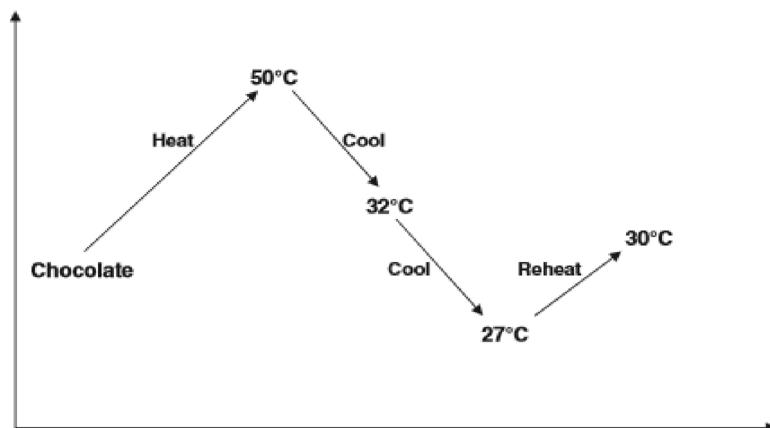

Figure 3. 3. Tempering process in chocolate (Source: Afoakwa, 2010).

Chocolate melts until 50 °C to melt all of the fatties, and then get cooled 32°C to crystallization point, 27°C for crystallization to obtain the correct form of stable crystals. Finally, the chocolate is reheated at 29-31 °C to melt out unstable crystals (Talbot, 2008).

However, the tempering process in milk chocolate is slightly different from dark chocolate because of the effect the crystal formation of milk fat molecules. The tempering process in milk chocolate is around 29.4 °C because of having a lower melting point. Chocolate is poured for moulding at 29- 30 °C as a liquid for being well- shape (Afoakwa, 2010).

Well-tempered chocolate has some properties;

- ✓ Color
- ✓ Well shape
- ✓ Gloss
- ✓ Stable product-harder
- ✓ Contraction from the mould
- ✓ Longer shelf-life
- ✓ Heat resistant (during the packaging).

As it was given in the Introduction chapter, functional food products have been developed for psychiatric disorders; dairy products were taken great interest at the initial stage of functional food development; later other products such as fruits and vegetables, cereals, bakery and snack food products have gained attention.

Consumption of certain amounts of chocolate may be beneficial to prevent these psychiatric disorders (Parker & Crawford , 2007). Chocolate may interact with a range of neurotransmitter systems that contribute to appetite, reward and mood regulation (Parker, Parker, & Brotchie, Mood state effects of chocolate, 2006). There are two patents related to chocolate products with probiotic content that prevent from high cholesterol and gastrointestinal diseases. There have been two patents present regarding *B. infantis* 35624 and *L. paracasei* probiotics. These patents have demonstrated the cholesterol-lowering effects of both probiotics in the chocolate, in separate studies, as well as their gastrointestinal disease inhibitor effect (Patent No. 0330151, USA) (Patent No. 102206, China). 10g chocolate enriched with 28 mg GABA was found to reducing stress (Nakamura, Takishima, Kometani , & Yokogoshi, 2009). In the literature, various studies have been carried out using different probiotic strains in milk chocolate, bitter chocolate, and white chocolate (Table 3.2).

Table 3. 2. Chocolate samples containing probiotics

Chocolate Types	Probiotics	Probiotic concentration and form	Added probiotics	Probiotics storage	Viability rate of probiotics	Reference
White chocolate	*Lb. paracasei* and *Lb. acidophilus*	9 log CFU/25 g, freeze-dried powder	After conching	at 13-15 ºC	Two strains are satisfied after the storage time.	(Konar, et al., 2018).
Dark chocolate	*Lb. rhamnosus, Lb. paracasei, Lb. casei* and *Lb. reuteri*	8-9 log CFU one dose in 15 g chocolate	After melted	at 18 ±2 ºC	*Lb. rhamnosus* and *Lb. paracasei* gave better results during the shelf life	(Succi, et al., 2017).
Milk and dark chocolate	*Lb. acidophilus* LH5, *Streptococcus thermophilus* ST3, and *B. breve* BR2	9-8 log CFU/g 3.5 g/kg	After tempering	at 4 ºC and 20 ºC	*B. breve* is less durable than the other probiotic bacteria.	(Lalicic- Petronijevic , Popov-Raljic, Lazic, Pezo, & Viktor, 2017).
Milk chocolate	*Lb. acidophilus* NCFM*, B. lactis* HN019, and *Lb. rhamnosus* HN001	At least 6-7 log CFU/g 2.5 DCU / kg, freeze dried powder	After milling	at 20 ºC	Found 90 % types of *Lactobacillus*	(Bulatovic, Zaric, Krunic, Boric, & Rakin, 2016).
Milk and dark chocolate	*B. lactis* HN019 and *Lb. acidophilus* NCFM	8 log CFU 1 g of freeze-dried powder	After tempering	at 15 ± 2 ºC and 20-30 ºC	*B.lactis* strain live was slightly longer than *Lb.acidophilus* strain	(Klindt-Toldam , et al., 2016).
Dark chocolate	*B. indicus* HU36 and dietary fibers	6.08 log CFU/g , freeze-dried powder	After melted	at 18 ºC	Bacteria were found over 5-log CFU/g at the end of storage.	(Erdem, et al., 2014).
Dark chocolate	*Lb. plantarum*	8 log CFU/g	After melted	at 4 ºC	After the storage days, bacteria were decreased 6.5 log CFU/g	(Foong, Lee , Ramlı, Tan, & Ayob , 2013)
Dark chocolate	*Lb. rhamnosus* R0011	7 log CFU/g, freeze-dried	nd	at 4 °C and 20 °C	There is a viability loss of approximately 0.2 log CFU per g	(Raymond & Champagne , 2015).

(cont. on next page)

Table 3.3 (cont.)

Chocolate Types	Probiotics	Probiotic concentration and form	Added probiotics	Probiotics storage	Viability rate of probiotics	Reference
Dark chocolate	*Lb. rhamnosus* and *B.longum*	8-10 log CFU/g, freeze-dried and microencapsulation by spray-coating technology	After melted	at 4 °C	There is a viability loss of approximately 0.15 log CFU/g	(Champagne, Raymond, Guertin, & Belanger, 2015).
Milk chocolate	*Lb. brevis subsp. coagulans*	6 log CFU/g, freeze-dried	After melted	at 4 °C	It was evaluated simulated digestion system	(Yonejima, et al., 2015).
Milk and dark chocolate	*Lb. rhamnosus* IMC 501® and *Lb. paracasei* IMC 502®	9 log CFU/g, freeze-dried	nd	at room temperature	Milk chocolate is effective for viability rate of the probiotics	(Coman M. M., et al., 2012).
Milk and dark chocolate	*Lb. acidophilus* NCFM® and *B. lactis* HNO19	8 log CFU/g, freeze-dried	After tempering	nd	Viability of *Lb. acidophilus* was found longer	(Lalicic- Petronijevic , et al., 2015).
Milk chocolate	*Lb. casei NCDC 298*	8 log CFU/g, freeze-dried	nd	at 7 °C	Viability of bacteria was found acceptable during storage time	(Mandal, Hatı, Puniya , Sıngh, & Sıngh, 2012)
Milk and dark chocolate	*Lb. helveticus* and *B.longum*	9 log CFU, freeze-dried powder, spry drying technique	nd	nd	Milk chocolate is effective for viability rate of the probiotics	(Possemiers, Marzorati, Verstraete, & Van de Wiele, 2010).
Dark Chocolate	*Lb. rhamnosus* RIUM	8 log CFU / g, extrusion technique	nd	at 4 °C and 20 °C	There is a viability loss of encapsulated bacteria of approximately 0.49 log CFU/g and a viability loss of free-cell bacteria about 0.21 log CFU/g	(Erginkaya, Sarıkodal, Özkütük, Konuray, & Turhan, 2019)

nd: not define

In these studies, the bioavailability of probiotics throughout their shelf life, the sensory and structural effects on the product and the effects on the GI system were examined. These studies were also investigated to compare with survival rate of Lactic acid bacteria and *Bifidobacteria* in chocolate products. In addition, they focused on finding good storage condition and survival rate of bacteria for different non-dairy products such as ice-cream, chocolate, salami and jam. Some important points can be drawn from the result of these studies. These probiotics should be suitable for industrial working conditions, able to sustain their long-term viability in storage conditions and to remain stable (Patterson & Burkholder, Application of prebiotics and probiotics in poultry production., 2003; Fernandez, Boris , & Barbes , 2003). Moreover, foods containing probiotic bacteria may not be taken in sufficient quantities in the body when consumed. Various factors such as storage state and product development also affect the survival of probiotic bacteria; hence, the probiotic dose in the products is important (Coman M. M., et al., 2012; Douglas & Sanders , 2008; Kalliomaki , et al., 2010).

CHAPTER 4

MATERIAL AND METHOD

4.1. Material

Reference cultures were provided from Northern Regional Research Laboratory (NRLL) and Spanish Type Culture Collection (CECT) in this thesis (Table 4.1). Lactic acid bacteria strains with probiotic properties were provided from the culture collection of the Molecular Food Microbiology Laboratory (IYTE-GMB-GMLKK) of Food Engineering Department, İzmir Institute of Technology. Probiotic cultures preserved in glycerol stocks at -80ºC were inoculated on 1% to MRS broth, incubated at 37ºC for 24 hours, and then were refreshed.

Table 4. 1. Used reference cultures

No	References Bacteria	References Code	References
1	*Lactobacillus helveticus*	NRLL B- 4526	Agricultural Research Service Culture Collection
2	*Lactobacillus rhamnosus*	NRRL B- 442	(Muyyarikkandy, Alqahtani, Mandoiu, & Amalaradjou, 2018).
3	*Lactobacillus delbrueckii* subsp. *bulgaricus*	NRRL B- 548	(Muyyarikkandy, Alqahtani, Mandoiu, & Amalaradjou, 2018).
4	*Lactococcus lactis* subsp. *lactis*	CECT- 4432	Agricultural Research Service Culture Collection

All raw materials for chocolate were provided from ETİ Food Industrials and Commerce Inc, Eskişehir. Raw materials of milk chocolate is sugar, milk powder, cocoa powder, cocoa oil, fat-free milk powder, lecithin, and vanillin.

4.2. Method

In this part of the thesis, methods are described to select GABA-producer bacteria, microencapsulation and quality parameters of functional chocolate.

4.2.1. Selection of GABA Producer Probiotic Bacteria

Three different methods were used to determine GABA producer bacteria by using qualitative method for preselection and quantitative techniques.

4.2.1.1. Stock Cultures Refreshed

LAB which was preserved in glycerol stock at -80ºC, were cultured two times at 30 or 37 ºC for 24 hours in MRS broth, and refreshed as inoculum in the fermentation trials.

4.2.1.2. Complex pH Indicator Method

Complex pH indicator was used as a qualitative method to determine whether GABA was produced by the bacteria. 0.1 g of methylene blue and 0.2 g of methyl red were dissolved in 100 ml ethanol (100%). The two solutions were mixed together at 1:1 (v/v) ratio to prepare complex pH indicator and was found magenta color. Then the 0.1ml of complex pH mixture was added to 4 ml of the reaction solution to observe the color change (Figure 4.1). If color change observed from magenta to green, the bacteria was glutamate decarboxylase (GAD) producing bacterium (Yang, et al., 2006).

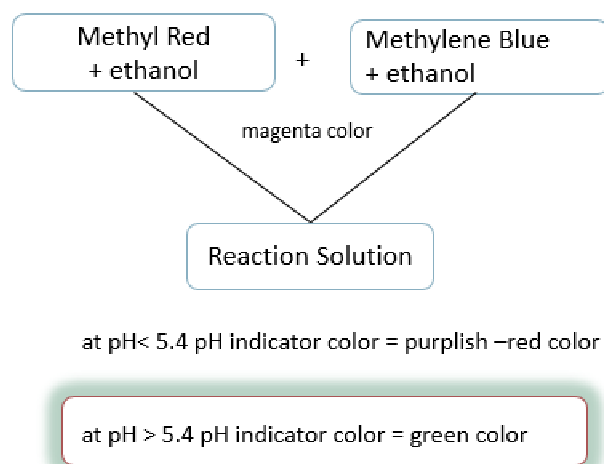

Figure 4.1. Evaluation of complex pH indicator.

4.2.1.3. Screening LAB for GABA Production Abilities Using High-Performance Liquid Chromatography (HPLC) Method

GABA contents were determined using a Perkin 200 series HPLC system (PerkinElmer 200 HPLC, Shelton, USA) as quantitatively. All information about the specifications of HPLC system, analytical terms and mobile phase program is shown in Table 4.2, Table 4.3 and Table 4.4 HPLC method for the measurement of GABA concentration used in this thesis is the modification of the method described by (Tajabadi, et al., 2015).

Table 4. 2. Specifications of HPLC system for determination of GABA producer LAB

System Specifications	
System	PerkinElmer 200 series
Detector Type	Diode array detector (DAD)
Autosampler	LR23329C
Column Type	ACE 5 C18 (250*4.6 mm, 5 µm)

Table 4. 3. Analytical terms of HPLC system for determination of GABA producer LAB

Analytical Conditions	
Injection Volume	20 µL
Column Temperature	26°C
Flow Rate	1 µL / min
Wavelength	254 nm

Table 4. 4. HPLC mobile phase program profile for determination of GABA producer LAB

Mobile Phase Program		
Time (min)	% Mobile Phase A	% Mobile Phase B
0.5	98	2
15	93	7
19	90	10
32	67	13
33	0	100
37	0	100

One hundred ml of bacterial samples were centrifuged at 5.000 g for 15 min at 4°C. Then pellets were washed in NaCl solution (0.85 %) three times. The pellets were resuspended in 10 times 50 mM of L-glutamic acid (Sigma, G1251)(containing 0.1 mmol/L of Tween 80) and adjusted pH 4.7 by 0.5 mol/L HCl. The samples were incubated at the incubator without shaking through at 37 ºC for 24, 48, 90 and 60 h and the supernatants were collected by centrifugation at 5.000 g for 15 min at 4°C to measure by complex pH indicator and HPLC (Yang, et al., 2006). After the incubation 100-µl supernatant was filtered through the 0.22 µl filter. The resulting filtrate was solved in 20 µl ethanol-water- triethylamine solution (2:2:1) and was evaporated using a vacuum pump under 300 millitorr pressure. After evaporation, samples with added solution ethanol-water-triethylamine –phenyl isothiocyanate (7:1:1:1), were incubated for 20 min. at room temperature, so that phenyl isothiocyanate can lead to derivatization of GABA. Samples were evaporated under 300 millitorr pressure again after the incubation. Evaporated samples were diluted and were analyzed using C18 column (250 x 4.6 mm internal diameter and 5 µm diameter) from HPLC. Two different mobile phases were used for this analysis. Mobile phase A was a mix: 10 g sodium acetate three hydrates was solved in 900 ml deionized water and were prepared to add 500-µl triethylamine. Phase's pH was calibrated using acetic acid to 5.8. In addition, Mobile phase B consisted of acetonitrile- deionized water (6:4).

4.2.1.4. Screening LAB for GABA Production Abilities Using Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) Method

GABA contents were determined using a Shimadzu LC-20AD series HPLC system (Shimadzu LC-20AD HPLC, Japan) for quantitative measurements. All information about the specifications of HPLC system, analytical terms and mobile phase program are shown in Table 4.5, Table 4.6 and Table 4.7 GABA concentration was measured by amino acid analysis using HPLC (Hayaloğlu, Topçu, & Koca, 2011). Firstly, samples were prepared with dissolving in 50 mM L-glutamic acid were incubated at the

incubator without shaking. After adjusted pH to 5.0, the bacterial strains were incubated through at 37 ºC for 24, 48, 72 and 96h and supernatants were used to measure.

Table 4. 5. Specifications of RP-HPLC system for determination of GABA producer LAB

System Specifications	
System	Shimadzu LC-20AD
Detector Type	Diode array detector (DAD)
Autosampler	Shimadzu SIL-20A HT
Column Type	Pico. Tag Column (3.9 x 300 mm,4μm) (WATERS)

Table 4. 6. Analytical terms of RP-HPLC system for determination of GABA producer LAB

Analytical Conditions	
Injection Volume	30 μL
Column Temperature	45°C
Flow Rate	1 mL / min
Wavelength	254 nm

Table 4. 7. RP-HPLC mobile phase program profile for determination of GABA producer LAB

Mobile Phase Program		
Time (min)	% Mobile Phase A	% Mobile Phase B
0.01	95	5
10.00	60	40
15.00	0	100
19.00	0	100
19.01	95	5
20	Stop	

After incubation, 1.0 ml culture were added in 10 ml 0.1 N HCl. This mixture was homogenized and incubated in an ultrasonic bath at room temperature for 20 minutes. The samples were centrifuged 3000 *g* for 10 min. at 4 ± 1°C at the end of the incubation.

