REAL-TIME PCR AS A MOLECULAR TOOL FOR THE ENUMERATION OF PROBIOTICS IN COMMERCIAL PRODUCTS

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ABSTRACT

REAL-TIME PCR AS A MOLECULAR TOOL FOR THE ENUMERATION OF PROBIOTICS IN COMMERCIAL PRODUCTS

Quantitative Real-Time PCR (qPCR) assays targeting the 16S rDNA was developed as a genus and species specific detection tool for *Bifidobacterium and Lactobacillus, and Bifidobacterium animalis* subsp. *lactis* BB-12 and *Lactobacillus acidophilus* LA-5, respectively. Standard curves were established to quantify these probiotic bacteria. The linear regression of standard curves indicated high correlations between the log numbers of pure probiotic culture cells and the C_t values. The assay had a high efficiency and the limit of detection was estimated to be 1.54 ng DNA (corresponding to 10^4 cells). Results show that qPCR method may be very useful as a rapid, sensitive and specific tool for detecting and quantifying *B. animalis* subsp. *lactis* BB-12 and *L. acidophilus* LA-5 in probiotic supplements.

FTIR spectroscopy was used for the first time to determine the ratios of different microorganisms in commercial probiotic supplements. FTIR analysis was also performed for the pure probiotic cultures of *B. animalis* subsp. *lactis* BB-12 and *L. acidophilus* LA-5. Results obtained in this study showed that FTIR spectroscopy is potentially a rapid method for determining probiotic cell components and their ratios in the supplements and verification their detection and identification.

ÖZET

TİCARİ ÜRÜNLERDEKİ PROBİYOTİKLERİN SAYIMI İÇİN MOLEKÜLER BİR ARAÇ OLARAK GERÇEK ZAMANLI PCR

16S rDNA'yı hedefleyen Kantitatif Real-Time PCR (qPCR) analizleri sırasıyla *Bifidobacterium*, *Lactobacillus* ve *Bifidobacterium animalis* subsp. *lactis* BB-12, *Lactobacillus acidophilus* LA-5 cins ve tür spesifik saptama aracı olarak geliştirilmiştir. Standart eğriler bu probiyotik bakterilerin sayısını ölçmek için oluşturuldu. Standart eğrilerin lineer regresyonu probiyotik kültür hücrelerinin log sayılarıyla Ct değerleri arasında yüksek korelasyon göstermektedir. Analiz yüksek verimlidir ve saptama limiti 1,54 ng DNA (10^4 hücreye karşılık gelen DNA miktarı) olarak tahmin edilmiştir. Sonuçlar qPCR yönteminin *B. animalis* subsp. *lactis* BB-12 ve *L. acidophilus* LA-5 probiyotik takviyelerinin saptanması ve miktarının belirlenmesi için hızlı, duyarlı ve spesifik bir araç olarak çok yararlı olabileceğini göstermektedir.

FTIR spektroskopisi ticari probiyotik ürünlerdeki farklı mikroorganizmaların oranlarını belirlemek için ilk defa kullanılmıştır. FTIR analizi ayrıca *B. animalis* subsp *lactis* BB-12 ve *L acidophilus* LA-5 saf probiyotik kültürler için yapılmıştır. Bu çalışmada elde edilen sonuçlar, FTIR spektroskopisinin probiyotiklerin hücre bileşenleri ve bu bileşenlerin oranlarının belirlenmesinde ve ayrıca saptanması ve tanımlamasında hızlı bir yöntem olarak potansiyeli olduğunu göstermiştir.

It is dedicated to my mother.

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LIST OF ABBREVIATIONS

subsp. :	Subspecies
spp :	Species
min :	Minute
g :	Gram
mg :	Miligram
L :	Liter
µl :	Microliter
h :	Hour
rpm :	Round per minute
LAB :	Lactic Acid Bacteria
B. :	*Bifidobacterium*
L. :	*Lactobacillus*
EPS :	Exopolysaccharide
cfu :	Colony forming unit
UNG:	Uracil N-glycosylase
Mb :	Megabase
DNA :	Deoxyribonucleic Acid
RNA :	Ribonucleic Acid
dNTP :	Deoxynucleotide triphosphate
kbp :	Kilo base pair
PCR :	Polymerase Chain Reaction
MRS :	de Man, Rogosa and Sharpe Medium
EDTA :	Ethylene Diamide Tetra Acetic Acid
TE :	Tris-EDTA
TAE :	Tris Acetate EDTA
SDS :	Sodium Dodecyl Sulfate
G:	Probiotic Gold
Atp:	Probiotic ATP
SEM:	Scanning Electron Microscopy

CHAPTER 1

INTRODUCTION

1.1. Probiotics

Probiotics word is from Greek origin and its mean is "for life". Probiotics terms were used first time in 1965 by Lilly and Stillwell to identify substance that supports other microorganisms' growth. These terms were described in fields ranging from history until today finally defined as when ingested adequate amounts they have positive effects in gastrointestinal tract on the host (Gomes and Malcata 1999). First fermented food beneficial effects on the metabolism have been suggested by Nobel prize-winner Russian scientist Elie Metchnikoff in the early 20th century. Although the beginning of fermented products studies are very old, studies on probiotics has increased rapidly in the last 10-15 years (Sanders 1999). Probiotics are living organisms that beneficially affect the health of the host by sustaining a balanced intestinal flora and stimulating its immune system. Dead bacteria, products isolated from bacteria, or end products of bacterial growth also may give lots of benefits, but these derivatives are not regarded to be probiotics since they are not alive when administered. Probiotics are isolated generally from human and animal intestinal tracts. Native bacteria are not consider as probiotics until the bacteria are isolated, purified, and demonstrated to have a health benefit when administered. Nowadays many microorganisms are used as probiotics. Most commonly used as probiotics are the group of lactic acid bacteria consist of *enterococci*, *lactobacilli* and *bifidobacteria* (Salminen, Ouwehand, Isolauri 2002). Key microbial species used as human probiotics are listed in Table 1.1.

There are a various array of probiotic products on the market worldwide. Yogurt sometimes can contain probiotic bacteria, but the market has expanded beyond yogurt. Cheese, fermented and unfermented milks, juices, smoothies, cereal, nutrition bars, and infant/toddler formula all are food vehicles for probiotic transfer. Also probiotics are sold as dietary supplements, medical foods, and drugs. Probiotic microorganisms are usually from the *Lactobacillus* and *Bifidobacterium* genus, and may contribute to our

health in many different ways. Shortening of the duration of rotavirus diarrhea, relief of signs and symptoms of lactose intolerance, decreasing the risk of allergy in atopic individuals, cancer prevention, lowering of serum cholesterol levels, prevention of urogenital infections, and synthesis and enhancement of the bioavailability of nutrients are some benefits that connected with probiotics (Coşkun 2006).

Table 1.1. Key genera and species of microbes studied and used as probiotics (Source: Sanders, Morelli, and Bush 2001)

Genus	Species
Lactobacillus	*acidophilus* *brevis* *delbruelckii* *fermentum* *gasseri* *paracasei* *plantarum* *reuteri* *rhamnosus* *salivarus*
Bifidobacterium	*adolescentis* *animalis* *bifidum* *infantis* *lognum*
Streptecoccus	*thermephilus* *salivarus*
Enterococcus	*faecium*
Escherichia	*coli*
Bacillus	*coagulans* *clasuii*
Saccharomyces	*cerevisiae*

1. 1. 1. Regulatory Considerations of Probiotics

In both the USA and Europe, supermarkets and pharmacies are brimming with probiotics - products containing live micro-organisms claiming they improve health. For a number of products, claims are based on insufficient research, underpowered studies, or mixed research results, yet individual consumers find that the products have benefit to them. The use of probiotics in products can result in several regulatory categories, 4 of which are fundamental: “food” or food ingredient, “medical food,” “dietary supplement,” and “drug” or “biological product.” Foods are the only FDA-regulated

product categorized not by “intended use” but rather simply by “use”; a “food” is circularly defined as “an article used for food” (Degnan, 2008). Probiotics are microbes that are claimed to promote health and well-being when added to foods. However, the European Food Safety Authority (EFSA) has so far advised negatively about health claims for probiotics. European regulatory framework is still not harmonized as probiotics are regulated by the Food Products Directive and Regulation if to be marketed as food supplement (Regulation 178/2002/EC; Directive 2000/13/EU) and under Herbal Medicinal Products Directive (2004/24/EC) if marketed as traditional herbal products.

1. 1. 2. Efficiency of Probiotics

There are many impacts of probiotics on human health. Probiotics activate as phosphatase that increase the absorption of the protein in breast milk. Some species produce vitamin B1, vitamin B12, vitamin C and folic acid. The production of this vitamin increases the nutritional properties of fermented milk products. In vitro conditions, some probiotics has shown antibacterial activity against to *E. coli, S. aureus, S. typhi, S. dysenteriae, C. albicans* species. Probiotics of proven antibacterial activity originate from producing organic acids such as lactic acid and acetic acid and also bacteriostatic agents and peroxides. These acids inhibit proliferation of microorganisms by lowering the pH in the intestinal environment. Probiotics shows beneficial effects by regulating the balance microbial intestinal tract of humans and animals. They reduce the number of pathogenic bacteria by producing antimicrobial compounds, competing for colonization and competing for nutrients. They can also change microbial metabolism (enzymatic activity) by producing of enzymes that regulate the digestive system, reducing ammonium, amine and toxic enzymes production, improving the function of the intestinal wall. They increase of antibody titers and macrophage activity to improve the immune system. And there are also therapeutic effects of probiotics. For example; probiotics produce anti-tumorigenic or anti-mutagenic compounds and inhibits the formation of cancer, while generation of possible carcinogens, regulate of intestinal micro flora (Rafter 2002, 2003). Probiotics strengthen the protective mucosal barrier in the intestinal system. Thus reducing gut permeability, inhibit passing of allergic substances to blood. And also it has been determined that probiotics reduce serum

cholesterol levels in mice and pigs (Grunewald, 1982; Gilliland et al., 1985). Other effects of probiotics are prevention of pathogens, stabilization the flora of the urogenital system and ensuring that restructuring comes, activating the serum immune systems (Roberfroid, 2000).

1. 1. 3. Genus *Bifidobacterium*

Bifidobacterium is a genus of gram-positive, non-motile, often anaerobic bacteria. They generally live in gastrointestinal tract, vagina (Schell 2002, Mayo 2010) and mouth (*B. dentium*) of mammals, including humans. *Bifidobacteria* are one of the major genera of bacteria that constitute the colon flora in mammals. Some *Bifidobacteria* are used as probiotics. *Bifidobacterium adolescentis, Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium longum, Bifidobacterium thermophilum* species are most commonly use as probiotic. As with all probiotics, *Bifidobacterium* is only classified as a probiotic when it is: administered live; capable of surviving the administering process and afterwards growing; and administered in an amount demonstrated to provide health benefits on the host. There are lots of physiological effects and clinical benefits of *Bifidobacteria*. They may protect the host from potentially harmful substance have been proposed, by production of inhibitory substances, blockade of adhesion sites and stimulation of immunity (Lievin 2000). They stimulate the immune system through non-specific modes of action, resulting in enhance immune responsiveness to a wide variety of antigens. They can also protect host from carcinogenic activity by reducing the production and/or activity of potential carcinogens (Orrhage 1994, Saikali 2004).

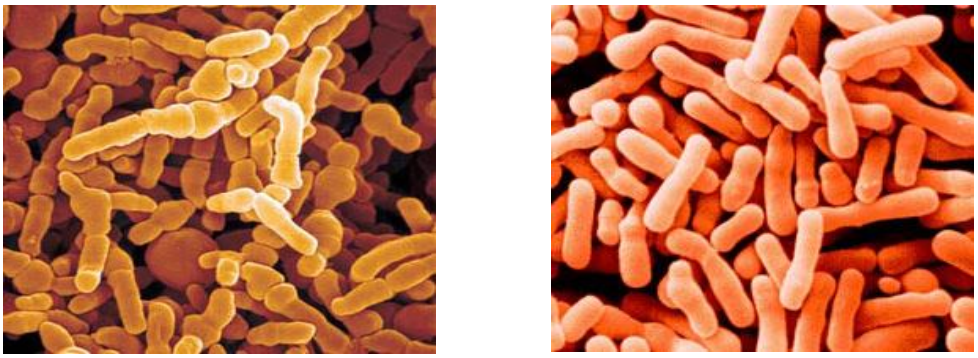


Figure 1.1. *Bifidobacterium animalis-lactis, Bifidobacterium-infantis*

1.1.4. Genus *Lactobacillus*

Lactobacillus is a genus of gram-positive facultative anaerobic or microaerophilic rod-shaped bacteria (Figure 1.2.) (Makarova 2006). They are a main part of the lactic acid bacteria group. In humans and mammals they are part of the vaginal microbiota (Petrova at al. 2015). Lots of *Lactobacillus* species are considered as probiotic like *Lactobacillus bulgaricus, Lactobacillus lactis, Lactobacillus acidophilus, Lactobacillus gasseri, Lactobacillus cellebiosus, Lactobacillus delbrueckii, Lactobacillus reuteri, Lactobacillus curvatus, Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus johsonli, Lactobacillus rhamnosus, Lactobacillus helveticus, and Lactobacillus salivarius.* Some *Lactobacillus* species are used as starter cultures in industry for controlled fermentation in the production of yogurt, cheese, sauerkraut, pickles, beer, wine, cider, kimchi, cocoa, kefir and other fermented foods, as well as animal feeds. The antibacterial and anti-fungal activities of "*Lactobacillus*" rely upon production of bacteriocins and low-molecular weight compounds which inhibits these microorganisms (Inglin, Raffael C. 2015). Most *Lactobacillus* species in mammals are regarded as harmless. *Lactobacilli* live in the urinary, digestive and genital tracts of mammals. *Lactobacillus* is probably effective for preventing diarrhea in children, or bacterial vaginal infections. Some strains of *Lactobacillus* spp. and other lactic acid bacteria may have potential therapeutic properties including anti-inflammatory and anti-cancer activities. It was proved the protective effects of some strains of these bacteria anti-tumor and anti-cancer effects in mice (Chen at al. 2009).

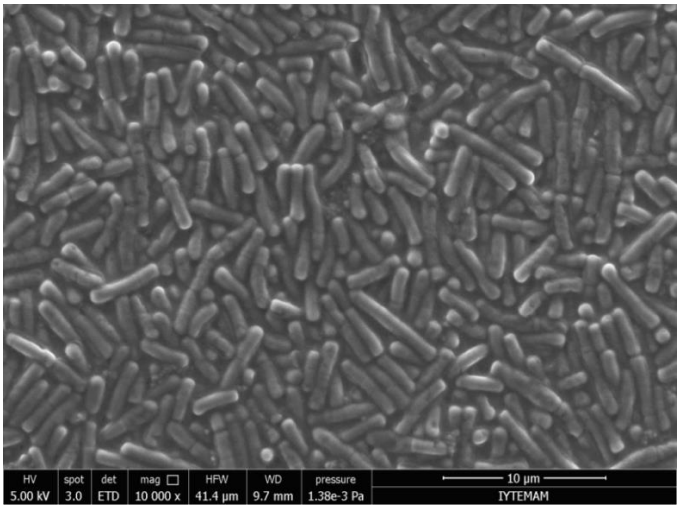


Figure 1.2. *Lactobacillus acidophilus* SEM scanning

1.1.5. Prebiotics Synbiotics

Prebiotics could be define that “a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon.” A candidate prebiotics must fulfill to some criteria for considering as prebiotics. They must be having resistance to gastric acid, enzymatic digestion and intestinal absorption, fermented by the intestinal microbiota, selectively stimulate of growth and activity of intestinal bacteria (Michael de Vrese 2008). So, natural and synthetic fructooligosaccharides, galactooligosaccharides, and oligosaccharides could be considered as prebiotics (Saito et al. 1992). Prebiotic carbohydrates are dietary fibers, as they are not digested by human enzymes. But they could be fermented by the flora of the large intestine. In this way, they increase biomass, feces weights, and feces frequency, have a positive effect on constipation and on the health of the mucosa of the large intestine Synbiotic is the term that used when a product contains both probiotics and prebiotics. Since the word intend to synergism, this term should be source for products in which the prebiotic compound selectively favors the probiotic compound. It means that a product containing oligofructose and probiotic *Bifidobacteria* would complete the definition. Growth-promotion of potentially protective bacteria and/or the inhibition of potentially pathogenic microorganisms, as well as stabilization of the intestinal environment by lowering the pH and release of short-chain organic acids, have been investigated and con-firmed frequently in in vitro and in vivo trials are positive effects of synbiotics on the intestinal flora. Andersson et al. 2001 defined synbiotics as mixtures of probiotics and prebiotics that useful affect the host by evolving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract of the host. This expression, however, should only be used in the case of a true “synergistic” mutual support. Most food items containing both probiotic bacteria and prebiotic carbohydrates do not complete this criterion.

1.2. Application of Probiotics

Probiotic foods are sold in grocery stores and supermarkets. And also food supplements are available most of pharmacies and health food shops. Probiotics are added to both fresh and fermented dairy products (e.g., milk, fermented milk, and

yogurt). The packages are traditional single or multipack cups or tubs, instead of larger sizes. The market for food applications of probiotics is obviously larger than that for probiotics sold in capsules, sachets, and other pharmaceutical forms. Yogurt-type drinks are the fastest-growing product category, but the variety of probiotic food applications is not limited to milk-based products. Probiotic fruit juices, berry soups, and soy- and cereal-based fermented products are also sold. Probiotic foods are produced by adding the probiotic strains at the same time with the standard cultures in the fermentation tank. In an alternative process, fermentation takes place one by one, and probiotic cultures (e.g., *Lactobacillus acidophilus* and *Bifidobacterium* species) are collect to form the final product (Saxelin 2008).

1.2.1. Non-Dairy Applications

Lactic acid fermentations have been practiced since long and a number of conventional fermented products based up non-dairy substrates like cereals, vegetables etc**.** Non-dairy probiotics are viable both in the form of beverages and fermented foods. These are some of the traditional, non-dairy based fermented foods including large amounts of health promoting microorganisms: Sauerkraut (fermented cabbage or acidic cabbage), Suan Tsai or Suan Cai or Chinese fermented vegetables, kimchi, brined cucumber, fermented cassava, Stinky tofu or stinky soybean curd, Nigerian ogi (fermented maize or sorghum porridge), sourdough bread, boza, pozol. And these are non- traditional non-dairy probiotics: fortified fruit juices, Saccharified rice yoghurt (Vasudha and Mishra 2012).

