DEVELOPING PROBIOTIC LOZENGES TO IMPROVE ORAL HEALTH

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ABSTRACT

DEVELOPING PROBIOTIC LOZENGES TO IMPROVE ORAL HEALTH

Recently, there is a great need to overcome complaints about oral health from children, mental and physically handicapped people who are inadequate in oral hygiene and after chemotherapy of cancer patients. With reduced body resistance, opportunistic *Streptococcus mutans* and *Candida albicans* in the mouth become dominant, causing disruption of oral health. Therefore, the effect of lactic acid bacteria on pathogens was investigated in order to protect oral health with the thesis study. *Lactobacillus pentosus* NRRL-B 227 was determined among the probiotic bacteria tested for this purpose and its activity on the pathogen *Streptoccocus mutans* ATCC 25175 and *Candida albicans* DSMZ 5817 was found in broth microdilution, agar overlay and planktonic culture assays except disc diffusion test. To reduce the number of pathogens in oral microflora, lozenges containing *L. pentosus* were developed. Three different lozenges with encapsulated and free bacteria and control lozenge were produced, kept at different temperatures; 4°C and 25°C. No significant decrease in viability of the encapsulated probiotic strain after lozenge production and storage at 4°C was observed, the probiotic amount in the lozenge initially counted as 7.84 log CFU/g, while 7.73 log CFU/g at the end of 3 months shelf life. However, lozenges stored at 25°C probiotics lost their vitality after one month. Additionally, lozenges containing free bacteria have lost viability rapidly. Color and water activity were observed differently in the formulations ($p < 0.05$). The formulations maintained their microbiological safety during storage. Lozenge with *L. pentosus* NRRL-B 227 has a significant potential for improving oral health and provides an alternative to the diversification of products containing probiotics.

ÖZET

AĞIZ SAĞLIĞINI İYİLEŞTİRMEK İÇİN PROBİYOTİK PASTİL GELİŞTİRİLMESİ

Son yıllarda ağız hijyenini sağlamada yetersiz kalan çocukların, zihinsel ve bedensel engelli insanların ve kanser hastalarının kemoterapi sonrası ağız sağlığı konusundaki şikayetlerinin giderilmesine önemli ölçüde ihtiyaç vardır. Azalan vücut direnci ile birlikte ağız florasında fırsatçı *Streptococcus mutans* ve *Candida albicans* hızla baskın hale gelerek ağız ve diş sağlığının bozulmasına neden olmaktadır. Bu nedenle, yapılan tez çalışması ile ağız sağlığını korumak için laktik asit bakterilerinin patojenler üzerindeki etkisi araştırılmıştır. Bu amaçla denenen probiyotik bakteriler arasından *Lactobacillus pentosus* NRRL-B 227'nin kullanımına karar verilmiştir ve yapılan inhibisyon metotlarından disk difüzyon testi dışında broth mikrodilüsyon, agar overlay ve planktonik kültür testi çalışmalarında patojen *Streptoccocus mutans* ATCC 25175 ve *Candida albicans* DSMZ 5817'nin üzerindeki etkinliği ortaya çıkarılmıştır. Oral mikroflorada patojen sayısını azaltmak için *L. pentosus* içeren fonksiyonel pastil ürünü geliştirilmiştir. Kapsüllenmiş ve kapsüllenmemiş bakteri içeren ve bakteri içermeyen üç farklı pastil üretilerek 4°C ve 25°C olmak üzere farklı sıcaklıklarda muhafaza edilmiştir. Kapsüllenmiş probiyotik suşun pastil üretiminden sonra ve 4°C'de depolama sırasında yaşama kabiliyetinde önemli bir düşüş gözlemlenmemiştir, başlangıçta 7.84 log CFU/g iken 3 aylık raf ömrü sonunda 7.73 log CFU/g olarak sayılmıştır. Ancak 25°C'de depolanan pastillerde 1 ay sonra bakteri canlılığını önemli ölçüde kaybetmiştir. Ayrıca kontrol olarak üretilen kapsüllenmemiş formda bakteri içeren pastillerde canlılık hızlı bir şekilde düşmüştür. Formülasyonlar su aktivitesi ve renk açısından farklı bulunmuştur ($p < 0.05$). Tüm formülasyonlar depolama süresince mikrobiyolojik açıdan güvenli olarak kalmıştır. *L. pentosus* NRRL-B 227 suşuna sahip pastil, ağız ve diş sağlığının iyileştirilmesi açısından önemli bir potansiyele sahiptir ve probiyotik içeren ürünlerin çeşitlendirilmesine bir alternatif sunar.

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CHAPTER 1

INTRODUCTION

The protection of human oral health is very important in protecting overall health. Children and disabled people are inadequate to protect their oral health. In addition to them cancer patients who are on chemotherapy treatment complaints about oral infections and dental diseases are quite large. Therefore there is need to overcome complaints on the oral hygiene for preschoolers and school-age children, people with mental and physical disabilities, chemotherapy patients. The tooth and caries initiator *Streptococcus mutans* and opportunistic pathogen *Candida albicans* are problems that must be overcome in oral and dental health. Decays in the early ages leave irreversible damage and affect the health of individuals as well as their social lives. As a result of the fall of immunity, the opportunistic *C. albicans* cause candidiasis in the mouth. To prevent the development of these pathogens in oral microflora should be regularly performed cleaning of the mouth and teeth. However, cleaning for mentally and physically handicapped people and preschool children may not be done regularly and truly. In addition, because of the weakening of the immune system of chemotherapy patients, products are needed to improve oral health.

Probiotics are microorganisms that have beneficial effects in improving human and animal health when taken in sufficient amounts (Food and Health Agricultural Organization of the United Nations and World Health Organization, 2002). Probiotics have great importance to prevent various health problems from reducing cholesterol to preventing obesity (Zhang, et al., 2017).

Treatment of bacterial and fungal infections is challenging due to the drug resistance development. Therefore, the use of probiotics instead of drugs has come to the fore. Probiotics inhibit the growth or suppression of the development of infectious or pathogen microorganisms by the mechanism of antimicrobial action. The action mechanism of probiotics is consist of three forms; to produce antimicrobial compounds, to reduce the

number of pathogens and harmful bacteria by competing for nutrients and colonization zones, production of enzymes that promote digestive system, reducing the production of ammonia, amine or toxic enzymes and improving the function of the intestinal wall and changing microbial metabolism (enzymatic activity), to improve the immune system by increasing antibody level and macrophage activity (Savadogo, Quattara, Basssole, & Traoer, 2006).

Nowadays, consumption of probiotic-containing foods is a common approach. Most food products containing probiotic microorganisms are classified as functional food products, which appear as a significant portion of these foods. With the increase in consumer awareness, demand for probiotic functional foods is increasing. (Granato, Branco, Cruz, Faria, & Nazzaro, 2010).

There is a concurrence that nourishment has a major influence on reducing the risk of disease and increasing welfare, and hence functional foods are emphasized in the food sector. Although yet the dairy products are pioneers of functional foods (Sanchez, Reyes-Gavilan, Margolles, & Gueimonde, 2009), other food products such as probiotic apple snacks (Akman, Uysal, Uçak Özkaya, Tornuk, & Durak, 2019), probiotic impregnated olives have functional properties and exert health benefit effects on human health. The minimum amount required to obtain health benefits from probiotics is 10^6 CFU/mL during consumption (Prado, Parada, Pandey, & Soccol, 2008). Microencapsulation is often used to protect beneficial microorganisms against stress caused by environmental factors. Microencapsulation can provide adequate protection, especially for the survival of probiotics in gastrointestinal conditions and foods (Weinbreck, Bodnar, & Marco, 2010).

Probiotic cells have been shown to be effective in inhibiting oral pathogens. Various products have been used as carriers of probiotics so that these microorganisms adhere in the mouth and multiply in oral microflora. These products are food supplements such as lozenges and tablets, as well as by adding probiotic cells to dairy products, yoghurt and cheese products have been used to improve oral and dental health.

Lozenges are simply produced candies; when they are slowly dissolve in the mouth that can provide various compounds (Edwards, 2001). Lozenges are retained in the mouth

longer; they can have an impact on reducing the risks of decays and other disorders that represent potency to carrying probiotics (Witzler, Pinto, Valdez, Castro, & Cavallini, 2017).

In the lozenge production process, water-soluble fillers and binders are used, and these substances should be preferable to taste. Glucose and mannitol, especially sorbitol, are widely used fillers. Gelatin is usually used as binder. There are three main lozenge types; hard lozenges, soft lozenges and chewable lozenges. While chewable lozenges in the child population are popular, soft lozenges have gained popularity due to ease of preparation and applicability to various drugs. Hard sugar lozenges contain sucrose and other sugar mixtures in an amorphous form. (Shinde, et al., 2014).

Foods and beverages containing sugar cause demineralization in the teeth while foods and beverages containing sugar alcohol such as mannitol, sorbitol, xylitol, maltitol, erythritol, polydextrose, isomaltulose, isomalt, sucralose or lactitol do not affect the mineralization of the teeth and do not cause dental erosion (EFSA, 2011). Polyols promote remineralization of teeth when used as a replacement of sugar after meal. Besides helping to oral health, polyols have low glycemic properties; inducing a low blood sugar rise, helping to regulate and maintain blood sugar levels (Grembecka, 2015). Sorbitol ($C_6H_{14}O_6$), systematic name is d-Glucitol can be classified depend on its functionality like mannitol; sweetener, thickener, stabilizer, humectant, bulking agent (Deis & Kearsley, 2012); (Jamieson, 2012). Mannitol and sorbitol are resistant to digestion of oral bacteria that prevent from the raise in the mouth acidity. Therefore, according to the FDA and European Commission, foods including these sugar alcohols can have label stating “does not promote tooth decay” (EFSA, 2011). JECFA also have approved the using of them as food additives that are regarded as safe (Grembecka, 2015).

The main aim of the thesis is to identify a competitive probiotic lactic acid bacterium that will contribute to the improvement of individuals’ oral and dental health who are insufficient to provide oral hygiene and to develop lozenges as a functional product containing this probiotic. Thus, *Streptococcus mutans* and *Candida albicans* will be eliminated by using probiotic which provides antimicrobial character and competitive advantage in oral microflora. For this purpose, the inhibitory effect of 23 different lactic

acid bacteria on *Streptococcus mutans* and *Candida albicans* using 4 different methods was examined. A probiotic lactic acid bacterium which has been confirmed to be effective on oral pathogens has been microencapsulated so as to preserve its viability for a long time.

CHAPTER 2

ORAL HEALTH

2.1. Oral Microbiota

The mouth cavity, which has the most complex microflora of the human body, contains more than 700 microorganisms (Kuramitsu, He, Lux, Anderson, & Shi, 2007); (Tong, et al., 2011). More than 400 of these species were periodontal, 300 species were identified from other oral parts such as tongue, oral mucosa membranes, caries lesions, endodontic infections (Paster, Olsen, Aas, & Dewhirst, 2000). Streptococci consist of 20% of these microorganisms; bacteria, viruses, archaea and fungi. In general, it is known that human oral and dental health is not only influenced by inhabitant bacteria but also the individual's age and health, lifestyle and nutritional status (Stamatova & Meurman, 2009).

A study about the phylogenetic distribution of oral microbiota has shown 619 taxa are present in human mouth cavity. 96% of the taxa consist of six major phyla, Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Spirochaetes and Fusobacteria. Remaining 4% of the taxa include, Euryarchaeota, Chloroflexi, Chlamydia, SR1, Tenericutes, Synergistetes, and TM7. From Firmicutes phylum and Bacilli class, the genus Streptococcus, whose species are the most common dominant bacterial species in the mouth (Dewhirst, et al., 2010).

In the oral cavity where resident microorganisms have been demonstrated to bring about several infectious diseases such as tooth caries, gum diseases, endodontic infections, tonsillitis and alveolar osteitis. Furthermore, various studies have shown effects of periodontic pathogenic bacteria in enhancing systemic diseases (Seymour, Ford, Cullinan, Leishman, & Yamazaki, 2007) containing respiratory, diabetes and cardiovascular cases (Hajishengallis, 2015), stroke (Joshipura, Hung, Rimm, Willett, & Ascherio, 2003), preterm birth (Offenbacher, et al., 1998) and pneumonia (Awano, et al., 2008).

2.2. Oral Disorders

Dental caries, caused by impaired balance of oral microflora, are considered to be one of the most common and contagious diseases of human beings. For the formation of caries, three factors must be present, including cariogenic bacteria, predisposed tooth surface, and nutrients to promote bacterial growth. There are over 300 bacteria species in the oral cavity. In these species, *Streptococcus mutans*, a cariogenic organism alone, cause caries (Çakır, Gürhan, & Attar, 2010).

Streptococcus mutans, which has the greatest influence on tooth decay (Nicolas & Lavoie, 2011), produces biofilms on the tooth surface by synthesizing glucuronide-insoluble glucan and also reduces the pH of the saliva by synthesizing acid from sucrose and causes demineralization. Therefore, preventing the overgrowth of *S. mutans* in oral microflora is an important step to reduce tooth decay (Çakır, Gürhan, & Attar, 2010); (Kutsch & Young, 2011); (Tong, et al., 2011); (Kalakonda, Pathakota, Jayakumar, Koppolu, & Lakshmi, 2016)

Molecular analysis of oral microbiota of pre-school children has shown that *Streptococcus mutans* is an important cause of early-onset caries (Becker, et al., 2002). Oral odor is caused by the impaired balance of the variable flora in the oral cavity. The growth of pathogen is inevitable when the microbial balance is deteriorated by various causes.

The breakdown of the proteins found in the saliva by the action of the pathogens causes the formation of the odor of volatile sulfur compounds such as hydrogen sulphide (Elahi, Pang, Ashman, & Clancy, 2005). Besides, *Porphyromonas gingivalis* and *Fusobacterium nucleatum* usually leads to various forms of periodontal diseases (Moore & Moore, 2000); (Ximenez-Fyvie, Haffajee, & Socransky, 2000), which are inflammatory diseases that affect tissues supporting gums and teeth. These diseases start with gum inflammation called gingivitis. If they are not treated, periodontitis progresses and irreversible damage occurs (Orbak & Zihni, 2006); (Barlow, 2010).

2.2.1. Dental Caries

Interaction between fermentable carbohydrates and bacteria, which have ability of adhere to tooth surface and production of acids from sugars, lead to dental caries. In time, the acids cause to demineralization of tooth's enamel and dentin. The first sign of the tooth decay is white spot lesion, if the demineralization environment is decreased or prevented, the white spot lesion may not progress and may remineralize. The outermost part of the white spot lesion is usually referred to as the surface zone. The surface zone relatively resist because of remineralization from calcium, phosphate and fluoride in saliva. The most demineralized part of the lesion is body lesion. If demineralization environment is continued, the surface enamel will be weakened and cavitation will form. When cavitation occurs, pathogen bacteria can easily invade the underlying dentin and unfortunately they do not get under control by protective treatments.

High amount of cariogenic bacteria, consumption of sugar frequently, insufficient salivary flow, inadequate oral hygiene and poverty are the some risk factors that cause dental caries. These risk factors must be reduced and must be taken precaution against caries, otherwise dental caries occur. The caries lead to high cost treatments, painful times, hospitalizations, stimulating other diseases and even death, and also effect on quality of life such as eating problems and being ashamed to smile (Tinanoff, 2018).

2.2.1.1. Caries Microbiology

Gram-positive bacterium *Streptococcus mutans* contributes to caries due to its capability to attach on surfaces of tooth, producing high amount of acid, and resistance at low pH conditions (Coykendall, 1997).

Due to frequent carbohydrate consumption, low pH values in dental plaque may lead to changes in biofilm which adhere to the tooth by preferring bacteria that can survive and growth in acidic environment (Marsh, 2003). Therefore, bacterial acid production is

both a main element in tooth demineralization and influences microbial composition of plaques.

Besides the major cariogenic pathogen *S. mutans*, some bacteria, which are acidogenic and aciduric, have ability to form biofilm and help to cariogenic actions, these are Lactobacillus species, Veillonella, Actinomyces, Bifidobacterium, Scardovia, Fusobacterium, Prevetella, Candida, etc. (Gross, et al., 2012).

Molecular analysis shows that the key source of mutans streptococci colonization of their children is mothers (Douglass & Tinanoff, 2008). At early ages, the colonization of *S.mutans* is crucial risk factor for decay initiation (Berkowitz, 2006).

For reducing dental caries, frequency sugar consumption must be reduced; teeth must be brushed twice daily. Brushing teeth physically is insufficient, so fluoride-containing toothpaste must be used (Santos, Oliveira, & Nadanovsky, 2013). Yet, this type of toothpastes may cause fluorosis in children; to prevent it, children should brush very small amount of toothpaste (Wright, et al., 2014).