1 ml of the supernatant of the samples were taken and 1 ml of 40 % (w/v) trichloroacetic acid (TCA) solution was added. After the mix with vortex, it was kept in ice water at 4 ± 1°C. The resulting suspension was centrifuged (Hettich 320R) at 20.000 *g* x 10 min. at 4 ± 1°C. Deproteinized supernatants of 25µL were placed in eppendorf tube and freeze-dried under vacuum. 20 µl methanol 1 M sodium acetate - triethylamine solution (2:2:1) was added to the dried sample and was freeze-dried using a vacuum pump. After the process, samples with added solution methanol-deionized water-triethylamine–phenyl isothiocyanate (7:1:1:1), were incubated for 20 min at room temperature, so that phenyl isothiocyanate can lead to derivatization of GABA. Samples were dried under vacuum again after the incubation. Dried samples were diluted and then analyzed using Waters column (3.9 x 300 mm internal diameter) by HPLC. The derivatized samples were dissolved with 10 mM sodium acetate buffer (pH 6.4 adjusted acetic acid) containing 5% acetonitrile.

Two different mobile phases were used for this analysis. Mobile phase A mixture contain: 70 mM sodium acetate, 2.5% acetonitrile dissolved in 900 ml ultra pure water; and phase's pH was calibrated using acetic acid to 6.55. In addition, Mobile phase B consisted of acetonitrile- ultra pure water- methanol (9:8:3, v/v/v). Both solvents were prepared by adding 10 mg / L disodium ethylene diamine tetra acetic acid (Na_2EDTA). GABA standards (Sigma) were used to identify GABA.

4.2.2. Microencapsulation of Probiotic Bacteria

Lb. rhamnosus NRRL B-442 probiotic bacteria were microencapsulated by using the emulsion method (Cabuk & Harsa, 2015). During the first step of microencapsulation by emulsion, probiotic bacteria were inoculated into 10 ml of MRS broth media with 0.1% ratio and incubated at 37 ºC for 24 h under anaerobic conditions. The probiotic bacteria were incubated by the second inoculation process into 10 ml of MRS broth at 37ºC for 16-18 h. At the end of incubation, the cell pellets were harvested by centrifugation at 5.000 rpm at 4 ºC for 15 min. After the centrifugation, the precipitate was separated from the supernatant.

4.2.2.1. Preparation of Whey Protein Concentrate (WPC)-Pullulan Microcapsules

WPC (9%, w/v) into distilled water was mixed at room temperature so that it could completely dissolve. The protein solution was stirred using magnetic stirrer for almost 3 h to provide a proper dissolution and after hydration, the solution was denatured at 80 ^{o}C for 30 min. The denatured solution was cooled. Pullulan (13%) was dissolved in water and was mixed for 3 h in magnetic stirrer (Çabuk, Development of whey protein-pullulan microcapsules for the encapsulation of Lactobacillus acidophilus NRRL-B 4495 as a functional food ingredient, 2014). Denatured WPC solution was mixed with the pullulan solution and final protein solution concentration was 9% (w/v). WPC-pullulan were mixed with *Lb. rhamnosus* NRRL B-442 probiotic microorganisms (10^{11} CFU/g).

After the formation of WPC-pullulan matrix, water-in-oil emulsions were prepared 60% sunflower oil and 40% aqueous phase. The aqueous phase consisted of WPC-pullulan mix containing microorganism into the oil phase including 1% soybean lecithin. A homogenizer (IKA T25 D, Germany) at 3000 rpm for 5 min prepared the emulsion. Then, the emulsion was continued homogenized in $CaCl_2$ solution (100 mM) at 3400 rpm for 2 min and was continued to mix at 160 rpm for 30 min in orbital stirrer (IKA 125 B, Germany) to harden the microcapsule. At the end of the process, microcapsules were separated from oil phase using centrifuge at 1000 rpm for 1 h.

4.2.2.2. Freeze Drying

Microcapsules were frozen under -20 ^{o}C. Microencapsulated probiotics were freeze-dried in a Lablanco freeze-dryer using a standard lyophilization program (Freezone 18, Kansas, USA,) e.g. -55 ^{o}C and at 0.050 mbar vacuum for 48 hours. In addition, they were kept at 4 ^{o}C in the screw-capped glass bottle for further experiments.

4.2.2.3. Enumeration of Encapsulated Bacteria

Firstly, microcapsules should need to be separated for enumeration of microencapsulated bacteria. Thus, diluted samples at 1/1 ratio was homogenized at 11.000 rpm for 5 min, then samples were inoculated into a petri plate and left for incubation under anaerobic conditions at 37 ^{o}C for 48 h by the pour plate technique (Teoh, Mirhosseini, Mustafa , Hussin , & Manap , 2011).

Lyophilized microcapsules diluted at 1/1 ratio, swelling was achieved at room temperature for 5 min. After microcapsules were broken, the viable cell number was evaluated under anaerobic condition by the pour plate technique. Viable cell number was determined as colony forming units per gram (CFU/g) and microencapsulation yield can be calculated by using following equation (4.1);

$$\text{Microencapsulation yield (\%)} = 100 \times \frac{\text{CFU/g after encapsulation}}{\text{CFU/g before encapsulation}} \tag{4.1}$$

4.2.2.4. Change in Bacterial Viability during Storage Time

Microencapsulated bacteria and free-cell bacteria in these samples were preserved at 4°C to protect from any change in bacterial viability; and survival rates of both forms of bacteria over 2-months storage period was observed.

4.2.2.5. Enumeration of Probiotics

Count of the bacteria was performed in duplicates and 2 repeat following the standard plating methodology. Total counts of viable probiotic bacteria were identified as log CFU/g.Survival rate of the bacteria after freeze-drying process was calculated according to the formula (4.2);

$$\text{Degree of survival (\%)} = 100 \times (N/N_0) \tag{4.2}$$

N_0: the number of bacteria in the milk chocolate before lyophilization - just produced (initial time).

N: the number of bacteria in the milk chocolate after lyophilization (end of storage time).

Where N and N_0 present after freeze-drying process and initial viable count of bacteria, respectively (Çabuk, 2014).

4.3. Chocolate Preparation

The chocolate contains 50.7 g carbohydrate, 8.3 g protein, 3.4 g dietary fiber and 34.5g fatty per 100 g (ETİ Food Industrials and Commerce Inc). Three different chocolate types were prepared, which were control (without probiotic) (C), chocolate including microcapsule-probiotic (M) and chocolate containing free-cell probiotic (F).

Tempering process was carried out for three types of chocolate in order to improve the unstable crystals structure of cocoa butter. The tempering was followed in four steps: chocolate masses were heated at 45°C to melt all fats, slowly cooled at 25 ^{o}C with stirring to stable crystals and then reheated at 29- 31 ^{o}C to melt out unstable crystals. After tempering, chocolate was moulded in the forms of 11.67 ± 0.5 g cubes and these samples were cooled. Microencapsulated probiotic and cell-free microcapsules were added before moulding. All chocolate samples were stored at room temperature and at the cooling temperature of 4°C to determinate probiotic viability under various storage conditions (Klindt-Toldam , et al., 2016). All of the chocolate samples were measured after 7 days of storage time in order to attain the chocolate stabilization (Bulatovic, Zaric, Krunic, Boric, & Rakin, 2016).

4.3.1. Chemical Analysis

Moisture and ash analysis of chocolate samples were evaluated as chemical analysis.

4.3.1.1. Moisture Analysis

According to (AOAC , 1990), the moisture content of the chocolate was determined gravimetrically by oven drying at 105±2 ^{o}C for 24 h to reach weight equilibration. The moisture content (MC) was estimated by the equation (4.3). Mean values from 2 repeat and duplicates measurements and standard errors were evaluated.

$$MC\ (\%) = [(W_{wet} - W_{dry})/W_{wet}]*100 \qquad (4.3)$$

W_{wet}: the weight of the wet chocolate samples

W_{dry}: the weight of fully dry chocolate samples.

4.3.1.2 Ash Analysis

Ash content of dry sample chocolate was determined according to (AOAC , 1990). 5-10 g chocolate samples were weighed into 25-50 mm porcelain crucible, which was cooled and was reached weight equilibration after it was warmed to 600°C. The crucible was risen gradually to 600 ^{o}C and held for 4 h in a protherm furnace. Afterwards, the crucible was cooled to a room temperature into a desiccator. The crucible containing ashes was left for drying in the water bath (IKA HB 4 B, Germany) after 96% ethyl alcohol was added into ashes. Ash samples were cooled in a desiccator and weight until the equilibration was reached, total ashes content was estimated from the equation (4.4). Mean values from 2 repeat and duplicate measurements and standard errors were calculated according to the results.

$$\text{Total Ashes Content}\ (\%) = 100*\ (M_2 - M_1/M) \quad (g/100g) \qquad (4.4)$$

M_2: Weight of empty crucible + ashes (g)

M_1: Weight of empty crucible (g)

M: Weight of test sample (g)

4.3.2. Microbiological Analysis

Yeast, Mold, total coliform and *Salmonella* counts were done in order to test whether chocolate was produced under hygienic conditions. The viability of lactic acid bacteria in chocolate products were determined.

4.3.2.1. Yeast and Mold Count

After dilutions were prepared for chocolate samples, these diluted samples were added to Yeast Extract Glucose Chloramphenicol (YGC) agar medium by pour plate methods and incubation result count was enumerated at 20-25 ºC for 3-5 days (Ünlütürk & Turantaş, 2002).

4.3.2.2. Viability of LAB

Quantities of lactic acid bacteria in the chocolate samples were evaluated immediately after the chocolate preparation (initial count) and 7, 14, 30 and 60 days of storage at 4±2°C and room temperature. Total cell count of encapsulated bacteria was also evaluated immediately after freeze-drying and 7, 14, 30 and 60 days of storage at 4± 2°C. Total lactic acid bacteria counts were determined using Man, Rogosa, and Sharpe (MRS) agar medium. After serial dilutions were prepared for the samples, petri dishes were sown and incubated at 37°C for 24-48 hours in anaerobic conditions. Counting was enumerated on petri containing 30-300 colonies and the results were determined as colony forming unit (CFU) / ml.

4.3.2.3. *Salmonella* Count

Peptone water (225 ml) was prepared and 25 g sample was weighed to determine the presence of *Salmonella*. Samples were homogenized with stomacher bag and were incubated for 24 h at 37 ± 1°C. Then, the medium was transferred to Rappaport and Vassiliadis (RVS) Broth, a selective pre-enrichment medium, incubated at 35 ± 1°C for 24 hours, then cultured on Brilliant Green Agar from developmental medium and incubated for 24 hours at 35 ± 1 °C. The confirmation test for *Salmonella* presence was conducted by sowing the sample on Triple Sugar Iron Agar (BAM , 1998) where subjected brown, gray or black colonies would have been appeared in the case of *Salmonella* contamination.

4.3.2.4. Total Coliform Count

For *Coliform* group microorganism identification, VRB agar was planted by spread plate technique. For this, VRB agar was added 10 gr of 100 ml erlenmeyer flasks, after physiological water addition, the mixture was mixed homogeneously. Then dilutions of 1/10, 1/100 were made in these samples and then incubated for 24-48 h (BAM , 1998).

4.3.3. Physical Analysis

Color and texture profile analysis of chocolate samples were evaluated as physical analysis.

4.3.3.1. Color Analysis

Color of the chocolate samples was measured with a Chroma Meter (Model CR-400 Konica Minolta Sensing Inc., Japan). Chocolate samples, which were kept through 7

days of period at room temperatures, and were measured after tempering process (Koca, 2011).

Color measurements were made at 20 °C to determine the L * (bright), a * (green to red) and b * (blue to yellow) values of the chocolate samples. Mean values from 5 repeat and 2 replicates measurements and standard errors were utilized (Afoakwa, 2010).

4.3.3.2. Texture Profile Analysis

The hardness and fracturability of chocolate samples were measured by using the Texture Analyzer Model (TA-XT PLUS, Stable Micro Systems, Godalming, UK) with a load cell of 50 N. Hardness is the maximum penetrating force (g) necessary to for the needle to penetrate into the chocolate samples (26x 20 mm and depth 20 mm). The hardness measurements of chocolate samples were done by needle probe at the temperature value of 25 °C (Table 4.8). The samples were chosen as the same dimensions and smooth surfaces.

Table 4. 8. Texture analyzer conditions

Methods Conditions	**Value**
Test Mode	**Compression**
Pre- test speed	1.0 mm/s
Test speed	2.0 mm/s
Post-test speed	10.0 mm/s
Rupture Distance	17 mm
Trigger Type	Auto Force
Trigger Force	5.0 g
Rupture Mode	Close
Tare Mode	Automatic
Advanced Options	Open
Control Oven	Disabled

The fracturability was reported as the maximum load (g) required for fracturing tempered chocolate samples (26x 20 mm and depth 20 mm). Until the chocolate samples

were broken, the probe descends at 10 mm/min. The one point bend test was made for chocolate samples. These analyses were done with three replications as mean values. In addition, standard errors were calculated for both analyses (Yücekutlu, 2015). Mean values from 5 repeat measurements and standard errors were calculated.

4.3.4. Sensory Analysis

Sensorial evaluation was performed and repeated twice for probiotic chocolate samples; at least 20 panelists consisting of the faculty members and students of İzmir Institute of Technology, Food Engineering Department (both experienced and inexperienced) were present for evaluation (Erdem, et al., 2014).

Panelists were asked to define probiotic chocolate samples for their sweetness, appearance, greasiness, color, odor, texture, taste/flavor and general liking. The scale was given between 1-5 score which 1 is the lowest and 5 is the highest (Farzanmehr & Abbasi, 2009).

4.4. Static *In-Vitro* Simulated Digestion Method

First, the activity of the enzymes used in the simulated digestion system was determined based on the methods described by Minekus et al. (2014). After determination of enzyme activity, simulated digestion process was carried out.

4.4.1. Pancreatic Lipase Activity

The pancreatic lipase activity was determined using pH-stat titration method. First, assay solution, titration solution and enzyme solution were prepared. 5 or 10 mg of lipase enzyme powder was dissolved in 5 or 10 ml purified water and kept on ice,

eventually concentration of the enzyme solution was 1 mg/ml which was tested. The aqueous solution was prepared that 7.20 mg Tris-(hydroxymethyl)-amino methane, 1800 mg NaCl, 40 mg $CaCl_2$ and 420 mg sodium taurodeoxycholate (biliary salts) were dissolved in 200 ml purified water. 2.0 g NaOH was dissolved in 500 ml purified water (0.1 N NaOH) as titration solution. 14.5 ml of assay solution and 0.5 ml of tributyrin were added in titration vessel on the mechanical stirrer at 37°C. Then, 10 to 100-µL pancreatic lipase solution (8xUSP) (containing colipase) was prepared. NaOH addition is necessary to maintain the pH of titrating solution at pH 8.0, 37°C. These processes provide measuring linear kinetics of free fatty release for at least 5 minutes. Moreover, 15 ml of the aqueous and 0.5 ml of tributyrin were added in the titration vessel to mix using magnetic stirring at 37°C to prepare blank solution.

In addition, pancreatic lipase activity was calculated according to equation 4.4.

$$\text{Units/mg powder} = \frac{R\,(NaOH) \times 1000}{v \times [E]} \qquad (4.4)$$

R(NaOH): rate of NaOH delivery in µmole NaOH per min (µmole free fatty acid released per min.).

v: volume of the of prepared enzyme solution added in a pH-stat vessel

[E]: concentration of prepared enzyme solution (mg powder/ ml)

4.4.2. *In-Vitro* Simulated Digestion Process

The digestion procedure used to test bioaccessibility in chocolate samples, according to Paz-Yepez C. et al. (2019) methods. Three digestion fluids were prepared, which are salivary, gastric and intestinal. These fluids were used fresh from the stock solution. There is also three-stage to evaluate *in-vitro* digestion; oral stage, gastric stage and intestinal stage. Experimental condition was evaluated in triplicate.

Oral stage: simulated salivary fluids (SSF) were prepared for the stage containing 15.1 mmol/L, KCl, 3.7 mmol/ L KH_2PO_4, 13.6 mmol/ L of $NaHCO_3$, 0.15 mmol/ L of $MgCl_2(H2O)_6$, 0.06 mmol/ L of $(NH_4)_2CO_3$ and 1.5 mmol/ L of $CaCl_2$. The simulated

fluid was adjusted to pH 8 at 37°C. Free-cell bacteria and chocolate samples (including microencapsulated bacteria and free-cell bacteria) were mechanically broken down by a mortar. 5ml salivary fluid and 5ml samples were homogenized and then incubated for 3 min at 37°C. α-Amylase was added as part of the saliva stock solution to achieve a concentration in the saliva mixture of 75 Unit/ ml.

Gastric stage: simulated gastric fluid (SGF) were prepared containing 6.91 mmol/ L of KCl, 0.9 mmol/L of KH_2PO_4, 25 mmol/ L of $NaHCO_3$, 47.2 mmol/ L of NaCl, 0.1 mmol/ L of $MgCl_2(H2O)_6$, 0.5 mmol/ L of $(NH_4)_2CO_3$ and 0.15 mmol/ L of $CaCl_2$ (pH 3.0). After the oral stage, this gastric solution was added including the oral bolus in a ratio 1:1 (v/w). Pepsin was added into the gastric fluid to achieve a concentration in gastric mixture of 2000 Unit / ml and adjusted to pH 3.0 with HCl (1N). Each sample was flipped from top to bottom at 55 rpm for 120 min at 37 °C and incubated.