1.2.1.1. Supplements

Lactobacilli and *Bifidobacteria* are generally used as probiotic supplements in probiotic products. In addition, *Enterococcus* species, *Bacillus* species, *Escherichia coli*, *Saccharomyces boulardii* (a yeast), and other species are used in food supplements (Table 1. 2). A wider array of probiotic strains, either singly or in combination, is used in supplements in foods. Lots of product formulations exist: hard gelatin or vegetable capsules, tablets with or without enterocoating (e.g., those that dissolve in neutral

conditions), chewable tablets, and sachets. Supplement formulations may also include other active components, including vitamins and prebiotics (Saxelin 2008).

Table 1.2. The most common probiotic food supplements
(Source: Saxelin 2008)

Lactobacillus and Bifidobacterium species
Streptecoccus thermophilus
Lactobacillus delbruelckii subps. *bulgaricus*
Enterococcus faecium
Enterococcus faecalis
Bacillus subtilis
Bacillus clausii
Escherichia coli strain *nissle*
Saccharomyces boulardii and other yeast

1.2.1.2. Cereal Based Foods

There are wide arrays of traditional non-dairy fermented beverages produced around the world. Many of traditional non-dairy fermented beverages are non-alcoholic produced with cereals as principal raw material (Prado et al. 2008). According to Lamsal and Faubion (2009), cereal and cereal component-based foods provide opportunities to contain probiotics, prebiotics, and fiber in human diet. Cereals include water-soluble fiber (such as β-glucan and arabinoxylan), oligosaccharides (such as galacto- and fructo-oligosaccharides) and resistant starch, and therefore have been claimed to fulfill the prebiotic concept. All grains are also sources of many phytochemicals, including phytoestrogens, phenolic compounds, antioxidants, phytic acid and sterols (Katina et al., 2007). For enzymatic degradation of phytate and releases minerals such as manganese (which is an important growth factor of LAB), iron, zinc and calcium; fermentation of cereals supplies at optimum pH conditions. Strains of *Lactobacillus* have been recognized as complex microorganisms that are necessary fermentable carbohydrates; amino acids, B-group vitamins, nucleic acids and minerals to grow, and thus fermentation of cereals may represent an easy way to obtain a rich substrate that sustains the growth of probiotic bacteria. Cereal-based products have been improved primarily for the combined effect of probiotics, prebiotics, and dietary fibers. Boza, Pozol, Togwa, Kunun-zaki, Borde, Ogi are some of cereal based traditional foods that contain probiotic bacteria (Enujiugha and Adebanjo 2015).

1.2.1.3. Animal Feed

The ban of antibiotics as growth promoters (AGPs) has been a challenge for animal nutrition increasing the need to find alternative methods to control and prevent pathogenic bacterial colonization. Probiotics, prebiotics and synbiotics could be possible solutions for avoid of using antibiotics. The principle effects of these feed additives are the increase resistance to pathogenic bacteria colonization and improved host mucosa immunity, in this way resulting in a reduced pathogen load, an improved health status of the animals (Williams et al. 2001) and a lower risk of food-borne pathogens in foods. Furthermore, probiotic treatments sometimes positively affected the growth performance, improving the feed efficiency of the animal (Gaggìa et al. 2010). There was not a big interest in probiotics during the following decades until the 1960s and 1970s when were discovered for animal nutrition. The first potent products for animal nutrition to introduce the specific requirements for feed additives did not appear on the European market till the mid-1980s. Nowadays, modern animal nutrition has all range of defined strains of probiotics belonging to the groups of lactic acid bacteria, *Bacillus* spores and yeasts (Angela et al. 2004).

1.2.2. Dairy Applications

Dairy products generally contain *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacteria* (Table 1.3.).

Table 1.3. Probiotic bacteria that may be associated with milk products
(Source: Holzapfel et al 1998)

***Lactobacillus acidophilus* Group**	***Lactobacillus reuteri***
L. acidophilus	
L. amylovous	***Lactobacillus plantarium***
L. crispatus	
L. gasseri	
L. johnsonni	***Bifidobacterium species***
***Lactobacillus casei* group**	*lactis* *bifidum*
L. casei	*adolescendis* *breve*
L. paracasei	*animalis* *infantis*
L. rhamnossus	*lognum*

Milk or milk products provide a proper environment for these probiotic organisms (Gilliant 2014). Milk has a remarkable position in the development of probiotic foods because it contains Omega-3, phytosterols, isoflavins, conjugated linoleic acid, minerals, and vitamins. Dairy products such as ice cream, cheese, yogurt, Acidophilus-Bifidus-milk, Ayran, Kefir, Kumis, Doogh containing probiotics and dairy beverages (both fermented and non-fermented) have been considered as significant facility for the delivery of probiotics. In fermentation process, acids such as lactic acid, acetic acid and citric acid are naturally produced. These acids are commonly used as organic acids to increase organoleptic qualities. Lactic acid bacteria are more tolerant to acidity and organic acids than most of the pathogens and spoilage microorganisms. Probiotic bacteria consumption via dairy food products is a good way to re-establish the intestinal micro-floral balance. And also it may be more logical to consider probiotics as a prophylactic rather than a therapeutic treatment for intestinal infections. Since the more interest in probiotics, various types of probiotic products were suggested as carrier foods for probiotic micro-organisms by considering consumers can take in huge amounts of probiotic bacteria for the therapeutic effects (Homayouni 2012).

1.2.2.1. Yogurt

Yoghurt has been historically recognized to be ‘a healthy food’ with therapeutically benefits. In the recent years, there has been a significantly increase in the popularity of yoghurt especially probiotic yoghurt. The conventional yoghurt starter bacteria, *L. bulgaricus* and *Streptococcus thermophilus*, cannot live passage through intestinal tract and consequently so, they are not considered as probiotics. But if add *L. acidophilus* and *B. bifidum* into yoghurt, yogurt is obtain extra nutritional and physiological values. Probiotic yoghurt is obtained by adding probiotic starter cultures to traditional yogurt. Heat treated homogenized milk with an enhance protein content (3.6–3.8%) is inoculated with the conventional starter culture at 45°C or 37°C and incubated for 3.5 and 9 h, separately. The probiotic culture can be added before fermentation at the same time with the conventional yoghurt cultures or after fermentation to cooled (4°C) product before packaging. Bio-yoghurt, containing *L. acidophilus* and *B. bifidum* is a potential vehicle for delivery of these probiotic cells to consumers. *L. acidophilus* and *B. bifidum* have to keep viability and activity in yoghurt

as a probiotic at consumption time. Probiotic yoghurt includes metabolic products secreted excreted by microorganisms, that affect the viability *of L. acidophilus* and *B. bifidum*. The inhibition of *Bifidobacteria* in probiotic yoghurt is owing to antagonism effects between starter bacteria rather than hydrogen peroxide or organic acids (Dave and Shah, 1997). The proper procedure for probiotic yoghurt production is growing the *Bifidobacterium* spp. separately, followed by washing off of free metabolites and the transfer of the cells to the probiotic yoghurt. Oxygen toxicity is a significant problem for *Bifidobacterium* 23 because they are strictly anaerobic. Low initial oxygen ingredient in milk may gain the low redox potential necessitate in the early phase of incubation to provide healthy *Bifidobacteria* growth. Oxygen dissolves with ease in milk during yoghurt production and also crosses through packages during storage. To avoid the oxygen toxicity problem, it has been suggested to inoculate *S. thermophilus* and *Bifidobacterium* at the same time during fermentation *S. thermophilus* has a great oxygen utilization ability, which results in decreasing of dissolved oxygen in probiotic yoghurt and an increasing in viability of *Bifidobacteria*. Higher alive lactic acid bacteria rates were obtained at lower storage temperatures (Foschino et al., 1996). Low storage temperature constrict the growth of *L. bulgaricus* and therefore also over-acidification. *Bifidobacteria* are pretty less tolerant to low storage temperature when compared to *L. acidophilus* (Homayouni 2012).

1.2.2.2. Probiotic Dairy Beverages

Dairy beverages are produced from milk or its derivatives, in which the dairy base represents at least 51% (vol/vol) of the formulation, with or without the addition of other ingredients and can be go through to a fermentation process using yogurt cultures. The consumption of dairy beverages is mostly associated with the consumption of yogurt, because of their similar sensory properties and the healthy habits of consumers (Zhu et al., 2009). From the technological outlook, the primary difference between yogurt and fermented dairy beverages is the addition of whey to the latter, which results in lower viscosity. The physical properties of dairy products may be affected by factors such as composition and heat treatment of milk, breaking of the gel, the use of stabilizers, the microbial culture used (Nielsen, 1975; Parnell-Clunies et al., 1986; Hassan et al., 1996), and the storage conditions until the end of shelf life (Marafon et al.,

2011). Addition with probiotic bacteria and prebiotic ingredients show a new option to add further value to dairy beverages, as reported in various studies on their qualification as a food matrix (Oliveira et al., 2002; Castro et al., 2009; Zoellner et al., 2009). The use of whey in the production of dairy beverages could be a promising alternative for dairy industries, because dairy beverages are thought positively by consumers. The main human-health benefits of cheese whey are well known and contain the prevention of cancer, enhance in levels of glutathione, enhance in antimicrobial function, and enhance in the fullness response (Madureira et al., 2007). Increased quantities of whey in dairy products suggest greater use of this precious industrial by-product, with a useful environmental effect (Castro 2012).

1.2.2.3. Fruit and Vegetable Based Fermented Foods

Non-dairy probiotics are present both in the form of beverages and fermented foods. These products may be based up cereals, vegetables or fruits. Each product submits unique and specific health benefits based upon the substrate. Non-dairy based fermented foods containing large amounts of health promoting microorganisms:

Sauerkraut (fermented cabbage or acidic cabbage) directly translated from German: "sour herb" or "sour cabbage", produce by naturally fermenting finely chopped, salted (2-3% salt) cabbage. Spontaneous fermentation is carried out by natural microflora of cabbage that contains lactic acid bacteria, including *Leuconostoc, Lactobacillus,* and *Pediococcus* (Farnworth, 2003).

Suan Tsai or Suan Cai or Chinese fermented vegetables is a popular dish or seasoning food in China. It is well known for sour flavor. Suantsai can be produce from Chinese cabbage, cabbage or mustard. Most of these products are still produced by spontaneous fermentation even at household level, representing the flexibility and simplicity of handling this kind of fermented food. Recent probiotic species namely *Lactobacillus harbinensis* was isolated from conventional ‘Suan cai’ of China (Miyamoto et al., 2005).

Kimchi, or gimchi (kimchee/ kim chee), is also produced from vegetables and is popular in Korea. It includes functional ingredients like carotene and vitamin C (which can serve around 80% of recommended daily requirement), Vitamin B1/ B2, vitamin A, iron and calcium due to its ingredients red pepper, garlic, onion and ginger.

Through its health attributes kimchi has been listed in world's top 5 healthiest foods by magazine "Health". The health benefits that have been connected to kimchi include: anti-tumor activity, lipid/ hypercholesterolemia decrease activity, anti-oxidant activity (Kim et al., 2002). In addition to its active ingredients it also contains useful lactic acid bacteria which include different strains of *Lactobacillus plantarum*, *Lactobacillus sakei* and different species named as *Lactobacillus kimchii.*

Fermented cassava is used to make gari, a sour farinaceous meal and fufu, a fine paste starchy food that is made into porridge, in Nigeria. Gari is really popular food product because it comes in a ready-to-eat form. During fermentation, lactic acid bacteria metabolize the starch in the cassava pulp important production of organic acids, such as lactic acid, which lowers the pH (Coulin et al., 2006; Odunfa, 1985; Okafor and Ejiofor, 1990). The genera of lactic acid bacteria reported during gari making in West Africa include *Lactobacillus* and *Leuconostoc* (Kostinek et al., 2005).

Stinky tofu or stinky soybean curd is a very popular traditional fermented Chinese snack. It is a tofu which has been fermented in a mixture of fermented milk and a vegetable, meat and fish based brine, or some integration of the three. The microbial strains found in stinky brine vary according to the specific materials of the producers' adaptation (Liu et al., 2011). Huang and colleagues have isolated *Lactobacillus* genus and *Bacillus* genus from the fermented pickle (Huang et al., 2009).

Nigerian ogi (fermented maize or sorghum porridge) and Baba (fermented millet) steady food of Nigerian population made by lactic acid fermentation. Ogi is made by soaking sorghum or maize in water, wet-milling and sieving them. The paste is separated to sediment and kept for fermentation (Banigo et al., 1974). Traditionally, the fermentation takes place naturally by various microorganisms, in that *L. plantarum* and *L. confusus* as the significant players (Johansson et al., 1995).

Fermented rice noodle known as Sour Mifen in China and Khanom jeen in Thailand is very popular traditional food. These are not considered as potential probiotics since ingredient is cooked after fermentation and as per the standard definition of probiotics, microorganisms must be alive.

Sourdough bread is consumed generally in European centuries (Wahren, 1985). Besides to bread production, sourdough is being utilized for the manufacture of a range of products like cakes, crackers etc. these days (Ottogalli et al 1996; Foschino et al, 1999; Vogel et al., 1999). Sourdough fermentations are carried out by complex and

diverse microflora that consist of yeasts and lactic acid bacteria mainly *Lactobacillus sanfranciscensis*.

Tanzanian togwa (fermented sorghum, maize, millet or maize–sorghum beverage) is extensively consumed in Tanzania either in the form of porridge as weaning food or in diluted from as beverage. Mugula et al., 2003 have isolated and characterized the bacteria from traditional togwa which includes numerous species of *Lactobacillus* among which *Lactobacillus plantarum* was dominating, *Weissella confusa* and *Pediococcus pentosaceus*. Yeast species were found to be *Issatchenkia orientalis, Saccharomyces cerevisiae, Candida tropicalis* and *Candida pelliculosa*.

Ethiopian kombucha (fermented black tea or mushroom tea) is a sweetened black or green tea that is fermented with the help of yeasts and bacteria. The mass of yeast and bacteria forms a sac at the top of fermenting tea that appears like mushroom. The yeasts involved in kombucha fermentation are *Saccharomyces cerevisiae, Torulaspora delbrueckii, Candida stellata, Schizosaccharomyces pombe, Brettanomyces bruxellensis* and *Zygosaccharomyces bailii.*

Boza is a Bulgarian traditional cereal-based fermented beverage with a pleasant sweet-sour, breadlike taste. It is also consumed in some areas of Turkey, Albania and Romania. Different cereals (wheat, millet and rye) can be used for boza production, and fermentation is caused by natural mixtures of yeast and lactic acid bacteria. Hancioglu and Karapinar (1997) reported *Leuconostoc paramesenteroides*, which was predominant (26%), *Lactobacillus sanfrancisco, Lactobacillus coryniformis, Lactobacillus fermentum, Lactobacillus confusus, Leuconostoc mesenteroides* subsp. *mesenteroides, Leuconostoc mesenteroides* subsp. *dextranicum,* and *Leuconostoc oenos* in Turkish Boza (Sangita 2015).

1.3. Molecular Approaches to Study the Probiotics: Real-Time PCR

1.3.1. Molecular Methods to Study Probiotics

In recent years it has become progressively clear that the food industry and gastrointestinal microbiologists suppose sensitive and reliable methods to identify and characterize the probiotic ingredient of foods and the host's gastrointestinal (GI) tract. Particular interest is afforded to the lactic acid bacteria (LAB; especially *Lactobacillus*

spp. and *Bifidobacterium* spp.) due to: (i) the association of these organisms with health-promoting characteristic; (ii) their containment in numerous food products as 'probiotics'; and (iii) the requirements of legislative and industrial bodies, and also the consumer, with respect to safety, labeling and strain integrity (Charteris et al. 1997; Hozapfel et al. 2001). Additional areas of interest focus around contamination, food-borne pathogens, and any microbiological basis to GI disorders, or sensitivity to such illnesses. Molecular techniques have been studies of probiotics and human GI microflora for four main aims: (i) characterization of bacterial variety within samples; (ii) enumeration of phylogenetically related groups of bacteria; (iii) tracking or monitoring of specific organisms or populations, both quantitatively and qualitatively; and (iv) certain identification of isolates, especially probiotics. Probiotics are 'live microbial supplements' that are culturable and for this reason the full range of classification protocols is proper, including the genetic techniques (A. L. McCartney 2002).

1.3.2. Real-Time PCR

Viability is generally considered a prerequisite for optimal probiotic functionality (Maukonen et al. 2006). Many studies have shown that the viability of bacteria is not a simple question of cells being dead or alive. Traditionally, plate counting has been the method of option for viability determination, but there are many disadvantages, including the relatively long times required for the growth of colonies. The viable plate count method can be frustrated by clumping, inhibition by neighboring cells, and combination of the growth media used (Breeuwer and Abee 2000). Besides, many possible probiotic effects of bacteria rely on activity rather than cultivability, even dead cells can have some probiotic effect, such as immunomodulation. Hence, obtaining information about all individual bacteria and their physiological status is proper (Bunthof and Abee 2002). Four terms to describe the different stages of microorganisms are suggested by Kell et al. (1998): viable (active and readily culturable), dormant (inactive but ultimately culturable), active but non-culturable, and dead (inactive and non-culturable). Bunthof and Abee 2002 reported that such dormant population might exist in probiotic products and dairy starters. Further studies showed that probiotic bacteria may become dormant in fermented products during long storage (Lahtinen et al.

2005). Therefore, there is a big interest in the improving of rapid methods for cell viability determination (Breeuwer and Abee 2000; Grattepanche et al. 2005). Although culture-independent molecular methods for identification and enumeration of probiotic bacteria have been developed in recent years, they are still not generally used in routine laboratories. Among possible alternatives, quantitative real-time polymerase chain reaction (PCR have a potential to substitute conventional enumeration of probiotic bacteria (Furet et al. 2004). In real-time PCR, the quantity of DNA is measured after each cycle via fluorescent dyes that efficiency increasing fluorescent signal in direct ratio to the number of PCR product molecules (amplicons) created. Whole data gathered in the exponential phase of the reaction yield quantitative information on the starting amount of the amplification target. There are two common methods for the detection of PCR products in real-time PCR: (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA, and (2) sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter that allows detection only after hybridization of the probe with its complementary sequence.