2.2.1.2. *Streptococcus mutans*

Several studies have been conducted on virulence factors of *S. mutans*, which is the primary agent of tooth decay. The virulence factors of the microorganism were capable of the synthesis of water-insoluble glucans from the disaccharide, tolerance to low pH and lactic acid production (Kuramitsu, 1993).

This bacterium has the characteristics of two tooth decay factors required by a cariogenic organism: acid tolerance and production of acid. Acid tolerance indicates that the bacteria are resistant to low pH values, which is caused by various mechanisms, which hold the cytoplasm of bacteria at a stable physiological pH. The pathogen *S. mutans* can carry and ability to ferment a range of dietary carbohydrates, after fermentation it produces organic acids, especially lactic acid that easily demineralizes the teeth surface and structure. Another factor is the capability of synthesizing extracellular polymers of insoluble glucan

that serves as a saliva food source, and helps to retain on the tooth surface (Lamont & Egland, 2015).

2.2.2. Candidiasis

Candidiasis is a phenomenon of dysbiosis, resulting in the overgrowth of *Candida* and the imbalance in the oral microbiota (Ishikawa, et al., 2015). Species of *Candida* which are commensals and dominant in the oral cavity, they present in healthy individuals' oral microflora approximately 25%–75% (Barros, Ribeiro, & Rossoni, 2016). *Candida albicans*, common pathogen species in the mouth, is seen in immunocompromised individuals and patients, as well as healthy people also cause infections that negatively affect oral health (Mothibe & Patel, 2017). *C. albicans* is normally inhabited in the oral cavity and is potentially considered the most pathogenic fungus (Jarvensivu, Hietanen, Rautemaa, Sorsa, & Richardson, 2004).

2.2.2.1. *Candida albicans*

The main pathogenicity factor of *Candida albicans* is its cell wall since this part directly contacts with host cells. *C. albicans* includes substances which are significant for its virulence, such as mannoprotein derivatives that have immunosuppressive properties to increase the defense of fungus against host immune system (Chaffin, Lopez-Ribot, Casanova, Gozalbo, & Martinez, 1998).

Virulence factors of *C. albicans* are related to the common of pathogens involve: coaggregation and adhesion, intervention of immune system, phenotype shifting and various supporting drivers such as immunomodulation and antibiotic resistance. The usage of antibiotic is suppressed by the microorganisms which are vying against *Candida albicans* that makes Candida effortless to form colony (Nasution, 2013).

C. albicans can lead to two major infections types in human beings: life-threatening systemic infections and superficial infections, such as vaginal and oral candidiasis (Calderone & Clancy, 2012).

2.2.2.2. Symbiotic Relationship

The two important oral pathogens have a symbiotic relationship; *S. mutans* excretes lactic acid that can act as a carbon source for *C. albicans* growth, in turn the yeast decreases oxygen volume to levels preferred by *S. mutans* and supply growth premonitory considerations for the bacteria (Brogden & Guthmiller, 2008).

2.2.3. Cancer Patience's Oral Disorders

Cancer patients are treated by methods such as chemotherapy and radiotherapy. However, these tough treatments lead to some problems in the body, the most important example of which is mucositis. It usually occurs acutely after the first week of chemotherapy. It may also become more severe during treatment (Sonis, 2009). Many cancer patients have low quality of life because of mucositis and its side effects. Mucositic patients suffered from pain, physical and psychological discomforts and limitations (Martinez, et al., 2014). In a clinical treatment, radiotherapy + chemotherapy + placebo, radiotherapy + chemotherapy + probiotic combination were grouped for detection of the effect of probiotic combination on oral mucositis induced by chemotherapy and radiotherapy in nasopharyngeal cancer patients. It has been found that the immune response of the patient is significantly increased by the combination of probiotic bacteria and the modification of the intestinal microbiome reduces the incidence of oral mucositis (Jiang, 2018). In another study, *L. brevis* significantly decreases the severity of chemotherapy-induced mucosal inflammation of head and neck cancer patients and prevents them from developing (Sharma, et al., 2012).

CHAPTER 3

PROBIOTICS

3.1. Probiotic Microorganisms

According to the definition of World Health Organization; probiotics are living microorganisms, which show beneficial impacts on health of individuals when taken in sufficient quantities (Food and Health Agricultural Organization of the United Nations and World Health Organization, 2002). Genera showing probiotic properties include *Lactobacillus, Saccharomyces, Bifidobacterium, Streptococcus, Enterococcus, Pediococcus, Bacillus, Leuconostoc, Escherichia coli.* However, health positive effects have mainly been showed for specific probiotic species of these genera (Fijan, 2014). Having protective and therapeutic properties from disease, probiotics have an important place in the protection of the health of individuals (Parvez, Malik, Ah Kang, & Kim, 2006) containing developed resistance to infectious diseases (Rostami, Mousavi, Mousavi, & Shahsafi, 2018), alleviation of lactose intolerance (Roskar, et al., 2017), protection of bowel diseases, diarrhea, vaginal and urogenital infections (Tachedjian, Aldunate, Bradshaw, & Cone, 2017); (Tomas , Duhart, De Gregorio, Pingitore, & Nader-Macıas, 2011), decreased allergy and respiratory infections (Hatakka, et al., 2001), reduced serum cholesterol concentration (Zhang, et al., 2017), raised resistance to chemotherapy and reduced colon cancer risk (Dubey, Ghosh, Bishayee, & Khuda-Bukhsh, 2016).

3.1.1. Lactic Acid Bacteria

The lactic acid bacteria have been used in the fermentation of food throughout the centuries, as starter cultures, for improving flavor and texture, and as well for ability to inhibit the development of spoilage and pathogenic microorganisms (Abee, 1995); (Stiles,

1996). To produce fermented food products, lactic acid bacteria are used, due to their fermentative properties (Saxelin, Tynkkynen, Mattila-Sandholm, & de Vos, 2005). They are gram-positive, catalase-negative, facultative anaerobic and motile. The lactic acid bacteria do not form spores and do not constitute cytochrome.

Lactic acid bacteria consist of are *Lactobacillus, Lactococcus, Bifidobacterium, Tetragonococcus, Vagococcus, Weissella, Streptococcus, Leuconostoc, Aerococcus, Oenococcus, Carnobacterium, Enterococcus, Sporolactobacillus,* and *Pediococcus* (Yerlikaya, 2019). Some specific lactic acid bacteria strains have been widely characterized as probiotics; these are *Lactobacillus* and *Bifidobacterium* (Sanchez, Ruiz, Gueimonde, Ruas-Madiedo, & Margolles, 2012). The reason is that, they have many important properties such as; high tolerance to bile and acid capability of adhere to the gut surface, resistance to low pH like gastric juice, capability of inhibition of potentially pathogenic species, resistance of antibiotics and elimination of cholesterol (Curto, Mandalari, Dainty, Faulks, & Wickham, 2011); (Tulumoğlu, et al., 2013).

Lactobacilli are very extensive in nature and they are commonly isolated from various different matrices, such as fermented products (Grigoryan, Bazukyan, & Trchounian, 2018), plants (Kawasaki, et al., 2011), soil (Chen, Yanagida, & Shinohara, 2005) and human gut (Wang, et al., 2010) and human feces (Archer & Halami, 2015). Yogurt, cheese and other fermented dairy products are the main source of probiotics (Guarner, et al., 2005). In healthy human beings, Lactobacilli are usually present 10^3–10^4 CFU/g in the oral cavity, 10^3–10^7 CFU/g in the ileum, 10^4–10^8 CFU/g in the colon and 10^7–10^8 CFU/g in the vagina (Merk, Borelli, & Korting, 2005); (Bernardeau, Vernoux, Henri-Dubernet, & Gueguen, 2008).

3.2. Anti-Pathogenic Action of Probiotics

The mechanism of the anti-pathogenic action; firstly probiotics adhere to the surface and produce extracellular antimicrobial substances via the metabolizing of mainly carbohydrates, proteins and other materials into the substantial components such as,

bacteriocins, organic acids, hydrogen peroxide and low-molecular-mass peptides, which can inhibit or kill pathogenic microorganisms (de Melo Pereira, de Oliveira Coelho, Junior, Thomaz-Soccol, & Soccol, 2018); (Prabhurajeshwar & Chandrakanth, 2019).

3.2.1. Bacterial Adhesion

Microorganism adhesion occurred between bacterial cell membrane and interactive surfaces. Probiotic bacteria adhesion generally depends on extracellular compounds, protein profiles and hydrophobicity cell surface features (Collado, Gueimonde, Hemandez, Sanz, & Salminen, 2005). Along with proper adhesion, thanks to the sufficient amount of cell mass provide to aggregation, and thus probiotics exert beneficial activities. Probiotics aggregation form barrier or biofilm, it ensures protection of host system and prevention of colonization of pathogenic organisms (Inturri, Stivala, Furneri, & Blandino, 2016). Lactobacilli have well ability of adherence onto epithelial cells, as well as have high ability of aggregation (Nikolic, Jovcic, Kojic, & Topisirovic, 2010).

3.2.2. Probiotics Aggregation and Coaggregation

The first stage of bacterial colonization is adherence to tissues that affects subsequent stages of infectious diseases or commensalism. The capability of bacterial adherence to host mucosal surfaces is important to use as probiotics (Food and Health Agricultural Organization of the United Nations and World Health Organization, 2002). Autoaggregation among the same species and coaggregation between different species are considered important for the intestinal and oral microbiota in which probiotics are active (Collado, Meriluoto, & Salminen, 2008). Probiotics can prevent pathogenic bacteria from sticking to the mucosa by creating a barrier by coaggregation with pathogens or through autoaggregation. Therefore, the ability of bacteria to adhere and aggregation is a

prerequisite in the selection of probiotic strain to improve oral health (Piwat, Sophatha, & Teanpaisan, 2015).

Competition for nutrients and space, coaggregation with pathogens and stimulation of immune system are comprised of the other mechanisms of probiotic antagonism (Lebeer, Vanderleyden, & De Keersmaecker, 2008). Based on the properties of microorganisms, these antagonistic actions change depends on the species (Martin, et al., 2009); (Vera-Pingitore, et al., 2016); (Veron, Di Risio, Isla, & Torres, 2017). Coaggregation allows the accumulation of pathogens together with probiotic microorganisms and catalyzes the removal of pathogens by feces (Soleimani, Kermanshahi, Yakhchali, & Sattari, 2010); (Vidhyasagar & Jeevaratnam, 2013).

3.2.3. Biofilm Formation

Biofilm is a structure where bacteria adhere to a surface and live in certain integrity and maintain their vitality by communicating with each other. Microorganisms are located in a matrix containing a number of nucleic acids, polysaccharides and proteins, also known as extracellular polymeric substances, forming the biofilm structure (Post, Stoodley, Hall-Stoodley, & Ehrlich, 2004). Although biofilm formation is perceived as a condition unique to pathogens, beneficial bacteria such as lactic acid bacteria also form biofilms. However, biofilm-forming lactic acid bacteria can be used as a biofilm to protect against persistent biofilms of pathogens. *Lactobacillus casei* and *Lactobacillus plantarum* have biofilm which exhibits antagonistic effect against methicillin-resistant *Staphylococcus aureus*. Effective biofilm formation was observed at the end of 48 hours using the tissue culture plate method. As an inhibition test, agar well diffusion and agar surface diffusion methods were used (Kumar, Alam, Rani, Ehtesham, & Hasnain, 2017). *Lactobacillus plantarum* subsp. *plantarum* JCM1149, *Lactobacillus fructivorans* JCM 1117 and *Lactobacillus brevis* JCM1059 bacteria form biofilms, and also the biofilm of *L. plantarum* M606 bacteria isolated from onion is more resistant to acid than free (planktonic) form (Kubota, Senda, Nomura, Tokuda, & Uchiyama, 2008). Bacteriocin-producing *Lactobacillus plantarum*

35d, *Enterococcus casseliflavus* IM 416K1 and bacteriocin-nonproducing *L. plantarum* 396/1, *Enterococcus faecalis* JH2-2 bacteria form biofilms. *L. plantarum* 35d and *L. plantarum* 396/1 showed inhibiting effect on *Listeria monocytogenes* NCTC 10888 pathogen (Guerrieri, et al., 2009).

3.2.4. Antimicrobial Byproducts

Several studies demonstrated that products produced by bacteria can have similar impacts on barrier function and pathways, without live microorganisms. These microbial products are called as postbiotics. Postbiotics are metabolic byproducts produced from probiotics, have biological action in the host (Patel & Denning, 2013). They consist of not only organic acids, bacteriocins, ethanol, acetaldehydes, hydrogen peroxide and diacetyl, but also heat-killed probiotic microorganisms that also attend biological activities (Islam, 2016). It is stated that because of the inhibitory effect on pathogens, metabolic byproducts can be used instead of antibiotics (Ooi, Mazlan, Foo, Mohamad, & Rahim, 2015). These non-viable bacterial products resist hydrolysis via enzymes produced by mammalian, they are non-pathogenic and non-toxic (Figueroa-Gonzalez, Cruz-Guerrero, & Quijano, 2011).

3.2.4.1. Bacteriocin

Bacteriocin is a low molecular weight protein or peptide, which is biologically active, it is yielded by not only gram-positive bacteria and also gram-negative bacteria, has capability of inhibit to the development of pathogens (Prabhurajeshwar & Chandrakanth, 2019); (Diep, Straum, Kjos, Torres, & Nes, 2009). Bacteriocins possess antimicrobial activities, but they are different from antibiotics. The biggest distinction among bacteriocins with antibiotics is that the activities of bacteriocins are limited to strains related to producer strains and particularly same strains. However, antibiotics have a wider range of activities and do not affect their preference for these nearly related strains, even if

their activity is limited. In addition, bacteriocin is synthesized ribosomally during the primary stage of growth, although antibiotic is generally secondary metabolite (Beasley & Saris, 2004).

In general, the action of gram-positive bacteria' bacteriocins is directly against other gram-positive strains. However, under normal conditions, bacteriocins synthesized by gram-positive strains do not have an inhibitory impact on gram-negative species (McAuliffe, Ross, & Hill, 2001). In gram positive bacteria, such as lactobacilli, bacteriocins are small in size, destabilizing the integrity of the cytoplasmic membrane, disrupting membrane potential and/or infiltrating cellular solutes and killing target microorganisms, which ultimately causes cell death (Nes, et al., 1996); (Diep & Nes, 2002). Classification of bacteriocin carried out according to their physicochemical features; class l and class ll. Class l is called lantibiotic (lanthionine-containing antibiotic) family; bacteriocins consist of modified amino acids derived from post-translational modifications and contain intramolecular thioether ring structures. Nisin and epidermin are common members of lantibiotics (McAuliffe, Ross, & Hill, 2001); (Chatterjee, Paul, Xie, & van der Donk, 2005). Class II is called a non-lantibiotic family; bacteriocins include unmodified peptides, moreover thioester bridges and circular forms (Nes, et al., 1996); (Eijsink, et al., 2002); (Diep & Nes, 2002). Reuterin, which is produced by *Lactobacillus reuteri,* and diacetyl combination showed synergistic effect against growth of *Listeria monocytogenes* (Langal, et al., 2014).

Generally, bacteriocins cause cell death by inhibition of biosynthesis of cell wall and/or impairing the membrane by pore formation (Cotter, Hill, & Ross, 2005). The antimicrobial activity of *Lactobacillus paracasei* SD1 in the oral microflora was obtained by purification of the supernatant. Purification of the active compound was accomplished by ammonium sulphate precipitation. Paracasin SD1, the bacteriocin of *Lactobacillus paracasei* SD1, showed an active antimicrobial action against pathogens (*Porphyromonas gingivalis Streptococcus mutans, S. sobrinus,* and *Aggregatibacter actinomycetemcomitans*). This feature makes paracasin SD1 a suitable candidate, especially for the prevention and / or treatment of oral diseases (Wannun, Piwat, & Teanpaisan, 2014). In their study, Busarcevic & Dalgalarrondo (2012), showed that probiotic

Lactobacillus salivarius BGHO1 and its bacteriocins have a great potential for their use as antimicrobial drugs. Live and heat-inactivated *L. rhamnosus* and *L. paracasei* inhibited the adhesion of *S. mutans* and *S. oralis* on the titanium surface and pathogens' biofilm formation. In addition to them, the supernatant of *L. paracasei* reduced the biofilm formation of streptococci (Cinandrini, Campana, & Baffone, 2017). It has been observed that reduction in *S. mutans* adhesion, which has been left to incubate with *L. salivarius*, heat inactivated (Sanudo, Lugue, Diaz-Ropero, Fonolla, & Banuelos, 2017). (Bhupesh, Jalpan, & Dhaval, 2017), a toothpaste formulation was formed using bacteriocin produced by the probiotic *Lactobacillus acidophilus* and the antibacterial activity was confirmed with testing on *Escherichia coli* and *Staphylococcus aureus*.