Intestinal stage: Simulated intestinal fluid (SIF) were prepared including 6.8 mmol/ L of KCl, 0.8 of KH_2PO_4, 85 mmol/ L of $NaHCO_3$, 38.4 mmol/ L of NaCl, 0.33 mmol/ L of $MgCl_2(H2O)_6$, 0.6 mmol/ L of $CaCl_2$ (pH 7.0) and also bile salts (1 mM) and pancreatin (2000 LU/g of fat) . This solution and gastric chime were mixed in a ratio 1:1 (v/w) and adjust pH 7.0 with NaOH (1N). Each sample was flipped from top to bottom at 55 rpm for 120 min at 37 °C. If the pH of the solution drops to 5.7, this may indicate that the lipase is inactive. Therefore, the pH value was monitored through the digestion process. Samples taken from the simulated fluids at the end of each stage were inoculated on MRS agar. Thus, viability of bacteria in chocolate samples were evaluated at the end of the static experiments to simulate the environment of mouth, stomach and intestines; results were estimated by using equation 4.2. Mean values from dublicate measurements and standard errors were calculated.

4.5. Morphology of Microcapsules

The morphology of chocolate samples with free-cell and microencapsulated bacteria and its simulated gastric fluids were examined by using environmental scanning electron microscopy (ESEM).

4.6. Statistical Analysis

The results of the analyses performed to identify the differences between the chocolate produced. One-way analysis of variance (ANOVA) using the "Minitab 18 Statistical Software" program was applied to the microencapsulated and free-cell bacteria with the control group among the chocolate produced. In addition, the differences between the data averages of the application groups were compared by the Tukey test and the data of the comparison groups were tested according to the $\alpha = 0.05$ confidence interval.

CHAPTER 5

RESULTS AND DISCUSSION

5.1. Screening of Probiotic Bacteria for GABA Producing Abilities

The LAB strains were screened to determine their ability to produce GABA from L-glutamic acid for the period of 24, 48, 72 and 96 h. These incubation times were determined based on previous researches (Siragusa , et al., 2007; Li, Gao, Cao, & Xu, 2008; Di Cagno, et al., 2010; Komatsuzaki, Shima , Kawamotoa , Momosed , & Kimurab, 2005; Hayat, et al., 2015). Moreover, GABA- producing ability is effective between pH 4.0 and 5.0 (Barla, et al., 2016). Therefore, a total of 4 reference LAB strains were incubated with L- glutamic acid pH 4.7 and 5.0 for 24, 48, 72 and 96 h to find the highest GABA-producing ability in this thesis (Table 4.1). The strains screened in this study were selected since their therapeutic effects for neuropsychiatric disorders were elaborated in Chapter 2 Table 2.4.

Firstly, qualitative screening was carried out. An acidic condition is obtained to activate the GAD enzyme by adjusting the pH to 4.7 in the pH indicator method. In the case of *S. salivarius* ssp. *thermophilus* STX-2, rise of pH was seen after incubation at its optimum growth conditions and consequently green color changes were observed based on the acid-base analysis (Yang, et al., 2006). GABA was expected to be excreted into the extracellular environment throughout GAD gene expression under these conditions. Therefore, the color change was occurred because of alkalization conditioning of the medium (Diana, Tres, Quilez, Llombart, & Rafecas, 2014). In this study, only green color of GABA standard was observed, while the medium of bacteria showed the pink color. According to the complex pH indicator analysis result, all bacterial cultures used in the study were not produced GABA. Figure 5.1 shows the pH changes of bacteria after incubation. For example, *Lactobacillus rhamnosus* NRRL B- 442 pH was 3.75 with a pink color change; GABA producing ability cannot be confirmed according to this qualitative method. However, the same strain were found to be GABA producer when RP-HPLC method was conducted.

In the literature, *Lb. brevis* NCL912, *Lb. brevis* DPC6108, *Lb. brevis* PM17, *Lb. brevis* IFO 12005 have an optimum pH value of 5.0 for 48 h, pH 6.8 for 9h, pH 4.7 for 24h, pH 5.2 for 48h, respectively to produce GABA (Li, Gao, Cao, & Xu, 2008; Barrett, Ross, O'Toole, Fitzgerald, & Stanton, 2012; Siragusa , et al., 2007; Yokoyama, Hiramatsu, & Hayakawa, 2002). *Lb. delbrueckii subsp. bulgaricus* PR1 showed GABA production at pH 4.7 for 24h and *Lb. delbrueckii ssp. lactis* produces at pH 4.0 for 24 h (Siragusa , et al., 2007; Tsai, Chiu, Ho, Lin, & Wu, 2013). *Lb. helveticus* PR4, B26-W and ND01 strains have the optimum pH value for GABA production between 3.5- 4.6 for 30h and 120h, respectively (Nejati, et al., 2013; Sun T. , et al., 2009). It was found that *Lb. rhamnosus* FC3-6 produced GABA at pH 4.7 for 120 h at the end of fermentation period. *Lc. lactis* CECT-8184, DIBCA13, DIBCA2 and PU1 strains isolated from cheese generated GABA of high quantity at pH 4.7-6.8 for between 24h and 120h (Diana, Tres, Quilez, Llombart, & Rafecas, 2014; Nejati, et al., 2013). Moreover, pH 4.0 and 24h were found the best conditions for *Lc. lactis subsp. lactis* PU1 (Coda , Rizzello , & Gobbetti , 2010; Rizzello, Cassone , Cagno , & Gobbetti , 2008). Therefore, it can be said that color change is not a dependable criteria since every bacterial strain has its own optimum pH, it is only the indication as the pH value of the medium changes.

In this thesis, not only the qualitative screening, HPLC and RP-HPLC methods were used to determine GABA concentrations of probiotic bacteria for quantitative measurements. The mean values of all samples were calculated from double repeated measurements and standard errors**.** Sample preparation methods were different for each HPLC methods as described in detail in Materials and Method 4.2.1. section, whereas 50mM L-glutamic acid (1.0 %) was used as a substrate at each for both.

Firstly, GABA production abilities of bacterial strains were measured using C18 column by HPLC method. In case of GABA standard, chromatogram pike of retention time was found approximately 30 min. However, peaks of standard GABA were not resolved well in the range of 500 ppm and lower, interferences were shown in between the peaks, Gaussian type peaks were not seen to obtain quantitative data. Therefore, chromatogram results showed that higher GABA peak were belong to *Lactobacillus rhamnosus* NRRL- B442, fermented at pH 3.65 at 37ºC for 90 h (Appendix A). Other bacterial samples having different fermentation times were also measured by HPLC under same parameters. Not all the probiotic bacteria produced GABA.

There have been many research in literature to determine GABA concentration by HPLC using C8 and C18 columns, that can be suitable for protein and peptides (Rosetti & Lombard, 1996; Ko, Lin , & Tsai, 2013; Horanni & Engelhardt, 2013; Tajabadi, et al., 2015). Successful resolutions for GABA were obtained according to these studies, e.g. LiChrospher 100 RP-18 column (250 x 4.6 mm, 5µm), Mightysil C18 column (250 x 4.6 mm, 5µm), Hypersil ODS C18 reverse-phase column (250 x 4.6 mm, 5µm), SB-C-8 (2.1 x 50 mm) and Kinetex C18 column (100 x 2.10 mm, 2.6µm).

Therefore, on the outset of this thesis, ACE C18 column (250 x 4.6 mm, 5µm) were used to identify GABA concentrations. It was decided to be used as highlighted that ACE C18 column with hydrophobic characteristics is generally used for protein and peptides, by above research studies. Nevertheless, this operation was not resulted with success to extract quantitative data even though many pre-derivatization methods and programs have been tried.

Afterwards different type of column, Pico.Tag column (3.9 x 300 mm, 4µm), based on silica- packing materials, was used in able to obtain quantitative data for GABA. The Pico.Tag column is generally used the analysis for free-amino acids, amino acids of peptides and protein hydrolysates. Pico.Tag column involves in following two steps for analysis of food samples. These steps include the acid hydrolysis to release amino acids, and pre-column derivatization of food samples to separate the phenylthiocarbamyl amino acid derivatives by using HPLC. In this study, phenylisothiocyanate was used for pre-column derivatization of GABA standard and bacteria samples. The amine group of amino acid bonds to carbon group of phenylisothiocyanate and as a result of phenytfhiocarbamyl amino acid is produced.

It was found that the separation of the phenytfhiocarbamyl derivatives of the amino acids takes approximately 12 min. with the Pico.Tag method using sodium-based buffers. Thus, it was supported that Pico.Tag column can be used as an alternative of the ion-exchange analyzer (White, Hart, & Fry, 1996) since ion-exchange chromatography is conventionally best separation method for amino acid analysis.

The derivatization method used for Pico.Tag method was also been tried for comparing results obtained earlier by pH indication method. At the end of fermentation time for each probiotic bacteria alteration of pH values were recorded. As seen from Figure 5.1, optimum pH of all strains for GABA production was observed in the range of

pH 3.65 and 4.88. *Lactobacillus rhamnosus* NRRL B-442 had the lowest pH optimum throughout the fermentation period among four LAB strains.

The calibration curve was determined by Pico.Tag method using RP-HPLC for five different concentrations 20, 50,100, 200, 500 mg/L of GABA standard as seen in Appendix B. The regression coefficient (R^2) was found greater than 0.99 for this curve. GABA concentrations belong to four-probiotic bacteria were calculated according to GABA calibration curve equation. Chromatogram pike of retention time of GABA standard was found at 6.08 min (Appendix B).

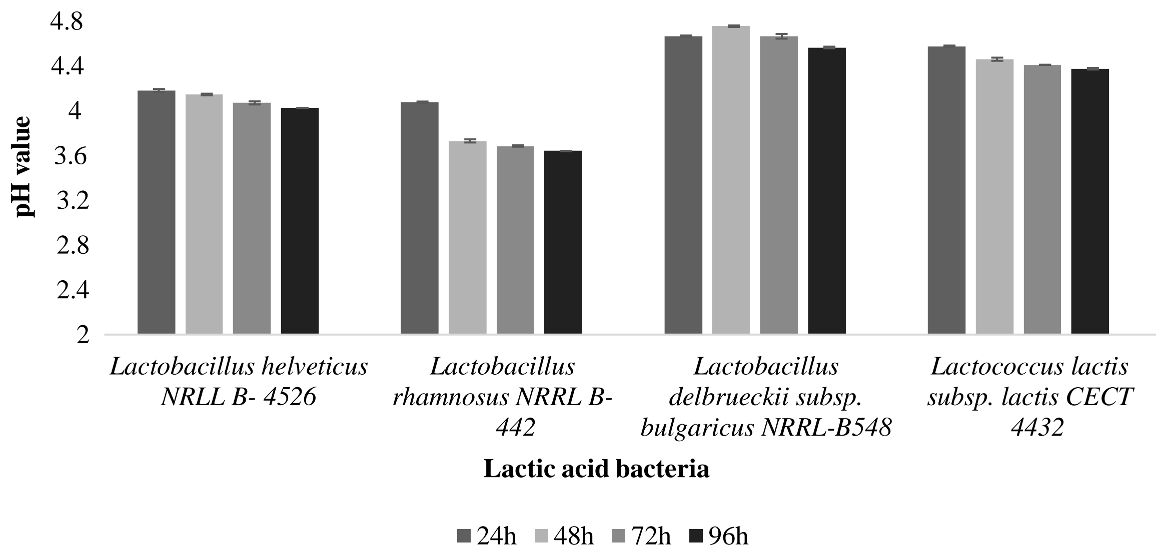

Figure 5. 1. Qualitative pH indication method of LAB strains to estimate GABA secretion.

Lactobacillus rhamnosus NRRL B- 442 produced the highest GABA concentration to be approximately 58,800 mg/L at pH 4.07 at 37ºC for 24 h at the end of fermentation period, which had the highest GABA concentration among other LAB strains (Table 5.1). The chromatograms obtained for four LAB strains at different fermentation periods of 24h, 48h, 72h and 96h were given in Appendix B. *Lb. helveticus* NRRL-4526 had similar GABA concentrations at 24h, 48h and 72h. However, GABA was not detected by *Lb. helveticus* NRRL-4526 at the end of 96 h incubation time. GABA-production ability of *Lb. delbrueckii* subsp. *bulgaricus* NRRL-B548 and *Lc. lactis* subsp.

lactis CECT 4432 was not found at noticeable difference owing to their fermentation time of 24 – 96 h. *Lb.delbrueckii* subsp. *bulgaricus* NRRL-B548 produce GABA between the concentration values of 48,160±151 and 55,147±263 mg/L.

In the literature, there have been many studies investigated GABA content of LAB. For example, *Lb. paracasei NFRI* from isolated fermented fish had GABA content of 31145.3 mg/kg (Komatsuzaki, Shima , Kawamotoa , Momosed , & Kimurab, 2005). *Lactobacillus* subsp. *OPK 2-59, Lactobacillus buchneri* M*S, Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *lactis* B had GABA concentrations in between 180 and 25883.12 mg/kg (Lu , Xie , & Gu , 2009; Seok , et al., 2008; Lu , Xie , Gu , & Han, 2008). *Lactobacillus paracasei* PF6*, Lactobacillus plantarum* C48*, Lactobacillus delbrueckii* subsp. *bulgaricus* PR1, *Lactococcus lactic* PU1 and *Lactobacillus brevis* PM17 produced GABA at the concentrations of 99.9 mg/kg, 16 mg/kg, 63.0 g/kg, 36.0 mg/kg and 15 mg/kg, respectively (Siragusa , et al., 2007; Coda , Rizzello , & Gobbetti , 2010). In another study, it was found that *Lactobacillus brevis* NCL912 consisted of 103719.1 mg/kg GABA (Li, Qiu , Gao , & Cao , 2010). In another study, *Lactobacillus plantarum* Taj-Apis362 was isolated from honeybees produced 1.76 mM GABA (Tajabadi, et al., 2015). In addition, *Lactobacillus brevis* strain synthesized 4599.2 mg/kg GABA (Huang , Mei , Wu , & Lin , 2007). *Lb. rhamnosus* FC3-6, *Lb. helveticus* PR4, *Lb. helveticus* B26-W, *Lb.casei* 2749 and *Lc. lactis* DIBCA13 produced GABA in the concentration of 16.3 mg/kg, 10.3 mg/kg, 16.9 mg/kg, 24.7 mg/kg and 10.1 mg/kg respectively at 37 ^{o}C (Nejati, et al., 2013). In another study, it was found that two different *Lb. helveticus* strains of E2303 and ND01 produced GABA between 56.44 and 113.35 mg/L (Sun T. S., et al., 2009).

Table 5. 1. GABA concentration of probiotic bacteria

Probiotic bacteria	GABA (mg/ L)			
	24h	48 h	72 h	96 h
Lb helveticus NRLL B-4526	48,993±286^{a}	50,277±211^{a}	50,680±258^{a}	-
Lb.rhamnosus NRRL B- 442	58,881±985^{a}	57,524±269^{ab}	54,893±837^{bc}	52,950±105^{c}
Lb.delbrueckii subsp. *bulgaricus* NRRL-B548	55,147±263^{a}	48,160±151^{a}	50,117±162^{a}	51,288±414^{a}
Lc. lactis subsp. *lactis* CECT 4432	44,947±232^{a}	48,082±320^{a}	49,166±162^{a}	50,177±101^{a}

Means indicated with different small letters represent significant differences on the same line (P < 0.05).

Lb. brevis strains of 12005, CECT 8183, CECT 8181 and CECT 8182 isolated from 2 different cheese (goat and sheep cheese) generated GABA at high quantity 0.83, 0.96, 0.94 and 0.94 mM (Diana, Tres, Quilez, Llombart, & Rafecas, 2014). *Lb. plantarum* DSM19463, which is another bacterium isolated from cheese, synthesized GABA of 0.89 mM (Di Cagno, et al., 2010). It was found that *Lb. rhamnosus* GG produced the highest GABA concentration of 0.44 mg/ml at the end of 36 h between different fermenation times (Song & Yu, 2018).

Recently, many clinical studies focused on consuming probiotic bacteria instead of various drugs such as lorazepam and diazepam with pharmacological properties acts on GABA receptors (Cryan & Kaupmann , 2005; Rudolph & Möhler , 2004; Singewald, Schmuckermair, Whittle, Holmes, & Ressler, 2015). Different probiotic bacteria have been indicated to decrease anxiety symptoms (Pirbaglou , et al., 2016; Wallace & Milev, 2017). For example, it was supported in a clinical study that consumption of *Lactobacillus rhamnosus* could decrease anxiety disorder because of the effect of GABA neurotransmitter in CNS (Bravo, et al., 2011). In other words, clinical studies were presented to support that *Lb. rhamnosus* regulate GABAergic system and emotional behavior. It means that consumption of probiotic bacteria was effective on neuropsychiatric disorders (Bravo, et al., 2011; Enticott, Rinehart, Tonge, Bradshaw, & Fitzgerald, 2010).

5.2. Microencapsulated Probiotic Bacteria

It is known that factors like storage conditions of a product, food process conditions, and the gastrointestinal system limits the viability of probiotic bacteria. Therefore, microencapsulation is known as one of the effective method conserving bacteria against these factors (Gadhiya, Patel, & Prajapati, 2015).