1.3.2.1. Non-specific Real-Time Chemistry

A DNA-binding dye binds to all double-stranded (ds) DNA in PCR, causing fluorescence of the dye. An enhancing in DNA product during PCR hence leads to an increase in fluorescence intensity measured at each cycle but, dsDNA dyes such as SYBR Green will bind to all dsDNA PCR products, including nonspecific PCR products (such as Primer dimer). This can potentially combine with, proper monitoring of the intended for target sequence. In real-time PCR with dsDNA dyes the reaction is regulated as standard, with the addition of fluorescent dsDNA dye. Then the reaction is run in a real-time PCR tool, and after each cycle, the volume of fluorescence is evaluated with a detector; the dye only fluoresces when bound to the dsDNA (i.e., the PCR product). This method has the benefits of only needing a pair of primers to fulfill the amplification that keeps cheaper; but, only one target sequence can be evaluated in a tube (Ririe K.M. et al 1997).

1.3.2.2. Specific Real-Time Chemistry

Fluorescent reporter probes can identify only the DNA that consisting of sequence complementary to the probe. So use of the reporter probe remarkably improves specificity, and allows execution the technique even in the presence of other dsDNA. With, using different-coloured labels, fluorescent probes can be used in multiplex assays for monitoring different target sequences in the same tube. The specificity of fluorescent reporter probes avoids interference of measurements caused by primer dimers as well, that are unwanted potential by-products in PCR, but fluorescent reporter probes do not prohibit the inhibitory effect of the primer dimers that may suppress accumulation of the wanted products in the reaction. The procedure depends on a DNA-based probe with a fluorescent reporter at one end and a quencher of fluorescence at the reverse end of the probe. The big affinity of the reporter to the quencher prevents detection of its fluorescence; corruption of the probe by the 5' to 3' exonuclease activity of the Taq polymerase breaks the reporter-quencher affinity and in this way allows unquenched emission of fluorescence that can be detected after stimulation with a laser. An enhancing in the product targeted by the reporter probe at each PCR cycle for this reason causes a commensurate increase in fluorescence owing to the breakdown of the probe and release of the reporter.

1.4. Spectroscopic Methods to Study the Probiotics: FTIR

1.4.1. Spectroscopic Methods to Study Probiotics

Conventional methods used to analyze lactic acid bacteria and probiotics including plate counts, molecular biology (He, H.J et al. 2015). While these methods are very beneficial and reliable, they are invasive and requires too much time, and so not proper for the increasing demands of real time and on-line analyses of big amounts of samples. The improving of strong equipment has definitely contributed to complete these necessities. Infrared and Raman spectroscopies have several benefits over other methodologies, easy using, minimal or no preparation of samples, quick registration of spectra in situ and in real time without using chemical reagents (environmentally friendly methodologies) (Karoui 2013). Additionally, almost every sample (i.e., liquids, solutions, pastes, powders, films, fibers, gases and surfaces) can be studied by carefully

selecting appropriate sampling techniques. Using infrared and Raman spectroscopies for the studying of biological systems, including lactic acid bacteria and probiotics, is an increasingly expanding approach. Actually, taxonomic studies, monitoring probiotics production, evaluation of biochemical and biophysical bacterial properties are really important applications. The interaction of matter with light in the infrared region of the electromagnetic spectrum gives boom to the experimental spectra. The spectrum stands for the transmitted, reflected or dispersed radiation as a function of energy or a magnitude proportional to it (wavenumber, frequency, wavelength, energy). The interactions of infrared radiation with matter may be understood in terms of difference in molecular dipoles related with vibrations and rotations (Mauricio 2015).

1.4.2. FTIR: Fourier Transform Infrared Spectroscopy

FTIR stands for Fourier Transform Infrared, the prior method of infrared spectroscopy. In infrared spectroscopy, IR radiation is passed through a sample. The sample absorbs some of the infrared radiation and some of it is traversed (transmitted). The resulting spectrum stands for the molecular absorption and transmission, composing a molecular fingerprint of the sample (Table 1. 4.). Like a fingerprint there is not two unique molecular structures produce the same infrared spectrum. This makes infrared spectroscopy beneficial for several types of analysis. FTIR technique is use to gain an infrared spectrum of absorption or emission of a solid, liquid or gas. High spectral resolution data over a wide spectral range simultaneously collect by FTIR spectrometer. This offers a big benefit over a dispersive spectrometer that measures intensity over a narrow range of wavelengths at once (Naumann et al 1991). FTIR spectra of bacteria are specific to a given strain and indicate the spectral characteristics of cell components, like fatty acids, membrane and intracellular proteins, polysaccharides, and nucleic acids (Mariey et al., 2001). The advantages of the using FTIR spectroscopic analysis of microorganisms are can be summarized as: giving information about basic molecular properties of material, has fast and easy using if compare other methods, Gun light screening and sequence analysis than 5 minutes of samples requires less time and low cost, very little sample amount is enough for measurement, Multivariate statistical analysis are used for the interpretation of the light series, In Microbiology; it can be used in determination of the bacterial composition and

cell components, taxonomic classification, determination of the amount of microorganisms, process control, gives information about the microbiological quality control, epidemiological studies and hygiene control (Mouwen 2005).

Table 1. 4. Characteristic IR (infrared) bands
(Source: Naumann D. et al. 1991)

Wavenumber (cm^{-1})	**Assignment**
3640-3610	Hydroxyl (O-H)
~3500	νO-H
3500-3300	Amines (N-H)
~3200	Amide A of proteins
3100-3000	Aromatic rings (C-H)
3080-3020	Alkenes (C-H)
2960-2850	Alkanes (C-H)
2959	$\nu(C\text{-}H_3)$ as
2934	$\nu(C\text{-}H_2)$ as
2921	ν(C-H2) as (fatty acids)
2898	νC-H (triple bond)
2872	ν(C-H3)s
2500-1900	*Triple bonds*
1900-1500	*Double bonds*
1741–1715	$\nu(C\text{-}H_2)$s (fatty acids)
~1695	νC=O (carbonic and nucleic acids)
~1685, ~1675	Amide I from antiparallel β-sheets and β-turns of proteins
~1655	Amide I of α-helices of proteins
~1637	Amide I of β-sheets of proteins
1548	Amide II of proteins
1515	"Tyrosine" band
1500	*Deformation/heavy atoms*
1468	$\delta(C\text{-}H_2)$
~1400	ν(C-O)s of COO−
1310–1240	Amide III of proteins
1250–1220	$\nu(P{=}O)$as of PO_2^-
1200–900	C-O-C, C-O dominated by ring vibrations of carbohydrates C-O-P, P-O-P
1085	$\nu(P{=}O)$s of PO_2^-
720	C-H rocking of $>CH_2$
900–600	"Fingerprint region"

a ν: stretching; δ: bending; s: symmetric; as: asymmetric. Amide A, I, II and III are typical bands of proteins. Amide A corresponds to νN-H; Amide I, to νC=O of amide groups; Amide II, to νC-N + δN-H coupled out of face, and Amide III, to νC-N + δN-H coupled in face.

CHAPTER 2

AIM OF DISSERTATION

Currently probiotics are used commonly in our daily life. *Bifidobacterium animalis* subsp. *lactis* and *Lactobacillus acidophilus* are significant part of probiotic bacteria. Enumeration and identification of these bacteria from commercial products by molecular and conventional techniques is the object of the study. Real Time PCR and FTIR technologies will be used as molecular techniques for identification and enumeration. It is expected to find end of this study:

• All commercial products that will be studied are expected to confirm their bacterial species and bacterial counts.

• Pure cultures will be obtained from commercial products by cultivating in plates. And also live cells count will be determined in commercial products. These findings will be used to detect live/dead cell ratio by comparing real time PCR results.

• Real Time applications will be used to determine for quantification of total bacteria count in different commercial products and total *Bifidobacterium* and *Lactobacillus* counts will be determinate separately as genus specific and species specific. Also, Real Time PCR as a rapid and reliable method that is expected confirms plate count results in a short time.

• FTIR spectroscopy will be used for determination the ratios of different microorganisms in commercial probiotic supplements. And also *Bifidobacterium animalis* subsp. *lactis* will be compared with commercial product that contains *Bifidobacterium animalis* subsp. *lactis* bacterial strain. Thus it is expected to confirm commercial products content.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

3.1.1. Probiotic Bacterial Strains

Gram-positive, non-motile, anaerobic, nonpathogenic *Bifidobacterium animalis* subsp*. lactis* (BB-12) *and Lactobacillus acidophilus* (LA-5) were handled during the study.

B. animalis subsp*. lactis* (BB-12®) is originated from Chr. Hansen's collection of dairy cultures. It is used worldwide since 1985 as an ingredient in food and dietary supplements, with no reported consumer illness or injury. *Bifidobacterium* (BB-12®) has received a Generally Recognized as Safe (GRAS) status by the Food and Drug Administration (FDA) in the US. In Europe, *B. animalis* has been granted Qualified Presumption of Safety (QPS) status since 2007 by the European Food Safety Authority (EFSA) - a status granted on species level. The large number of clinical studies and scientific publications contains which the BB-12® probiotic strain may have beneficial effects regarding the gastrointestinal and immune areas. Alone, the BB-12® probiotic strain; may reduce the risk of an upset stomach, enhance the immune response, reduce respiratory tract infections, support bowel function, reduce crying, fussiness and irritability in infants, alleviate symptoms of skin irritation. Combined, the BB-12® and LA-5® probiotic strains; may reduce the duration of stomach upset, support the re-colonization of the intestinal microbiota.

L. acidophilus (LA-5®) is originated from Chr. Hansen's collection of dairy cultures. It used worldwide since 1979 as an ingredient in food and dietary supplements, with no reported consumer illness or injury. In Europe, *Lactobacillus acidophilus* has been granted Qualified Presumption of Safety (QPS) status since 2007 by the EFSA) – a status granted on species level. The US FDA designated the LA-5® strain as GRAS.

Lactobacillus acidophilus (LA-5) and *Bifidobacterium animalis* subsp*. lactis* (BB-12) cultures were isolated from commercial products that were used in this thesis.

Table 3.1. Commercial supplements that were used in this study

Product	**Name of contains bacteria**	**Amount of bacteria**	**Other contents**	**Country of origin**
BAKSO Sachet	***Bifidobacterium animalis* subsp. *Lactis* (BB-12)**	10^9 cfu/sachet	Maltodextrin	Slovenia
BAKSO Capsule	***Lactobacillus acidophilus* (LA-5)** ***Bifidobacterium animalis* subsp. *lactis* (BB-12)**	2×10^9 cfu/capsule	Inulin	Slovenia
ProbioticGold	*Enteroccous faecium* ***Lactobacillus acidophilus*** *Lactobacillus rhamnosus* *Bifidobacterium bifidum* *Bidobacterim longum*	$2,5\times10^9$ cfu/sachet	Fructooligosaccharides, Lactulose, VitaminC (L- ascorbic acid), VitaminE (DL-a tocopheryl acetate, Vitamin A (Vitamin A acetate), VitaminB6 (Pyridoxine hydrochloride), VitaminB1 (thiamine hydrochloride), Vitamin B2 (riboflavin)	South Korea
ProbioticATP	*Lactobacillus casei* *Lactobacillus rhamnonosus* *Lactobacillus plantarum* ***Bifidobacterium lactis***	$2,5\times10^9$ cfu/sachet	Fructooligosaccharides, Galacto oligosaccharides, VitaminC (L-ascorbic acid), VitaminB6 (Pyridoxine hydrochloride), VitaminB1 (thiamine hydrochloride), Vitamin B2 (riboflavin)	South Korea

Four different commercial probiotic products were analyzed in this study (Table 3.1). Microorganisms declared on the label were *L.acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 at a concentration of 10^9 colony forming units (CFUs) of each strain per capsule or sachet. Apart from bacteria, the probiotic product BAKSO Sachet also contained Maltodextrin, BAKSO Capsule Inulin, Probiotic Gold Fructooligosaccharides, Lactulose, VitaminC (L- ascorbic acid), VitaminE (DL-a tocopheryl acetate, Vitamin A (Vitamin A acetate), VitaminB6 (Pyridoxine hydrochloride), VitaminB1 (thiamine hydrochloride), Vitamin B2 (riboflavin) and ProbioticATP Fructooligosaccharides, Galacto oligosaccharides, VitaminC (L- ascorbic acid), VitaminB6 (Pyridoxine hydrochloride), VitaminB1 (thiamine hydrochloride), Vitamin B2 (riboflavin).

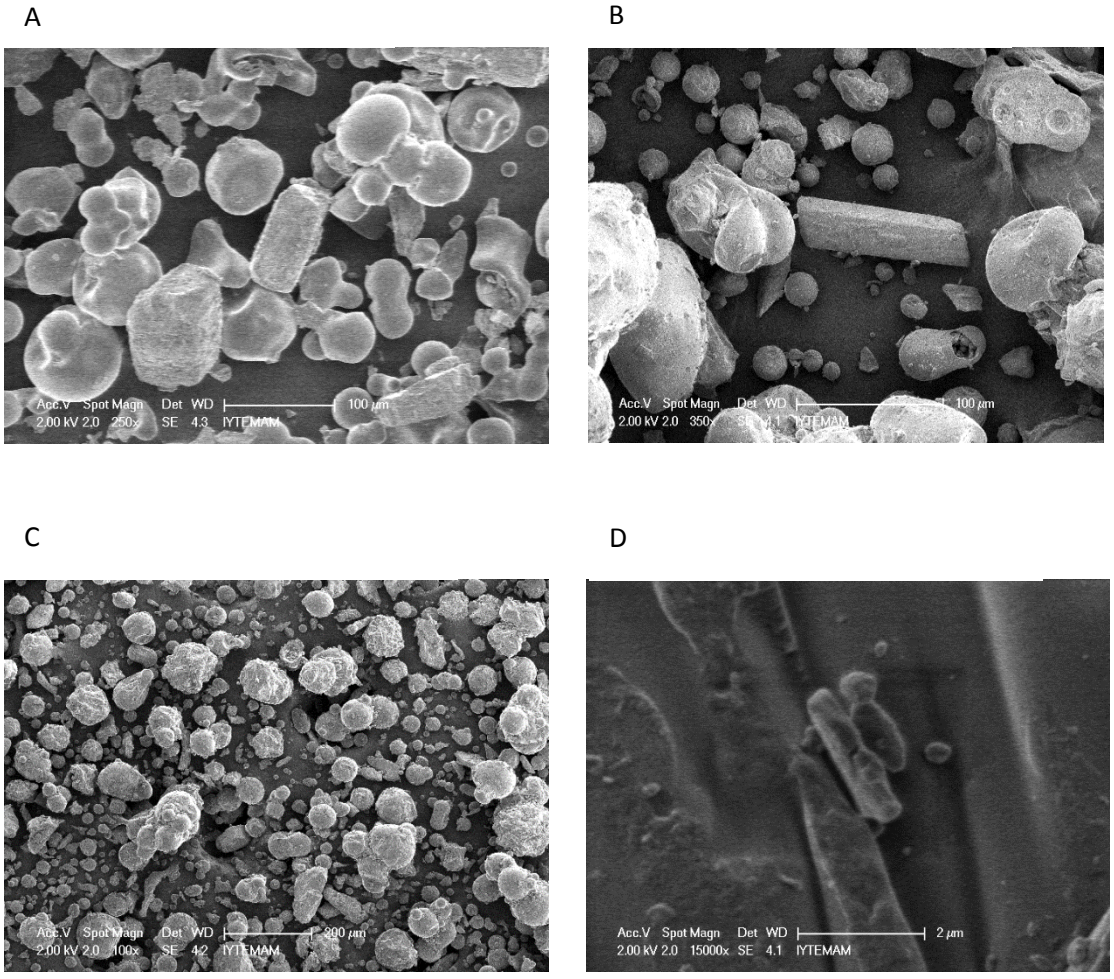


Figure 3.1. SEM scanning of probiotic products
A: Probiotic ATP B: Probiotic Gold C: Bakso Capsule D: Bakso Sachet

3.2. Methods

3.2.1. Pure Cultures and Growth Conditions

3.2.1.1. Microbial Growth Condition

Growth of *L. acidophilus* may not occur at as higher than a temperature as 45°C, but optimum growth condition within 35-40°C. It has acid tolerance varies from 0.3% to 1.9% titratable acidity, and its optimum pH lying at 5.5-6.0.

Bifidobacterium optimum pH for growth is 6-7, with almost no growth at pH 4.5-5.0 or below or at pH 8.0-8.5 or above. Optimum growth temperature is 37-41°C in anaerobic conditions, with maximum growth at 43-45°C and almost no growth at 25-28°C or below (Ana 1999).

3.2.1.2. Dose Preparations

1 capsule or sachet probiotic commercial product was dispersed 1/1000 g/l peptone water and diluted as 10^{-1}, 10-^{2} 10^{-3} 10^{-4} 10-^{5}, 10^{-6}, 10^{-7}, 10^{-8}. 100 µl sample of each dilution was cultivated to plates.

3.2.2. Cultural Counting

Plate count agar was used for counting of total viable cells (bacteria yeast and mold). PCA agar was measured then proper amount of water was added to agar. After the pH control, PCA solution was autoclaved. Each probiotic product sample was added 8 ml peptone water and diluted as 10^{-1}, 10^{-2}, and 10^{-3}. Then, 1 ml sample was cultivated to plate. Each sample was cultivated twice at 37°C for 3 days. End of the 3 days number bacteria was counted.

Potato Dextrose Agar was used for counting total yeast and mold. PDA agar was measured then proper amount of water was added to agar. After the pH control, PDA solution was autoclaved. Each probiotic product sample was added 8 ml peptone water and dilute as 10^{-1}, 10^{-2}, and 10^{-3}. Then, 1 ml sample was cultivated to plate. Each

sample plated twice and cultivated at 37°C for 3 days. End of the 3 days counted increase in number bacteria.

MRS agar was used for counting total *Lactobacillus* and *Bifidobaterium* species. MRS agar was measured then proper amount of water was added to agar. After the pH control, MRS solution was autoclaved. Each probiotic product sample was added 8 ml peptone water and diluted as 10^{-5}, 10^{-6} and 10^{-7}. Then, 1ml sample was cultivated to plate. Each sample was cultivated twice at 37°C for 3 days under anaerobic and aerobic conditions.