3.2.4.2. Defensins

Probiotics stimulate host anti-pathogenic defense pathways. For resistance of the adverse effect of pathogen microorganisms, the intestinal tract have some mechanisms, containing the producing of defensins, which are antimicrobial cationic peptides just like bacteriocins but defensins are produced by Panetch cells in small intestine crypts and intestinal epithelial cells. The synthesis of defensins can be stimulated by probiotics or they produce proteases to activate defensins in the intestine (Britton & Versalovic, 2008); (Figueroa-Gonzalez, Cruz-Guerrero, & Quijano, 2011).

3.2.4.3. Short Chain Fatty Acids

Among the fatty acids, short chain fatty acids with fewer than six carbon atoms are (Cook & Sellin, 1998) formic acid (C1), acetic acid (C2), propionic acid (C3), butyric acid (C4), isobutyric acid (C4), isovaleric acid (C5), hexanoic acid (C6). They help to sustain a favorable acidity in the colon that is essential for excreting bacterial enzymes, and also metabolizing of foreign substances and carcinogens in the intestine (Kareem, Ling, Chwen,

Foong, & Asmara, 2014). *Salmonella enterica, Clostridium difficile* and *Serovar typhimurium* are inhibited by short chain fatty acids produced via probiotics (Tejero-Sarinena, Barlow, Costabile, Gibson, & Rowland, 2013). A study showed that the short chain fatty acids had capability of inhibiting oral pathogens depends on structural characteristics of the bacterial species. The formic acid had the most powerful anti-pathogenic action against the number of oral pathogens (Huang, Alimova, Myers, & Ebersole, 2011).

3.2.4.4. Hydrogen Peroxide

Production of hydrogen peroxide (H2O2) occurs by lactic acid bacteria in the presence of oxygen (O2) from flavoprotein oxidases or nicotinamide adenine dinucleotide (NADH) peroxidase. Hydrogen peroxide leads to oxidizing of sulfhydryl groups that begins denaturation of a certain number of enzymes hence membrane lipids peroxidate, therefore, permeability of membrane of the pathogens increase and eventually, cell death (Ammor, Tauveron, Dufour, & Chevallier, 2006). Hydrogen peroxide can lead to the production of lethal free radicals, such as superoxide and hydroxyl radicals, they can harm DNA (Byczkowski & Gessner, 1988). In a study, *L. crispatus* F117 and *L. paracasei* strains F2 and F28 produced high level of H_2O_2, inhibited the growth of *Staphylococcus aureus* in a plate assay (Ocana, De Ruiz Holgado, & Nader-Macias, 1999).

3.2.4.5. Organic Acids

Organic acids are produced from the carbohydrate metabolized through the lactic acid bacteria. Based on lactic acid bacteria species properties, types and amount of organic acids vary during process of fermentation. The main products of carbohydrate metabolism are acetic acid and lactic acid. The decreasing of pH by lactic acid and acetic acid has bactericidal and bacteriostatic actions (Agrawal, 2005). Organic acid decreases the

cytoplasm pH and reduces its metabolic actions by diffusing the target organism from the membrane (Piard & Desmazeaud, 1991). A study showed that the growth of *Escherichia coli* O157:H7 was reduced by lactic acid, producing from some *Lactobacillus* strains (Ogawa, et al., 2001). Development of *Helicobacter pylori* was suppressed by *Lactobacillus casei* subsp. *Rhamnosus, Lactobacillus acidophilus, Lactobacillus bulgaricus,* and *Bifidobacterium bifidus* strains with action of a lactic acid, acetic acid and hydrochloric acid (Midolo, Lambert, Hull, Luo, & Grayson, 1995).

Lactic acid bacteria fermentation may yield with the main organic acid called as lactic acid; this acid remains in balance with its dissociated and undissociated forms and the degree of dissociation depends on pH (Lindgren & Dobrogosz, 1990). Low external pH is known to result in the acidification of the cell cytoplasm; on the contrary, lipophilic undissociated acid has been proposed to pass passively through the membrane (Kashket, 1987). The undissociated acid performs by changing the permeability of cell membrane or through disrupting the electrochemical proton gradient, which leads to failure of the substrate transport systems and stops metabolic activities (Ström, Schnürer, & Melin, 2005).

3.2.4.6. Carbon Dioxide

The main producers of carbon dioxide (CO_2) are heterofermentative lactic acid bacteria like *Lactobacillus fermentum.* CO_2 creates an anaerobic environment, which plays a role in inhibition of enzymatic decarboxylations, in addition that CO_2 accumulation in the cell membrane lipid can result in nonfunctional membrane permeability (Eklund, 1984). Carbon dioxide can efficaciously prevent a number of food spoilage microorganism's development, notably gram-negative psychotrophic bacteria (Farber, 1991). A previous study stated that lactobacilli and yeast produced carbon dioxide in kefir, and which promote to antimicrobial effect on aerobic microorganisms, due to generating anaerobic conditions (Chifiriuc, Cioaca, & Lazar, 2011).

3.2.4.7. Diacetyl

Diacetyl (2,3-butanodione) is an aroma component, is produced by lactic acid bacteria strains by citrate fermentation. Diacetyl reacts with arginine utilization for inhibition of the growth of gram-negative bacteria. Strains of *Listeria, Escherichia coli Salmonella, Aeromonas,* and *Yersinia* are inhibited with 344 μg/mL diacetyl (Jay, 1982); (Jay, 1986). According to a previous study, antimicrobial activity of diacetyl on gram-negative bacteria is higher than on gram-positive bacteria (Langal, et al., 2014). A study results represent that the flavor compound diacetyl has antimicrobial action on inhibition of growth of *Staphylococcus aureus*, which is gram-positive foodborne pathogen, thus diacetyl improve food safety. In addition the antimicrobial activity could increase with treatment of heat (Bowles, Sackitey, & William, 1995). In another study was carried out with *Escherichia coli, Listeria monocytogenes* and *Staphylococcus aureus* and results showed that *E. coli* was very sensitive to diacetyl, whereas *L. monocytogenes* was more resistant than other microorganisms (Lanciotti, Patrignani, Bagnolini, Guerzoni, & Gardini, 2003). To control of *Salmonella typhimurium* and *Escherichia coli* O157:H7 diacetyl was used, which is produced by *Pediococcus acidilactici* that is starter culture of salami fermentation. Within this study the bactericidal effect of diacetyl was proven (Kang & Fung, 1999).

3.2.4.8. Biosurfactants

Biosurfactants are amphiphilic components; they are yielded by microorganisms with emphasized emulsifying and surface activities (Singh, Van Hamme, & Ward, 2007). These microbial surfactants, which have anti-adhesive activity, involve a number of surface-active molecules classified by their microbial origin and chemical composition. They contain lipopolysaccharides, polysaccharide–protein complexes, glycolipids, protein-like substances, lipopeptides, neutral lipids phospholipids and fatty acids. Biosurfactants isolated from *Streptococcus thermophilus* A and *Lactococcus lactis* 53 demonstrated

significant antimicrobial activity against a number of yeast and bacteria (Rodrigues, Teixeira, Van der Mei, & Oliveira, 2006). The strategy of antimicrobial action is prevention of cell adhesion and cell colonization on the surface, this activity can be used both food industry and biomedical field (Meylheuc, et al., 2006). The colonization of different pathogens for instance *Streptococcus agalactiae Staphylococcus aureus* and *Staphylococcus epidermidis* are reduced with biosurfactants on several materials. A biosurfactant obtain from *Lactobacillus paracasei*, which showed antimicrobial and anti-adhesive effects on a number of pathogenic microorganisms (Gudina, Teixeira, & Rodrigues, 2010).

3.3. *Lactobacillus* Species are They Good or Bad for Maintaining Oral Health?

Some previous studies have shown that some lactobacilli strains cause the progression of tooth decays (Teanpaisan, et al., 2007), which is since some strains have ability of production of acid. However, according to the other findings, all strains do not produce acid and so do not demonstrate tooth decay effect (Piwat, Teaspaisan, Thitasomakul, Thearmontree, & Dahlen, 2010). Furthermore, many studies have stated that some strains have probiotic effect on improving oral and dental health (Nase, et al., 2001); (Teanpaisan & Piwat, 2014).

3.4. Probiotics Improve Oral Health

In the past years, probiotics are generally related to bowel health and most clinical studies have centered on the prevention or treatment of gastrointestinal infections and diseases; but in recent years it has been suggested that probiotics have important effects in protecting and improving oral health with the increased interest in these microorganisms (Haukioja, 2010); (Jain & Sharma, 2012). *Lactobacillus* and *Bifidobacteria* are the most

common probiotic strains (Parvez, Malik, Ah Kang, & Kim, 2006). According to experimental studies and clinical trials these two taxa have the potential to control the growth of oral microorganisms, including the cariogenic streptococci (Meurman, 2005). Probiotics have ability to reduce *Streptococcus mutans* risk, decrease gingivitis and periodontitis, and decline cytokine concentrations, which mediate in inflammatory processes (Flichy-Fernandez, et al., 2015).

Probiotics which could change the oral microbiome might provide clinical management of gum diseases which is called periodontitis as well, with two potential benefits (Saha, Tomaro-Duchesneau, Tabrizian, & Prakash, 2013). The first one is combating dysbiosis through suppression of periodontal pathogens' growth. Other benefit is modulating active illness associated with low immunity or inflammatory pathways to decrease the devastating gum diseases inflammation and cause immune homeostasis, which might be sustained by the host for long (Allaker & Stephen, 2017).

Some studies conducted by Laleman, et al., (2014); Wattanarat, et al., (2015); Jindal, Pandey, Agarwal, & Singh, (2011); Nase, et al., (2001); Nagaraiappa, et al., (2015) concluded that using probiotics, *S. mutans* counts can be decreased over time and this can have a preventive influence on tooth decay, but short-term periods make it impossible to continue to be effective after probiotic-therapy has been stopped. Additionally, appropriate probiotic species, duration of treatment, concentration and suitable delivery vehicle to be used should be determined (Seminario-Amez, Lopez-Lopez, Estrugo-Devesa, Avuso-Montero, & Jane-Salaa, 2017).

3.4.1. Probiotic Mechanism to Inhibit Oral Pathogen Growth

To limit or prevent tooth decay, the probiotic bacteria must compete with cariogenic bacteria to prevent the proliferation of them, which must be integrated into the bacterium that binds to the tooth surface and produces dental biofilm (Bonifait, Chandad, & Grenier, 2009). Biofilm is a structure in which bacteria adhere to a surface, live in certain integrity, and communicate with each other to protect their vitality. Microorganisms are housed in a

matrix containing a number of polysaccharides, nucleic acids and proteins known as extracellular polymeric substances, which make up the biofilm structure (Post, Stoodley, Hall-Stoodley, & Ehrlich, 2004). Although the ability to form biofilms is perceived as unique to pathogens, probiotic bacteria such as lactic acid bacteria also form biofilms. In addition, lactic acid bacteria that form biofilms can be used as protective biofilms against persistent biofilm of pathogens (Kumar, Alam, Rani, Ehtesham, & Hasnain, 2017).

3.4.2. Probiotics and Their Antimicrobial Substances

Probiotic microorganisms, which have bactericidal and bacteriostatic effects on pathogens, have ability to produce different antimicrobial substances; bacteriocins, organic acids, carbon peroxide, diacetyl and hydrogen peroxide (Meurman, 2005); (Tong, et al., 2011). Antimicrobial peptides or proteins produced by bacteria are called bacteriocins. In recent years, the use and development of a new generation of antimicrobial agents, such as bacteriocins, has great prospects for inhibiting and overproduction of resistant pathogens as a result of frequent use of antimicrobial drugs (Yang, Lin, Sung, & Fang, 2014). Additionally, systemic use of antibiotics which may lead to gastrointestinal side effects (Becker D. E., 2013), and allergic reactions (Becker D. E., 2013); (Meurman & Stamatova, 2007); (Laleman & Teughels, 2015). For this reason, alternative therapies can give satisfactory results without risking the disease (Bennadi, 2013). Treatments must be conducted regarding the use of probiotic microorganisms especially in immunocompromised people (Samot & Badet, 2013). In a new study, Rossoni, et al., (2018) has shown that most Lactobacillus strains isolated from the oral cavity of non-carious people have bioactive substances that inhibit *S. mutans* growth in planktonic cultures.

In dental and periodontal healthcare field, usage of probiotic microorganisms has shown beneficial impacts. Many clinical studies typically have focused on investigating *Streptococcus mutans* counts, salivary flow, gingival or plaque scores, and pocket depth to confirm probiotic's effectiveness (Wescombe, Hale, & Heng, 2012); (Saha, Tomaro-

Duchesneau, Tabrizian, & Prakash, 2013). Samot, Lebreton & Badet (2011), have tested lactobacilli strains capable of adhesion on oral surfaces, which have been demonstrated that good antimicrobial characteristics of probiotic species are necessary to eliminate or prevent pathogenic bacteria. Lactic acid bacteria can produce organic acids derived from carbohydrate fermentation that can interfere with *in vivo*, growth of surrounding microorganisms by reducing pH of the ecosystem (Shookkhee, Chulasiri, & Prachyabrued, 2001). In addition, some species of various probiotic strains produce hydrogen peroxide or bacteriocins, which are well known bacterial antagonistic modes (Ito, et al., 2003); (Dobson, Cotter, Ross, & Hill, 2012).

3.5. Probiotic Products to Improve Oral Health

Many products containing probiotic bacteria have been produced to improve oral and dental health.

3.5.1. Lozenge

It has been shown that in a study conducted with $1x10^8$ CFU/g *L. reuteri* by Keller, Hasslof, Dahlen, Stecksen-Blicks & Twetman (2012) and Keller & Twetman (2012), there was no significant effect on *Streptococcus mutans* while reducing the amount of the pathogenic bacteria in saliva in a study conducted by Çağlar, Kuşçu, Çildir, Kuvvetli, & Sandallı (2008). Lozenge supplemented with *Streptococcus salivarius*, significantly reduced plaque scores and *S. mutans* counts (Burton, et al., 2013). In addition to prevention of tooth decays, lozenges have also been studied for the treatment of periodontal diseases. *L. reuteri* fortified lozenge reduced gingival inflammations, gingival bleeding, probing pocket depth (Vivekananda, Vandana, & Bhat, 2010); (Tekce, et al., 2015). A course of *L. rhamnosus* and *Bifidobacterium animalis* containing lozenges taken by healthy individuals were reported to show decreased both plaque accumulation and gingival inflammation, but

had no significant changes in the salivary ecology (Toiviainen, et al., 2015). Lozenge including *L. brevis* also decreased plaque accumulation, gingival inflammation and probing pocket depth (Shah, Gujjari, & Chandrasekhar, 2013). In another research, lozenges produced with *Lactobacillus reuteri* reported that it assisted in the treatment of scaling and root planning of chronic periodontitis. Substantial falls were recorded in salivary *Porphyromonas gingivalis*, supragingival and subgingival plaque in the treatment group. Nevertheless, overall plaque scores were significantly reduced when in comparison to the group receiving clinical curation and consuming placebo lozenges (Teughels, et al., 2013). *L. brevis* CD2 lozenges were tried to treatment of halitosis, but unsuccessful to demonstrate a development in breath volatile sulfur compound concentrations (Marchetti, et al., 2015).

3.5.2. Milk

L. rhamnosus fortified milk, reduced caries development (Stecksen-Blicks, Siostrom, & Twetman, 2009); (Rodriguez, et al., 2016), decreased salivary *S. mutans* (Juneia & Kakade, 2012); however, in some studies, milk included the same probiotic demonstrated no impact on caries-related bacterial levels in saliva and level of supragingival plaque (Lexner, Blomqvist, Dahlen, & Twetman, 2010). *L. paracasei* reinforced milk and milk powder, reduced the *S. mutans* counts and increase lactobacilli numbers (Ritthagol, Saetang, & Teanpaisan, 2014); (Teanpaisan & Piwat, 2014); (Wattanarat, et al., 2015). Milk drink prepared with *L. casei*, reduced gingival crevicular fluid volume and bleeding on probing levels (Slawik, et al., 2011). Nase, et al. (2001) found that consumption of milk containing *L. rhamnosus* may be considered as an option in maintaining oral health at an early age. In a research carried out to stop halitosis, consuming *Lactobacillus casei* Shirota milk did not demonstrate important alterations volatile sulfur compound concentration in the breath or organoleptic scores, in spite of availability of the probiotic bacterium in the tongue surface throughout curation process (Sutula, Coulthwaite, Thomas, & Verran, 2013).