In this study, bacteria cultures were microencapsulated by using water-in-oil emulsion technique as described in Figure 5.2, following step by hardening of the microcapsules. Microencapsulation was carried out by choosing *Lb. rhamnosus* NRRL B-442 strain as for the highest GABA-producer, it was incubated at 37 ºC for 23 h, according to its growth curve (Appendix C).

Lb. rhamnosus NRRL- B442 was added at the concentration approximately $1.2x10^{11}$ CFU/mL into the emulsion. Immediately after the bacteria were microencapsulated, freeze-drying was applied for the bacteria having the concentration of 10^9 CFU/g and stored at 4ºC for future experiments (Figure 5.3). During freeze-drying process 2 log decrease in bacteria count was obtained; therefore microencapsulation efficiency of *Lb. rhamnosus* probiotic bacteria were found to be as 81.81% .

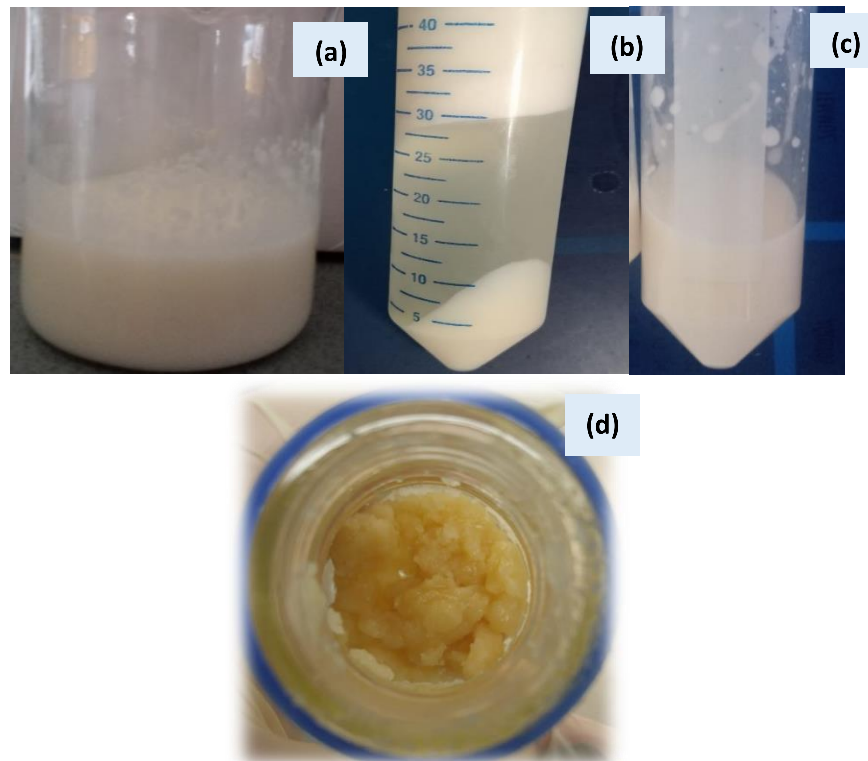

Figure 5. 2. Microencapsulation of *Lb. rhamnosus* NRRL- B442 strain by emulsion technique

(a): WPC+ pullulan + probiotic bacteria solution
(b): (a) + $CaCl_2$ solution + oil emulsion
(c): Microencapsulated probiotic bacteria, after emulsion was separated
(d): Microencapsulated and freeze-dried bacteria

In Figure 5.3, microencapsulated bacteria were followed by a decline of viable cell counts from 9.01 log CFU/g to 8.41 log CFU/g after 60 days of storage at 4ºC. The survival of freeze-drying *Lb. rhamnosus* NRRL- B442 was found as 93.34% for 8 weeks. Same microencapsulation method was used by Çabuk (2014), where the survival rate of *Lactobacillus acidophilus* was found as 92% for 4 weeks.

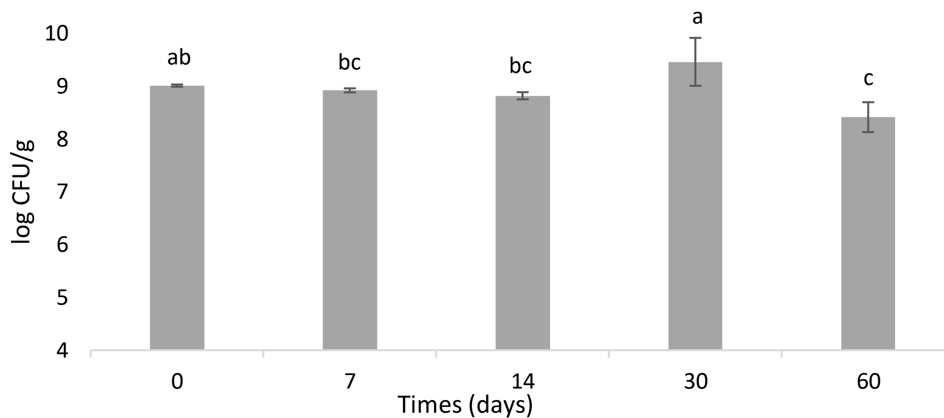

Figure 5. 3. Survival of microencapsulated *Lb. rhamnosus* NRRL- B442 during storage.

5.3. Chocolate Preparation

The initial microencapsulated bacteria count was 10^9 CFU/ml in chocolate formulation. After microencapsulated probiotic was added into the chocolate, its count decreased approximately 2 log. Thus, final concentration in chocolate was found as 10^7 CFU/g. Ultimate count of microencapsulated *Lb. rhamnosus* NRRL-B442 viable cells in chocolate samples were 10^7 CFU/g based on the dilution effect of 1.0 % that 1g microencapsulated cells were added in 100 g chocolate sample. In addition, free-cells of bacterial cell suspension were added at the concentration of 10^9 CFU/ml that should resemble to 7 log CFU/g used for microencapsulated cells. Similar decrease in the cell numbers were observed immediately after probiotic chocolate was produced (Bulatovic, Zaric, Krunic, Boric, & Rakin, 2016; Erginkaya, Sarıkodal, Özkütük, Konuray, & Turhan, 2019; Coman M. M., et al., 2012; Lalicic- Petronijevic , Popov-Raljic, Lazic, Pezo, & Viktor, 2017). These chocolates were stored both at 4ºC and at room temperature for 2 months. The survival rate of *Lb. rhamnosus* after 60 days in milk chocolate at 25ºC was remarkably decreased for F and M chocolate samples. However, it was observed that the degree of bacterial survival during storage was higher at 4ºC. Therefore, keeping the F and M samples at 4ºC could be preferred since it is the best storage condition for preserving the viability of the probiotic bacteria (Khanafari , Porgham, & Ebrahimi , 2012; Konar, et al., 2018).

In this thesis, F and M samples had almost the same survival rate of bacteria during storage time. Moreover, M sample had a slightly lower degree of survival e.g. 79% and F sample had a survival rate of 83% in the chocolate, results are shown in Figure 5.4. It was also known that the degree of probiotic survival in different types of chocolate was depended on a strain character. In the literature, the survival rate of *Lactobacillus* strains in chocolate was found to be 90% for 6 months (Bulatovic, Zaric, Krunic, Boric, & Rakin, 2016). The survival rate of *Lb. rhamnosus* PTCC1637 cells were found high in the chocolate at 4ºC for 25 days (Khanafari , Porgham, & Ebrahimi , 2012). Viable cells of *B. indicus* HU36 count was found in the range of 88-91% in the chocolate (Erdem, et al., 2014).

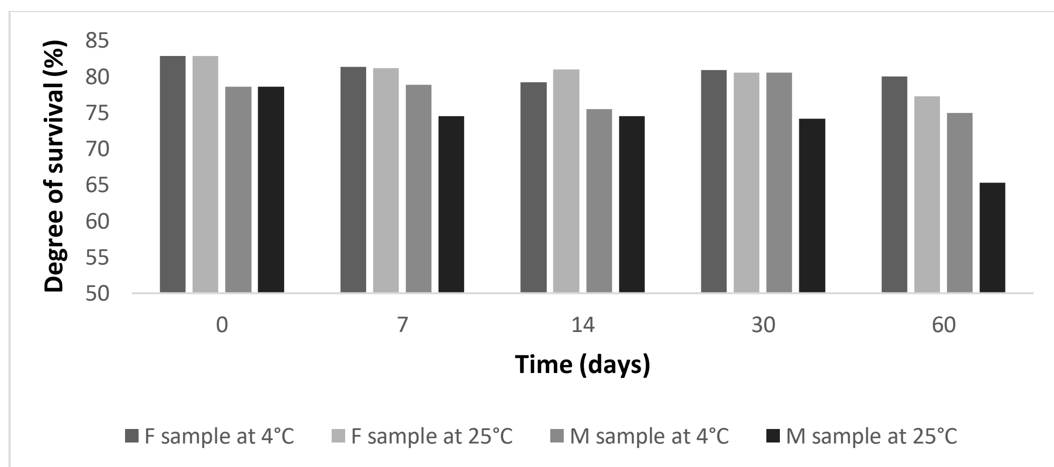

Figure 5. 4. Degree of survival (%) of *Lb. rhamnosus* NRRL B-442 during storage time.

In this study, F chocolate samples had slightly higher viable probiotic count than M samples. Table 5.2 demonstrated information about the viability of *Lactobacillus rhamnosus* NRRL B-442 in chocolate samples during storage. The number of microencapsulated bacteria (M) in the chocolate was merely lower at the beginning of storage (0th day) than free-cells (F). It is shown that microencapsulation of cells develop resistance to stress factors such as chocolate melting temperatures and food composition (Ding & Shah, 2008; Konar, Toker, Oba, & Sagdıc, 2016). Small differences in cell counts of F and M samples have been found, it can be attributed to the harsh conditions of chocolate production process; immediate mixing of chocolate at melting temperature.

Table 5. 2.Viability of *Lactobacillus rhamnosus* NRRL B-442 free-cell and microencapsulation in milk chocolate samples (log CFU/ 10g).

Sample	**Storage time**					**Storage Temperatures**
	0th day	**7th days**	**14th days**	**30th days**	**60th days**	
F	7.457±0.07^{aA}	7.318±0.09^{aAB}	7.132±0.14^{aB}	7.284±0.11^{aAB}	7.204±0.17^{aB}	4°C
	7.457±0.07^{aA}	7.307±0.22^{aA}	7.288±0.22^{aA}	7.252±0.10^{aA}	6.953±0.19^{aB}	25°C
M	7.076±0.13^{bABC}	7.096±0.12^{aAB}	6.795±0.11^{bBC}	7.248±0.28^{aA}	6.747±0.32^{Ac}	4°C
	7.076±0.13^{bA}	6.708±0.14^{bA}	6.706±0.16^{bA}	6.677±0.13^{bA}	5.879±0.65^{Bb}	25°C

a, b: Means indicated with different small letters represent significant differences on the same column (P < 0.05).
A, B, C: Means indicated with different capital letters present significant differences on the same line (P < 0.05).

It was observed that viable cell counts in F and M chocolate samples increased after 30 days, while after 7, 14 and 60 days viable cells count in the samples decreased when stored at 4°C. The effect of coating materials during microencapsulation process on cell viability was found statistically significant between days at 4°C (P<0.05). While bacteria count had the highest survival at the end of 30th days, the count was the lowest at 60th days under 4°C. However, the survival of bacteria in M sample at 25°C reduced significantly at the end of 60 days. Moreover, microencapsulated bacteria and M samples at 4°C were similarly increased and reduced in storage condition, but the viability of count probiotic bacteria in M at 25°C reduced remarkably (Figure 5.5).

It was observed that microencapsulated bacteria and M sample had similar changes among different days at 4°C. Dealing with F samples, the survival of free-cell bacteria was found statistically different between storage days at 4°C (P<0.05). However, it slowly decreased at 25 °C storage conditions. Moreover, F samples were not found statistically different between at various storage temperature (P>0.05). Furthermore, F and M samples was found statistically different from each other at storage conditions of 25 °C (P<0.05). However, they were found to be significantly different for 0 and 14 days at 4°C, but it was found that there was no statistically significant difference between the samples for 7, 30 and 60 days (P>0.05) (Figure 5.6). The viability of the M and F samples during refrigerated storage were found to be the best condition. Other experimental

studies also supported this finding (Khanafari , Porgham, & Ebrahimi , 2012; Erginkaya, Sarıkodal, Özkütük, Konuray, & Turhan, 2019; Lalicic- Petronijevic , Popov-Raljic, Lazic, Pezo, & Viktor, 2017; Lalicic- Petronijevic , et al., 2015).

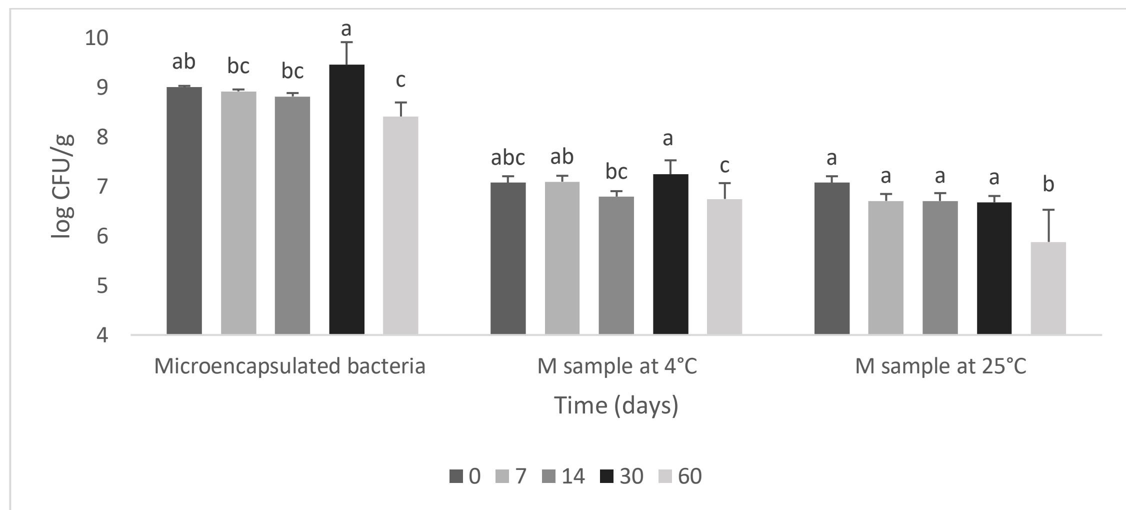

Figure 5. 5. The viable cell count of microencapsulated *Lb. rhamnosus* NRRL B-442 and M samples at 4 ºC and 25ºC.

The value in columns represents the difference in content of the different samples of microencapsulated probiotic in chocolate and without chocolate. Mean values in the group of columns, labelled with the same letter are not statistically different ($p < 0.05$).

To illustrate, a study showed that the total number of *Lactobacillus* bacteria cells in dark chocolate was found as 6-7 log CFU/g for 12 months at 4°C (Nebesny, Zyzelewicz, Motyl, & Libudzisz, 2007). Lactic acid bacteria can be continued their metabolic activities at mesophilic temperatures such as 27ºC and 30ºC and produced various metabolites. Thus, the bacteria pass from the exponential phase to the death phase and lose their vitality over time. Depending on this knowledge, products with probiotics should be preferred stored at lower temperatures to prevent their metabolic activities (Khanafari , Porgham, & Ebrahimi , 2012; Dharmasena, 2012).

In this study it was observed that free and encapsulated bacteria were added in chocolate, the survival rate of the bacteria was gradually reduced. M samples at 4ºC reduced approximately 0.329 log CFU/g and F samples at 4ºC decreased almost 0.253 log CFU/g at the end of storage. The viability of freeze-dried form of *Lb. acidophilus* NCFM and B. *lactis* HNO19 in chocolate samples were observed with the loss of 1.1-1.6 log CFU/g at the end of 14 months (Klindt-Toldam , et al., 2016). The combination of *Lb. brevis* F19 and *Lb.rhamnosus* GG in dark chocolate were decreased approximately 1.0 and 0.4 log CFU per dose during 90 days (Succi, et al., 2017).

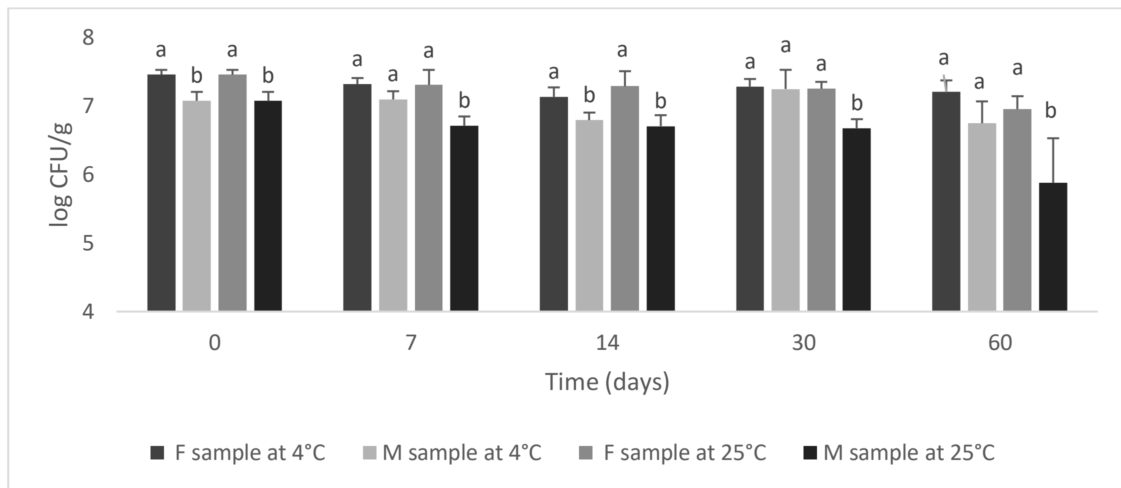

Figure 5. 6. Survival of *Lb.rhamnosus* NRRL-B 422 in F and M samples during storage (a) at 4 ºC and (b) 25 ºC.