3.2.2.1. Growth and Maintenance of the Cell Culture

The purified bacteria with homogeneous cell morphology were stored in a -80°C deep freezer in MRS in 20% (v/v) glycerol for preservation. The glycerol stocks were prepared by mixing 0.5 ml of active cultures with 0.5 ml of fresh, sterile MRS medium with 40% glycerol in Eppendorf tubes. Then, the resulting suspension subsumed 20% glycerol. The frozen stocks were prepared and only one set was used for one test for avoiding any contamination or loss of activity.

3.2.3. DNA-Extraction

3.2.3.1. DNA Extraction Using Kit

Bacteria DNA preparation kit was used for DNA purification from bacteria to use conventional PCR in this study. First of all, for cell resuspension; 500 µl of cultured bacteria cells were centrifuged at 10000 g for 1 minute, discarded the supernatant, resuspended the cell pellet in 300 µl of Resuspension Buffer, 2 µl of Lysozyme Solution was added. Solution was mixed well by inverting several times and incubated tube at 37 °C for 1 hour. Then pellet was centrifuged at 10000 g for 1 minute and discarded the supernatant. Then for cell Lyses; 300 µl Lysis Buffer and 2 µl RNase were added to cell pellet, vortexed vigorously for 30-60 seconds. 8 µl Proteinase K was added and mixed by pipetting, incubated at 60 °C for 10 minutes and cool down for 5 minutes for inhibition of Dnases. Then 300 µl Binding Buffer was added and vortexed briefly, placed the tube on ice for 5 minutes, centrifuged for 5 minutes at 10000 g. For column

activation; a spin column was placed into a 2 ml collection tube, added 100 µl Activation Buffer into a spin column, centrifuged at 10000g for 30 second, discarded the flow-thorough. For column loading, the supernatant was pipetted directly into the spin column, centrifuged for 1 minute at 10000 g, and discarded the flow-through. For primary washing; 500 µl washing buffer was added into the spin column, centrifuged for 30 second at 10000g, discarded the flow-thorough. For secondary washing; 500 µl washing buffer was added into the spin column, centrifuged for 30 second at 10000g, discarded the flow-through, centrifuged again at 10000g for 1 minute to remove residual washing buffer, discarded the 2 ml wash tube and place the column in the elution tube. For elution of DNA; 40-50 µl elution buffer was added into the center of column, incubated at room temperature for 1 minute, centrifuged at10000g for 2 minutes, DNA was stored at 4°C.

3.2.3.2. Manuel DNA Extraction

Genomic DNA was isolated for qPCR using the following procedure for qPCR (Cardinal et al. 1997).

- Overnight activation of 10ml MRS broth
- Harvesting cells at 6.000 rpm for 5 min.
- Removing the liquid phase, washing pellet with 500µl 1xTE buffer (pH 8) and then centrifugation at 6.000 rpm for 5 min
- Suspending cells in 200µl 1xTE buffer (pH 8) containing 25% sucrose and 30mg/ml lysozyme and mixing gently.
- Incubation for 1 h at 37°C.
- Addition of 370µl 1xTE buffer (pH 8) containing 1mg/ml Proteinase K
- Addition of 30 µl SDS
- Incubation for 1 h at 37°C.
- Addition of 100µl 5M NaCl solution and 80µl CTAB/NaCl solution (10% cetytrimethylammonium bromide, 0,7M NaCl)
- Incubation for 10 min at 65°C
- Adding 750 µl chloroform/isoamyl alcohol (24/1, v/v) and mixing effectively
- Centrifugation at 6.000 rpm for 5 min
- Transferring the upper aqueous phase into a new Eppendorf tube.

• 2nd Chloroform/isoamyl alcohol extraction and centrifugation at 6000 rpm for 5 min
• Transferring the upper aqueous phase into a new Eppendorf tube.
• DNA precipitation by the addition of an equal volume (750µl) of isopropanol
• If DNA wool is observed, taking the wool with a yellow tip and transferring into a new Eppendorf tube containing 500 µl 70% ethanol
• If DNA wool is not observed, centrifugation at 6.000 rpm for 10 min to pellet
•DNA and washing with 500 µl 70% ethanol
• Pelleting DNA with centrifugation at 6.000 rpm for 10 min after washing
• Removing the ethanol and drying the pellet at 37°C for 10 min
• Dissolving pellet in 100µl 1xTE buffer (pH 8) containing 100µg/ml RNase
• Incubation for 1 h at 37°C. Adjusting the volume to 400µl with 1xTE buffer (pH 8)
• Dissolving DNA with alternating cold-heat shock twice (80°C for 10 minutes, and -20° C for 20 minute.

3.2.4. Protocols Optimization for qPCR

PCR amplifications were performed in a 25-ul reaction volume, containing SYBR Green q PCR Green-Master with UNG (Invitrogen Corporation, Carlsbad, California, USA), 0.6 ul each primer (Table 3.2. and Table 3.3.) and 3 ul of genomic DNA extract. The PCR amplification was performed with Real Time PCR Lightcycler 480 Roche (Indianapolis, US) instrument and also LightCycler® 480 Multiwell Plate 96 was used as plate.

3.2.4.1. Primer Selection

Genus specific and species specific primers was used to determine for quantification of *Bifidobacterum* and *Lactobacillus*, and also the numbers of the *Bifidobacterium animalis* subsp. *lactis* and *Lactobacillus acidophilus* in different commercial probiotic supplements (Table 3.2. and 3.3.).

Table 3.2. Primers that were used for real time PCR

Source: F-Lac, R-Lac: Walter et al. (2001), Acido lac: Kao et al. (2007), Bifid-F Bifid-R: Matsuki et. al.(2002) , B_ani-f B_ani-r: Junick and Blaut (2012)

Name	**5'-3' Sequence**	**Oligo type**	**Scale**	**Purified by**	**Yield nmol**	**MW g/mol**	**Tm °C**	**For conc. Pmol/µl**	**Add µl/ µl added**	**Oligo sent**
F-Lac	GCAGCAGTAGGGAATCTTCCA	DNA	0,01umol	RP-FCP	13.2	6455.3	55.9	100	132	Dry
R-Lac	GCATTYCACCGCTACACATG	DNA	0,01umol	RP-FCP	13.9	6029.5	54.5	100	139	Dry
Acido	TGAACCAACAGATTCACTTC	DNA	0,01umol	RP-FCP	13.4	6045	48.2	100	134	Dry
Lac	TGACGACAGCCATGCACCA	DNA	0,01umol	RP-FCP	14.1	5766.8	58.9	100	141	Dry
Bifid-F	CTCCTGGAAACGGGTGG	DNA	0,01umol	RP-FCP	16.9	5251.5	54.8	100	169	Dry
Bifid-R	GGTGTTCTTCCCGATATCTACA	DNA	0,01umol	RP-FCP	12.7	6676.4	52.3	100	127	Dry
B_ani-f	GACCAATGCGGAAGACCAG	DNA	0,01umol	RP-FCP	13.3	5815.8	55.6	100	133	Dry
B_ani-r	GTTGTTGAGAATCAGCGTGG	DNA	0,01umol	RP-FCP	13.3	6228.1	53.3	100	133	Dry

Table 3.3. Primers that were used for real time PCR
Source: Lac_acido_F Lac_acido_R :Cani et al (2008), BifTOT-F BifTOT-R: (Rinttilä et al 203004), B_ani_F B_ani_R: (Requena et al., 2002)

Name	**5'-3' Sequence**	**Oligo type**	**Scale**	**Purified by**	**Yield nmol**	**MW g/mol**	**Tm °C**	**For conc. Pmol/ µl**	**Add µl/ µl added**	**Oligo sent**
Lac_acido_F	GAGGCAGCAGTAGGGAATCTTC	DNA	0,01umol	RP-FCP	10.1	6824.5	56.7	100	101	Dry
Lac_acido_R	GGCCAGTTACTACCTCTATCCTTCTTC	DNA	0,01umol	RP-FCP	9.5	8112.3	57.2	100	95	Dry
BifTOT-F	TGCCGTCYGGTGTGAAAG	DNA	0,01umol	RP-FCP	12.8	5563.2	55.8	100	128	Dry
BifTOT-R	CCACATCCAGCRTCCAC	DNA	0,01umol	RP-FCP	14.9	5052.3	53.8	100	149	Dry
B_ani-F	GCGCTGGGCTGCTCTGGAAGC	DNA	0,01umol	RP-FCP	12.2	6479.2	66.7	100	122	Dry
B_ani_R	TGGCGAGCTCATCGACATACT	DNA	0,01umol	RP-FCP	11.1	6406.2	57.2	100	111	Dry

3.2.4.2. Standard Curve Preparation

Standard curves were obtained from amplifying extracted genomic DNA of 10^6 CFU mL^{-1} *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 cultures with genus (*Lactobacillus* and *Bifidobacterium*) and species (*L. acidophilus* and *B. lactis*) specific primers (Table 3.2.). Amplified products were quantified in Nanodrop (Thermo Scientific NanoDrop 8000) Serial 10-fold dilutions of target amplicons were prepared to detect the sensitivity and a minimal limit of detection qPCR. Genomic DNA was isolated using the procedures that mentioned above in section 3.2.3.2.

3.2.4.3. Preparation of qPCR Master Mix

The preparation of a master mix is prominent in qPCR reactions to reduce pipetting errors. Master Mix was prepared double set for per sample according to standard kit procedures (Table 3.4.**)**.

Table 3.4. qPCR master mix contents

Component	**20 µl assay**	**Final Conc.**
q PCR Green-Master with UNG	10 µl	1 X
Primer forward (10 µl)	0.6 µl	300 nm
Primer reverse (10 µl)	0.6 µl	300 nm
Template DNA	3 µl	<500 ng/assay
PCR-grade water	Fill up to 20 µl	-

Dispensing the Master Mix:

Master Mix was vortexed thoroughly to assure homogeneity, and then dispensed into qPCR wells of the PCR well plate.

Addition of Template DNA:

The remaining 3 µl of sample DNA was added to each reaction vessel containing master mix and cap or seal the tubes. Tubes were centrifuged before cycling to remove possible bubbles and qPCR was achieved according to SybrGreen chemistry conditions (Table 3.5**.**).

Table 3.5. qPCR cycles of SybrGreen chemistry

UNG treatment	50 °C	2 min	1X
Initial denaturation and polymerase activation	95°C	2 min	1X
Denaturation	95°C	15 sec	40X
Annealing	50°C	20 sec	40X
Elongation	72°C	30 sec	40X

3.2.5. *L. acidophilus* LA-5 Culture Preparation for Growth Curve

L. acidophilus LA-5 culture was grown in MRS broth media for 16 hours. 100 µl inoculum of this culture was added into 50 ml MRS broth, and then 100 µl of this mixture was used to determine for standard growth curve.

3.2.6. DNA Amplification for Confirmation Bacteria

For confirmation of *Lactobacillus acidophilus* that obtained from commercial products, EGE1 and EGE2 primers were also used. Forward primer is complementary to the 5'end of 16S rDNA, and the reverse primer is complementary to the 3' end of 16S rDNA region.

Forward Primer: EGE1: 5'-AGAGTTTGATCCTGGCTCAG-3'

Reverse Primer: EGE2: 5'CTACGGCTACCTTGTTACCA-3'

The PCR Conditions:

Step 1: 94°C for 5 min

Step 2: 94°C for 1 min (denaturation) 40 Cycle

Step 3: 56°C for 1 min (annealing)

Step 4: 72°C for 1 min (elongation)

Step 5: 72°C for 10 min

The final reaction volume of polymerase chain reaction was 50 µl and the amount of DNA template used was 500 ng. The recipe of the reaction mixture was given in the Appendix C. Obtained sequences were compared with the sequences deposited to NCBI database with BLAST analyze. *Lactobacillus acidophilus* that obtain

from commercial product were confirmed end of the BLAST analyze and obtained accession number as KX581698.

Separation of Amplification Products for Qualitative PCR:

Preparation of Agarose Gel: 0.8 g of agarose was dissolved in 100 µl 1x TAE buffer by boiling. After boiling, it was cooled to 45°C with water. 15 µl ethidium bromide solutions (10 mg/ml) was added and stirred. The agarose gel was poured into the gel casting stand and the combs were placed.

Loading of Agarose Gel: 10 microliters of PCR products were mixed with 2 µl of gel loading dye. The samples were loaded into the wells, starting from the second well on the gel. A DNA size-marker (100 kb, Fermentas) was loaded into the first well.

Electrophoresis of the Products PCR products was electrophoresed at 80 V for 30 min. Amplification products were visualized in a gel documentation system. The presence of DNA fragments with the size of 0-1500 bp indicated that the amplification was achieved (Figure 3.2.).

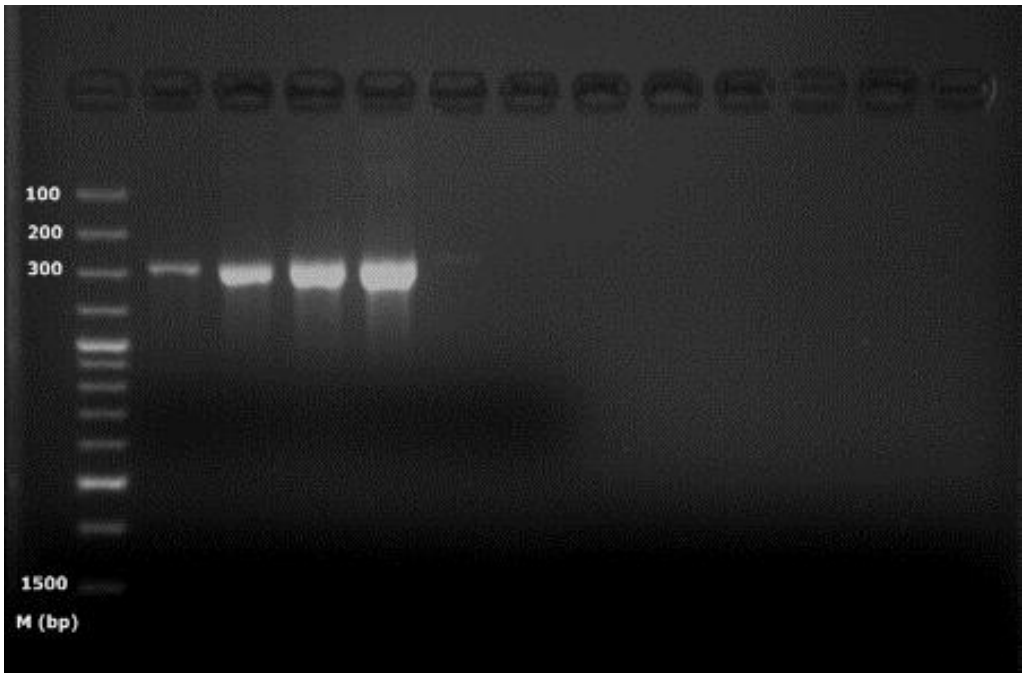


Figure 3.2. Confirmation gel screening of pure *Lactobacillus acidophilus* that was obtained from commercial products with qualitative PCR.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1. Cultural Count

MRS Agar was used for counting total *Lactobacillus* and *Bifidobaterium* in commercial products Bakso Sachet, Bakso Capsule, Probiotic ATP and Probiotic Gold (Table 4.1.).

Table 4.1. Plate count results of commercial probiotic supplement products

Products	Dilution rates			
	-5	-6	-7	-8
Bakso Sachet Sample 1	>300	343	42	NC
Bakso Sachet Sample 2	>300	325	39	NC
Bakso Sachet Li Sample 1	>300	396	35	NC
Bakso Sachet Li Sample 2	>300	326	30	NC
Bakso Capsule Sample 1	>300	211	24	NC
Bakso Capsule Sample 2	>300	182	15	NC
Bakso Capsule Li Sample 1	>300	260	24	NC
Bakso Capsule Li Sample 2	>300	260	29	NC
Probiotic Gold Sample 1	NC	>300	50	17
Probiotic Gold Sample 2	NC	>300	68	29
Probiotic ATP Sample 1	NC	38	2	ND
Probiotic ATP Sample 2	NC	35	1	ND

NC: Not counted, ND: Not detected any bacteria

Samples were added separately into 8 ml peptone (1 g/l) water and l-sistein HCl (2 g/l) for detecting l-sistein HCl effects in growth rate. Then samples were diluted as 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}. Then 1ml of each diluted sample was cultivated to MRS plate and incubated at 37°C for 3 days. Each sample was cultivated twice. It was not detected any significant differences between using peptone (1 g/l) water count and l-sistein HCl (Li) count (Table 4.1.). And also it was not detected any contaminant microorganism in commercial products according to PDA and PCA counting results.

4.1.1. Plate Count Calculations

Increase numbers of bacteria are counted and the numbers of bacteria counts are calculated for each commercial product.