3.5.3. Ice cream

Ice cream became a functional food by adding various probiotic bacteria such as *Bifidobacterium lactis* (Çağlar, et al., 2008), *B. animalis* (Singh, Damle, & Chawla, 2011), a combination of *B. lactis, L. casei* and *L. acidophilus* (Chinnappa, Konde, Konde, Raj, & Beena, 2013) and within one daily intake, a significant decrease in salivary *S. mutans* was observed. Ashwin, et al., (2015) found that use of *B. lactis* (Bb-12) and *L. acidophilus* (La-5) fortified ice cream, a reduction in the count of *S. mutans* colony forming unit during the administration of probiotics for 6 months. Short term consuming ice cream including bifidobacteria can decline number of *S. mutans* bacteria in younger individuals (Nagaraiappa, et al., 2015).

3.5.4. Yogurt

Yogurt containing *B. animalis* (Çıldır, et al., 2009) and both of *L. bulgaricus* and *S. thermophilus* (Ferrazzano, Cantile, Sangianantoni, Amato, & Ingenito, 2011) reduced *S. mutans* in saliva samples. In addition, *B. lactis* fortified yogurt reduced total microbial counts in dental plaque (Pinto, Cenci, Azevedo, Epifanio, & Jones, 2014). On the contrary, *B. animalis* enforced yogurt consumption among healthy children did not decrease salivary *Lactobacilli* and *S. mutans* levels (Nozari, Motamedifar, Seifi, Htamizargaran, & Ranjbar, 2015).

3.5.5. Tablets

For 14 days, the usage of probiotic *L. brevis* tablets retards the development of gingivitis (Lee, Kim, Ko, Quwehand, & Ma, 2015). Taipale, Pienihakkinen, Salminen, Jokela, & Söderling, (2012) demonstrated that using *B. lactis* (Bb-12) tablets during 24

months, early administration of probiotic strain does not represent its permanent colonization in the oral cavity. There is insignificant changes respect to the counts of pathogen *S. mutans*. Additionally, Taipale, Pienihakkinen, Alanen, Jokela, & Söderling, (2013) found that *B. lactis* (Bb-12) tablets do not differ significantly in the incidence of tooth decays. *L. salivarius* tablets usage for 8 weeks that demonstrated probiotics can be a good option for oral health care in patients at high periodontal disease risk (Shimauchi, et al., 2008). *L. reuteri* fortified tablet used daily consumption, decreased various periodontal pathogens selected in the subgingival microbiota (Iniesta, et al., 2012). Use of probiotic *L. reuteri* tablets during 12 weeks, decreased *Candida* counts in mouth cavity (Kraft-Bodi, Jorgensen, Keller, Kragelund, & Twetman, 2015).

3.5.6. Other Products

Consisting of *L. rhamnosus, Bifidobacterium* and *B. coagulans* powder, which is included $1.25x10^9$ microorganisms, reduced salivary *S. mutans* during intervention period (Jindal, Pandey, Agarwal, & Singh, 2011). According to Holz, et al., (2013) candy enriched with 1 or 2 mg *L. paracasei* demonstrated to fall in salivary *S. mutans*. However, *L. paracasei* fortified cereal shown no effect on the abundance of tooth decay, *S. mutans* or *Lactobacilli* throughout consumption of one daily for 9 months (Hasslof, West, Videhult, Brandelius, & Stecksen-Blicks, 2013). Preparation of gum with 0.02ml (0.5 McFarland) *L. reuteri* decreased counts of *Lactobacillus* and saliva pH (Biria, Eslami, Taghipour, & Akbarzadeh Baghban, 2014). It has been shown that drops prepared with *L. reuteri* reduce caries prevalence and gingivitis score if it is dropped five times daily for one year (Stensson, et al., 2014). Drops which are prepared with combining *L. rhamnosus, L. reuteri* and *B. infantis*, reduced salivary *S. mutans* (Tehrani, Akhlaghi, Talebian, Emami, & Keyhani, 2016). *B. animalis* (Bhalla, Ingle, Kaur, & Yadav, 2015) and *L. acidophilus* (Srivastava, Saha, Kumari, & Mohd, 2016) supplemented curd demonstrated a significant decline in salivary *S. mutans* and increase salivary pH. It has been proved that rinse solution prepared with *L. salivarius* and *L. reuteri*, improved plaque index, modified gingival and bleeding index (Penala, et al., 2016). Using sachet prepared with *L. rhamnosus*

demonstrated great reduction in probing pocket depth (Morales, et al., 2016). Researches using *Lactobacillus salivarus* WB21 for a short-term studies by people suffering bad breath problem found that improvement in periodontal health and also a decline in breath volatile sulfur compounds (Iwamoto, et al., 2001); (Suzuki, et al., 2014). Consumption of cheese including *L. rhamnosus GG, L. rhamnosus LC705* and *Propionibacterium freudenreichii ssp. shermanii JS* by a group of older individuals for 16 weeks, any changes were not detected in mucosal lesions; however, the number of oral *Candida* counts declined (Hatakka, et al., 2007). In a study among young people, it was found that probiotic cheese and control cheese did not cause a significant reduction in the number of salivary *Candida* (Ahola, et al., 2002).

Lactobacillus rhamnosus, L. paracasei and *L. reuteri* are highly reliable probiotic bacteria due to their anti-caries effects (Jindal, Pandey, Singh, & Pandey, 2012). Probiotics such as *L. rhamnosus* HS111, *Bifidobacterium bifidum, L. acidophilus* HS101 inhibit candidiasis and *Candida* infections by reducing the amount of *Candida* in the oral mucosa (Ishikawa, et al., 2015). Vivekananda, Vandana, & Bhat, (2010) confirmed plaque inhibition, anti-inflammatory and antimicrobial effects of *L. reuteri* by *in vivo* studies. *L. reuteri* ATCC 55730, *L. reuteri* ATCC PTA 5289 is effective against gum inflammation (Twetman, et al., 2009). *L. rhamnosus* GG produces antimicrobial metabolites that have great properties affected on *Streptococcus mutans* which has adverse impact on human oral health (Meurman, 2005). Shimauchi, et al., (2008) demonstrated that curation with probiotic *L. salivarius* enhances in smokers' probing depth and plaque index. Hence, they deduced that the therapy with probiotic is important option to maintain dental and periodontal health in patients.

Table 3.1. List of studies with probiotics conducted for their effects in oral cavity

Probiotic	Pathogen	Product	Reference
Enterococcus faecium CRL 183	*Streptococcus mutans*	Diet lozenge	Witzler et al. 2017 (*in vitro*)
Lactobacillus salivarius CECT 5713	*Streptococcus mutans*		Sanudo et al., 2017 (*in vitro/ in vivo)*
L. fermentum, L. plantarum , L.	*Streptococcus mutants, Candida*		K ojima et al., 2016 (*in*

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Table 3.1 (cont)

paracasei, L. gasseri and L. salivarius	*albicans* and *Porphyro monas gingivalis*		*vitro*)
L. rhamnosus, L. acidophilus, L. casei and L. reuteri	*Streptococcus salivarius, S. mutans, S, oralis*		Taheur et al., 2016 (*in vitro*)
L. *rhamnosus* HS111, *L. acidophilus* HS101, *Bifidobacterium bifidum*	*Candida*		Ishikawa et al., 2015 (*in vivo*)
Lactobacillus salivarius, Lactobacillus reuteri		Capsule	Penala et al., 2015 (*in vivo*)
Lactobacillus brevis CD2		Lozenge	Campus et al., 2013 (*in vivo*)
L. plantarum, L. paracacesi, L. rhamnosus and L. brevis			Samot et al., 2013 (*in vitro*)
Lactobacillus rhamnosus, L. paracasei and L. reuteri			Jindal et al., 2012 (*in vivo*)
Lactococcus lactis	*Streptococcus mutans*		Tong et al., 2011 (*in vitro*)
Lactobacillus brevis CD2		Lozenge	Sharma et al., 2011 (*in vivo*)
L. plantarum 299v, *L. plantarum* 931, *L. rhamnosus* GG, *L. rhamnosus* LB21, *L. paracasei* F19 and *L. reuteri* PTA 5289, *L. reuteri* ATCC 55730 and *L. acidophilus* La5	*Streptococcus mutans* and *Candida*		Hasslöf et al., 2010 (*in vitro*)
Lactobacillus reuteri DSM17938, *Lactobacillus reuteri* ATCC PTA 5289		Tablet	Vivekananda et al., 2010 (*in vivo*)
Lactobacillus reuteri ATCC 55730, *Lactobacillus reuteri* ATCC PTA 5289		Chewing gum	Twetman et al., 2009 (*in vivo*)
Bifidobacterium lactis Bb-12	Mutans streptococci and lactobacilli	Ice-cream	Çağlar et al., 2008 (*in vivo*)
L. rhamnosus GG, *L. rhamnosus* LC705, *Propionibacterium freudenreichii ssp. shermanii* JS	*Candida*	Cheese	Hatakka et al., 2007 (*in vivo*)
Lactobacillus reuteri ATCC 55730	Mutans streptococci and lactobacilli	Straws or tablets	Çağlar et al., 2006 (*in vivo*)
Bifidobacterium DN-173010	Mutans streptococci and lactobacilli	Youghurt	Çağlar et al., 2005 (*in vivo*)
Lactobacillus reuteri	*Streptococcus mutans*	Bovine milk	Nikawa et al., 2004 (*in vivo*)
Lactobacillus rhamnosus GG		Milk	Näse et al., 2001 (*in vivo*)

CHAPTER 4

MICROENCAPSULATION

4.1. Microencapsulation Techniques

Taking into account the positive effects of probiotic bacteria on health, probiotic supplements are made of nutrients to increase nutritional value of food and to bring functionality to food. To maintain the beneficial effects of bacteria, it is necessary to maintain their vitality and this is achieved only by microencapsulation.

Probiotic food products must be reliable and contain sufficient amount of probiotic species for consumption. Probiotic microorganisms must survive throughout the food production stages and maintain their viability throughout the food shelf life. Microencapsulation should be carried out to maintain the viability of probiotics during the production process, packaging and storage of foods (Tripathi & Giri, 2014).

Microencapsulation is the process of coating microorganisms with a suitable substance to provide appropriate microorganism release in the gut environment (Mortazavian, et al., 2008). Materials used to encapsulate probiotic cells include different polysaccharides such as gelatin, alginate, plant / microbial gums, hemicellulose, chitosan, pectin, starch, K-carrageenan, cellulose acetate phthalate, milk proteins and fats (Burgain, Gaiani, Linder, & Scher, 2011).

Techniques for encapsulating probiotic cells are extrusion, emulsion and spray drying. The most suitable method for probiotic cell encapsulation is the emulsion technique (Heidebach, Först, & Kulozik, 2012).

Microencapsulation processes make sure the long-term vitality of the bacteria and the preservation of the number of live microorganisms. Microencapsulation system is frequently preferred in food production stages. This system is a technology that protects

sensitive microorganisms and components in food with edible polymer materials (Eslami, Davarpanah, & Vahabzadeh, 2017). Figure 4.1 shows the effects of microencapsulation processes for protection of probiotic microorganisms from harsh environmental conditions.

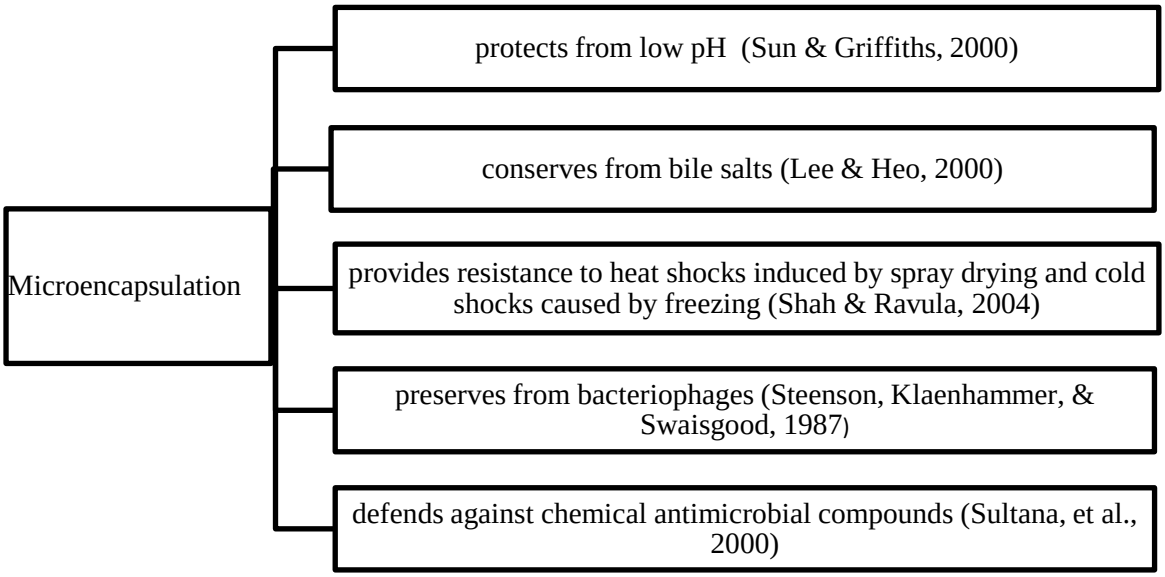

Figure 4. 1. Microencapsulation protects probiotics from harsh environmental conditions

4.1.1. Spray Drying Method

Spray drying is a method of producing a dry powder by quickly drying the liquid or slurry with a hot gas. In this technique, solution is dried, including the polymer matrix and the probiotic live cells. Gum arabic and starch are suitable for spray drying because they tend to form spherical microparticles throughout the drying process (Chen & Chen, 2007). Although it has a relatively inexpensive procedure, high temperature application significantly affects the viability of bacteria (De Voss, Faas, Spasojevic, & Sikkema, 2010).

4.1.2. Emulsion Method

Emulsion is a chemical method used to encapsulation of probiotic live microorganisms using encapsulating materials such as alginate, carrageenan and pectin. In this method, the relation between continuous and discontinuous phases is the main factor. In addition, since encapsulation takes place in an emulsion, an emulsifier and a surfactant are required. Then, calcium chloride is added to the emulsion as a solidifying agent (Kailasapathy, 2009); (De Voss, Faas, Spasojevic, & Sikkema, 2010). The technique increases the survival rate of microorganisms (Chen & Chen, 2007). *Lactobacillus delbrueckii*, microencapsulated by double emulsion method, maintained its viability while maintaining bacterial functions (Eslami, Davarpanah, & Vahabzadeh, 2017). *Lactobacillus rhamnosus* is encapsulated by double emulsion technique using sweet whey as an emulsifier. The encapsulated cells exhibited significant resistance to acid and bile salts in the gastro-intestinal tract. In addition, this double emulsion method provides the environment for the bacteria growth with the use of sweet whey (Pimentel-Gonzalez, Campos-Montiel, Lobato-Calleros, Pedroza-Islas, & Vernon-Cartera, 2009). *Lactobacillus bulgaricus* is encapsulated in the whey protein with the emulsion technique, maintaining viability in the gastrointestinal tract conditions (Chen, Li, Liu, & Meng, 2017).

4.1.3. Extrusion Method

In the extrusion technique, probiotic alginate and carrageenan are used as hydrocolloids to get into live cells. Microencapsulation by extrusion of probiotic cells involves reflecting the solution containing cells at a high pressure from a nozzle. Extrusion is a process that does not damage the probiotic cells and gives high vitality (Kailasapathy, 2002); (Krasaekoopt, Bhandari, & Deeth, 2003). Since the efficiency is relatively low, the disadvantage of this method is scaling. In a recent study, has used two different methods for the encapsulation of *Enterococcus faecium* CRL 183 has been used, these are extrusion and complex coacervation. As a result, the complex coacervation method was found to be

effective in sustaining the viability of *E. faecium*, at room temperature (Witzler, Pinto, Valdez, Castro, & Cavallini, 2017).

4.2. Microencapsulation Materials

Edible polymer materials are used for coating agent; alginate, chitosan and whey are widely used in the process.

4.2.1. Alginate

Alginate, which is a natural polysaccharide, obtained from brown algae containing a linear chain of 1 → f4 linked R-l-guluronic acid (G) and β-d-mannuronic acid (M) residues (Chen & Subirade, 2006). Alginate is a biocompatible, non-toxic and low cost biomaterial (Krasaekoopt, Bhandari, & Deeth, 2003). As a result of encapsulation of *Lactobacillus acidophilus* DD910 and *Bifidobacterium lactis* DD920 bacteria using a calcium-alginate polymer, it was shown that there was 2 and 1 log-less losses after 7 weeks, compared to those whose cell numbers were not encapsulated (Kailasapathy, 2006).