The probiotic load is acceptable between 6-8 log CFU/g for dairy products (Maragkoudakis, et al., 2006). Other studies are also supported this since the minimum count of probiotics at the time of intake requires ≥ 6 log CFU/g for having therapeutic effects (Doleyres & Lacroix, 2005; Konar, et al., 2018). Hence, F and M chocolate sample can be acceptable since they contain adequate amount probiotic cells (Table 5.2). Moreover, probiotic microorganisms in chocolate can also be used without microencapsulation because similar survival rates were obtained for F and M samples during storage period. However, some studies supported that microencapsulation technique was effective on the survival of bacteria during the GI tract (Possemiers, Marzorati, Verstraete, & Van de Wiele, 2010; Yonejima, et al., 2015; Coman M. M., et al., 2012; Champagne, Raymond, Guertin, & Belanger, 2015; Succi, et al., 2017; Klindt-Toldam , et al., 2016).

It was known that chocolate has protective properties for bacteria because of its lipid fraction of cocoa butter and high phenolic content (it is affected on reduced oxidative stress) (Burgain, Gaiani, Linder, & Scher, 2011; Lahtinen, Ouwehand, Salminen , Forssell, & Myllarinen, 2007; Yonejima, et al., 2015; Pedroso, Dogenski , Thomazini, Heinemann, & Favaro-Trindade, 2013). Thus, chocolate structure can represent a good carrier properties for preserving bacteria.

Some studies investigated the survival of probiotics in different chocolate types when tested in simulated GI tract. As was shown earlier in Table 3.2, there have been

many successful applications of probiotics, therefore "chocolate with probiotic" can be thought as a novel approach. However, limited study has been focused on food products containing probiotics to overcome neuropsychiatric disorders. For instance, in the double-blind clinical study it was found that consuming a milky-drink including *Lactobacillus casei* Shirota was effective on the treatment of depressed mood-like (Benton, Williams, & Brown, 2007). Chocolate can be other hand preferred as a good food source because studies reported that daily intake of chocolate has being add a positive effect on mood state and this property can be enhanced by entegrating probiotic power (Parker, Parker, & Brotchie, 2006; Pase , et al., 2013).

5.4. Moisture Analysis and Ash Analysis

While control chocolate (without bacteria) (C) and chocolate contains microencapsulated bacteria (M) were not found significantly different between each other ($p>0.05$), chocolate with free-cell probiotic bacteria (F) was determined slightly different from the other chocolate samples ($p<0.05$) (Table 5.3).

Table 5. 3. Chemical analysis results of chocolate samples

Sample	Moisture Count (%)	Ash Count (%)
C	1.664 ± 0.03^{b}	3.766 ± 1.396^{a}
F	1.809 ± 0.07^{a}	3.058 ± 0.68^{a}
M	1.678 ± 0.03^{b}	3.70 ± 2.24^{a}

Means indicated with different letters in the same column are different ($p<0.05$).

The result of the moisture analysis, C and M were found at similar moisture content, whereas the moisture of chocolate, containing free bacteria cell, was found the slightly at a higher value. According to the literature, moisture content is significant for chocolate products since moisture content in the product leads to agglomeration and gritty

structure by the sugar particles causing to increase viscosity. Molten chocolate has generally moisture content value of 0.5-1.5% (Afoakwa, 2010). Moreover, it was found that chocolate having moisture range at 3.0-4.0 % increase markedly yield value of chocolate and viscosity (Chevalley, 1999). Moreover, low moisture food products contain less amount of pathogens (Possemiers, Marzorati, Verstraete, & Van de Wiele, 2010). According to these findings, it was detected that the moisture range of all chocolate samples was not affected adversely other properties of chocolate.

Ash analysis results of chocolate samples were given in Table 5.3. These samples were not found statistically different (P>0.05) from each other in terms of ash content. In addition, it was found that the addition of probiotic bacteria, both encapsulated and free-cell bacteria, into chocolate products were not effective in terms of ash and moisture content.

5.5. Microbiological Properties of Chocolate

Table 5.4 presents the results of the count of yeast and mold, *Salmonella* and total *Coliform* as microbiological analysis. Control chocolate, chocolate including free-cells of bacteria (F) and chocolate including microencapsulated bacteria (M) were tested with these analyses.

Table 5. 4. The result of microbiological analysis

Sample	**Yeast and Mold (CFU/g)**	***Salmonella* (CFU/ 25g)**	**Total Coliform (CFU/g)**
C	**<10**	**-**	**<10**
F	**<10**	**-**	**<10**
M	**<10**	**-**	**<10**

At the end of the analysis, *Coliform*, *Salmonella* and mold – yeast were not be detected. Moreover, it is known that *Salmonella* is a crucial criterion for chocolate products in terms of Turkish Food Codex at Microbiological Criteria Regulation (Figure 5.7).

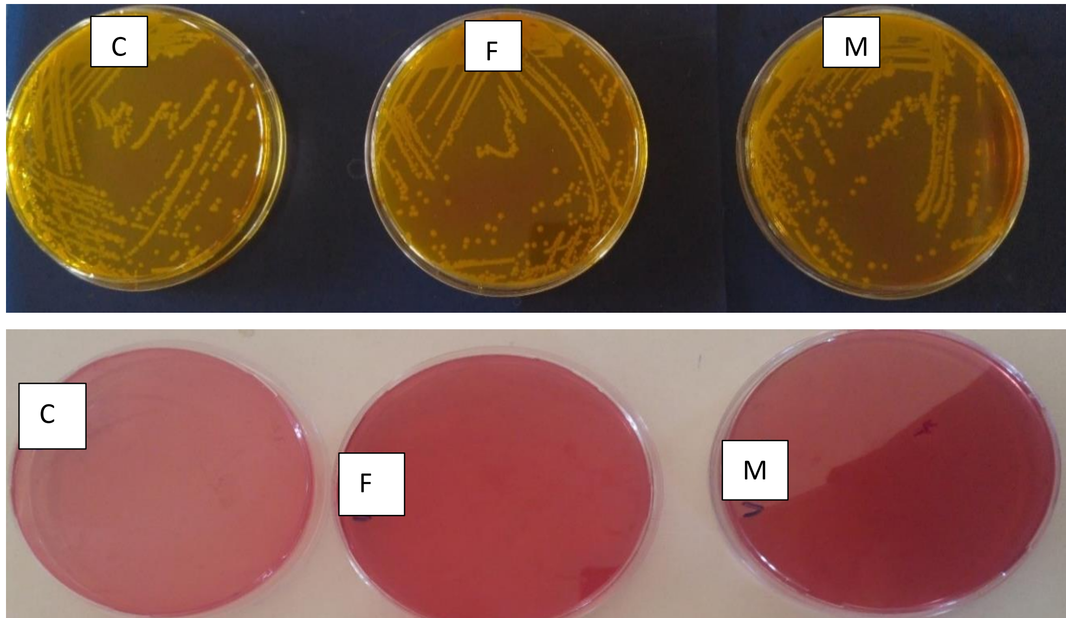

Figure 5. 7. The result of *Salmonella* and total coliform analysis.

Result of the microbiological analysis indicates that chocolate samples were produced under hygienic conditions.

5.6. Physical Analysis

Color and texture analysis of chocolate samples were evaluated for determining the differences occurred during the processes.

5.6.1. Color Analysis

L*value (luminance) show between 0 (black) and (100 white) of the sample in color. At the end of the statistical analysis, it was not found statistically significant ($p>0.05$) to add free-cell bacteria (F) and microencapsulated bacteria to chocolate (M) (Table 5.5). L* values were found not significant in all chocolate samples that contain milky matrix. Therefore L* values were found as similar mean values for all the samples.

a* values demonstrate the greenness or redness of a sample in color determination and b* values illustrate the yellowness or blueness of the sample in color. As seen from Table 5.5, F sample, control chocolate (C) and M sample ad respectively higher a* and b* values. It means that F sample had higher red and yellow color, whereas the M sample had lower red and yellow color. Overall, it was found that all of a* and b* values were different from each other ($p<0.05$). However, ΔE (total color difference) was calculated by using the color parameters for the corresponding two different chocolate samples and control sample (without bacteria) as the reference. The existence of probiotic bacteria in chocolate did not indicate a significant effect ($p>0.05$).

Table 5. 5. Color analysis conclusion of chocolate

Sample	L*	a*	b*	ΔE*
C	32.05±0.28[a]	10.14±0.10[b]	11.51±0.17[b]	*
F	31.69±0.98[a]	10.86±0.41[a]	12.31±0.72[a]	2.329±0.96[a]
M	31.47±0.84[a]	9.83±0.15[c]	11.07±0.29[c]	1.575±1.16[a]

Means shown with different letters in the same column are different ($p <0.05$).

5.6.2. Texture Profile Analysis

In a study with milk chocolate having optimal hardness was demonstrated as a good carrier to produce chocolate containing probiotics experimentally (Bulatovic, Zaric, Krunic, Boric, & Rakin, 2016). In this thesis study, texture analysis was evaluated for hardness and fracturability properties for 7 days after chocolate was prepared at the end of storage time. F and M samples did not show noticeable significance in 7 days. Also, there was no difference between chocolate with bacteria and control chocolate. Similar results were observed in another studies (Konar, et al., 2018; Nebesny, Zyzelewicz, Motyl, & Libudzisz, 2007).

In addition, all chocolate samples were not significantly different from each other ($P>0.05$). However, M, F and C chocolate samples were significantly different after 60 days ($P<0.05$). Compared to C sample, F sample had higher hardness value and M sample

was found slightly high (Figure 5.8). Hardness of M chocolate samples were slightly reduced at the end of storage time, as it was in C samples. This case can be attributed to the emulsion structure of microcapsules which can be easily integrated to chocolate fatty structure. On the other hand, F sample was increased in hardness. It was shown that free-cell bacteria are influenced on chocolate hardness because of migration of fat (Bulatovic, Zaric, Krunic, Boric, & Rakin, 2016).

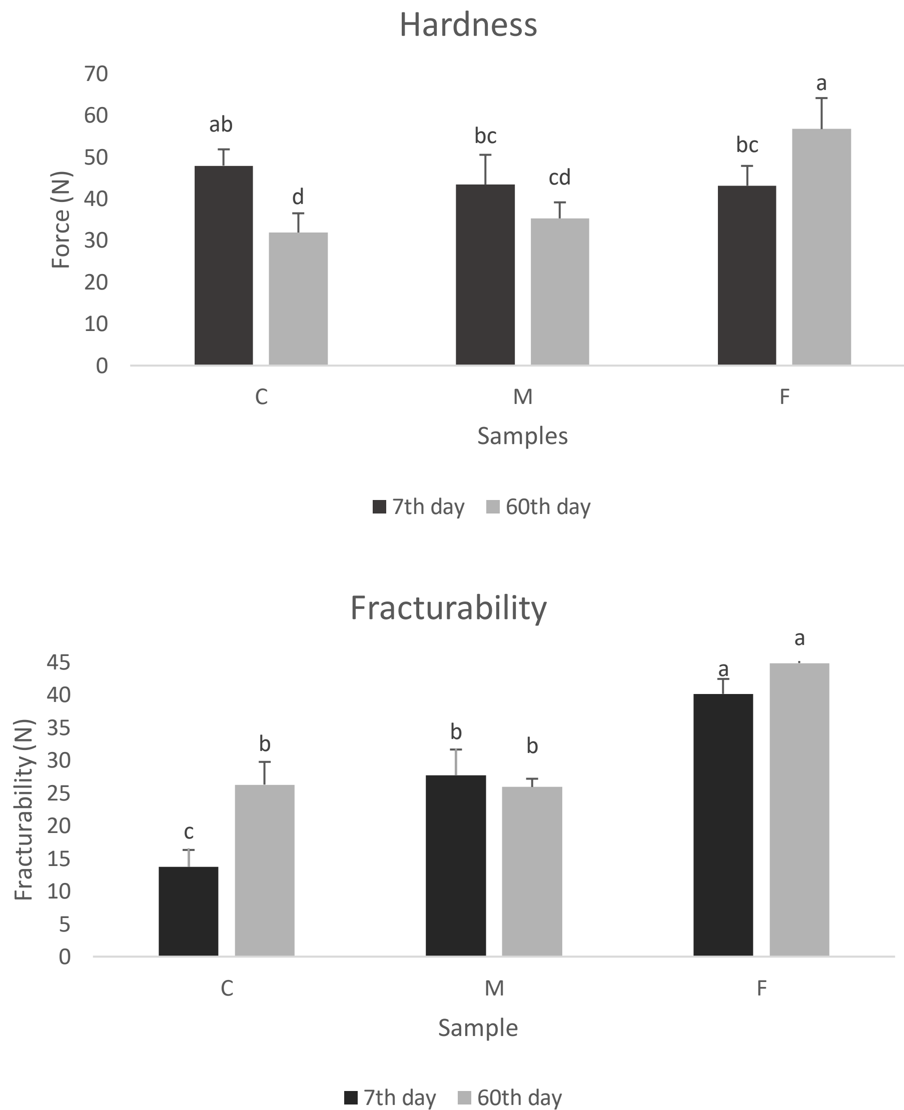

Figure 5. 8. The result of texture analysis after 7 days and 60 days of storage.

Fracturability represents a low degree of cohesiveness and the high degree of the hardness in the product. Therefore, it refers to force needed to compress product and compress before it breaks between teeth (De Clercq, et al., 2012; Szczesniak, 2002). While fracturability degree of F chocolate sample was found at the higher value, C

chocolate sample had the lowest fracturability value. The three samples were statistically notable different from each other ($p<0.05$). This property did not change significantly during storage time for F and M samples. While F sample was remained unchanged with higher fracturability, C and M samples kept the same fracturability value until the end of storage time. Similar results were reported by Konar et al. (2018) and Nebensy et al. (2007).

As a conclusion withdrawn from the texture analysis results, F samples having free-cells of bacteria, a hydrophilic phase- difficult to interact with the hydrophobic structure of chocolate- caused high fracturability and hardness. Since chocolate fracturability can be related to hardness, correlations in between were evaluated using Pearson correlation. Correlations were made between fracturability, hardness and bacterial count; and a significant correlation ($p<0.05$) were deduced with a coefficient of higher than 71%. Normally, obtaining sufficient correlation is difficult since chocolate quality parameters were affected by various uncontrollable factors during production therefore, standardization is challenging.

5.7. Sensory Analysis

The sensory analysis made after the microbiology analysis to be sure of chocolate samples did not contain any pathogenic bacteria. Chocolate samples were introduced by three different numbers to be evaluated by panelist (Figure 5.9). These chocolate samples, except control, were consisted of 7.0 log CFU/g probiotic bacteria.

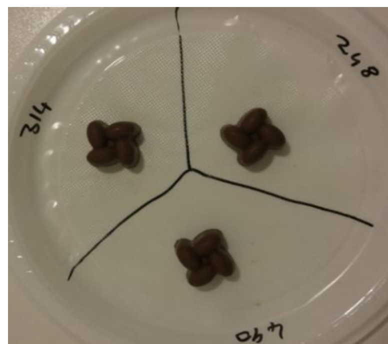

Figure 5. 9. Chocolate samples for sensory analysis.

It was found that color, texture, taste and general liking parameters were found mostly similar for F and M samples as well as control chocolate samples. According to

these properties of chocolate, it was no statistical difference between chocolate groups ($p>0.05$). However, control chocolate was preferred as the best in terms of odor property by the panelists. While C chocolate had the highest odor, F chocolate had less odor property to C chocolate and M chocolate. Hence, there are statistical differences in chocolate samples ($p<0.05$).

Table 5. 6. The result of sensory analysis

Sample	Color	Odor	Texture	Taste	General Liking
C	4.28±0.60[a]	4.28±0.68[a]	4.10±0.63[a]	4.45±0.60[a]	4.38±0.59[a]
F	4.225±0.62[a]	3.80±0.65[ab]	4.03±0.70[a]	4.18±0.84[a]	4.18±0.71[a]
M	4.28±0.55[a]	3.95±0.64[b]	3.98±0.73[a]	4.15±0.89[a]	4.15±0.70[a]

Means indicated with different letters in the same column are different ($p<0.05$).