Bakso Sachet Li count results calculations

$[(35+30)/2]*10^7=3.25*10^8$

$3.25*10^8*5=\sim10^9$ cfu/ml (1/5 is from first dilution)

Bakso Sachet count results calculations

$[(42+39)/2]*10^7=4.05*10^8$

$4.05*10^8*5=\sim10^9$ cfu/ml (1/5 is from first dilution)

Bakso Capsule Li count results calculations

$[(260+260)/2]*10^6=2.6*10^8$

$2.60*10^8*5=\sim10^9$ cfu/ml (1/5 is from first dilution)

Bakso Capsule count results calculations

$[(211+182)/2]*10^6=1{,}96*10^8$

$1{,}96*10^8*5=\sim10^9$ cfu/ml (1/5 is from first dilution)

Probiotic Gold count results calculations

$[(50+68)/2]*10^7=5.9*10^8$

$5.9*10^8*5=\sim10^9$ cfu/ml (1/5 is from first dilution)

Probiotic ATP count results calculations

$[(38+35)/2]*10^6=3.65*10^7$

$3.65*10^7*5=\sim10^8$ cfu/ml (1/5 is from first dilution)

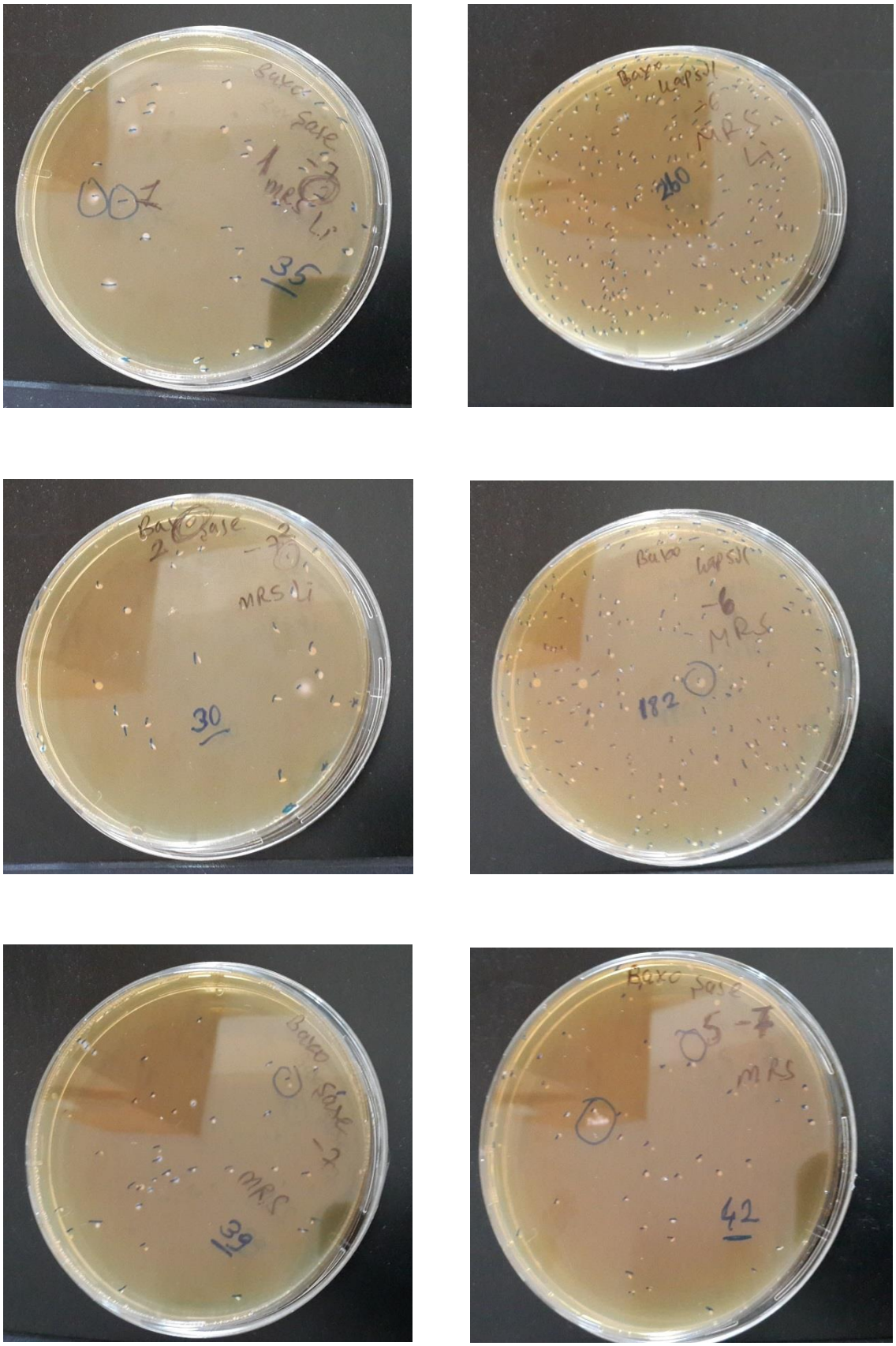
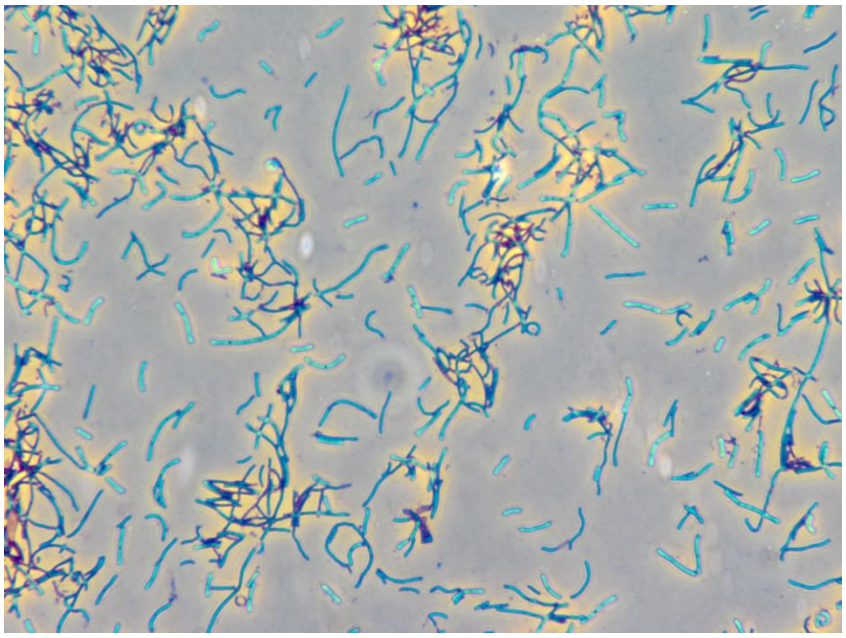
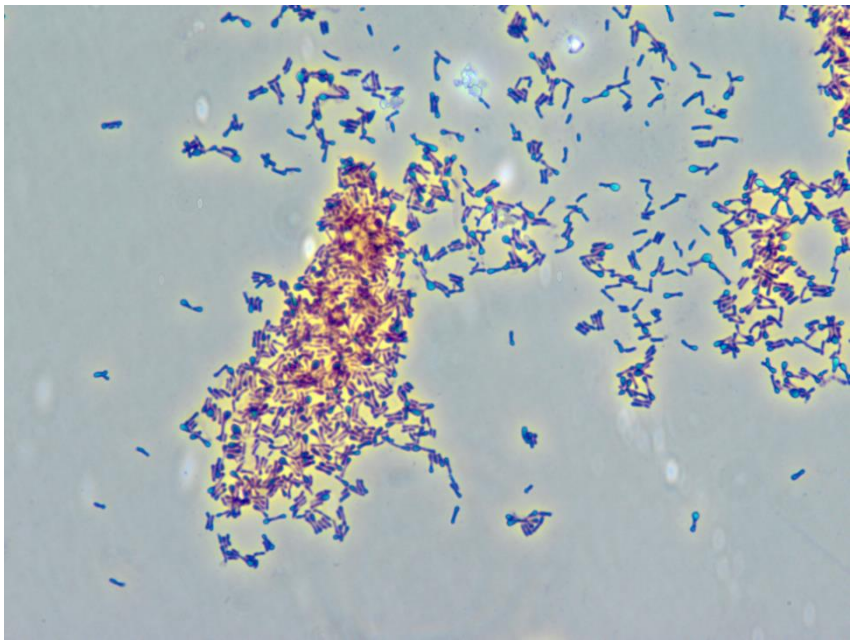


Figure 4.1. Photographs of the MRS plate count results



(a)



(b)

Figure 4.2. Light microscope photographs of the **(a)** *L. acidophilus* LA-5 and **(b)** *B. animalis* subsp. *lactis*.

4.2. qPCR

4.2.1. DNA Extraction of *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 Cultures and Commercial Products

Long term preserved isolates were cultured at 10 ml MRS Broth and genomic DNA was isolated using the (Cardinal et al. 1997) procedure. Each commercial product and cultured bacteria were extracted according to bacteria DNA extraction method as mentioned in section 3.2.3.2 (Table 4.2 and 4.3.).

Table 4.2. DNA amount of commercial products

Sample id	Conc.	units	A260	A280	260/280	260/230
G1	23.43	ng/ µl	0.469	0.344	1.36	0.40
G2	28.30	ng/ µl	0.566	0.415	1.36	0.41
Atp1	8.492	ng/ µl	0.170	0.095	1.80	0.46
Atp2	11.12	ng/ µl	0.222	0.156	1.42	0.40
B1	29.25	ng/ µl	0.585	0.408	1.44	0.42
B2	32.99	ng/ µl	0.660	0.480	1.38	0.45

Table 4.3. DNA extraction results cultured *Lactobacillus* and *Bifidobacterium* that were used in real time PCR process

Sample id	Cons.	units	A260	A280	260/280	260/230
B	194.7	ng/ µl	3.894	2.859	1.36	0.57
L	154.8	ng/ µl	3.096	2.332	1.33	0.46

4.3. *L. acidophilus* LA-5 Growth in MRS Broth

Lactobacillus acidophilus cultured bacteria were grown for 16 hours in broth media. 100 µl sample of this sample is added 50 ml MRS broth. 100 µl of this sample is used for varioskan application. According to varioskan application results *Lactobacillus acidophilus* growth curve was generated (Figure 4.3.).

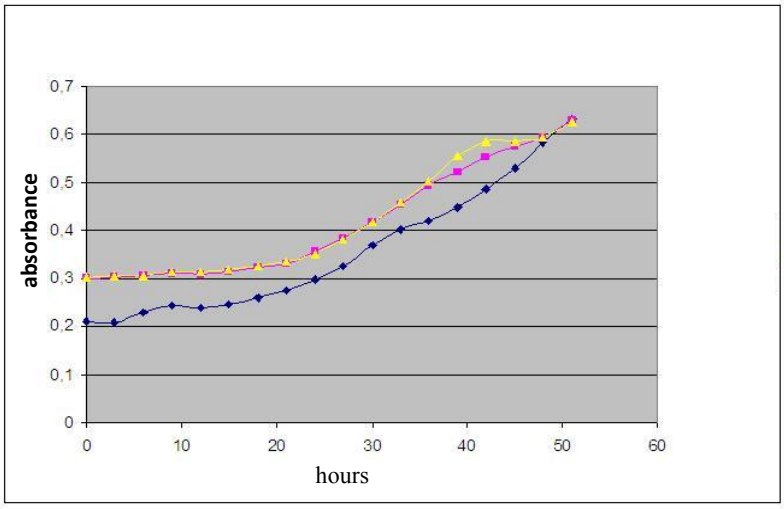


Figure 4.3. *Lactobacillus acidophilus* growth curve (Absorbance at 600 nm)

4.4. *L. acidophilus* LA-5 Growth in Fruit Juices

L. acidophilus LA-5 culture was grown in MRS broth for 16 hours. 100 µl inoculum of this was added into 50 ml MRS broth, 50 ml orange juice, 50 ml apple juice and 50 ml cherry juice. 100 µl of this sample was used for determination of growth (Figure 4.4.).

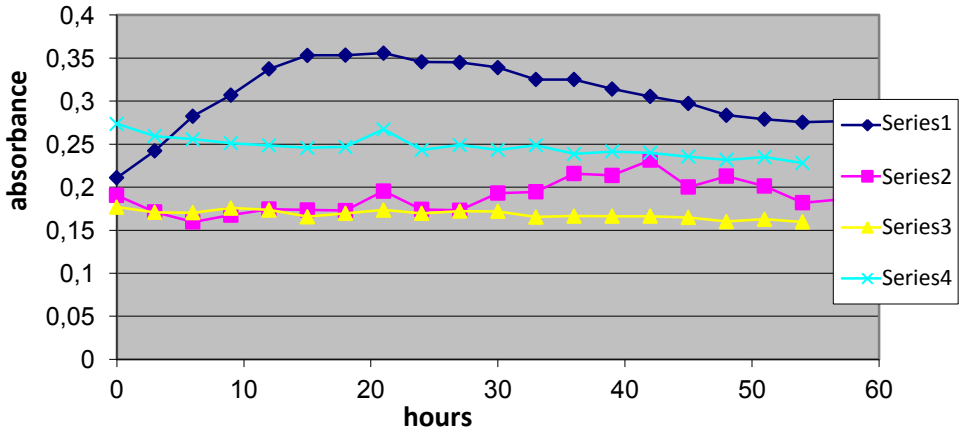


Figure 4.4. Growth curves of *L. acidophilus* LA-5 in MRS broth (Series1), orange juice (Series2), and apple juice (Series3), cherry juice (Series4).

According to growth curve in fruit juice, *L. acidophilus bacteria* can survive in fruit juice environment.

4.5. *L. acidophilus* LA-5 Plate Count

Lactobacillus acidophilus LA-5 culture was grown in MRS broth media for 16 hours. Diluted 10^{-5}, 10^{-6}, and 10^{-7} and cultivated triple in MRS Agar plates, then incubated at 37°C for 3 days. At the end of the 3 days colonies grown were counted (Table 4.4.)

Table 4.4. *L. acidophilus* count in MRS agar

Dilution	**Number of Count**		
-5	>300	>300	>300
-6	56	33	56
-7	5	7	7

4.6. Anaerobic *B. animalis* subsp. *lactis* BB-12 Plate Count

B. animalis subsp. *lactis* BB-12 culture was grown in MRS broth media for 16 hours. 1ml samples were added to 9 ml peptone water. So samples were diluted as 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}. 100 µl samples were added in MRS Agar plate. They were incubated in anaerobic jar for 30 hours and counted at 0, 5, 10, 24, 30th hours (Table 4.5.).

Table 4.5. *B. animalis* count in MRS agar under anaerobic conditions

Time / Dilution	**Control**	**T_0**	**T_5**	**T_{10}**	**T_{24}**	**T_{30}**
-4	>300	18	125	>300	>300	>300
-5	>300	5	8	47	>300	>300
-6	124	*	2	4	102	>300
-7	8	*	*	*	30	135

4.6.1. Number of *B. animalis* subsp. *lactis* BB-12 under Anaerobic Conditions

Increasing number of *B. animalis* subsp. *lactis* BB-12 in anaerobic conditions was calculated by taking into consideration dilution rates (Table 4.6.) and growth curve was generated (Figure 4.5.).

Table 4.6. *B. animalis* subsp. *lactis* BB-12 count during growing in MRS agar under anaerobic conditions

Total Count of *B. animalis* subsp. *lactis* BB-12					
Control	t_0	t_5	t_{10}	t_{24}	t_{30}
1.24×10^8	1.8×10^6	1.25×10^7	4.7×10^7	1.02×10^9	1.35×10^{10}

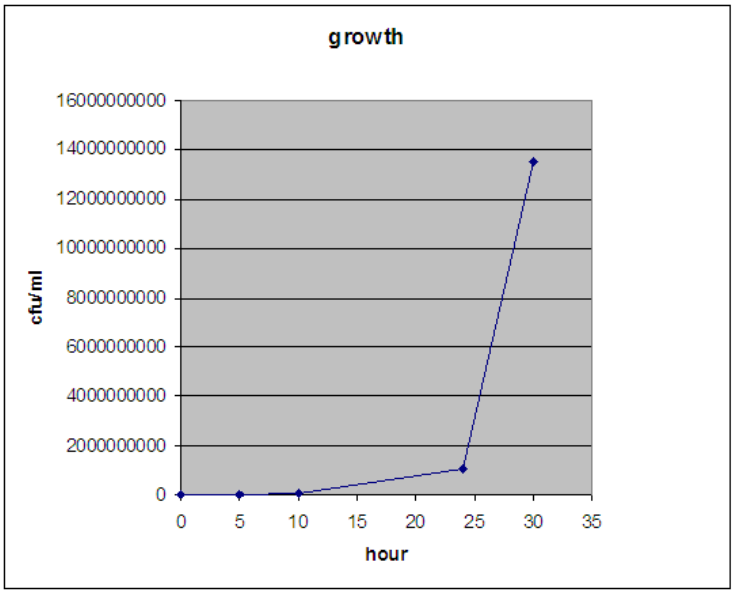


Figure 4.5. *B. animalis* subsp. *lactis* BB-12 growth curve under anerobic conditions.

4.7. Qualitative PCR for Primer Specificity

Qualitative PCR analysis was performed to test the specificity of the primer sets for the DNA extracted from pure cultures of *B. animalis* subsp. *lactis* BB-12 and *L. acidophilus* LA-5 and also DNA extracted from the commercial probiotic supplement

products. Amplified DNA products were subjected to the gel electrophoresis in agarose gel (1.5%) and visualized by ethidium bromide staining (Figure 4.6. and Figure 4.7.).

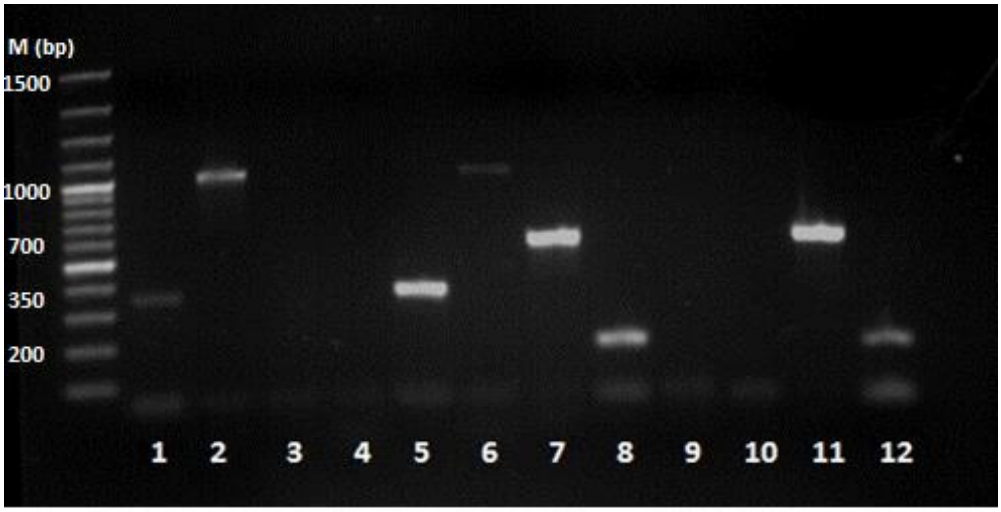


Figure 4.6. Gel application of *Lactocacillus* and *Bifidobacterium* primers **1.** 2× dNTP F-Lac-R-Lac *Lactobacillus* (genus specific) **2.** 2× dNTP Acido-Lac *Lactobacillus acidophilus* (species specific) **3**. 2× dNTP Negative control **4.** 2× dNTP Negative Control **5.** 10× dNTP F-Lac-R-Lac *Lactobacillus* (genus specific) **6**. 10× dNTP Acido- Lac *Lactobacillus acidophilus* (species specific) **7.** 2× dNTP Bifid-F Bifid-R *Bifidobacteria* (genus specific) **8.** 2× dNTP B_ani-f B_ani-r *Bifidobacterium animalis* subsp. *lactis* (species specific) **9.** 10× dNTP Negative Control **10.** 10× dNTP Negative Control. **11.** 10× dNTP Bifid-F Bifid-R *Bifidobacteria* (genus specific) **12**. 10× dNTP B_ani-f B_ani-r *Bifidobacterium animalis* (species specific).