4.2.2. Chitosan

Chitosan, produced from chitin, is a natural material obtained from chemical or microbiological processes of crustaceans. Due to its biocompatibility, non-toxic nature, ease of use, biodegradability and cheapness, it is frequently preferred for encapsulation processes (Sashiwa & Aiba, 2004). Microencapsulation of *Lactobacillus rhamnosus* GG in chitosan-coated alginate particles increased the survivability of bacteria during the gastrointestinal tract. Furthermore, this technique increased the cells tolerance to the heat treatment and under appropriate conditions allowed them to be metabolically active

(Abbaszadeh, Gandomi, Misaghi, Bokaei, & Noori, 2014). *Lactobacillus rhamnosus* GG continued the viability of apple juice for 90 days as a result of encapsulation of microorganism with chitosan and alginate. Furthermore, microencapsulated bacteria showed higher viability compared to free bacteria during gastrointestinal therapies (Gandomi, Abbaszadeh, Misaghi, Bokaie, & Noori, 2016).

4.2.3. Pullulan

Pullulan, which can be used in the encapsulation process, is a water-soluble, non-toxic, colorless, odorless, tasteless and non-heat-affected polysaccharide. It has a wide usage area in most industrial fields; food industry, paper industry and pharmacy, especially for its use as coating material. Although various oligosaccharides such as inulin and lactulose are among the commonly used prebiotics, other polysaccharides such as pullulan have been shown to increase the activity of probiotic microorganisms by showing prebiotic properties in the intestinal environment and thus show a positive functional effect on human health (Ganzevles, Kosters, Vliet, Stuart, & Jongh, 2007); (Leathers, 2003).

4.2.4. Whey Protein

The emulsion may contain milk proteins, probiotics are coated with an enzymatic source gel, since milk proteins are not only natural carriers for probiotics but also have great gelling properties (Livney, 2010). Encapsulation of *Lactobacillus acidophilus* LA-5 was performed use of pectin and whey protein, maintaining higher viability in non-encapsulated cells over a period of 35 days (Ribeiro, et al., 2014).

CHAPTER 5

MATERIALS AND METHODS

5.1. Materials

Commercial species of *Lactobacillus pentosus* NRRL-B 227, *Lactobacillus paracasei ssp. paracasei* NRRL-B 1560, *Lactobacillus paracasei ssp. paracasei* NRRL-B 4560, *Lactobacillus casei ssp. casei* NRRL-B 1922, *Lactobacillus casei* CH1, *Lactobacillus casei* NRRL-B 441, *Lactobacillus curvatus, Lactobacillus rhamnosus* NRRL-B 442, *Lactobacillus farciminis, Lactobacillus plantarum* DSM 1954, *Lactobacillus acidophilus* NRRL-B 1910, *Lactobacillus delbrueckii ssp. bulgaricus* NRRL-B 548, *Lactobacillus fermentum, Lactobacillus delbrueckii ssp. lactis* NRRL-B 735, *Lactobacillus delbrueckii* NRRL-B 4525, *Lactobacillus brevis* NRRL-B 1836, *Lactobacillus brevis* NRRL-B 1830, *Lactobacillus fructosus* NRRLB- 2041, *Lactobacillus fructosus* NRRLB-641, *Lactobacillus hilgardii* NRRL-B 1843, *Lactobacillus reuteri* NRRL-B 14170, *Lactobacillus coryniformis ssp. coryniformis* NRRL-B 4391 and *Lactobacillus coryniformis ssp. torquens* NRRL-B 4390 were obtained from the ARS Culture Collection (NRRL, USA).

Streptococcus mutans ATCC 25175 and *Candida albicans* DSMZ 5817 were supplied from Ege University Culture Collection. Pullulan was obtained from Hayarashibara Co. Ltd (Okayama, Japan). Whey protein concentrate and soybean lecithin were obtained from Alfasol (Turkey). Sunflower oil was supplied from a local market.

All chemicals were obtained from Sigma and Merck. Growth media of microorganisms; MRS medium (de Man Rogosa and Sharpe, Merck, Germany, Catalogue number: 110660), BHI (Brain Heart Infusion, Oxoid, England, Catalogue number: CM1135), Nutrient Broth (Applichem, Germany, Catalogue number: 413793.1210), VRB Agar (Violet Red Bile Agar, Merck, Germany, Catalogue number: 101406) and PDA

(Potato Dextrose Agar, Oxoid, England, Catalogue number: CM0139). In microencapsulation process, droplet hardening agent is $CaCl_2$ (Applichem, Germany, Catalogue number: 141221.1210). Ingredients of lozenge are sorbitol (Merck, Germany, Catalogue number: 107758), gelatin (Merck, Germany, Catalogue number: 104072).

5.2. Methods

Firstly, probiotic and pathogen cultures were prepared, and inhibition analyses were carried out. After selection of probiotic, it was microencapsulated. After that, lozenge production and characterization were analysed.

5.2.1. Culture Preparation

Probiotic and pathogen stock cultures were prepared properly.

5.2.1.1. Probiotic Culture Preparation

A certain amount of bacteria from the stock culture maintained at -80°C was inoculated into MRS medium (de Man Rogosa and Sharpe, Merck, Germany) and incubated at 37^0C, for 24 h, under anaerobic conditions. After 24 h, a certain amount of bacteria was again inoculated into MRS medium and incubated at 37^0C, for 16 h. After the incubation, each of the probiotic bacteria tubes will be centrifuged at 5000 rpm for 15 minutes at 4^0C through a 0.2 micrometer filter to obtain the supernatant.

5.2.1.2. Pathogen Culture Preparation

A certain amount of *Streptococcus mutans* ATCC 25175 from stock culture maintained at -80^{0}C, was inoculated into BHI (Brain Heart Infusion, Oxoid, England) and allowed to incubate at 37^{0}C, for 24 h, under anaerobic conditions.

An aliquot *Candida albicans* DSMZ 5817 from stock culture maintained at -80^{0}C, was inoculated into Nutrient Broth (Applichem, Germany) and allowed to incubate at 37^{0}C, for 24 h, under aerobic conditions.

5.2.2. Inhibition Analyses

Four different inhibition methods were used to show antimicrobial effect of probiotics against pathogens microorganisms.

5.2.2.1. Agar Disc Diffusion Method

The agar disc diffusion technique was carried out according to Kojima, Ohshima, Seneviratne & Maeda, (2016). A suspension of the pathogens (of approximately 1×10^8 CFU/mL) was adjusted to a McFarland standard, and then spread with swap onto Mueller-Hinton agar in a petri dish. The discs were impregnated with different lactic acid bacteria supernatant were placed onto the top surface of the agar. A tweezers was used for the discs' placement. After 24 hours incubation at 35°C, growth inhibition zones around the discs were measured to the millimeter. A clear circular region around a disc shows sensitivity to this probiotic supernatant. The same method carried out with antibiotic discs, which are Tetracycline (30μg), Rifampicin (5μg), Pefloxacin (5μg), Vancomycin (30μg), Gentamicin (10μg), Azithromycin (15μg), Lincomycin (2μg), Amoxicillin (25μg), Chloramphenicol (30μg), Streptomycin (10μg), Kanamycin (30μg), Cephalothin (30μg), Penicillin (10μg),

Ampicillin (10μg), Erythromycin (15μg). For both pathogens, the experiments were performed in parallel.

5.2.2.2. Broth Microdilution Method

The *in vitro* antimicrobial actions of the *Lactobacillus* supernatants were tested use of the broth microdilution technique according to the standards of the Clinical and Laboratory Standards Institute, USA (Wayne, 2008). 100 μL of the supernatants prepared from probiotic bacteria were transferred into 96-well microtiter plate, and 100 μL of *S. mutans* ATCC 2517 adjusted optical density (OD) to 0.2, was transferred into each wells. Then, two drops of paraffin liquid are instilled and anaerobically incubated on the Varioskan (Varioskan™ LUX Multimode Microplate Reader, Thermo Fisher Scientific, USA) device for 48 hours. The inhibition was observed as a result of the measurements performed at 30 min intervals at 37°C at 600nm. The inhibition of *C. albicans* DSMZ 5817 was determined by applying the Broth Microdilution method steps by adjusting the OD to 0.5. Each supernatant were tested three times.

5.2.2.3. Agar Overlay Test

The inhibitory activity of the *L. pentosus* NRRL-B 227 was investigated by the agar overlay technique performed by Simark-Mattsson et al. (2007). The surface of the MRS agar (de Man Rogosa and Sharpe, Merck, Germany) was inoculated with 10 μL of an overnight culture of *L. pentosus* tested (one point per dishes). Agar plates were allowed to incubate for 1 day anaerobically for colony growth at 37°C (Anaerobic jar, Merck KGaA, Darmstadt, Germany). Then, the top was covered with 15 mL of BHI (Brain Heart Infusion, Oxoid, England) agar and Nutrient agar (Applichem, Germany), which had been included 10% of the *S. mutans* ATCC 25175 and *C. albicans* DSMZ 5817 to be tested, respectively. Following 24 hours incubation at 37°C under anaerobic conditions, the clear

region around the lactobacilli colonies was considered positive inhibition and the diameter of the zones were measured in millimeters. For both pathogens, the experiment was performed in parallel.

5.2.2.4. Antibacterial Activity of *L. pentosus* NRRL-B 227 Supernatant against Pathogens in Planktonic Cultures

The antimicrobial activity of *Lactobacillus pentosus* NRRL-B 227 against *S. mutans* and *C. albicans* in planktonic cultures was carried out by making some arrangements on the method performed by Rossini, et al., (2018). Standardized *S. mutans, C. albicans* and *L. pentosus* cell suspensions were prepared. Next, 250µL of *S. mutans* suspension and 250µL of *L. pentosus* supernatant were then added into 1.5 mL of BHI broth and mixed. In the same way, 250µL of *C. albicans* suspension and 250µL of *L. pentosus* supernatant were added and mixed into 1.5 mL of Nutrient broth. In the control group, the cell suspension of *S. mutans* and *C. albicans* was cultured only with its own medium. These cultures were allowed to incubate at 37 °C for 24 h and 48 h in anaerobic conditions for *S. mutans* and in aerobic conditions for *C. albicans*. After that, the cultures were diluted and they plated on Brain Heart Infusion Agar and Nutrient Agar for growth of *S. mutans* and *C. albicans*, respectively. Plates were allowed to incubate for 48 hours at 37°C under anaerobic and aerobic conditions, and then colony forming units were counted (CFU/mL). This analysis was performed in parallel to two independent experiments.

5.2.3. Microencapsulation of Probiotic Culture and Freeze-drying

The *L. pentosus* NRRL-B 227 cells were collected through centrifugation at 5000 rpm for 15 minutes at 4 ^{0}C. The supernatant was decanted and *L. pentosus* cells were resuspended in whey protein concentration-pullulan solution.

5.2.3.1. Formation of Whey Protein Concentrate-Pullulan Wall Matrix

Whey protein concentrate and pullulan emulsion were prepared according to the methodology of Çabuk & Harsa, (2015). Briefly, whey protein concentrate (9% w/v) was stirred for about 3 hours with a magnetic stirrer in distilled water at room temperature and after dissolution the solution was denatured for 30 minutes, at 80°C. Then, denatured solution was cooled. The pullulan (13% w/v) was stirred for about 3 hours with a magnetic stirrer to assure dissolution in distilled water at room temperature.

5.2.3.2. Microcapsule Preparation

First, the oil-in-water emulsion was formed by emulsifying an internal aqueous phase (polymer complex of whey protein concentrate and pullulan) including bacteria cells into an oil phase, which contained 1% soybean lecithin as an emulsifier. The primary emulsion was become homogeny with an Ultra Turrax homogenizer for 5 minutes (Ultra Turrax, model T25, Janke & Kunkel, IKA Labortechnik, Staufen, Germany). The emulsion was then homogenized in a 0.1 M $CaCl_2$ solution (Applichem, Germany) for 2 minutes with the homogenizer again. After the microcapsules formation, this slurry was shaken for 30 minutes at 160 rpm for hardening of the microcapsules. The separation of hardened microcapsules from the solution and the oil phase was performed by centrifugation at 1000 rpm for 1 hour.

5.2.3.3. Enumeration of Bacteria

Viability of microencapsulated *L. pentosus* NRRL-B 227 cells were determined by pour plate technique using MRS medium. For the counting of microencapsulated bacteria, the microcapsules were added to the peptone water at a ratio of 1:10, which is homogenized

with homogenizer. Then, using the MRS agar, the pour plate technique was applied to the appropriate dilution. Plates were allowed to incubate under anaerobic conditions with using anaerobic kit (Thermo Scientific™ Oxoid AnaeroGen, England) at 37°C for 48 hours and the colonies were numbered.

The number of live cells was expressed as colony forming units per gram microcapsule (CFU/g), and the efficiency was expressed in Equation 1:

$$\text{Encapsulation efficiency (\%)} = 100 \times (N/N_0) \quad (1)$$

where N is the live cell count of *L. pentosus* after microencapsulation process, N_0 is the live cell count of *L. pentosus* before microencapsulation process.

5.2.3.4. Freeze Drying

First, the microcapsules were chilled at -20°C. The microcapsules were then freeze-dried via a Lablanco freeze dryer (Freezone 18, Kansas, USA) at -55°C for 48 hours and under 0.050 mBar vacuum. Then, the microcapsules were maintained at 4°C for future studies.

5.2.4. Production of Lozenge

The lozenges were prepared according to the Witzler, Pinto, Valdez, Castro, & Cavallini, (2017) with sorbitol (Merck, Germany), gelatin (Merck, Germany), peppermint oil (Naturlife,Turkey), microencapsulated and freeze-dried *L. pentosus* NRRL-B 227, and water.

Three different lozenge formulations were used: control formulation (CL) with sorbitol (89.60 g/100 g), gelatin (1.50 g/100 g), water (8.40 g/100 g) and peppermint oil (0.50 g/100 g); probiotic formulation containing microencapsulated cells (CPL) with

sorbitol (88.41 g/100 g), gelatin (1.47 g/100 g), water (8.25 g/100 g), microencapsulated cells of *L. pentosus* NRRL-B 227 (1.38 g/100 g) and peppermint oil (0.50 g/100 g); probiotic formulation containing free cells (FPL) with sorbitol (88.41 g/100 g), gelatin (1.47 g/100 g), water (8.25 g/100 g), free cells of *L. pentosus* NRRL-B 227 (1.38 g/100 g) and peppermint oil (0.50 g/100 g).

Lozenges were produced by proper mixing and molding of all components, then dried at 35°C for 20 hours (Edwards, 2001), coated with aluminum foil in a plastic bag and stored at room temperature and refrigeration temperature.

5.2.5. Lozenge Characterization

Lozenge characterization was evaluated with microbiological, physicochemical and sensory analyses.

5.2.5.1. Microbiologic Evaluation

Samples of each formulation were collected and 1:10 lozenges were suspended in peptone water. Then, serial dilutions were prepared and used for vitality analysis of lozenges.

The live cell number of *L. pentosus* NRRL-B 227 in the product was determined by cultivating on MRS agar with pour plate technique and then incubated at 37°C anaerobically for 48 hours. The weekly counts were expressed as log CFU/g (Rossi, et al., 2008).

Microbiological safety of lozenges was determined by *Escherichia coli*, yeasts and mold counts. *Escherichia coli* analysis was performed in VRB Agar (Violet Red Bile Agar, Merck, Germany) and incubated at 25^0C, for 48 h.

The yeast and mold analysis was carried out in PDA (Potato Dextrose Agar, Oxoid, England) and incubated at 30^0C, for 120 h.

5.2.5.2. Physicochemical Assessments

The three different lozenge formulations were assessed for their physicochemical properties. The pH, moisture content, color and water activity were determined shortly after the production, in duplicate.

5.2.5.2.1. Color Measurement

Color measurements of lozenges were determined by Konica Minolta colorimeter (model CR 410, Konica Minolta, Tokyo, Japan). The CIE Lab system defined in the L*, a*, b* rectangle coordinates, where L * symbolizes the lightness, a * symbolizes the red green and b * symbolizes yellow blue.

5.2.5.2.2. Water Activity and Moisture Content Measurement

The lozenge sample water activity was determined by a Hygrolab C1 water activity counter (Hygrolab C1, Rotronic, Bassersdorf, Switzerland) (Dianawati, Mishra, & Shah, 2012). To determine the lozenges moisture content, they were dried in 105°C for 24 hours (Rajam, Karthik, Parthasarathi, Joseph, & Anandharamakrishnan, 2012). The average moisture content was calculated with Equation 2:

$$\text{Moisture content (\%)} = [(W_{wet} - W_{dry})/(W_{wet})] \times 100 \quad (2)$$

Where wet lozenge sample weight is W_{wet} and dry lozenge sample weight is W_{dry}.