In this study, 5 score is referred to the best. C chocolate liked in all sensory parameters and F and M chocolate were close degrees to C chocolate. C chocolate had generally 4.10 and over score (Figure 5.10). It was determined that odor and color features of F chocolate had a lower score. M chocolate was slightly lower than C chocolate and C chocolate had the highest score among the sensory characteristics.

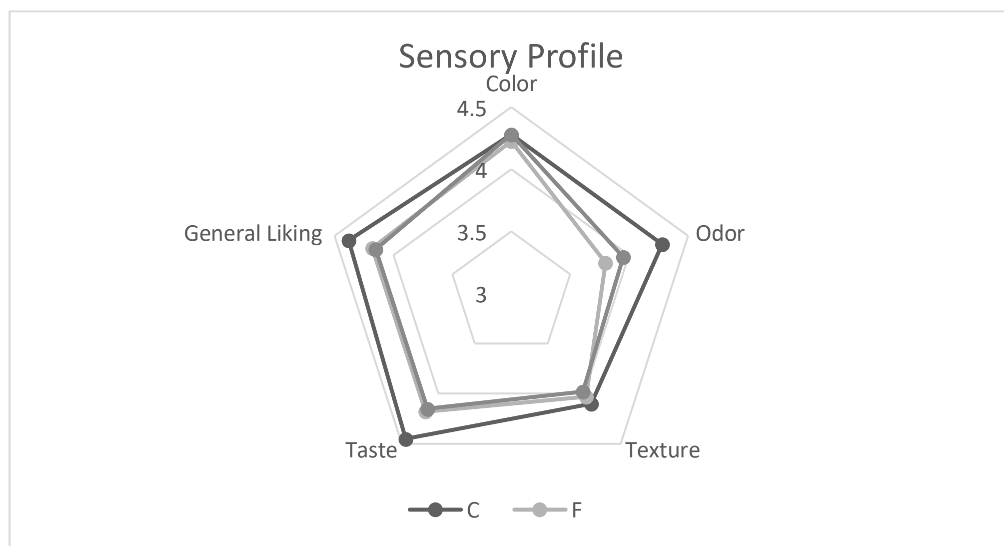

Figure 5. 10. Spider charts at 7 days of storage.

All in all, it was observed that probiotic bacteria usage in chocolate did not generally change the sensory characteristics of chocolate except odor property. However, the odor score can be ignored because of the lower score. In this study, general acceptability of chocolate with probiotic bacteria obtained by sensory evaluation were found as the same with control chocolate. It was found parallel that either free-cell bacteria or encapsulated bacteria were not significant for chocolate sensory features (Lalicic- Petronijevic , Popov-Raljic, Lazic, Pezo, & Viktor, 2017; Succi, et al., 2017; Klindt-Toldam , et al., 2016). Appreciating the taste and odor property is a critical factor for choosing the food product (Vermeir & Verbeke, 2006). According to sensory preferences of consumers, all of the samples had high scores between 3.8 and 5.0. Moreover, one of the most important quality parameters of chocolate is the particle size of chocolate (Afoakwa, 2010). In this thesis, addition of microcapsules did not affect the textural structure of the chocolate. As a result of sensory analysis, no difference has been found between microencapsulated chocolate and control chocolate by panelists. Therefore, integration of the emulsion matrix into the chocolate can be a suitable technique.

5.8. Static *In-vitro* Simulated Digestion

2000 U/ml of pancreatic lipase enzyme activity is identified for the intestinal stage to use SIF. The enzyme was fixed at 2000 LU/g of lipid. Thus, it was found that pancreatic enzyme from porcine pancreas (8x USP specifications, Sigma) is equal to 3.3 mg pancreatic powder. In this analysis, enzyme solution at 1.0 mg/ml concentration was tested using pH-stat titration method. These enzymatic activities were measured as triplicates and then calculated. Pepsin solution was prepared at a final concentration 3.0 mg/ml (Okuklu, 2014; Cabuk & Harsa, 2015).

After determination of enzymatic activities, M, F and C samples and free-cell bacteria were tested in simulated digestion system as duplicates. M, F samples and *Lb. rhamnosus* B-442 free-cell bacteria were tested in SSF, SGF and SIF to find the viability of the bacteria under the stress condition of digestion (Figure 5.11, 5.12 and 5.13).

Initially, free-cell bacteria were 9.0 log CFU/ml, while F and M samples were approximately 7.0 log CFU/g.

The SSF was adjusted to pH 8.0 and free-cell bacteria was not affected from SSF. Moreover, viability of bacteria in M and F samples were not statistically different ($p>0.5$), whereas survival rate of bacteria in M and F samples were found statistically different in SSF ($p<0.5$). The survival rate of bacteria was found as approximately 90% for M sample and 83 % F sample.

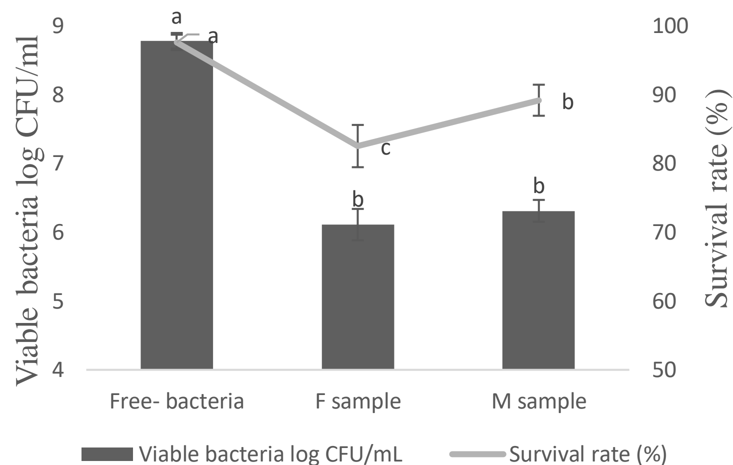

Figure 5. 11. Viable bacteria and survival rate of *Lb. rhamnosus* NRRL B-442 free, F and M samples in simulated saliva fluid (SSF) at 37ºC.

Probiotic bacteria e.g. *Lb. brevis* was integrated into different commercial products such as chocolate, yogurt and beverages. Researches were reported that chocolate had a protective effect against gastric condition and stress when compared to other commercial products (Yonejima, et al., 2015; Klindt-Toldam , et al., 2016; Succi, et al., 2017). This can be explained that chocolate butter has a protective effect for bacteria under stress factors such as bile, digestive enzymes and acid (Lahtinen, Ouwehand, Salminen , Forssell, & Myllarinen, 2007). Thus, determining the lipid digestibility of chocolate products in simulated gastrointestinal systems is important (Paz-Yepez, Peinado, Heredia , & Andres, 2019). There are some studies in the literature based on probiotic viability; e.g. viable count of *Lb. acidophilus* in milk chocolate was decreased 3.0 log during SGF experiment (Klindt-Toldam , et al., 2016). A study investigated the stability *Lb. acidophilus* and *B. animalis* strains and observed 3.0 log CFU/g, 0.6 log CFU/g declines, respectively after 180 min of SGF (Charteris, Kelly,

Morelli L., & Collins, 1998). Higher protection of probiotic bacteria in milk chocolate samples was observed under acidic conditions of SGF when compared to the ones in dark chocolate (Klindt-Toldam , et al., 2016).

Figure 5. 12. shows the viability and survival rate of *Lb. rhamnosus* NRRL B-442 for free, F and M samples in simulated gastric fluid (SGF) at 37ºC. All samples were tested in simulated gastric fluid for 120 min after adjusting pH to the value of 3.0. The survival rate of free-cells of *Lb. rhamnosus* NRRL B-442 remained constant over 90% ratio. In addition, not only the survival rate of bacteria in M samples but also viable bacteria of M samples was found at higher values than F samples, and all samples were noticeably different. Probiotic bacteria generally have high mortality rates below pH 3.0 (Mainville, Arcand, & Farnworth , 2005). However, survival of *Lb. rhamnosus* NRRL B-442 were found at high rates below pH 3.0 in this thesis study, therefore this strain is stable at low pH values.

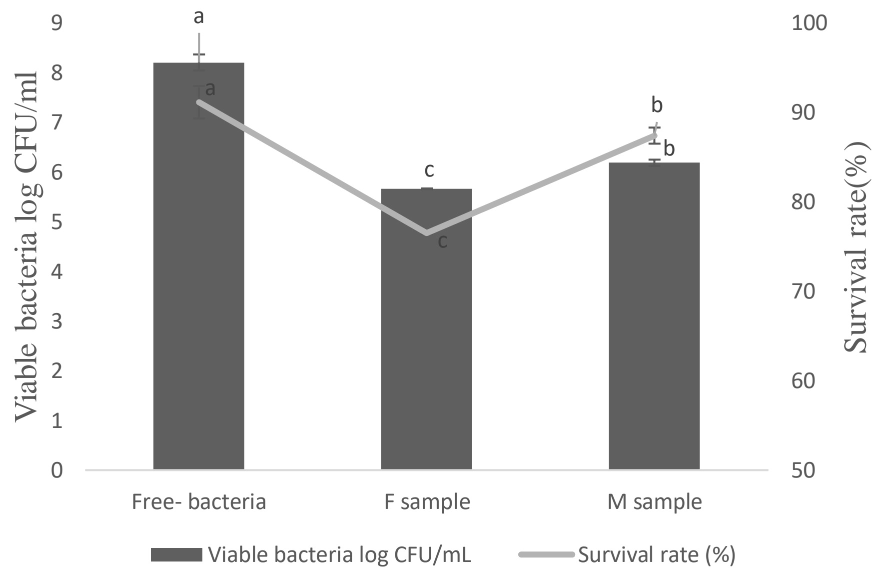

Figure 5. 12. Viability and survival rate of *Lb. rhamnosus* NRRL B-442 for free, F and M samples in simulated gastric fluid (SGF) at 37ºC.

Figure 5.13 shows the viability and survival rate of *Lb. rhamnosus* NRRL B-442 for free, F and M samples in simulated intestinal fluid (SIF) at 37ºC. As seen from this figure, *Lb. rhamnosus* NRRL B-442 was decreased from 9.0 log CFU/ml to 8.0 log CFU/ ml. It means that *Lb. rhamnosus* NRRL B-442 is resistant to low pH and gastric stress

conditions. Moreover, the survival rate of free-cell *Lb. rhamnosus* NRRL B-442 was found as 92% during simulated digestion system. Simulated digestion system experiments demonstrated that when this bacteria was added into chocolate as the forms of free-cell and microencapsulated, the survival ratios were found to be as 74 and 87 %, respectively.

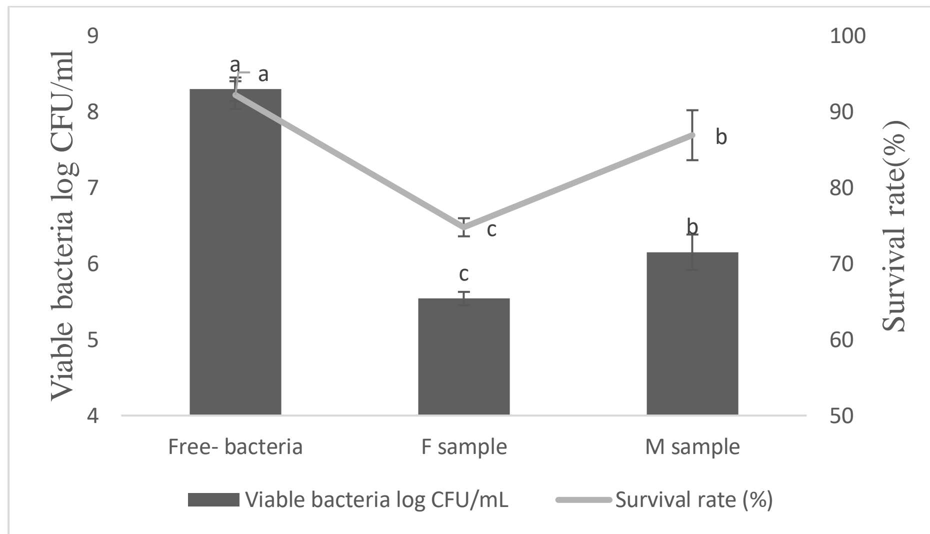

Figure 5. 13. Viable bacteria and survival rate of *Lb. rhamnosus* NRRL B-442 free, M and F samples in simulated intestinal fluid (SIF) at 37ºC.

Table 5.7 summarizes the results obtained from simulated *in-vitro* experiments. As seen, significantly different decrease was estimated between initial and final counts of F sample (P<0.5). Viability of probiotic bacteria in F samples were lost approximately 2 log CFU/ml during simulated *in vitro* analysis, whereas bacteria count in M samples decreased about 1 log CFU/ml.

Table 5. 7. Viability data of bacteria itself and in chocolate samples during sequential stages of *in-vitro* simulation analysis

Samples / *In vitro* Stages	Free-cell (*Lb.rhamnosus* NRRL B-442) (CFU/ml)	F sample (CFU/ml)	M sample (CFU/ml)
SSF	8.781±0.114^{a}	6.111±0.228^{a}	6.310±0.160^{a}
SGF	8.203±0.161^{b}	5.666±0.007^{b}	6.184±0.064^{a}
SIF	8.295±0.156^{b}	5.540±0.072^{b}	6.150±0.233^{a}

Means indicated with different letters in the same column are different (p <0.05).

Succi, et al., (2017) investigated probiotic integration into milk and dark chocolates. Viability of lyophilized forms of *Lb. paracasei* and *Lb. rhamnosus* GG were decreased about 5.5 log CFU/g and 6.0 log CFU/g under gastric conditions, respectively. However, approximately 4.0 log CFU/g decrease were found for the same strains integrated in dark chocolate at the end of SGF experiments. Besides, *Lb. rhamnosus* and *Lb. paracasei* were increased between 1.0 log and 0.7 log CFU/g in SIF (Succi, et al., 2017). In another study, survival rates of *Lb. helveticus* and *B. longum* in milk chocolate were found 91% and 80% after the simulated intestinal conditions, respectively (Possemiers, Marzorati, Verstraete, & Van de Wiele, 2010).

In this thesis study, M sample was found to be more stable during sequential simulated digestion. The viability of bacteria in these samples were found over 6.0 CFU/ml and this count is sufficient since it corresponds the desired levels of probiotic viability for functional foods described in the legislation document in Turkey (Official gazette, issue: 29960, 26/01/2017) (Appendix E).

5.9. Morphology of Microcapsules

The microstructure of free *Lb. rhamnosus* NRRL B-442 cell and chocolate samples before and after subjected to gastric and intestinal fluids sequentially were analyzed using environmental scanning electron microscopy (ESEM-Quanta 250 FEG, FEI). After all samples were lyophilized, they were cut and attached to double-sided carbon tapes without any pretreatment. The ESEM micrographs of free cell *Lb. rhamnosus* NRRL B-442 in simulated intestinal fluid was indicated in Figure 5. 14.

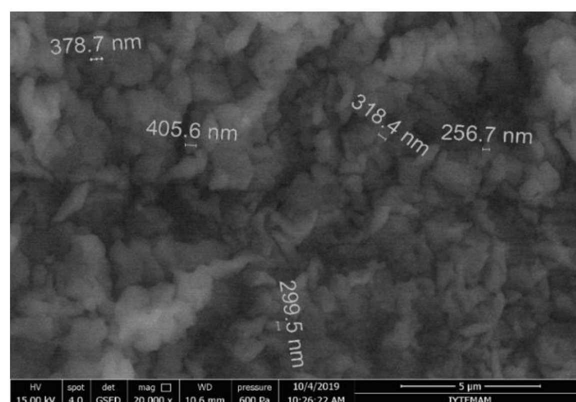

Figure 5. 14. ESEM micrographs of *Lb. rhamnosus* NRRL B-442 cell in SIF (x20000).

Free-cell bacteria of *Lb. rhamnosus* NRRL B-442 had dimensions between 256 and 400 nm in SIF. The ESEM micrographs of chocolate samples (Figure 5.15) and the samples in simulated gastric and intestinal fluid were seen in Figure 5. 16 and Figure 5. 17, respectively.