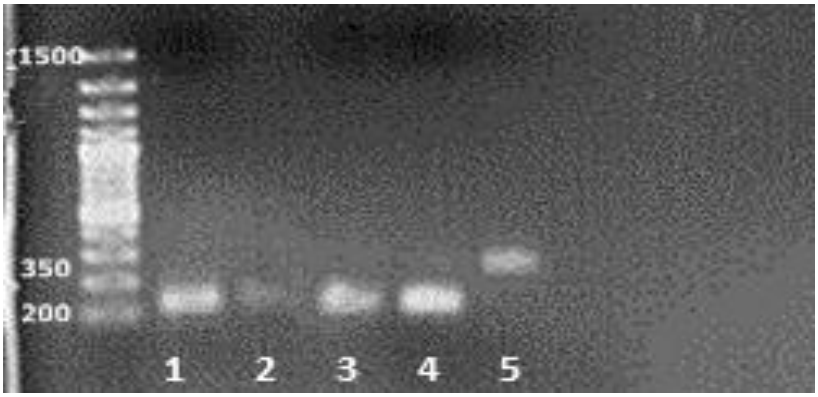


Figure 4.7. Gel application of *Lactocacillus* and *Bifidobacterium* primers **1. 2.** Lac_acido_F Lac_acido_R (species specific) **3.4.** B_ani-F B_ani-R (species specific) **5.** BifTOT-F BifTOT-R (Genus specific)

4.8. qPCR for *Lactobacillus*

DNAs were obtained from a *Lactobacillus* pure culture at a concentration of 10^6CFU mL^{-1}. Serial 10-fold dilutions were applied to determine the sensitivity of our qPCR (Figure 4.8). Each DNA dilution per PCR mixture was used to generate a standard curve and a minimal limit of detection (Figure 4.9). The assay sensitivity was more than 10^4CFU mL^{-1}, and the threshold values (C_T) values increased as the diluted genomic DNA concentration decreased.

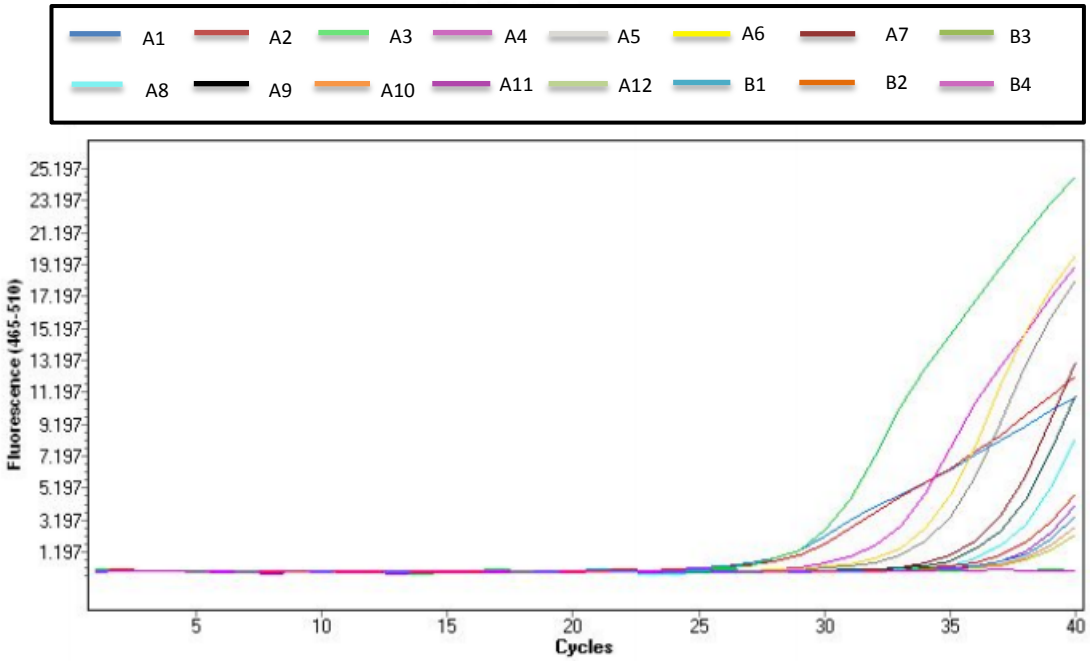


Figure 4.8. *Lactobacillus* 10^6 amplification curves. **A1 and A2:** Lac (F,R) primers for 10^6 CFU mL^{-1} genomic DNA, **A3 and A4:** 10^5 CFU mL^{-1} genomic DNA, **A5 and A6:** 10^4 CFU mL^{-1} genomic DNA, **A7 and A8**: 10^3 CFU mL-1 genomic DNA, **A9 and A10**: 10^2 CFU mL^{-1} genomic DNA, **A11 and A12:** 10^1 CFU mL^{-1} genomic DNA, **B1 and B2:** 10^0 CFU mL^{-1} genomic DNA, **B3 and B4:** Negative Control

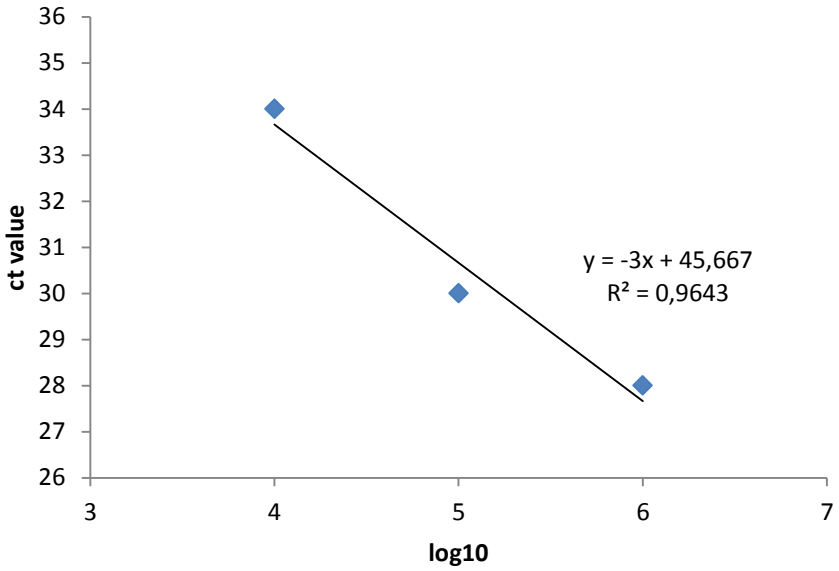


Figure 4.9. *Lactobacillus* 10^6 standard curve according to C_t values using Lac (F, R) Primers

This study qPCR assays could detect *B.animalis* or *L. acidophilus* 10^5 CFU/ml. Herbel et al 2013 describe an assay for the detection of *Lactobacillus* in yogurt samples with a detection limit identical with this study.

4.9. qPCR Standard Curve for *Lactobacillus*

DNAs were obtained from a 10^9 CFU mL^{-1} *L. acidophilus* culture. q PCR was performed for serial 10-fold dilutions of samples to generate a standard curve using Lac F, R (genus specific) primers (Figure 4.10). According to each C_t values of q PCR standard curve was generated for *Lactobacillus* (Figure 4.11).

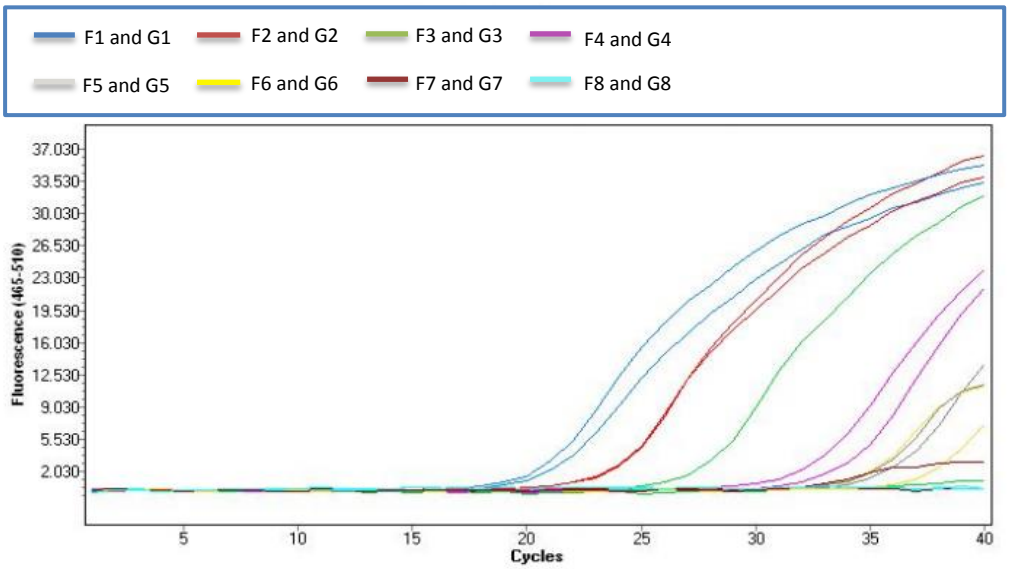


Figure 4.10. *Lactobacillus* 10^9 amplification curves **F1 and G1:** Lac (F,R) primers for 10^9 CFU mL^{-1} genomic DNA, **F2 and G2**:Lac (F,R) primers for 10^8 CFU mL^{-1} genomic DNA, **F3 and G3:** Lac (F,R) primers for 10^7 CFU mL^{-1} genomic DNA, **F4 and G4:** Lac (F,R) primers for 10^6 CFU mL^{-1} genomic DNA, **F5 and G5:** Lac (F,R) primers for 10^5 CFU mL^{-1} genomic DNA, **F6 and G6:** Lac (F,R) primers for 10^4 CFU mL^{-1} genomic DNA. **F7 and G7**: Negative Controls.

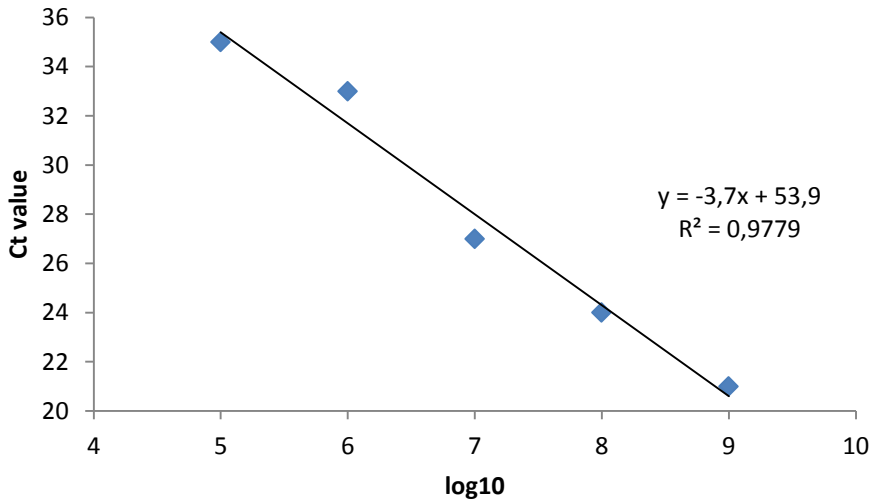


Figure 4.11. *Lactobacillus* 10^9 standard curve according to C_t values using Lac (F, R) Primers

According to the standard curve of *Lactobacillus*, equation and R^2 value were calculated (Table 4.7.).

Table 4.7. Equation and R^2 values for *Lactobacillus*

Target	Equation	R^2
Lactobacillus	Ct=-3.7x+53.9	0.977

Bifid-F Bifid-R, B_ani-f B_ani-r, Acido-Lac F Acido-Lac R primers didn't work in real time PCR it probably because their length regions are very long for qPCR. Thus other primers were applied in qPCR that mentioned in Table 3.3.

4.10. qPCR Standard Curve for *Bifidobacterium animalis*

DNAs were obtained from a 10^{10} CFU mL^{-1} *B.animalis* culture. q PCR was performed for serial 10-fold dilutions of samples to generate a standard curve using B_ani F, R (species specific) primers (Figure4.12). According to each C_t values of q PCR standard curve was generated for *Bifidobacterium animalis* (Figure 4.13).

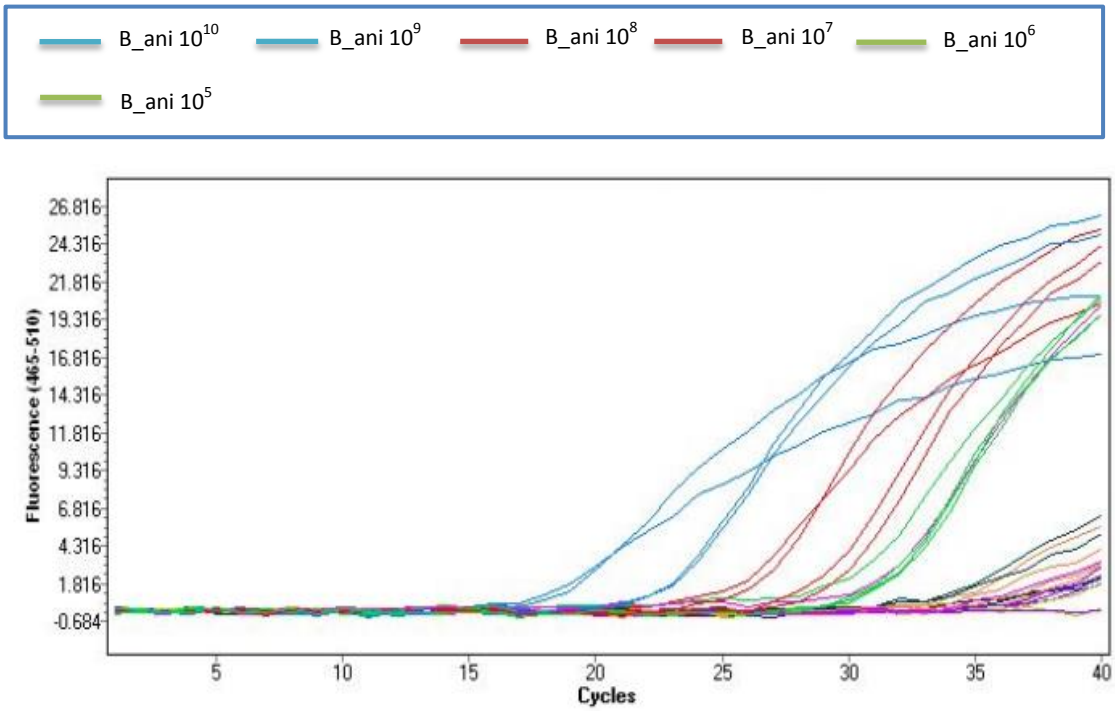


Figure 4.12. *Bifidobacterium animalis* 10^{10} amplification curves

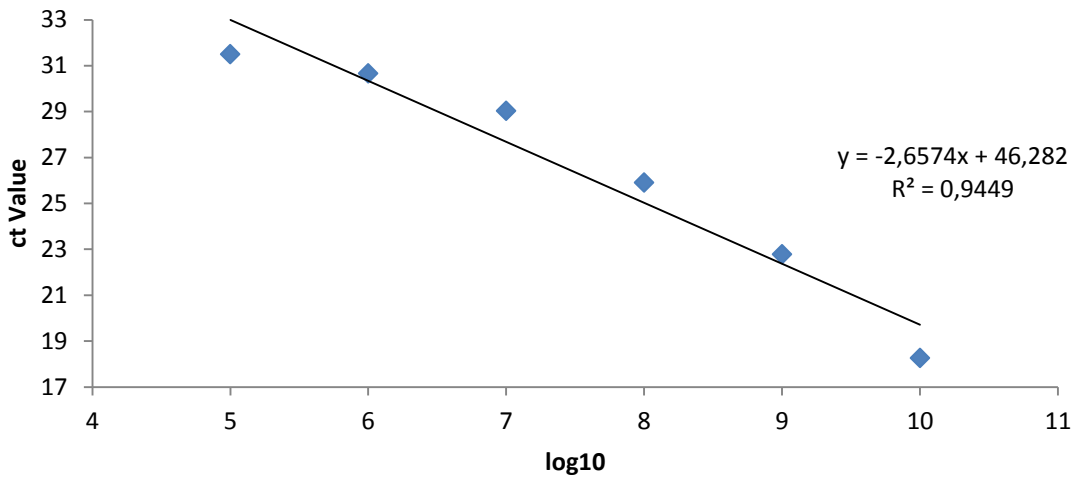


Figure 4.13. *Bifidobacterium animalis* 10^{10} standard curve according to C_t values using B_ani (F, R) Primers

According to the standard curve of *Bifidobacterium animalis,* equation and R^2 value were calculated (Table 4.8.).

Table 4.8. Equation and R^2 values for *Bifidobacterium animalis*

Target	Equation	R^2
Bifidobacterium animalis	Ct=-2.6574x+46.282	0.9449

4.11. Real Time Application with Commercial Products

DNAs were obtained from Probiyotic ATP, Probiyotic Gold and Bakso Capsule commercial products. Master mix and PCR cycle for primers has been performed as previously described in section 3.2.4.4. DNA (3µl) from each sample and Flac, Rlac (genus specific) primers (1 µl) were added PCR reactions. Figure 4.14. shows the results of each amplification curve of each product.