5.2.5.2.3. pH Measurement

The pH value was measured with a digital pH meter (Qualxtron®, Model 8010). For measurement, 3.0 g of lozenge sample was dissolved in 20.0 mL of distilled water.

5.2.5.3. Sensory Evaluation

The sensory panel consisted of 30 untrained people. Acceptance test of qualifications (appearance, flavor, color, texture, taste and general acceptance) using a 5-point hedonic scale (1 = not very liked and 5 = liked very much) (Meilgard, Civille, & Carr, 1988); (Stone & Sidel, 1993) sensory analysis was performed after one week the production of lozenges. Panelists evaluated 3 lozenge formulations at a time. Each lozenge sample was encoded with a 3-digit arbitrary number and presented appropriately to the panelists.

5.2.6. Statistical Analysis

All experiments were performed in parallel. Results were expressed with standard deviations. Data analysis was performed using Minitab 18.0 software (Minitab Inc., State College, PA, USA). Variance analysis (ANOVA) test and Tukey's test were used for the differences between the lozenge formulations.

CHAPTER 6

RESULTS AND DISCUSSION

6.1. Inhibition Analyses

Antimicrobial activity of probiotics was demonstrated with broth microdilution method, agar overlay test and planktonic culture assay, except agar disc diffusion technique.

6.1.1. Agar Disc Diffusion Method

23 reference strains of *Lactobacillus* were selected, including 2 strains of *L. paracasei*, 3 strains of *L. casei*, 3 strains of *L. delbrueckii*, 2 strains of *L. coryniformis*, 2 strains of *L. brevis*, 2 strains of *L. fructosus, L. pentosus, L. reuteri, L. plantarum, L. hilgardii, L. curvatus, L. rhamnosus, L. farciminis, L. acidophilus* and *L. fermentum.* All strains were screened for antibacterial action against *S. mutans* and *C. albicans* using agar disc diffusion method; however, which did not show any visible zone. This may be caused since discs were impregnated into low concentration of supernatant, so pathogens can easily become dominant in petri dishes. In addition to probiotics, antibiotic susceptible test was also carried out to investigate their antibiotic resistance; Figure 6.1 shows inhibition zone diameter of antibiotic discs on *S. mutans.* It was determined that many of antibiotic discs had inhibitory effect on *S. mutans*, but *C. albicans* had antibiotic resistance, these results are given in Table 6.1.

The effect of probiotics on pathogens was not observed by disc diffusion test, but antibiotic discs were effective only on *S.mutans. C. albicans* is resistant against antibiotics. Often the use of antibiotics causes microorganisms to resist them, so the use of antibiotics against *S. mutans* will lose its effectiveness after a period of time. However, the use of

probiotic as a solution to these two pathogenic microorganisms will positively affect both oral health and general body health.

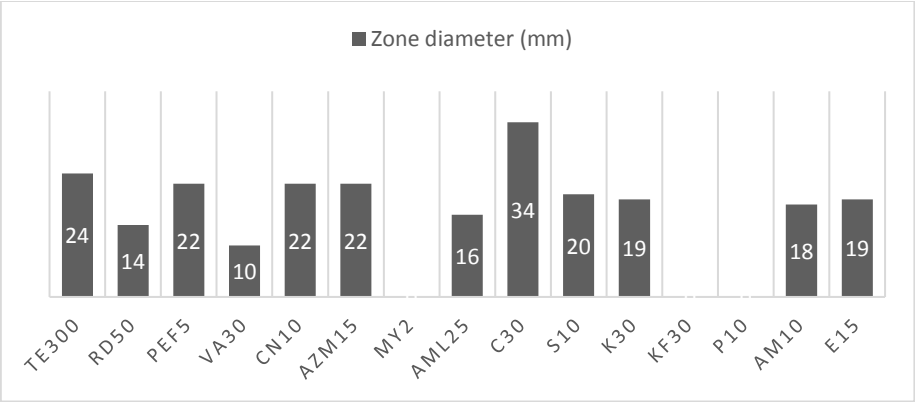

Figure 6.1. Inhibition zone diameters of antibiotic disc on *S. mutans*

Table 6.1. Antibiotic susceptibility profile of *S. mutans* and *C. albicans*

Antibiotic disc		**Inhibition zone**	
		S. mutans	***C. albicans***
TE300	Tetracycline 30µg	24mm (S)	No zone (R)
RD50	Rifampicin 5µg	14mm (S)	No zone (R)
PEF5	Pefloxacin 5µg	22mm (S)	No zone (R)
VA30	Vancomycin 30µg	10mm (S)	No zone (R)
CN10	Gentamicin 10µg	22mm (S)	No zone (R)
AZM15	Azithromycin 15µg	22mm (S)	No zone (R)
MY2	Lincomycin 2µg	No zone (R)	No zone (R)
AML25	Amoxicillin 25µg	16mm (S)	No zone (R)
C30	Chloramphenicol 30µg	34mm (S)	No zone (R)
S10	Streptomycin 10µg	20mm (S)	No zone (R)
K30	Kanamycin 30µg	19mm (S)	No zone (R)
KF30	Cephalothin 30µg	No zone (R)	No zone (R)
P10	Penicillin 10µg	No zone (R)	No zone (R)
AM10	Ampicillin 10µg	18mm (S)	No zone (R)
E15	Erythromycin 15	19mm (S)	No zone (R)

(R) = resistant, (S) = susceptible

Kojima, Ohshima, Seneviratne, & Maeda, (2016) showed that the cell free supernatant of lactobacilli strains: *L. gasseri, L. fermentum, L. plantarum, L. salivalius, L.*

paracasei produced a large growth inhibitory area for *Porphyromonas gingivalis*, which is also an oral pathogen, around the discs when compared to the negative control.

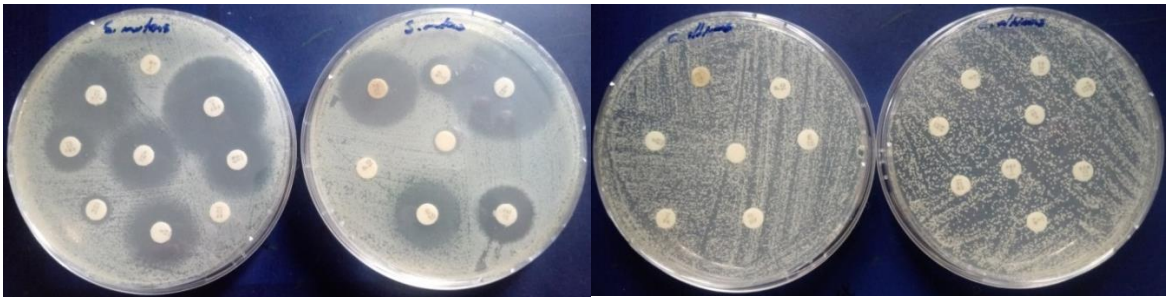

Figure 6.2. Appearance of inhibition zones in petri dishes

6.1.2. Broth Microdilution Method

After unsuccessful inhibition of pathogens by *Lactobacillus* species with agar disc diffusion method, broth microdilution method was carried out. 23 different reference *Lactobacillus* species in stock culture was tried for the inhibition of pathogens. All strains were screened for antimicrobial action against *S. mutans* and *C. albicans* using broth microdilution test. 11 of Lactobacillus strains (100%) screened showed antimicrobial activity against both *S. mutans* and *C. albicans*. Only *the Lactobacillus paracasei ssp. paracasei* NRRL-B 4560, *Lactobacillus delbrueckii* NRRL-B 4525, *Lactobacillus hilgardii* NRRL-B 1843, *Lactobacillus coryniformis ssp. torquens* NRRL-B 4390 strains had inhibitory effects on *S. mutans* after 48 hours in culture, others did not affect the growth of pathogens (Table 6.2).

Previous studies confirmed the results shown in Table 6.2. The most potent effect on the inhibition of oral pathogens was seen in *L. plantarum, L. paracacesi, L. rhamnosus* and *L. brevis* (Samot & Badet, 2013). *L. rhamnosus, L. acidophilus* and *L. casei* prevent the formation of a cariogenic environment in the mouth. *L. reuteri* has an inhibitory effect on the growth of oral pathogens (Taheur, et al., 2016). As probiotic bacteria *L. fermentum, L. plantarum* and *L. paracasei* have inhibition impacts on oral pathogens which are

Streptococcus mutants, *Candida albicans* and *Porphyromonas gingivalis* (Kojima, Ohshima, Seneviratne, & Maeda, 2016).

Table 6.2. Number of selected *Lactobacillus* species with complete inhibition against *S. mutans* and *C. albicans*.

No	References	References code	Effective inhibition on
1	*Lactobacillus paracasei ssp. paracasei*	NRRL-B 1560	*C. albicans & S. mutans*
2	*Lactobacillus paracasei ssp. paracasei*	NRRL-B 4560	*S. mutans*
3	*Lactobacillus casei ssp. casei*	NRRL-B 1922	*C. albicans & S. mutans*
4	*Lactobacillus casei*	CH1	
5	*Lactobacillus casei*	NRRL-B 441	*C. albicans & S. mutans*
6	*Lactobacillus curvatus*		
7	*Lactobacillus rhamnosus*	NRRL-B 442	*C. albicans & S. mutans*
8	*Lactobacillus farciminis*		*C. albicans & S. mutans*
9	*Lactobacillus plantarum*	DSM 1954	*C. albicans & S. mutans*
10	*Lactobacillus acidophilus*	NRRL-B 1910	*C. albicans & S. mutans*
11	*Lactobacillus delbrueckii ssp. bulgaricus*	NRRL-B 548	
12	*Lactobacillus fermentum*		
13	*Lactobacillus delbrueckii ssp. lactis*	NRRL-B 735	
14	*Lactobacillus delbrueckii*	NRRL-B 4525	*S. mutans*
15	*Lactobacillus pentosus*	NRRL-B 227	*C. albicans & S. mutans*
16	*Lactobacillus brevis*	NRRL-B 1836	*C. albicans & S. mutans*
17	*Lactobacillus brevis*	NRRL-B 1830	
18	*Lactobacillus fructosus*	NRRLB- 2041	
19	*Lactobacillus fructosus*	NRRLB- 641	
20	*Lactobacillus hilgardii*	NRRL-B 1843	*S. mutans*
21	*Lactobacillus reuteri*	NRRL-B 14170	*C. albicans & S. mutans*
22	*Lactobacillus coryniformis ssp. coryniformis*	NRRL-B 4391	*C. albicans& S. mutans*
23	*Lactobacillus coryniformis ssp. torquens*	NRRL-B 4390	*S. mutans*

It has been decided to carry out studies with *Lactobacillus pentosus* NRRL-B 227; since it inhibits the growth of both pathogens, *S. mutans* and *C. albicans*, which has not been used for improving oral health yet. The growth curve and microscopic image of *L. pentosus* NRRL-B 227 attached in Appendix A and B. Further studies were carried out on *L. pentosus* to show inhibitory effects. For this purpose, the supernatants of *L. pentosus* were used to find the minimum inhibitory concentration *via* broth microdilution technique.

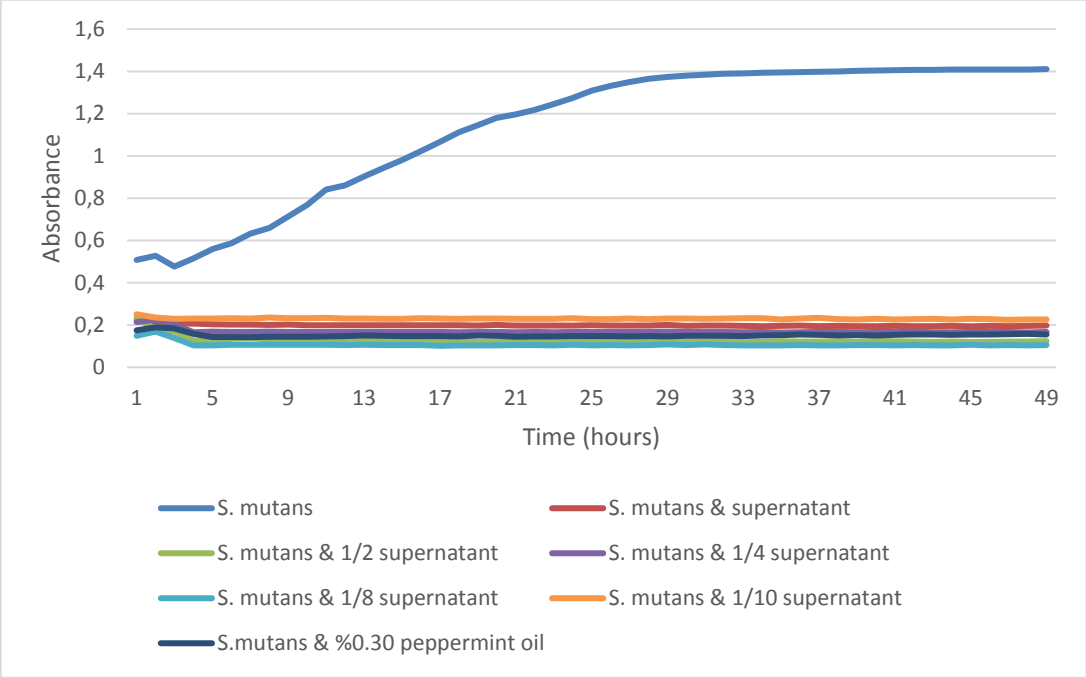

Figure 6.3. Inhibition of *S. mutans* by supernatant of *L. pentosus* at different concentrations

The absorbance of *S. mutans* and *C. albicans* were measured in 96 well-plates at the time scale during incubation time up to 48 h. In the experiments, a strong *Lactobacillus* supernatant inhibitory activity was found on *S. mutans* in samples taken every 30 minutes. All concentrations of *L. pentosus* supernatant tested (dilution of 1: 1, 1: 2, 1: 4, 1: 8, 1:10 supernatant) and the difference between *S. mutans* + BHI control group and *S. mutans* + *Lactobacillus* supernatant interaction group was shown by the Figure 6.3.

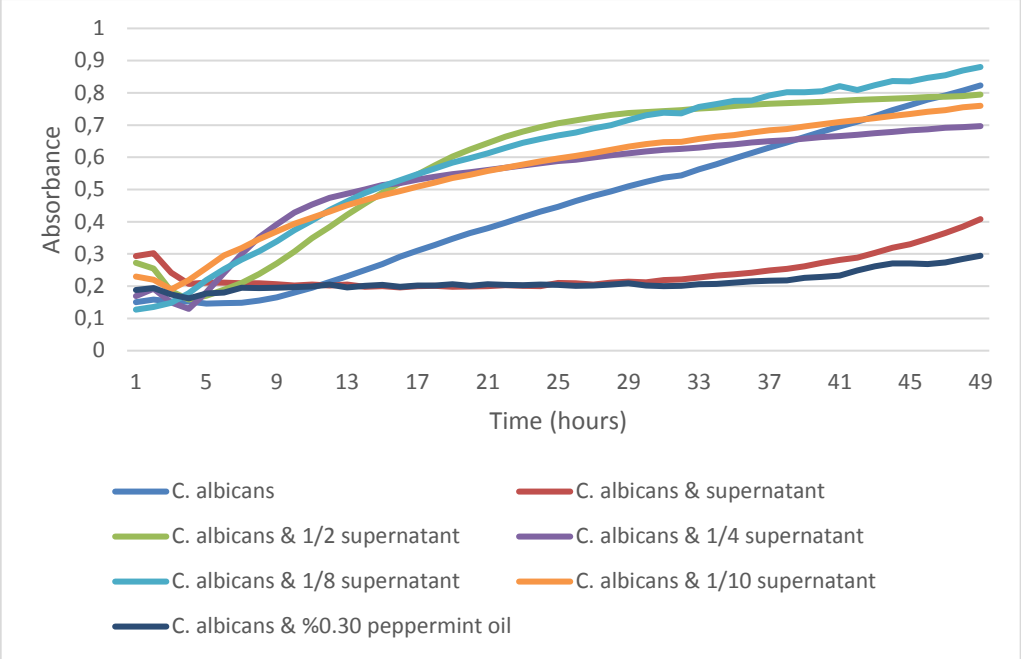

Figure 6.4. Inhibition of *C. albicans* by supernatant of *L. pentosus* at different concentrations

To determine whether the peppermint oil could exert an inhibitory effect on *S. mutans* and *C. albicans* interfere with the results, control group consisted only of *S. mutans* and 0.30% peppermint oil. As a result, peppermint oil has shown significant inhibitory effect on *S. mutans* (Figure 6.3).

After 30 h, *Lactobacillus* supernatant presented the largest fall in the measurement of optical density *C. albicans* cells determined by the absorbance value. *Lactobacillus* supernatant with different dilutions (1:1, 1:2, 1:4, 1:8, 1:10) and peppermint oil (%0.30) showed scattered absorbance curve (Figure 6.4), it was found that only 1:1 supernatant and peppermint oil had significant inhibitory effect on *C. albicans*.