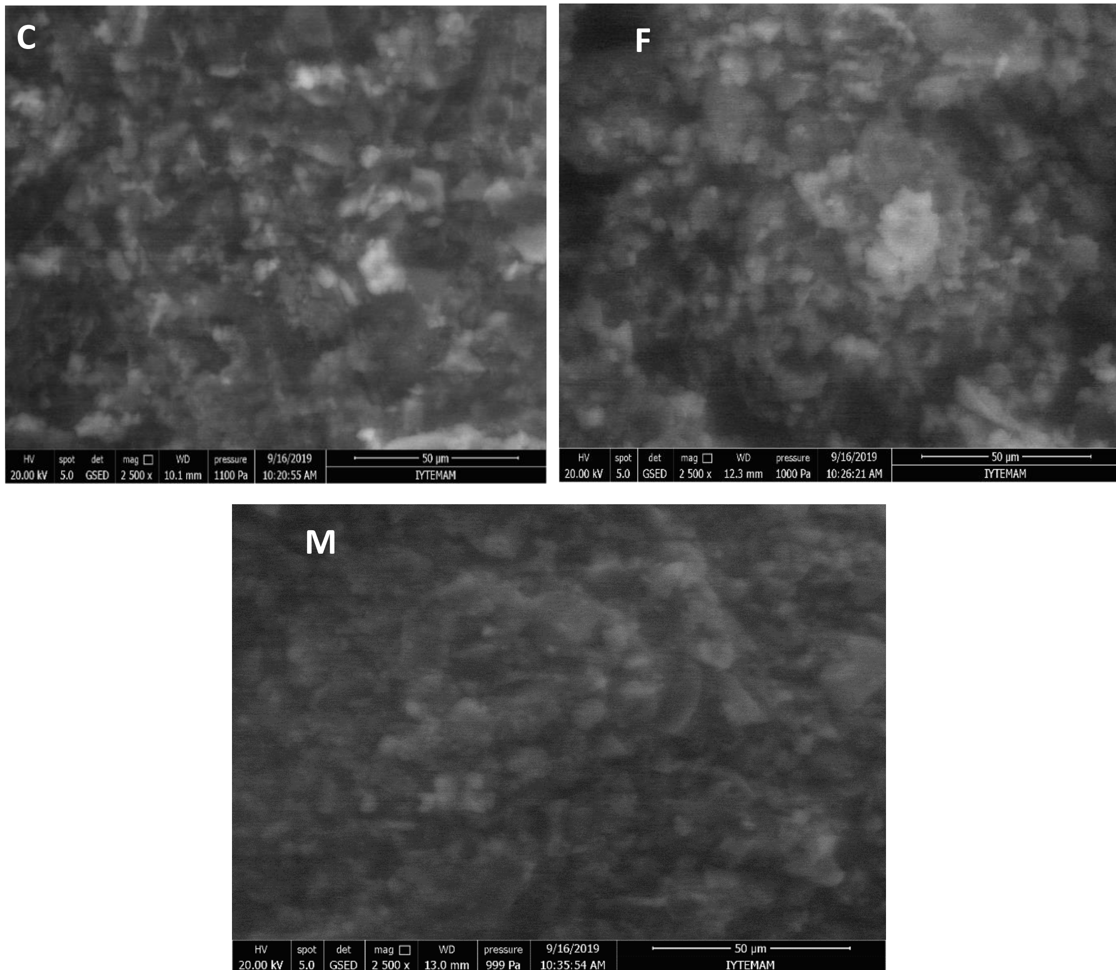

Figure 5. 15. ESEM micrographs of chocolate samples before *in-vitro* digestion (x2500).

According to ESEM images, the structure of chocolate samples shown in Figure 5.15 were not represent clear differences. This may be associated with buttery structure of chocolate samples. Both free and microencapsulated *Lb. rhamnosus* NRRL B-442 were thought to be integrated and distributed evenly inside the milk chocolate structure. The chocolate samples were evaluated in simulated gastric fluid (Figure 5.16) since carbohydrate and protein digestion progress when they are subjected to gastric fluid within the GI tract. Samples after SGF treatment were examined with ESEM.

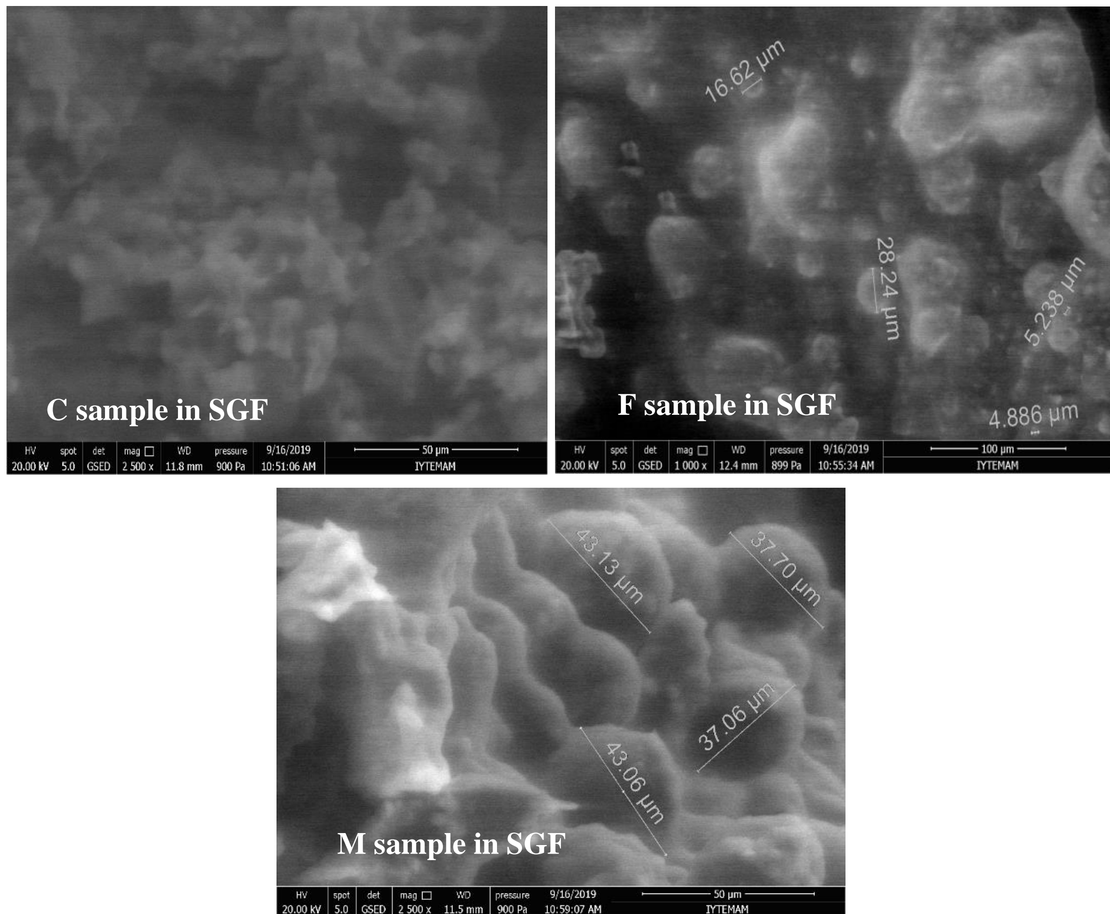

Figure 5. 16. ESEM micrographs of chocolate samples in simulated gastric fluid (x2500).

In Figure 5.16, topographical differences in size and shape can be seen for C, F and M samples; dimensions of microencapsulated bacteria was found to be 37 - 43 µm. Free bacteria in F samples had dimensions around 5 - 28 µm. Structure of C sample was totally different than the two samples of F and M.

The differences in sample structures seen from Figure 5.15 and 5.16 were clear and globular forms of bacteria could be observed. These structures become visible since degradation of protein and carbohydrates occurred by the action of pepsin enzyme during *in-vitro* digestion, and detaching and releasing of free and/or microencapsulated bacteria from chocolate matrix might have been happened.

Digestion of fats is known to be broken down in the body with the help of bile salts and pancreatic lipase enzyme (Golding & Wooster, 2010). Therefore, lipid digestion of the samples in simulated intestinal fluid were also examined with ESEM as given in Figure 5.17.

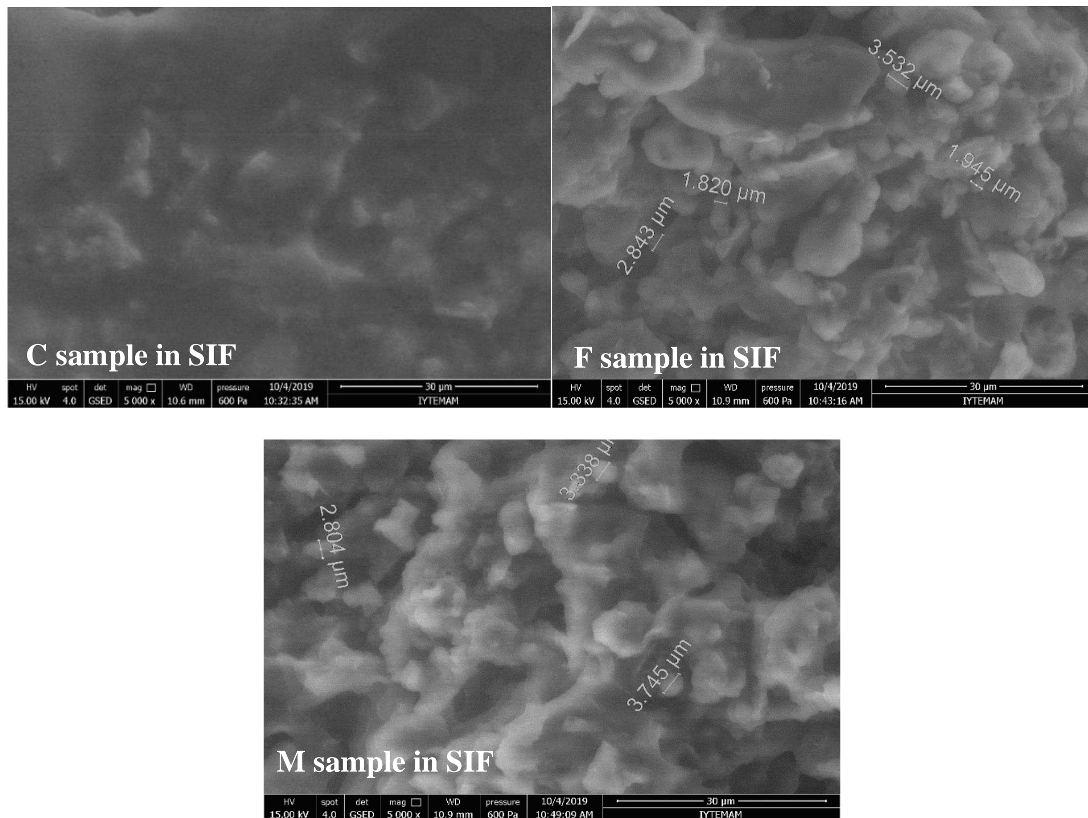

Figure 5. 17. ESEM micrographs of chocolate samples in simulated intestinal fluid (x5000).

Lb. rhamnosus NRRL B-442 strain in M and F samples had approximately 3.5 µm of size in SIF. The bacteria size was found lower than SGF based on breaking down chocolate fat. This can be attributed that fat globules were degraded during *in-vitro* digestion experiments.

Figures 5.16 and 5.17 indicate the bare structural differences in between C, F and M samples. Lysing of chocolate lipids occurred by the action of lyophilic pancreatic enzyme as seen in Figure 5.17. It was observed that M samples happened to shrink from 40 µm to 3 µm. Same encapsulation matrix (WPC-pullulan) was used to encapsulate *Lb. acidophilus* in the study by Cabuk (2015) observed that shrinkage occurred in microcapsules, the dimension decreased from 70 µm to 34 µm at the end of the SGF and that these coating materials were spherical in shape; data belong to SIF was not presented and the matrix does not contain chocolate. Therefore, most of the size reduction of microcapsules were occurred during SIF.

Overall, when ESEM images were examined, it can be said that chocolate can create an excellent barrier properties for probiotic cells during *in-vitro* SGF and SIF experiments. This can be attributed to the fatty matrix of chocolate that protects bacteria against harsh conditions.

CHAPTER 6

CONCLUSION

In this study, different lactic acid bacteria strains were screened for their GABA-producer abilities. GABA or glutamatergic effect on anxiety disorder is commonly used in pharmacotherapy; probiotic research in terms of GABA synthesis has been found in the literature, food products having GABA content is a novel concept yet. In literature, therapeutic effects of certain probiotic strains were observed on neuropsychiatric disorders in many clinical studies. Therefore, it was aimed to prepare functional milk chocolate, by incorporating GABA-producer probiotic, having a prominent effect on anxiety disorder.

Two different HPLC methods were used to determine GABA concentrations of bacteria. These measurements of GABA demonstrated that more effective results were obtained by RP-HPLC method using Pico.Tag column. Among the other four lactic acid bacterial strains *Lb. rhamnosus* NRRL B-422 fermented at 37°C for 24h was produced the highest GABA concentration as almost 59,000 mg/L. Hence, the functional chocolate product was prepared by using *Lb. rhamnosus* NRRL B-422.

In this study, *Lb. rhamnosus* NRRL B-422 was microencapsulated by WPC-pullulan water-in-oil emulsion technique. Survival rate of lyophilized microencapsulated bacteria was found over 90% at the end of 60 days at 4 °C. Free-cells and lyophilized microencapsulated forms of bacteria were incorporated into milk chocolate product. Survival rates of *Lb. rhamnosus* NRRL-B 422 in F and M samples were closed to each other as 83% and 79%, respectively at 4ºC for 60 days.

Moisture, ash and color analysis were not found significantly different ($p>0.05$). Sensory analysis was also shown similar result between the samples (C, F and M) by panelists. However, C and M samples had similar hardness and fracturability of chocolate, whereas hardness and fracturability of F samples had higher values.

Survival rate of *Lb. rhamnosus* NRRL B-442 was 90 % in simulated digestive system. Viability of bacteria in M was found more stable as over 6.0 log CFU/ml during

in-vitro conditions. While survival rate of bacteria in M sample was found as 87%, survival rate of bacteria in F sample was less than 75%. Count of bacteria in M and F samples was found statistically different ($P<0.5$).

Chocolate provided protective barrier to *Lb. rhamnosus* NRRL B-442 during simulated gastric and intestinal fluids supported by ESEM images. Usage of emulsion technique for microencapsulation of bacteria into chocolate was found suitable since the nature of chocolate matrix compatible with emulsion, according to ESEM images.

Overall, microencapsulated bacteria provide better textural properties of chocolate and higher survival rate of bacteria in chocolate during simulated digestion system compared to free-cell. Moreover, chocolate products can be a good carrier to protect the viability of bacteria against *in-vitro* stress condition depending on its fatty matrix according to ESEM images. This study offers future opportunities to alleviate anxiety disorder using GABA-producer bacteria in chocolate by clinical studies.

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APPENDIX A

THE HPLC CHROMATOGRAMS

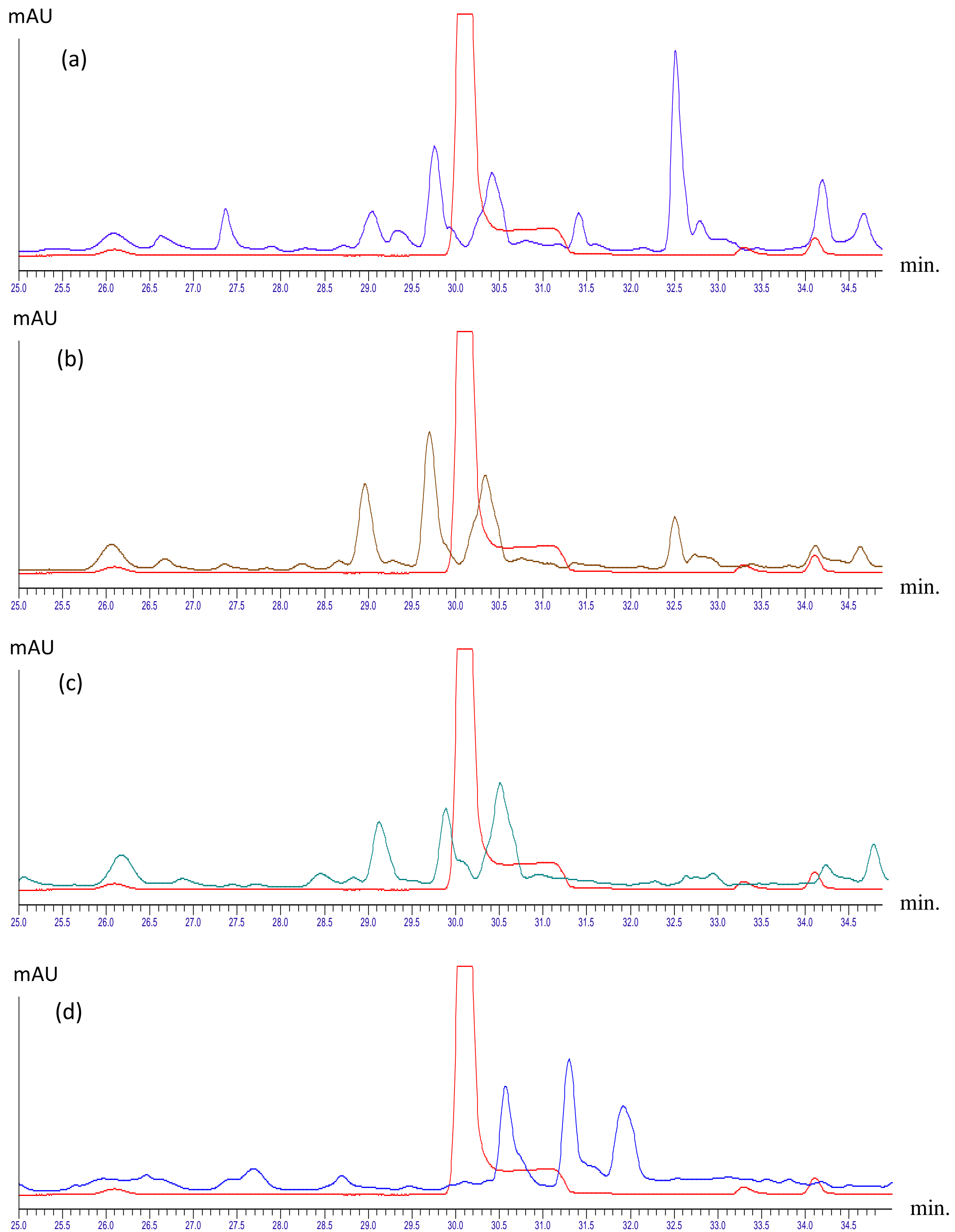
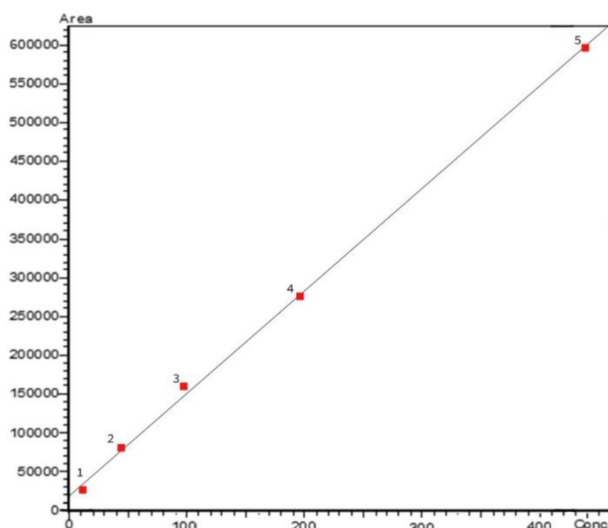


Figure A. 1. The HPLC chromatograms of GABA producer *Lb. rhamnosus* NRRL B-442 for 24 h (a), 48 h (b), 90 (c), 96(d).