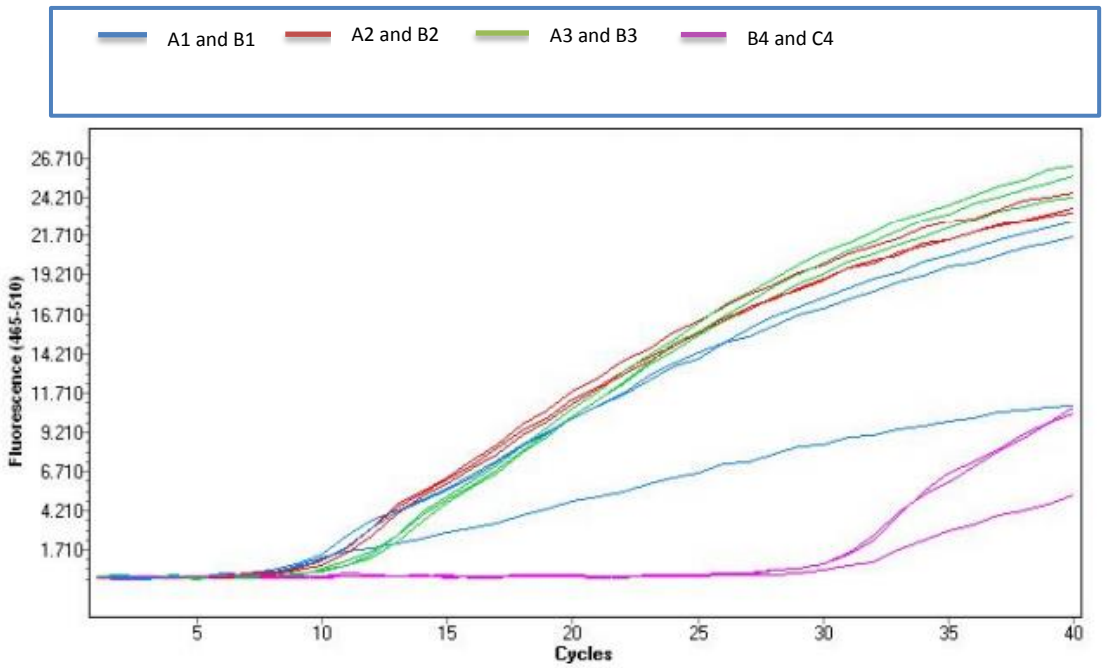


Figure 4.14. Amplification curve of probiotic products with using Flac Rlac primers **A1 and B1:** Probiotic ATP **A2 and B2:** Probiotic GOLD **A3 and B3:** Bakso Capsule **B4 and C4:** Negative Control

According to C_t value of each commercial product corresponding concentrations were calculated (Table 4.9.).

Table 4.9. *Lactobacillus* concentrations of probiotic products according to C_t values

Probiotic Product Name	Ct Value	Corresponding Concentration
Probiotic ATP	8.49	2.10^{12}
Probiotic GOLD	9.39	10^{12}
Bakso Capsule	10.83	6.10^{11}

DNAs were obtained from Probiotic ATP, Probiotic GOLD, Bakso Capsule and Bakso Sachet commercial products. Master mix and PCR cycle for primers has been performed previously described in section 3.2.4.4. DNA (3 µl) from relevant sample and Lac-acido, B_ani (species specific) primers (1 µl) were added PCR reactions. Figure 4.15. shows the results of each amplification curve of each product.

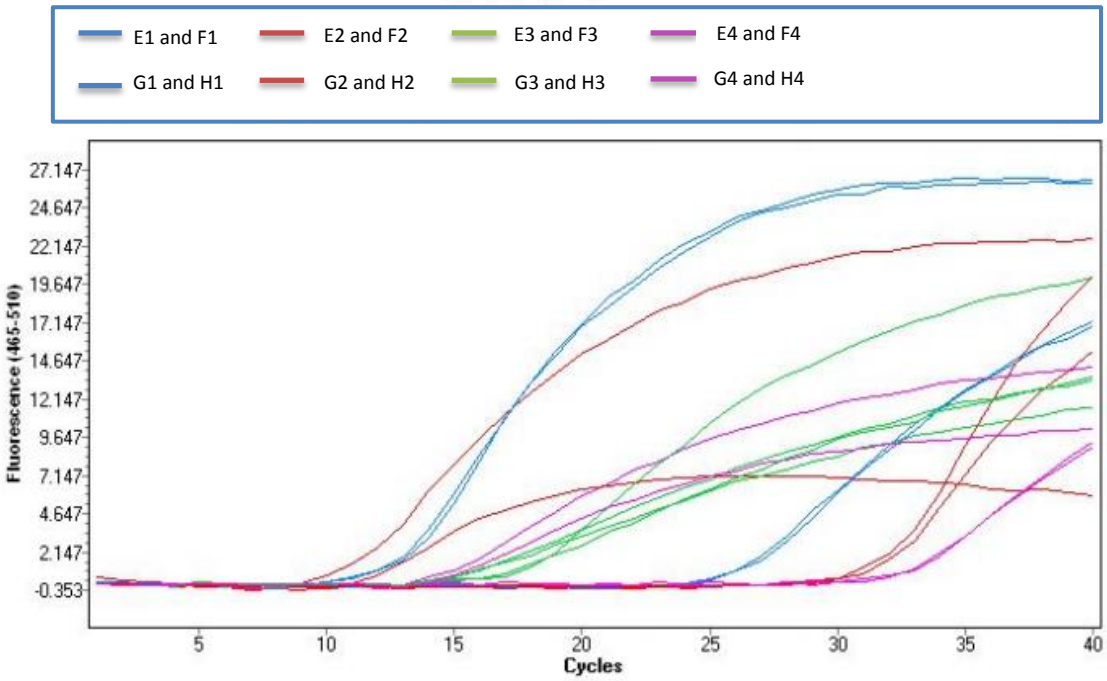


Figure 4.15. Amplification curve of probiotic products using Lac_acido, B_ani primers. **E1 and F1:** Probiotic ATP using Lac_acido F-R primers **E2 and F2:** Probiotic Gold using Lac_acido F-R primers **E3 and F3:** Probiotic ATP using B_ani F-R primers **E4 and F4:** Bakso Sachet using B_ani F-R primers. **G1 and H1:** Bakso Capsule using Lac_acido F-R primers. **G2 and H2** : Negative Control **G3 and H3:** Bakso Capsule using B_ani F-R primers **G4 and H4:** Negative Controls.

According to the q PCR C_t values corresponding concentrations of *Lactobacillus acidophilus* were calculated in commercial probiotic products (Table 4.10.).

Table 4.10. *Lactobacillus acidophilus* concentrations of probiotic products according to C_t values

Probiotic Product Name	Ct Value	Corresponding Concentration
Probiotic GOLD	10.71	6.10^{11}
Bakso Capsule	12.80	10^{11}

.DNAs were obtained from Probiotic Gold and Probiotic ATP commercial products. Master mix and PCR cycle for primers has been performed as previously described in section 3.2.4.4. DNA (3µl) from each sample and BifTOT (F,R) (genus specific) primers (1 µl) were added PCR reactions**.** Figure 4.16. shows the results of each amplification curve of each product.

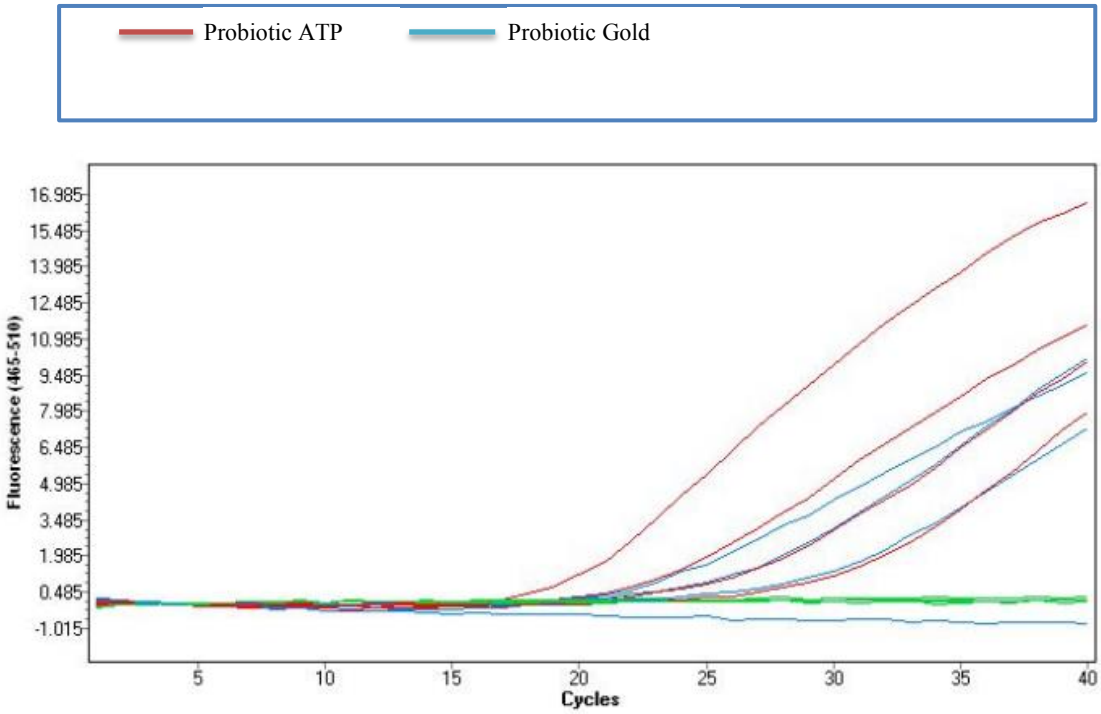


Figure 4.16. Amplification curve of probiotic products using BifTOT (F,R) primers

According to the q PCR Ct values (Figure 4.15 and 4.16) corresponding concentrations of *Bifidobacterium animalis* and total *Bifidobacterium* were calculated in commercial probiotic products (Table 4.11).

Table 4.11. *Bifidobacterium animalis and* total *Bifidobacterium* concentrations of probiotic products according to C_t values.

Probiotic Product Name	Ct Value	Corresponding Concentration
Bakso Sachet (*B. animalis*)	14.13	10^{12}
Bakso Capsule (*B. animalis*)	17.23	10^{11}
Probiotic Gold (total B)	19.78	10^{10}
Probiotic ATP (total B)	26.62	10^{7}

All commercial products were counted using cultural and molecular techniques (Table 4.12.). Molecular techniques allow detecting bacteria as genus and species specific.

Table 4.12. Comparison of cultural and molecular count results of commercial products

	Cultural Count		Molecular Count			
	Anaerobic	Aerobic	Total *L*	*L. acidophilus*	Total *B*	*B.animalis*
Probiotic Gold	NC	2.10^9	10^{12}	6.10^{11}	10^{10}	-
Probiotic ATP	NC	$1,5.10^8$	2.10^{12}	-	10^7	NC
Bakso Sachet	2.10^9	ND	-	-	10^{12}	10^{12}
Bakso Capsule	2.10^9	NC	6.10^{11}	10^{11}	10^{11}	10^{11}

NC: Not counted, ND: Not detected, -: Not Include that bacteria

Previous study (Drago et al. 2009) has indicated count of bacteria in different commercial probiotic products. But the probiotic products of that study were not in agreement with their relative labels and also contained contaminant organisms. On the contrary commercial products that were used in this study indicated an expected concentration for per unit. And also it was not detected any contaminant microorganism in any products.

4.12. FTIR Results

4.12.1. FTIR Results of Commercial Probiotic Supplement Products

FTIR Spectra of two commercial probiotic supplement products namely Bakso Sachet and Bakso Capsule were given in the Figures 4.17 and 4.18. As seen in the figures there are differences in the peaks, mainly in the ranges of 2900-3000, 1200-1700 and 650-800 cm^{-1}. These peaks stand for which bounds or groups are shown in Table 1.4.

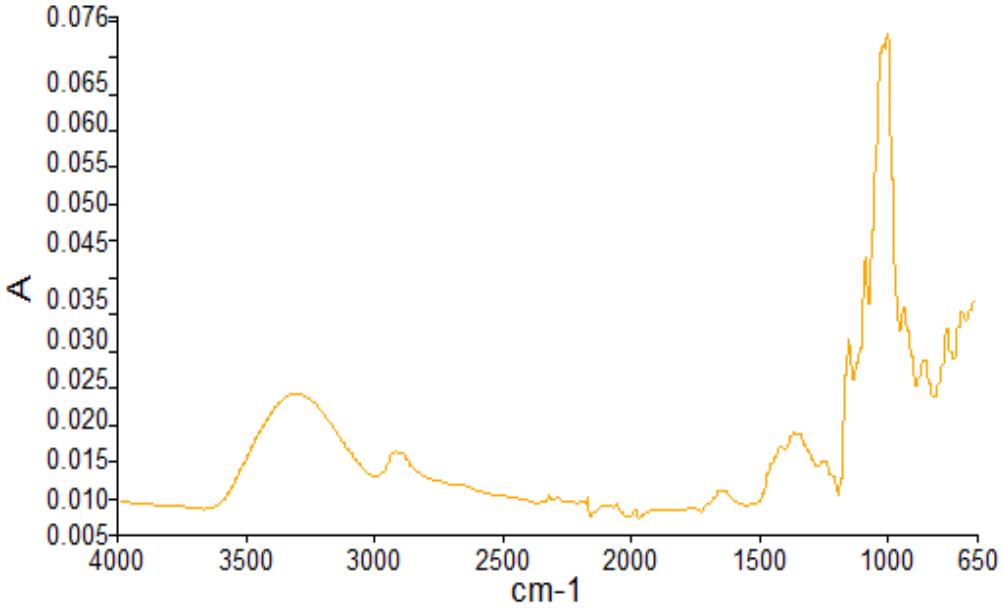


Figure 4.17. FTIR spectra of commercial probiotic product Bakso Sachet

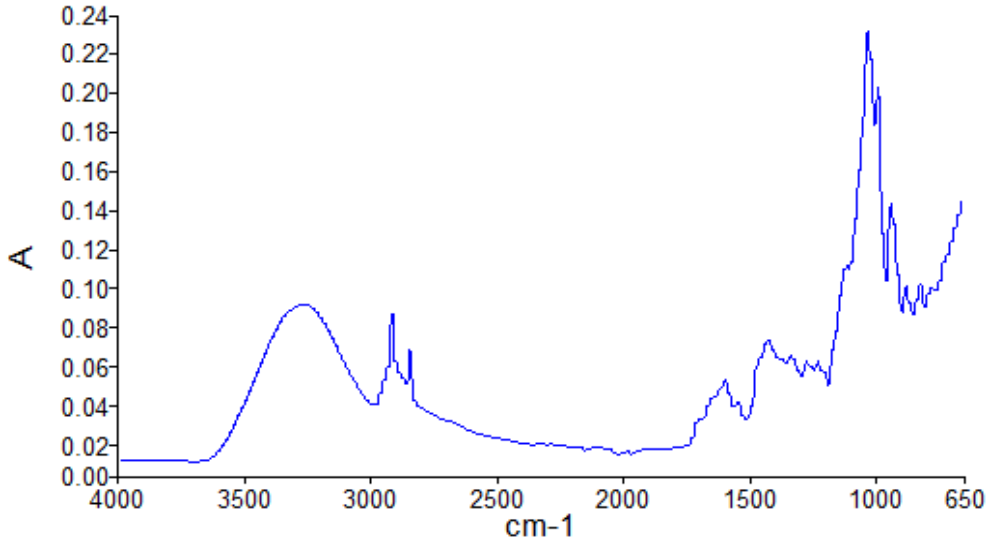


Figure 4.18. FTIR spectra of commercial probiotic product Bakso Capsule

FTIR Spectra representing commercial products Bakso Sachet and Bakso Capsule were compared in order to observe the differences in the frequencies for the assignment of functional groups (Figure 4.19.). These probiotic supplements were different from each other with a correlation coefficient 0.51.

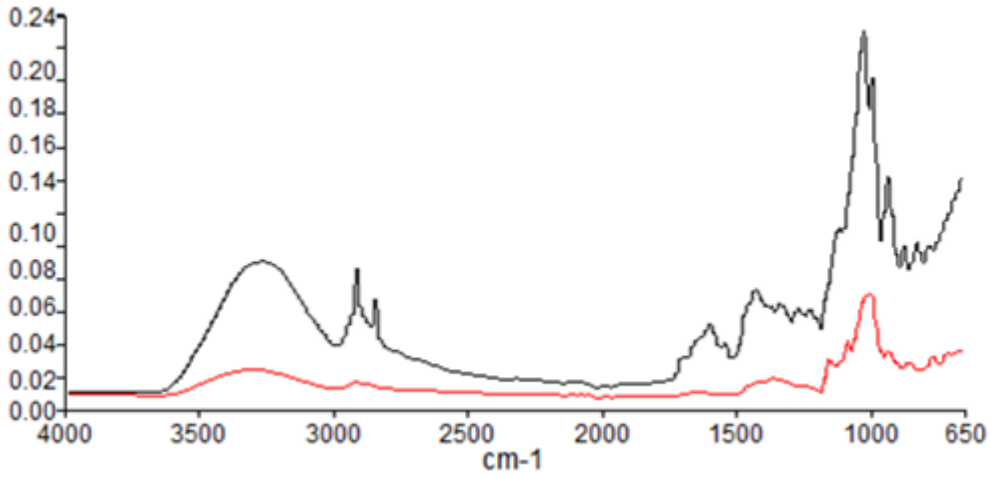


Figure 4.19. Comparison of FTIR spectra of commercial probiotic products Bakso Capsule and Sachets

These results show that the product contain approximately 50% *B. animalis* subsp. *lactis* and this finding confirm that product consists of both of the probiotics, namely *B. animalis* subsp. *lactis* BB-12.

FTIR Spectra of commercial probiotic supplement products namely Probiotic ATP and Probiotic Gold were given in the Figures 4.20. and 4.21. As seen in the figures there are differences in the peaks, mainly in the ranges of 1500-1900, 1468, 1400, 1240-1310 and 650-900 cm^{-1}, related with double bonds, $\delta(C\text{-}H_2)$, $\nu(C\text{-}O)s$ of COO^{-}, amide III of proteins, and "Fingerprint region", respectively.

FTIR spectra of commercial probiotic products Probiotic Gold and Probiotic ATP was given in the Figure 4.20. and 4.21. And also their comparison was given in the Figure 4.21. As seen in the Figure 4.22., although the spectra of both products seems alike each other, their contents are different and they differ from each other with a correlation 0.632 (Table 3.1.). While the product Probiotic Gold contains *L. acidophilus*, Probiotic ATP contains *B. lactis* according to the label.