These results indicated that *L. pentosus* released bioactive compounds, which can inhibit pathogens; *S. mutans* and *C. albicans* growth. Because of the great clinical importance of *S. mutans* amount in caries and *C. albicans* cells in candidiasis, as the method was demonstrated in this thesis, indicated the efficiency of the *Lactobacillus* supernatant on the growth of *S. mutans* and *C. albicans*.

Peppermint oil (*Menthae piperitae aetheroleum*) is obtained from fresh mint leaves. It is commonly used in pharmaceutical formulations, food products and cosmetic products. It heals headache, muscle pain, nerve pain, toothache and cures mouth inflammation, arthritis, itching, allergic rashes (Koo, Cha, Song, Chung, & Pan, 2013). Effect of peppermint oil on the pathogens mentioned above was used as a flavoring agent in lozenge formulation. It was also investigated with the broth microdilution method and its antimicrobial effect was confirmed.

6.1.3. Agar Overlay Test

In contrast to the method of agar disc diffusion method, the agar overlay method revealed inhibition zones of pathogens using *L. pentosus* NRRL-B 227. The zone diameters for *S.mutans* and *C. albicans* were measured as 15mm and 13mm, respectively and shown in Figure 6.5.

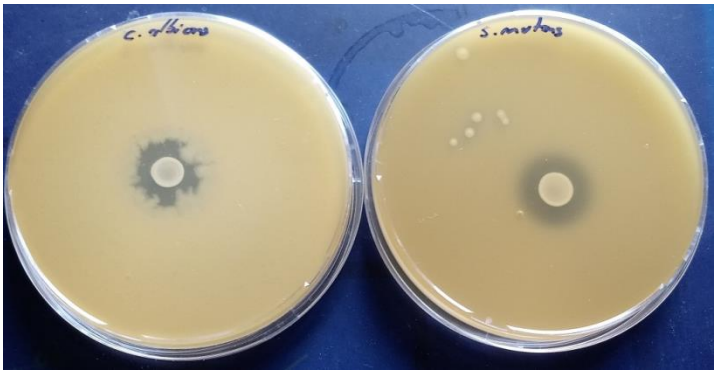

Figure 6.5. Inhibition zones of *C. albicans* and *S. mutans* caused by *L. pentosus* with agar overlay test

Simark-Mattsson, et al., (2007) evaluated the antimicrobial activity of *Lactobacillus* strains isolated from individuals with caries and non-caries using agar overlay interference tests. In the study, *Lactobacillus* strains were isolated from subjects without caries had

more inhibitory activity than *Lactobacillus* strains isolated from those with active caries against *S. mutans*. *L. paracasei*, *L. rhamnosus* and *L. plantarum* were selected since they have the highest antibacterial activity, and were able to completely inhibit *S. mutans* growth.

In order to prevent the formation of caries, it was studied to interfere with cariogenic pathogens colonization with probiotics. Nase, et al., (2001) in order to inhibit pathogen inhibition *in vivo*, *L. rhamnosus* GG tested and in the test group, less tooth decay and lower *S. mutans* were found. In addition, studies with *L. rhamnosus*, *Bifidobacterium* (Çağlar, et al., 2005), *Lactobacillus reuteri* (Çağlar, Kayaloğlu Çıldır, Ergeneli, Sandallı, & Twetman, 2006), *B. animalis* (Çıldır, et al., 2009), *L. paracasei* (Holz, et al., 2013) and *Lactobacillus casei* (Busscher, Mulder, & Van der Mei, 1999) have confirmed that the number of pathogens can be reduced, thus preventing dental caries.

6.1.4. Antibacterial Activity of *L. pentosus* Supernatant against Pathogens in Planktonic Cultures

L. pentosus NRRL-B 227 was screened for antimicrobial activity against *S. mutans* and *C. albicans* using planktonic cultures assay. For this aim, the indirect effect of *L. pentosus* was analyzed using the *L. pentosus* supernatant. *S. mutans* + *L. pentosus* supernatant interaction group was allowed to incubate for 24 and 48 h in BHI broth. For *C. albicans* + *L. pentosus* supernatant interaction group, *C. albicans* was incubated with *L. pentosus* supernatant for 24 and 48 h in Nutrient Broth. As a control, monoculture of *S. mutans* and *C. albicans* were also incubated.

After 24 or 48 hours in culture, *S. mutans* and *C. albicans* growth were determined by counting the colony-forming units (CFU/mL). Antimicrobial activity assay showed that control culture incubations of both species (without *L. pentosus* supernatant) contained $>10^6$ CFU/mL, although cultures containing 0.25 mg *Lactobacillus* supernatant showed no growth.

At the end of 24 hours with the broth microdilution method, the increase in the absorbance value of *C. albicans* may be the turbidity of the dead cells. This was confirmed by the study of planktonic culture. Analysis results are shown in Figure 6.6.

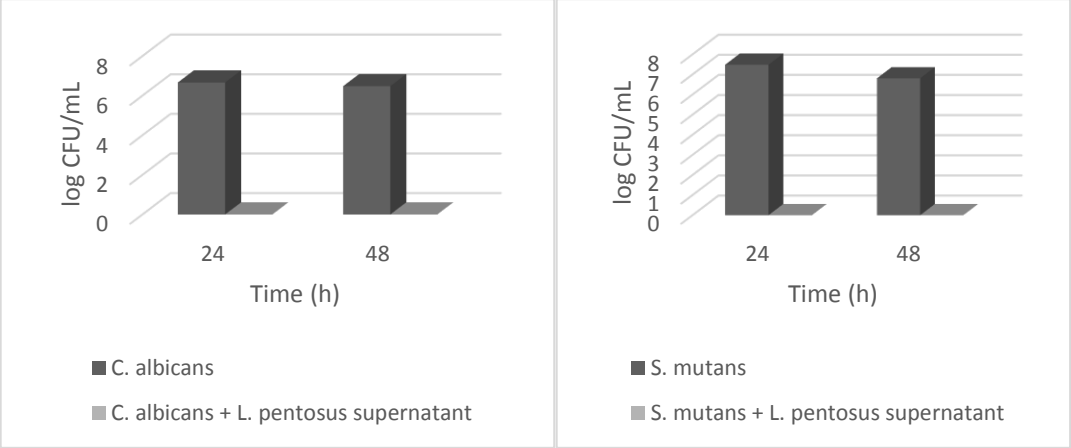

Figure 6.6. Planktonic growth of *Streptococcus mutans* and *Candida albicans* in the presence of *Lactobacillus pentosus* supernatant at the indicated concentration for 24 and 48 hours. Controls include growth medium without lactobacillus supernatant

In a recent study similar to this research, it has been observed that the highest antibacterial action against *S. mutans* with three species of *L. paracasei* and *L. fermentum*. These species reduced growth of *S. mutans* by more than 86% after 24 hours in planktonic culture (Rossoni, et al., 2018).

Dental caries and candidiasis are two common human infectious diseases. Recently, interest in the using probiotic cells for curation of these oral infections has increased. Before performing *in vivo* studies, a number of *in vitro* experimentation is required to identify a probiotic candidate. According to the results of various studies, the most powerful inhibitory effect was seen in *L. paracasei, L. plantarum, L. rhamnosus* and *L. brevis* (Koll-Klais, et al., 2005); (Koll, et al., 2008).

In this study, the use of *L. pentosus* NRRL-B 227 was preferred in contrast to the frequent probiotics that were frequently encountered in the literature. The lactic acid bacterium inhibited both oral pathogens tested by various methods. *L. pentosus* is a

probiotic with a strong anti-Candida activity, which has a significant antibiofilm activity that can be used not only in the food industries, but also in a wide range of applications as a biotherapeutic agent in the pharmaceutical industries (Aarti, et al., 2018). Mojgani, Hussaini, & Vaseji, (2015) observed high aggregation and adhesion features of *L. pentosus*. The probiotic also exhibits strong antimycotic action against *Candida albicans, Candida tropicalis* and *Candida krusei*. With similar studies were reported that *L. pentosus* has fungistatic effect against *Candida* (Okkers, Dicks, Silvester, Joubert, & Odendaal, 1999); (Voulgari, et al., 2010). *L. pentosus* has anti-pathogenic effect against some bacteria; *Salmonella, Escherichia coli* as well as antifungal effect against Aspergillus *oryzae* and *Aspergillus niger* (Casey, et al., 2004)*;* (Muhialdin, Hassan, & Sadon, 2011)*;* (Mogna, et al., 2012). A study showed that *L. pentosus* secretes a large amount of metabolites, which have a broad spectrum of anti-*Helicobacter pylori* activity; it is so important since *H. pylori* is multidrug-resistant (Zheng, et al., 2016). The results indicate that the probiotic strain can be used as an antibiotic-resistant probiotic with high aggregation properties and significant hydrophobicity, with resistance to low pH in simulated gastric juice and bile salt media (Aarti et al., 2018). *L. pentosus* is involved in the fermentation phase of many fermented products such as olives (Abriouel, Benomar, Perez-Pulido, Canamero, & Galvez, 2012). Thus, its daily consumption can be attained for all, and its addition to a functional product such as lozenge will be important to reach the amount of probiotic to be taken.

Recent reports suggest that dead cells or cell components can provide health-promoting effects, as well as live probiotic microorganisms (Sanders, 2003). In this study, it has been shown that not only live probiotic bacteria, but also lactobacilli supernatant can increase oral health by inhibiting pathogenic oral microorganisms.

6.2. Viable Cells Counts after Microencapsulation

The minimum amount of probiotic microorganisms to be beneficial for health is 6.0 log CFU/g (Prado, Parada, Pandey, & Soccol, 2008). No significant decrease was observed in the viability of *L. pentosus* NRRL-B 227 maintained at refrigerator temperature for 4

months by microencapsulation using the emulsion method ($p<0.05$). The initial cell count of *L. pentosus* was 11.05 log CFU/g, the cell count after microencapsulation was average 8,60 log CFU/g, so the efficiency of the microencapsulation process was 78-77%, maintained for 4 months as shown in Table 6.3.

Table 6. 3. Survival of *Lactobacillus pentosus* NRRL-B 227 after microencapsulation for 4 months

Time (months)	**Cell viability after microencapsulation (log CFU/g)**	**Cell survivability (%)**
0	8.6336 ± 0.0925^{A}	78.1320 ± 0.01^{A}
1	8.6672 ± 0.0198^{A}	78.4361 ± 0.012^{A}
2	8.5586 ± 0.0756^{A}	77.4534 ± 0.027^{A}
3	8.5862 ± 0.0146^{A}	77.7032 ± 0.03^{A}
4	8.5726 ± 0.0386^{A}	77.5801 ± 0.08^{A}

Means with different superscripts (A and B) within a column were significantly different ($P<0.05$).

Microencapsulation of *L. acidophilus* NRRL B-4495 was performed using the same emulsion technique. Initially, the amount of bacteria with 9.51 log CFU/g decreased to 7.87 log CFU/g at the end of 30 days and had a survival rate of 82 percent. The results of the study showed that microencapsulated cells with whey protein / pullulan complex, showed high resistance to bile salts and simulated gastric acid juice. (Çabuk & Harsa, 2015) .

The live numbers of concentrated and microencapsulated and after freeze-dried *L. pentosus* were 9.0 log CFU/g before being added to the probiotic lozenge formulation. The formulations were added in sufficient amounts to reach 7.0 log CFU/g of lozenges in CPL and FPL.

The each lozenge weight is about 3.0 g. A daily consumption one lozenge will be sufficient to reach 7.0 log CFU/g live probiotic cells, recommended to consume. Probiotic lozenges are expected to have a local effect in the oral cavity.

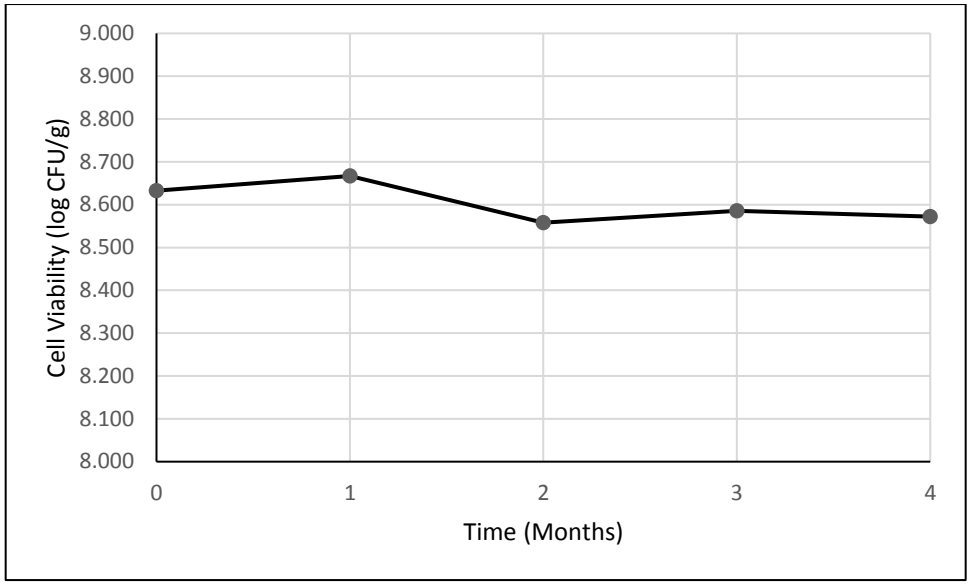

Figure 6.7. Survival of *L. pentosus* NRRL-B 227 after microencapsulation for 4 months

6.3. Microbiological Evaluation of Lozenges

Lozenge samples were evaluated microbiologically; determination of viable probiotic counts in lozenge formulations and investigation of safety of formulations.

6.3.1. Viable Counts

Survival of free and microencapsulated *L. pentosus* in the formulations is demonstrated in Table 6.4 and Figure 6.7. The live counts in free probiotic lozenge (FPL) formulations were decreased below 6.0 log CFU/g at the initial time. After 7 days, probiotic viability significantly decreased in both free probiotic formulations, which storage at refrigerator and room temperature, thus, the vitality tracking ended. The cell counts in capsulated probiotic lozenge (CPL) formulation sustained stability that might exhibit a protective microencapsulation effect. Viability of the cell stability was expected in CPL

formulations, because the microencapsulated cells represented high vitality for four months, at refrigeration temperature.

Table 6.4. Cell survival of free and microencapsulated *L. pentosus* NRRL-B 227 in lozenge at different temperatures for 90 days

Time(days)	CPL (-4ºC – 4ºC)	CPL (20ºC – 25ºC)	FPL (-4ºC – 4ºC)	FPL (20ºC – 25ºC)
0	7.8418 ± 0.013^{abA}	7.1590 ± 0.063^{cB}	5.6765 ± 0.019^{aA}	6.1210 ± 0.313^{aA}
7	7.8964 ± 0.046^{aB}	8.5501 ± 0.008^{aA}	4.2054 ± 0.001^{bA}	3.3865 ± 0.128^{bB}
14	7.8903 ± 0.055^{aB}	8.5127 ± 0.094^{aA}	-	-
21	7.7274 ± 0.040^{bA}	7.3512 ± 0.020^{cB}	-	-
28	7.8502 ± 0.043^{abA}	7.8165 ± 0.075^{bA}	-	-
42	7.7793 ± 0.038^{abA}	3.451 ± 0.1670^{dB}	-	-
56	7.7135 ± 0.021^{b}	-	-	-
70	7.7763 ± 0.012^{ab}	-	-	-
90	7.7312 ± 0.028^{b}	-	-	-

Means with different superscripts (a–c) within a column were significantly different ($P < 0.05$)

Witzler, Pinto, Valdez, Castro, & Cavallini, (2017) carried out a study on the production of a probiotic lozenge contain of *Enterococcus faecium* CRL 183. In their study microencapsulated cells with using extrusion technique maintained their viability for a long time, but in probiotic lozenge formulation viable counts showed a very slight reduction. Conversely, in a study by Toiviainen, et al., (2015) good viability results were obtained during the 4 weeks of treatment focusing on adult's oral microbiota, using sorbitol and xylitol tablets as *Bifidobacterium lactis* and *Lactobacillus rhamnosus* vehicle. Çağlar, Kuşçu, Çildir, Kuvvetli, & Sandallı, (2008) used isomalt lozenges as *Lactobacillus reuteri* vehicles, for improving of women oral microbiota and viability of bacterium was 8.0 log CFU/g throughout the 10-day treatment period.

6.3.2. Microbiological Safety

Microbiological safety of lozenges remained stable during their storage, were not observed neither *Escherichia coli,* nor molds/yeasts. These results were shown the

suitability of processing and conformity to the Turkish Food Codex about food additives section.