APPENDIX B

GABA CALIBRATION CURVE



Y = aX + b
a = 1172.488
b = 20490.61
R^2 = 0.9965058
R = 0.9982513
External Standard
Curve Fit Type:Linear
Origin:Not Forced
Weight:None
Mean RF: 1409.249
RF SD: 168.8416
RF %RSD : 11.98097

Figure B. 1. GABA calibration curve view

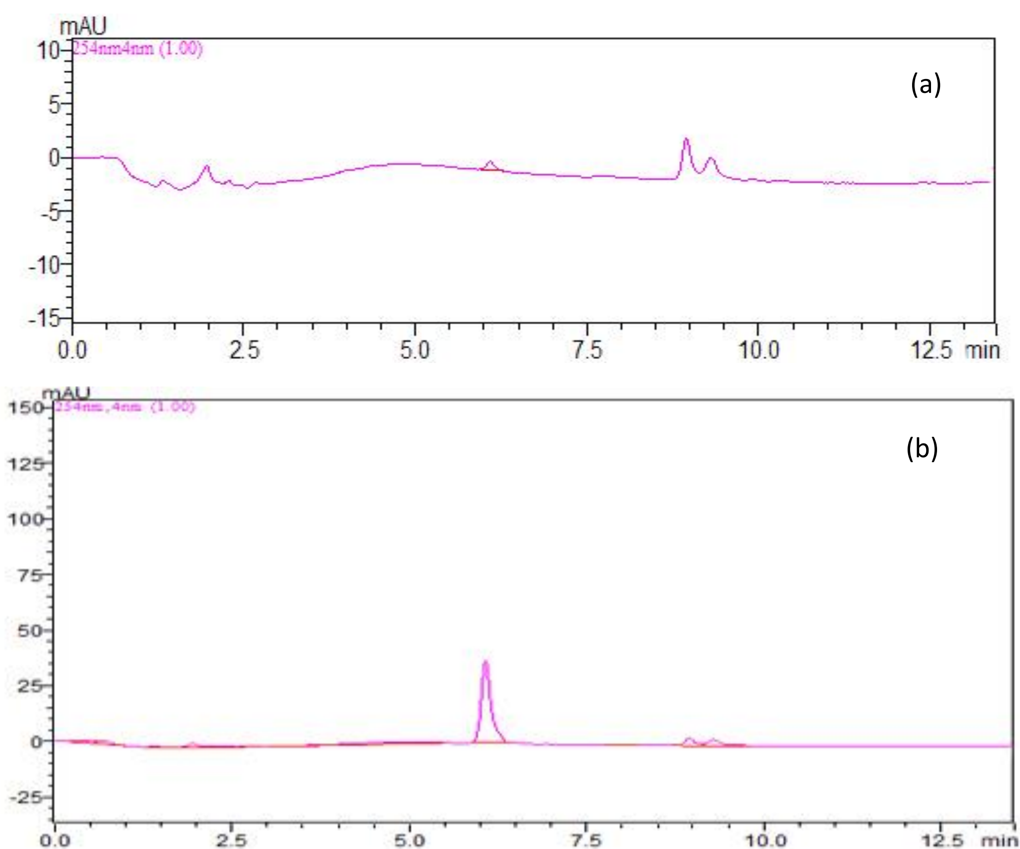


Figure B. 2. The RP-HPLC chromatograms of GABA standard 20 mg/L (a) and 2000 mg/L(b).

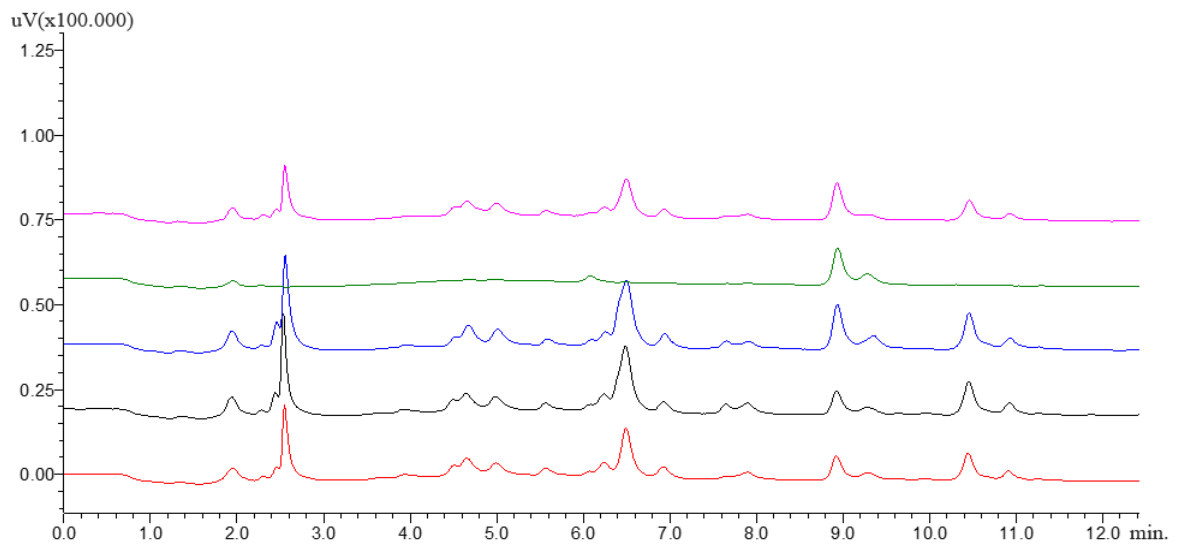


Figure B. 3. The RP-HPLC chromatograms of GABA producer bacteria for 24 h.

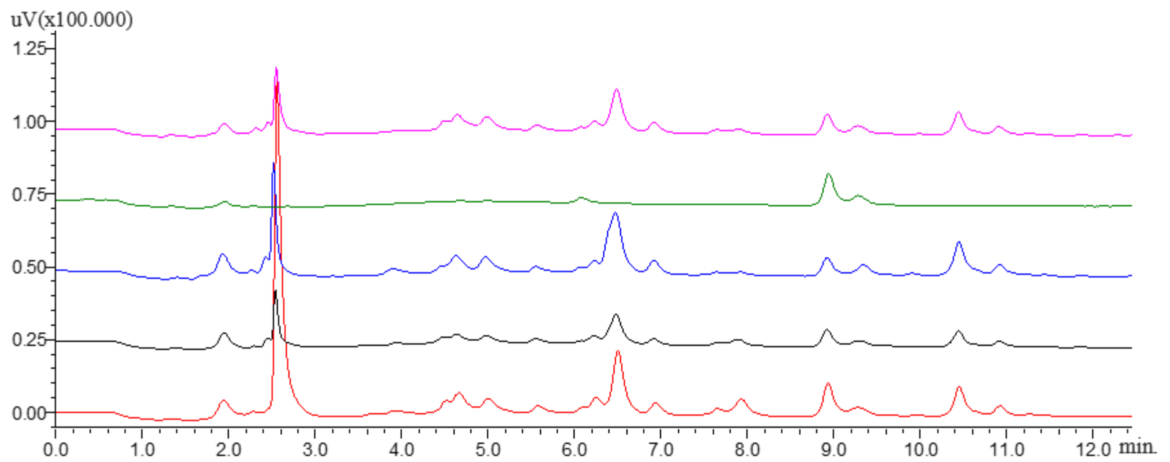


Figure B. 4. The RP-HPLC chromatograms of GABA producer bacteria for 48 h.

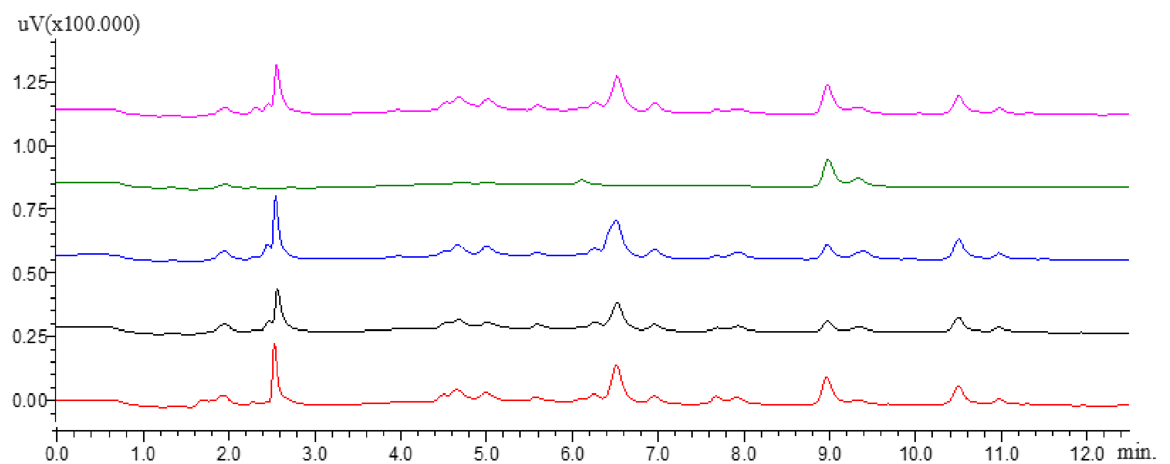


Figure B. 5. The RP-HPLC chromatograms of GABA producer bacteria for 72 h.

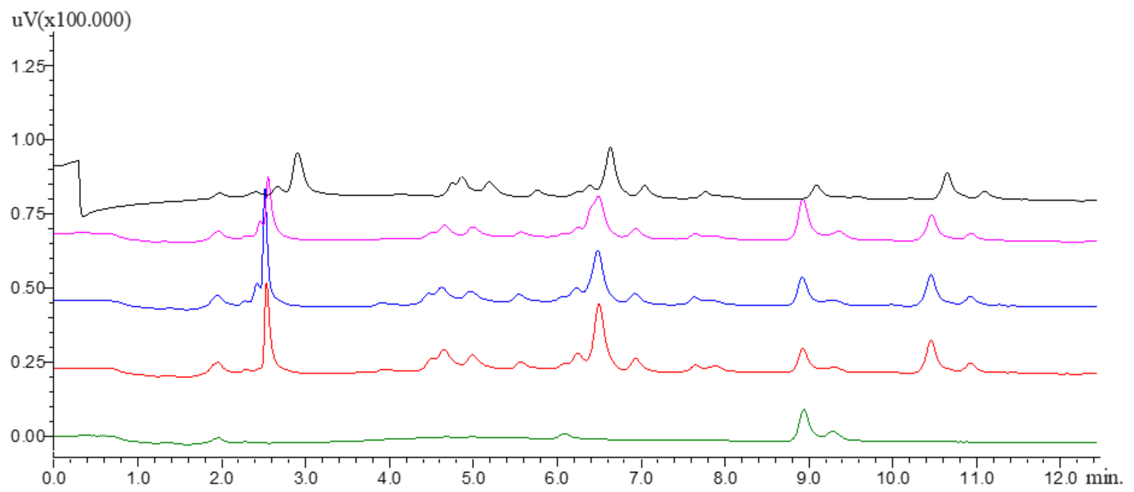


Figure B. 6. The RP-HPLC chromatograms of GABA producer bacteria for 96 h.

Black color → *Lb. helveticus* NRLL B- 4526

Pink color → *Lb. rhamnosus* NRRL B-B442

Blue color → *Lactobacillus delbrueckii* subsp. *bulgaricus* NRRL B-548

Red color → *Lc. lactis subsp lactis* CECT 4432

Green → 100 mg/L GABA standard

APPENDIX C

GROWTH CURVE

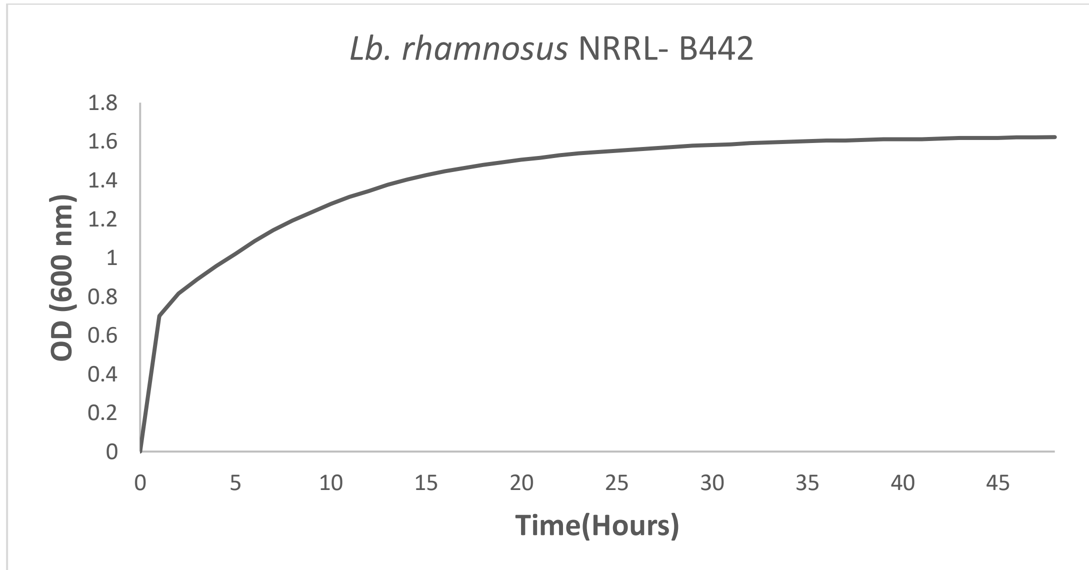


Figure C. 1. Growth curve of *Lb. rhamnosus* NRRL B-422 at 37 ºC for 48 h.

APPENDIX D

CHOCOLATES SENSORY EVALUATION FORM

Panelist Number_______

Dear Panelist,
A total of three pieces of milk chocolate will be presented to you. Please, you check the chocolate according to the presentation order. To tick up your thoughts about the properties of chocolate, simply put a cross (X) in one of the boxes. Before you start tasting the chocolate samples and taste the next chocolate, eat a bite of bread and drink some water.

Chocolate Number: _______

1. Tick your thinking about **the Color** of the chocolate

☐ The worst ☐ Dislike ☐ Liked and did not like ☐ Like ☐ The best

2. Tick your thinking about **the Odor** of the chocolate

☐ The worst ☐ Dislike ☐ Liked and did not like ☐ Like ☐ The best

3. Tick the chocolate with your finger and tick your thinking about **the Texture (structure)**

☐ The worst ☐ Dislike ☐ Liked and did not like ☐ Like ☐ The best

4. Tick your thinking about **the Taste** of the chocolate

☐ The worst ☐ Dislike ☐ Liked and did not like ☐ Like ☐ The best

5. Tick your thinking about your **General Liking** related to the chocolate.

☐ The worst ☐ Dislike ☐ Liked and did not like ☐ Like ☐ The best

Figure D. 1. Sensory evaluation form

APPENDIX E

HEALTH CLAIMS AND REGULATIONS

Ek-15

SAĞLIK BEYANLARI VE BEYAN KOŞULLARI

Besin öğesi / bileşen	Sağlık beyanı	Beyan koşulu
Probiyotik mikroorganizma	Bu gıda probiyotik mikroorganizma içerir. Probiyotik mikroorganizmalar sindirim sistemini düzenlemeye ve bağışıklık sistemini desteklemeye yardımcı olur.	Gıdanın en az $1.0x10^6$ kob/g canlı probiyotik mikroorganizma içermesi gerekir.

Figure E. 1. Health Claims about probiotics and functional foods of Turkish food codex (Source: Official gazette, issue: 29960, 26/01/2017).