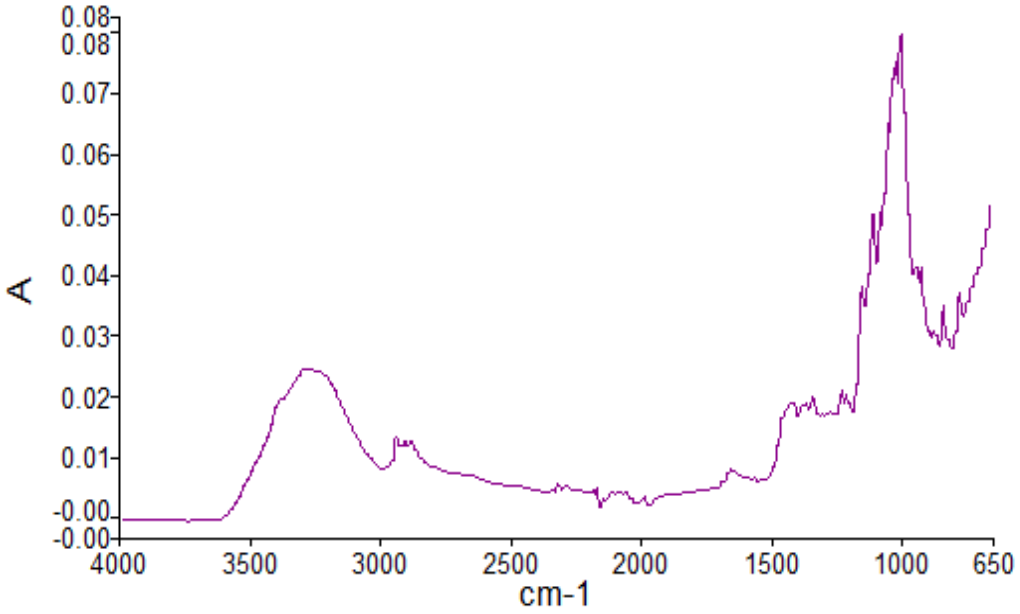


Figure 4.20. FTIR spectra of commercial probiotic product Probiotic ATP

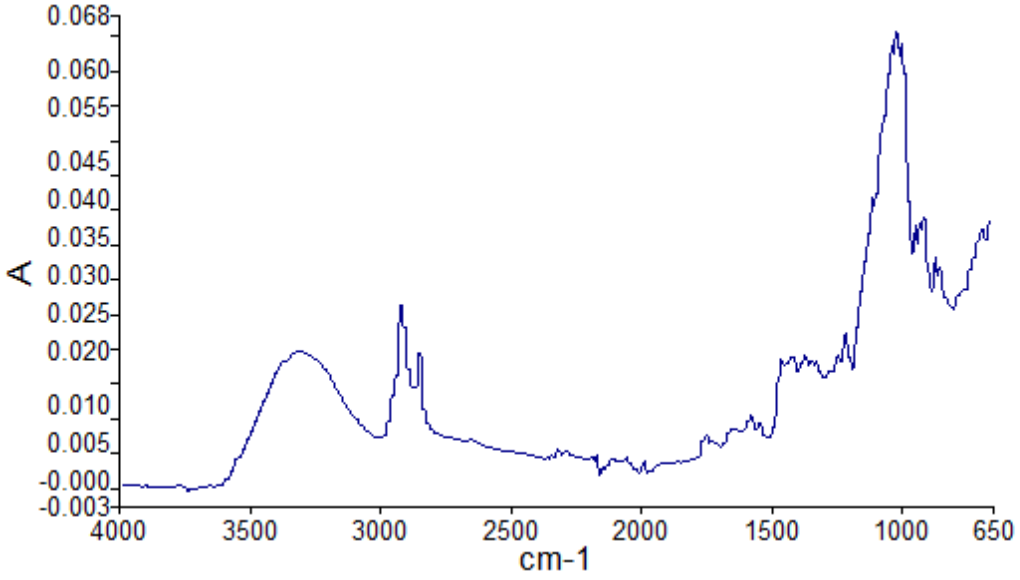


Figure 4.21. FTIR spectra of commercial probiotic product Probiotic Gold

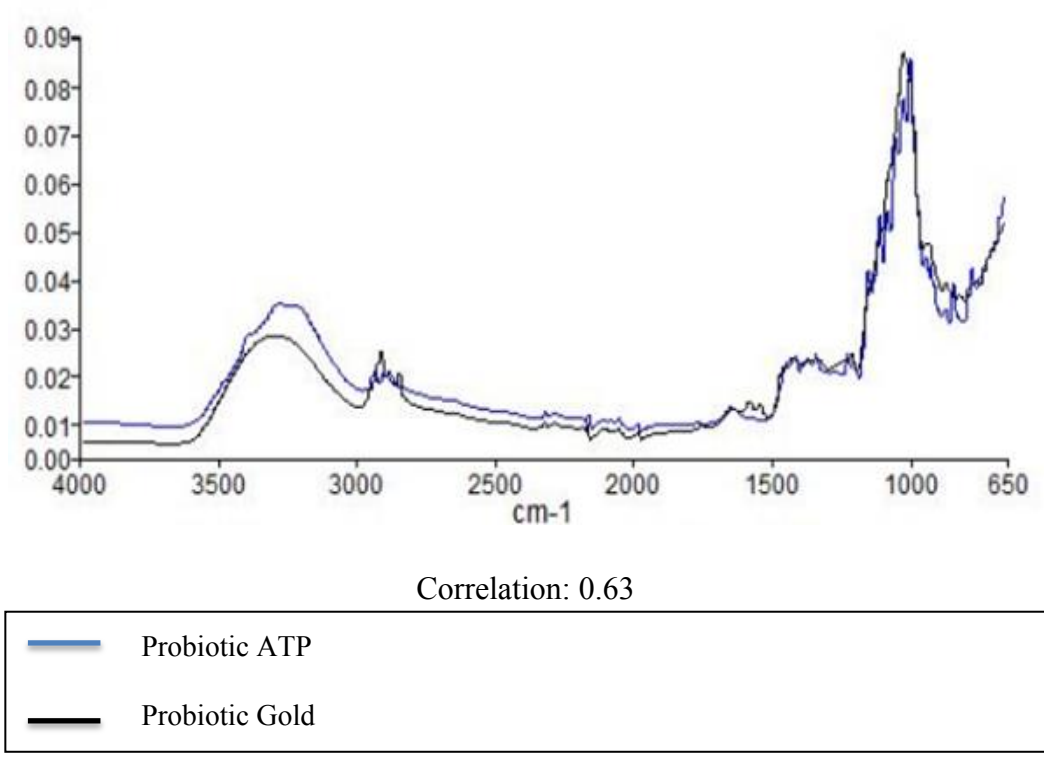


Figure 4.22. FTIR spectra comparison of commercial probiotic products Probiotic Gold and Probiotic ATP

Comparison of FTIR Spectra of Probiotic Gold and Bakso Sachet commercial probiotic supplement gave a 0.54 correlation as seen in the Figure 4.23. This result shows that the product contain approximately 54% *B. lactis.* These findings confirm that this product consists of both of the probiotics, namely *B. lactis* and *L. acidophilus* as claimed on the label.

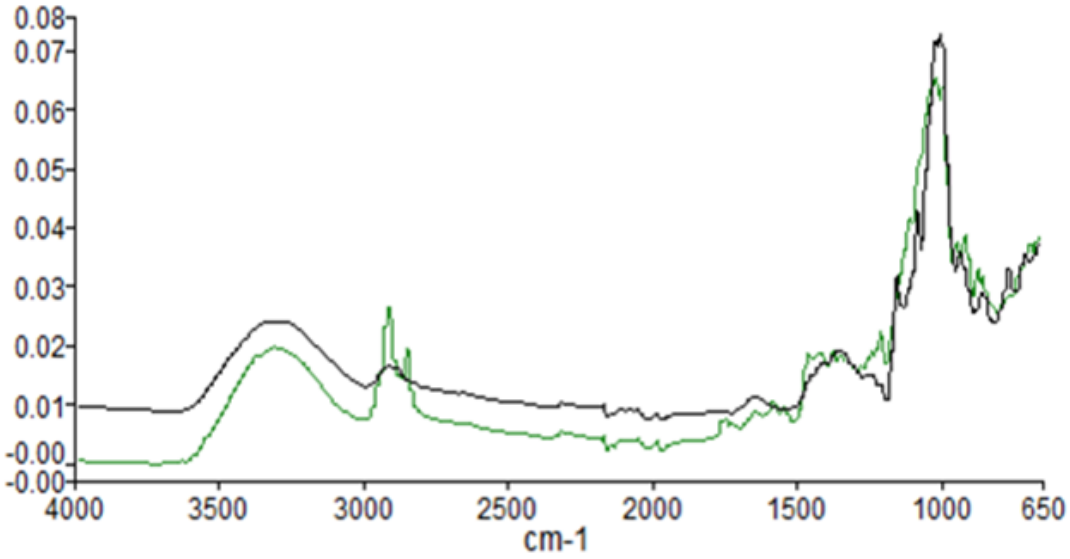


Figure 4.23. Comparison of FTIR spectra Probiotic Gold and Bakso Sachet

CHAPTER 5

CONCLUSION AND FUTURE PERSPECTIVE

Bifidobacterium animalis subsp. *lactis* BB-12 and *Lactobacillus acidophilus* LA-5 are the well-known probiotic bacteria. Enumeration and identification of these bacteria from commercial products by molecular and spectroscopic techniques was the object of the study. Pure cultures were used to validate the real time PCR and FTIR analysis. At the end of the study;

• qPCR method was used to determine for quantification of *Bifidobacterum* and *Lactobacillus*, and also the numbers of the *Bifidobacterium animalis* subsp. *lactis* and *Lactobacillus acidophilus* in different commercial probiotic supplements. Results obtained show qPCR have the potential as a rapid, sensitive, reproducible and reliable method that can be used to detect and differentiate the probiotic cells in commercial products within a short time.

• To the best of our knowledge FTIR spectroscopy was used for the first time to determine the ratios of different microorganisms in commercial probiotic supplements. FTIR results clearly indicate the potential of FTIR spectroscopy as a rapid, with a low price screening method for probiotic bacteria. In this study *Bifidobacterium animalis* subsp. *lactis* BB-12 was well detected in comparison with its species specific FTIR spectra the ratio and differentiated by FTIR in the commercial product that contains this probiotic.

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APPENDIX A

RECIPES FOR CULTURE MEDIA

A.1 MRS Broth

Ingredients	g/l
Pepton	10.0
Lab-Lemco meat extract	10.0
Yeast Extract	5.0
D(-) Glucose	20.0
Tween 80	1 ml
K_2HPO_4	2
Sodium acetate	5.0
Triammonium citrate	2.0
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Deionized water	1000 ml

A.2 MRS Agar

Ingredients	g/l
Pepton	10.0
Lab-Lemco meat extract	10.0
Yeast Extract	5.0
D(-) Glucose	20.0
Tween 80	1 ml
K_2HPO_4	2
Sodium acetate	5.0
Triammonium citrate	2.0
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Agar	15.0

Deionized water 1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was sterilized by autoclaving at 121°C for 15 min.

Table A.1. Chemicals

1	Agarose (standard)	AppliChem A2114
2	Chloroform	AppliChem A3830
3	Isoamyl alcohol	AppliChem A2610
4	PCR tubes	Greiner
5	EDTA	AppliChem A2937
6	Tris Base	Sigma T6066
7	SDS	AppliChem A0805
8	2-mercaptoethanole	Merck 8.05740
9	8-hydroxy chilonine	AppliChem
10	Phenol crystalline	AppliChem A1594
11	Isopropanol	AppliChem A3928
12	Ethanol (Molecular Biology Grade)	AppliChem A1151
13	Taq DNA Polymerase	MBI, Fermentas EP0401
14	Primers: Ege1	-
15	dNTP Set	MBI, Fermentas R0181
16	Sodium acetate	Sigma S-2889
17	Ethidium Bromide	AppliChem A1151
18	Taq I	Fermentas ER0671
19	Hae III	Fermentas ER0151
20	EcoR I	Fermentas ER0271
21	Lysozyme	AppliChem A3711
22	Ribonuclease A	AppliChem A3832
23	100bp DNA Ladder Gene RulerTM	Fermentas SM 0328
24	Glycerol	AppliChem A2926
25	Cetyltrimethylammonium bromide	AppliChem A0805
26	Proteinase K	AppliChem A3830

APPENDIX B

BUFFERS AND STOCK SOLUTIONS

B.1 1M Tris-HCl pH 7.2 and pH 8.0

121.1 g of Tris base was dissolved in 800 ml of deionized H_2O. pH was adjusted to the desired value by adding concentrated HCl. The approximate values of the amount of HCl required for the desired pH values are given below.

pH	HCl
7.4	70 ml
7.6	60 ml
8.0	42 ml

The solution was allowed to cool to room temperature before making final adjustments to the pH, and the volume of the solution was adjusted to 1 L with H2O. The pH of Tris solutions is temperature-dependent and decreases approx. 0.03 pH units for each 1°C increase in temperature. It was dispensed into aliquots and sterilized by autoclaving. If the 1 M solution had a yellow color, it was discarded and obtained Tris of better quality.

B.2 0.5 M EDTA pH 8.0

186.1 g of disodium EDTA•2H2O was added to 800 ml of deionized H2O. It was stirred vigorously on a magnetic stirrer. The pH was adjusted to 8.0 with 10N of NaOH (or approx. 20 g of NaOH pellets). Volume was adjusted to 1 L with deionized water. It was dispensed into aliquots and sterilized by autoclaving. The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to approx. 8.0 by the addition of NaOH.

B.3 50X TAE

242 g of Tris base was dissolved in deionized H_2O. 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0) were added to the solution. Lastly volume was adjusted to1 L with deionized water.

B.4 1X TAE

20 ml of 50 X TAE buffer was taken and the volume was adjusted to 1 L with deionized water. The 1x working solution was 40 mM Tris-acetate/1 mM EDTA.

B.5 3M NaCl

175.32 g NaCl was dissolved in deionized water and the volume was adjusted to 1L.

B.6 5M NaCl

292.2 g NaCl was dissolved in deionized water and the volume was adjusted to1L.

B.7 Ethidium Bromide Stock Solution (10mg/ml)

0.5 g ethidium bromide was dissolved in 50 ml deionized water and the solution was storred in dark bottle at room temperature.

B.8 3M Sodium Acetate pH 5,2

408.3 g of sodium acetate•$3H_2O$ was dissolved in 800 ml of deionized H_2O. The pH was adjusted to 5.2 with glacial acetic acid. The volume was adjusted to 1 L with deionized H_2O.

B.9 Chloroform-Isoamyl Alcohol Solution

96 ml of chloroform was mixed with 4 ml of isoamyl alcohol.

B.10 Phenol

Most batches of commercial liquefied phenol are clear and colorless and can be used in molecular techniques without redistillation, however some batches of

liquefied phenol are pink or yellow, and these should be rejected. Crystalline phenol was preferred in experiments. First, it was allowed to warm at room temperature and then it was melted at 68°C. Before use, phenol must be equilibrated to a pH of >7.8 because the DNA partitions into the organic phase at acid pH. Gloves, full face protection, and a lab coat should be weared when carrying out this procedure.

To the melted phenol, an equal volume of 0.5 M Tris-Cl (pH 8.0) buffer was added at room temperature. The mixture was stirred on a magnetic stirrer for 15 minutes. When the two phases have separated, the aqueous phase (upper phase) was removed with separation funnel. Then an equal volume of 0.1 M Tris-Cl (pH 8.0) was added to the phenol. The mixture was stirred on a magnetic stirrer for 15 min. The upper aqueous phase was removed as described before. The extractions were repeated until the pH of the phenolic phase is >7.8 (as measured with pH paper).

After the phenol was equilibrated and the final aqueous phase has been removed, phenol was divided to aliquots and 0.1 volume of 0.1 M Tris-Cl (pH 8.0) was added on top of each aliquot. The phenol solution should be stored in this form under 100 mM Tris-Cl (pH 8.0) in a light-tight bottle at -20°C. When needed, phenol was melted at room temperature. Hydroxyquinoline (to a final concentration of 0.1%), and ßmercaptoethanol (to a final concentration of 0.2%) were added before use. Hydroxyquinoline is an antioxidant, a partial inhibitor of RNase, and a weak chelator of metal ions. In addition, its yellow color provides a convenient way to identify the organic phase.

B.11 10% Sodium Dodecyl Sulfate (SDS)

100g of SDS was dissolved in 900ml of deionized water. Solution was heated to 68°C to dissolve. The pH was adjusted to 7.2 with the addition of a few drops of concentrated HCl. The volume was adjusted to 1L with deionized water.

B.12 CTAB/NaCl Solution:

4.1 g NaCl was dissolved in 80ml deionized water. 10g CTAB was added slowly while heating and stirring. The solution can be heated to 65°C to increase the dissolution. Lastly, the final volume was adjusted to 100ml.

B.13 10% Sodium Dodecyl Sulfate (SDS)

100 g of SDS was dissolved in 900 ml of deionized water. Solution was heated to 68°C to dissolve. The pH was adjusted to 7.2 with the addition of a few drops of concentrated HCl. The volume was adjusted to 1L with deionized water.

B.14 Gel Loading Dye

2 ml of 10XTBE and 6ml of glycerol was mixed in a falcon and the volume was adjusted to 20 ml with sterile deionized water. Bromophenol blue was added until the adequate color was obtained.

B.15 10X TBE Buffer

108 g of Tris base and 55 g of boric acid are mixed and dissolved in 800 ml of deionized water. 40ml o 0.5M EDTA (pH 8) was added. The volume was adjusted to 1L with deionized water.

B.16 PRIMER OF EGE1

590 µl primer EGE1 was dissolved in 295 µl of sterile deionized water to obtain 2µg/µl stock solution. 5µl of stock solution were then taken and mixed with 95µl sterile deionized water. The resulting solution had 100 µl, 10 picomole /µl concentration. Stock and working solutions were stored at -20°C.

APPENDIX C

PCR RECIPES

C.1 PCR Mixture

Mg free Taq DNA polymerase buffer 5µl

MgCl2 (25 Mm) 3µl

Sterile deionized water 32µl

Oligo forward 10 picomole/µl 1µl

Oligo reverse 10 picomole/µl 1µl

dNTP (2 mM each) 5 µl

C.2 dNTP (10X) and (2X)

Twenty microliters of each 100mM dATP, dCTP, dGTP, and dTTP are taken and mixed in an Eppendorf tube. 920 µl of sterile deionized water was added to a final concentration of 2 mM. Solution was mixed gently and stored at -20°C.