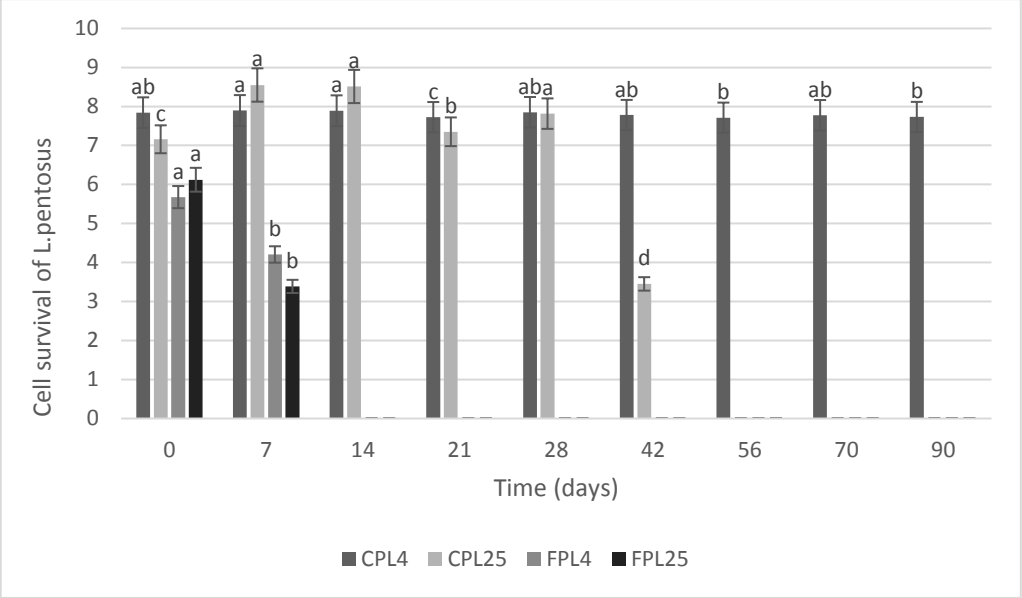

Figure 6.8. Cell survival of free and microencapsulated *L. pentosus* NRRL-B 227 in lozenge at different temperatures for 90 days

6.3.3. Physicochemical Evaluations

The results of lozenges' physicochemical evaluations are shown in Table 6.5. The color, moisture content (%), water activity and pH values showed differences among formulations CPL, FPL and CL ($p < 0.05$).

Adding the probiotic strain did not affect the lozenges luminosity. However, color analysis results showed CPL and CL formulations significantly lower a* parameter than FPL. On the other side, CPL formulation exhibited significantly higher b* parameter ($p < 0.05$).

The lowest levels of moisture content observed in CPL is probably related to containing encapsulated cells, but there was no significant differences among lozenges formulations.

When water activity values around 0.90 represent susceptibility to bacteria growth and around 0.80 represent ability to growth of molds and yeasts. To prevent microbial growth in foods, values below 0.60 are recommended. Recommended water activity range is 0.40 - 0.75 for lozenges (Bussiere & Serpelloni, 1985). According to Table 3, the water activities of lozenges were at suitable levels.

Lozenge formulations pH values did not demonstrate any significant differences; pH values changed 4.5 - 5.2.

Table 6.5. Physicochemical properties of lozenge formulations (CPL, FPL and CL)

	CPL	**FPL**	**CL**
Color	L*93.605± 0.1013^{A} a*-0.700± 0.03296^{B} b*1.9583± 0.1202^{A}	L*94.9833±0.18867^{A} a*-0.4766 ± 0.02359^{A} b* 0.69833 ± 0.1248^{B}	L*93.635±0.57767^{A} a*-0.6716 ± 0.03063^{B} b* 0.765 ± 0.03536^{B}
Moisture Content (%)	5.5020 ± 0.2090^{A}	5.8440 ± 0.7410^{A}	6.0560 ± 0.6030^{A}
Water Activity	0.4695 ± 0.0007^{AB}	0.6115 ± 0.0035^{A}	0.3880 ± 0.0834^{B}
pH	4.5550 ± 0.6580^{A}	5.1750 ± 0.1630^{A}	5.0850 ± 0.2330^{A}

Results are shown as means ± standard deviation.

Different capital letters on the same line show a significant difference by the Tukey's test ($p<0.05$).

CPL - Probiotic lozenge formulation, with the microencapsulated *L. pentosus*.

FPL - Probiotic lozenge formulation, with the *L. pentosus* free cells.

CL - Control lozenge formulation, without the probiotic strain.

L* = luminosity, black - white.

a* = green - red.

b* = blue - yellow.

6.3.4. Sensory Evaluation

Sensory evaluation of lozenges was performed during initial storage. The acceptability is shown in Table 6.6 and Figure 6.7.

Table 6.6. Sensory evaluation results of lozenge formulations CPL, FPL and CL

	CPL	FPL	CL
Appearance	3.069 ± 1.163^{A}	3.552 ± 1.021^{A}	3.724 ± 1.162^{A}
Color	3.655 ± 1.203^{A}	3.931 ± 1.067^{A}	3.966 ± 0.981^{A}
Flavor	3.310 ± 1.039^{A}	3.379 ± 0.942^{A}	3.172 ± 1.071^{A}
Taste	2.690 ± 1.312^{B}	3.517 ± 1.056^{A}	3.379 ± 1.147^{AB}
Texture	3.172 ± 1.104^{A}	3.724 ± 0.882^{A}	3.517 ± 0.785^{A}
Overall Acceptance	3.138 ± 1.217^{A}	3.483 ± 0.949^{A}	3.310 ± 0.930^{A}

Results are shown as means ± standard deviation.

Different capital letters on the same line show a significant difference by the Tukey's test ($p<0.05$), n = 30.

CPL - Probiotic lozenge formulation, with the microencapsulated *L. pentosus*.

FPL - Probiotic lozenge formulation, with the *L. pentosus* free cells.

CL - Control lozenge formulation, without the probiotic strain.

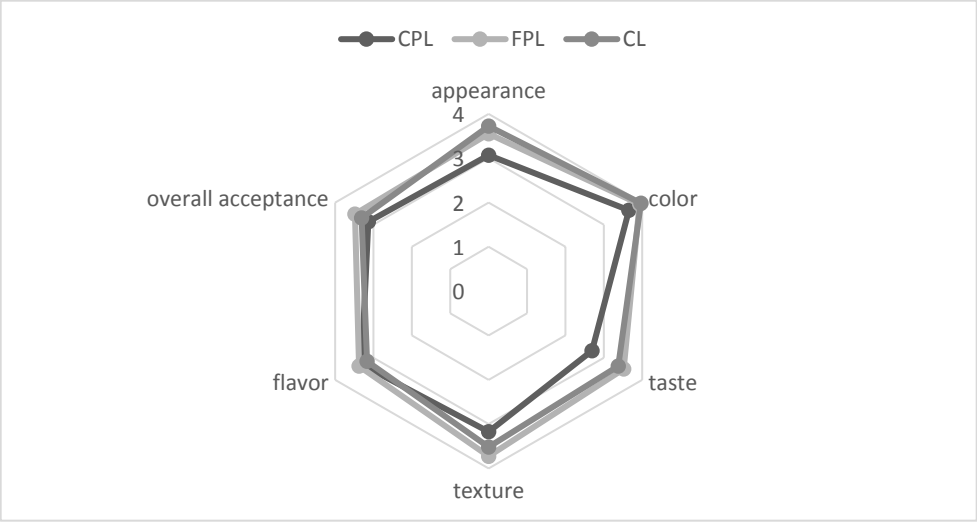

Figure 6.9. Spider diagram showing the results of sensory analysis

All formulations; CPL, FPL and CL were evaluated by panelists with above 3.0 points, ranging from “neither like nor dislike” to “like moderately”, however, encapsulated cell lozenge formulation achieved 2.69 for the taste. Consumers have stated that they have a positive purchase intention (certainly or possibly buy the product) for all formulations. The sensory analysis form performed is in Appendix C.

Lozenge formulations with encapsulated cells, free cells and no cells had inferior averages on appearance, color, flavor and texture, except taste ($p < 0.05$). This has been perceived as a defect by panelists as the addition of micro-encapsulated *L. pentosus* to lozenges has changed the taste of lozenges. However, the overall impression was that there was no difference between the formulations ($p < 0.05$).

CHAPTER 7

CONCLUSION

Various strains of lactic acid bacteria and different antimicrobial activity tests were used to investigate bacterial inhibition of oral pathogens. *Lactobacillus pentosus* NRRL-B 227 specifically inhibit species of *Streptococcus mutans* and *Candida albicans*, the probiotic would be expected to decrease the plaque biofilm development and therefore tooth decays and decrease the candidiasis risk without disruption of the normal oral microflora. Although the nature of antimicrobial activity remains unclear, *in vitro* assays of the antibacterial properties of *L. pentosus* have shown that it can be considered as a probiotic for the improvement of oral health. The microencapsulated *L. pentosus* stored at 4°C significantly retained its viability, and in the lozenge formulation there was only 0.11 log CFU/g decrease at the end of three months. On the other hand, probiotic lozenges stored at 25^0C cells did not survive, their viability decreased slightly after one month. In addition lozenges including free cells have lost viability rapidly. Microbiological safety of lozenges remained stable during their storage. In conclusion, the current study establishes a foundation for the production of probiotic lozenge that can be consumed by everyone, which can potentially be used to improve oral health that prevent the growth of oral pathogens without disrupting the balance of a healthy oral microflora. In future studies, the efficacy of lozenge can be assessed in simulated mouth and saliva media and in vivo studies.

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APPENDIX A

GROWTH CURVE

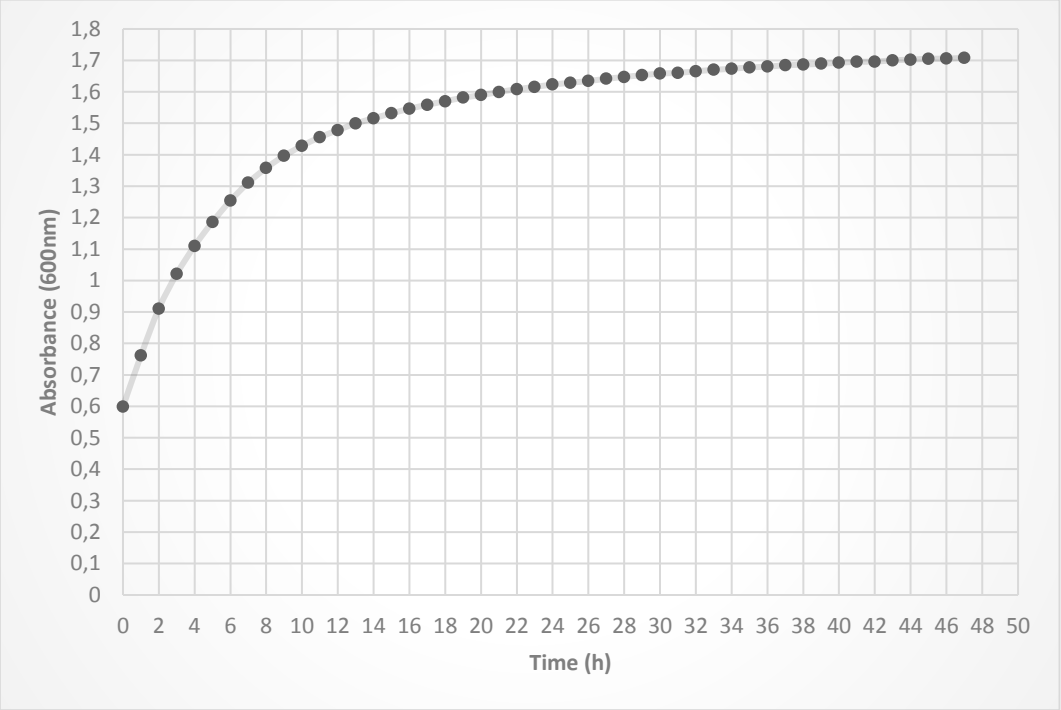

Figure A.1. Growth curve of *Lactobacillus pentosus.* NRRL-B 227

APPENDIX B

MICROSCOPIC IMAGE

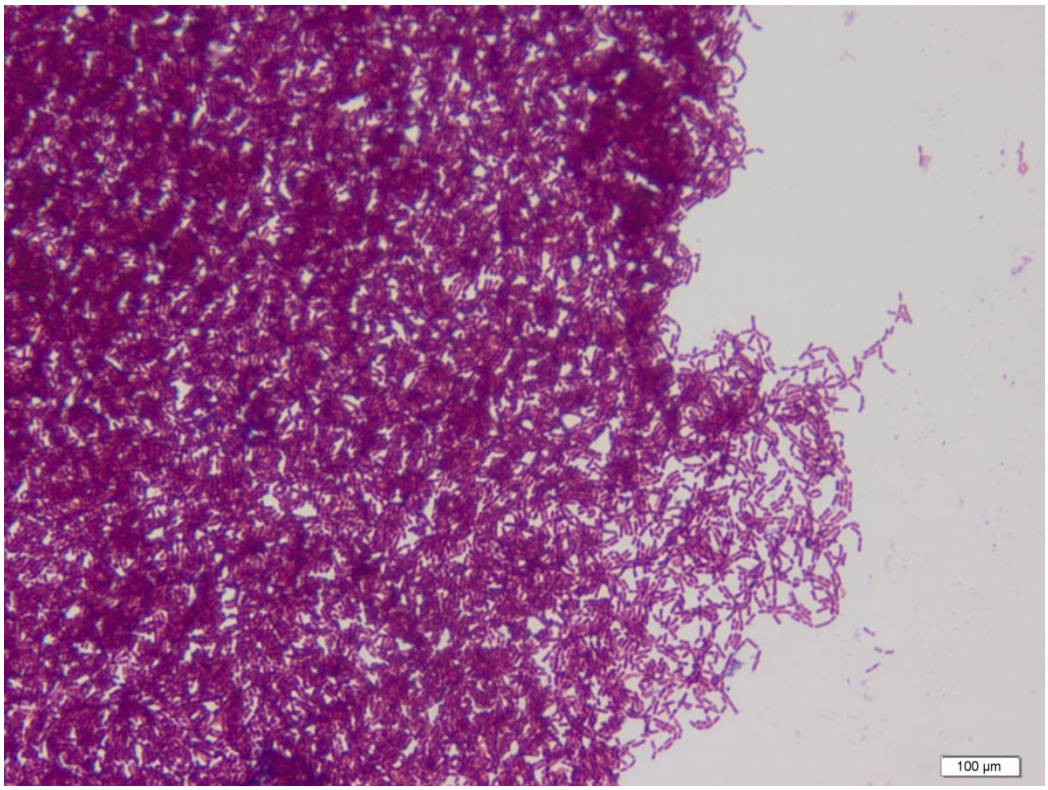

Figure B.1. Microscopic image of *Lactobacillus pentosus* NRRL-B 227

APPENDIX C

SENSORY EVALUATION TEST

Panelist Number_____

Dear Panelist,

In front of you are three lozenge samples. Taste the samples and sign (X) how much you like or dislike each of the characteristics. You can taste the samples more than once**.**

Lozenge Number: _____

1. Please taste the sample and sign the box that best describes how you feel about its appearance.

☐	☐	☐	☐	☐
Like very much	Like moderately	Neither like nor dislike	Dislike moderately	Dislike very much

2. Please taste the sample and sign the box that best describes how you feel about its color.

☐	☐	☐	☐	☐
Like very much	Like moderately	Neither like nor dislike	Dislike moderately	Dislike very much

3. Please taste the sample and sign the box that best describes how you feel about its flavor.

☐	☐	☐	☐	☐
Like very much	Like moderately	Neither like nor dislike	Dislike moderately	Dislike very much

4. Please taste the sample and sign the box that best describes how you feel about its texture/mouth feel.

☐	☐	☐	☐	☐
Like very much	Like moderately	Neither like nor dislike	Dislike moderately	Dislike very much

5. Please taste the sample and sign the box that best describes how you feel about its taste.

☐	☐	☐	☐	☐
Like very much	Like moderately	Neither like nor dislike	Dislike moderately	Dislike very much

6. Please taste the sample and sign the box that best describes how you feel about its overall acceptance.

☐	☐	☐	☐	☐
Like very much	Like moderately	Neither like nor dislike	Dislike moderately	Dislike very much

7. Please sign the box that best describes how you feel about buying this product.

☐	☐	☐	☐	☐
Would buy certainly	Would buy moderately	Neither buy nor do not buy	Would not buy moderately	Would not buy certainly

Panelist Age:

Panelist Sex:

Your Opinion:

Figure C.1. Sensory evaluation test