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Development of real-time PCR assay for detection of total bacteria in beverage emulsions

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Development of Real-Time PCR Assay for Detection of Total Bacteria in Beverage Emulsions

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Abstract

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Development of Real-Time PCR Assay for Detection of Total Bacteria in Beverage Emulsions

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The rapid growth and expansion of the soft drinks market and the necessity to meet and maintain the consumers' expectations of having high quality products safe for consumption, have drawn the attention to the need for rapid and sensitive methods for the detection of potential microbial contaminations. This has made the current conventional culture-based methods inconvenient due to the relatively long time they need to yield results, in addition to their relatively low sensitivity. In contrast, real-time PCR is a rapid and sensitive molecular detection technique, capable of providing quick detection and quantification methods of specific DNA sequences. In this study, a real-time PCR assay for the determination of total bacteria in one of the microbiologically sensitive constituents of soft drinks, called beverage emulsions, was successfully developed. This included the development of a modified DNA extraction protocol and the selection of a set of universal primers targeting a conserved region in the 16S rDNA of bacterial genome. The quantification strategy was based on a standard curve and a calculation method for the conversion of the determined DNA concentrations to bacterial cells numbers. This enabled the sensitive determination of total bacteria in beverage emulsions in the range between 10 fg/µL and 100 ng/µL, corresponding to 2 and 2 x 10^7 cells of *Escherichia coli*, respectively, in 6 to 8 hours instead of 7 days required by the pour plate method. Further optimization of the developed assay may allow the determination of viable bacterial cells, which will extend the scope of the developed assay applications in the beverage industry.

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List of Abbreviations

AAB	:	Acetic Acid Bacteria
ADI	:	Average Daily Intake
BHA	:	Butylated hydroxyl anisole
BHT	:	Butylated hydroxyl toluene
C	:	Cytosine
cDNA	:	Complementary Deoxyribonucleic Acid
CFU	:	Colony Forming Unit
DNA	:	Deoxyribonucleic Acid
dsDNA	:	Double-Stranded Deoxyribonucleic Acid
EU	:	European Union
G	:	Guanine
GHP	:	Good Hygienic Practices
GMP	:	Good Manufacturing Practices
HACCP	:	Hazard Analysis and Critical Control Point
HPLC	:	High Performance Liquid Chromatography
LAB	:	Lactic Acid Bacteria
LED	:	Light-Emitting Diode
NCBI	:	National Center for Biotechnology Information
NTC	:	No-Template-Control
PCR	:	Polymerase Chain Reaction
PCR-ELISA	:	Polymerase Chain Reaction-Enzyme-Linked Immunosorbent Assay
PET	:	Polyethylene Terephthalate
PNA	:	Peptide Nucleic Acid
Q	:	Quencher
R	:	Reporter
rDNA	:	Ribosomal Deoxyribonucleic Acid
RNA	:	Ribonucleic Acid
RTD	:	Ready-to-Drink

SAIB	:	Sucrose Acetate Isobutyrate
ssDNA	:	Single-Stranded Deoxyribonucleic Acid
TNTC	:	Too Numerous to Count
USA	:	United States of America
UV-Vis	:	Ultraviolet-Visible

Chapter One: Background and Literature Review

1.1Soft Drinks

Soft drinks can be defined in many different ways. However, they are generally described as "*beverages containing flavorings and/or fruit juices together with other constituents of technological or nutritional value designed to enhance the appearance and stability of the product to ensure its organoleptic properties remain intact during a reasonable shelf life*" [1].

1.1.1 Market and Consumption Trends

The market of the soft drinks is big and rapidly growing. They are available almost everywhere in the world. The global soft drinks volume sales has increased from 1,771 billion liters in 2011 to 1,974 billion liters in 2014, and it is expected to reach 2,128 billion liters by 2016 (Figure 1) [2]. Moreover, the projected growth of the soft drinks sales in 2014 was 3.8 %, compared to 3.6 % in 2011, and it is expected to be 3.9 % by 2016 (Figure 2) [3].

The world's largest soft drinks company, The Coca Cola Company [4], is leading the soft drinks market in the world (Figure 3). It has been operating since 1886 [4], and its core brand "Coca Cola®" has been ranked the third among the world's best brands in 2014 with a value of approximately 82 billion dollars, competing with the technology giants Apple Inc. (approximately 119 billion dollars) and Google (approximately 107 billion dollars) [5], showing how big the soft drinks market is.

Furthermore, according to its 2013/2014 sustainability report, the 20 billion-dollars portfolio of The Coca Cola Company includes more than 3,500 different products worldwide and more than 500 sparkling and still brands sold in the form of 1.9 billion servings a day and reaching people in nearly 200 countries [4]. Figure 4 shows the product portfolio distribution share of The Coca Cola Company worldwide in 2011 by category [6].

1.1.2 Development Trends

Different development trends in the manufacturing of soft drinks have been observed recently and the growth of the market is moving in different directions. One of the most significant trends is the use of non-calorific artificial sweeteners. Another trend is moving towards the search for unusual ingredients and new flavors such as botanical extracts (e.g. guarana and ginseng) for their indirect qualities, and ingredients with special nutritional or physiological effects (e.g. fruit juice, vitamins, minerals, and protein) [7], leading to an expansion of the soft drinks market in the area of functional drinks including energy drinks, sports drinks, wellness drinks, nutraceuticals, and soft drinks enriched with juices, vitamins and minerals. These drinks have been formulated to provide specific health or medical benefits such as improving immunity, enhancing heart health, and boosting energy. The market of these drinks is so diverse and varies according to age and gender with a trending focus on children, women and seniors [8].

1.1.3 Nutritional Significance

The expansion of the soft drinks market has drawn the attention of both the soft drink manufacturers and consumers to its health impact [8]. The nutritional significance of soft drinks in general comes from the fact that they are important vehicles for hydration. Because of their osmolality, they can be absorbed even more readily than water, hence replacing lost salts and energy easily and quenching thirst rapidly. They are also formulated to meet the nutritional needs, tastes, and physiological constraints of the population [7].

Soft drinks have three main nutritional significance areas. Energy is the first. Some soft drinks are formulated to provide the consumer with a rapid energy boost. The second area of nutritional significance is associated with the isotonic drinks which have osmolality equivalent to the body fluids and are used by the sportspeople because of their ability to promote rapid update of body salts and water leading to instant hydration. Third, many soft drinks have been formulated to low-calorie forms for those who wish to enjoy beverages while minimizing their calorific intake. Soft drinks manufacturers also claim some additional nutritional benefits such as delivering essential vitamins and minerals, especially to children [7].

1.1.4 Types

Soft drinks can be classified in many ways. This can be based on their functionality, sugar or juice content, main ingredients, carbonation level, or flavorings [8]. However, the most common way of soft drinks classification divides them into two main categories: ready-to-drink (RTD) products, and concentrated (also called dilute-to-taste or dilutable) products [7].

RTD products constitute the largest share of soft drinks production. They can be still or carbonated. Carbonated RTD products dominate the world's soft drinks market. Concentrated products are purchased in a concentrate form by the consumer who then adds water (carbonated if needed) to achieve the desired taste [7]. Recent studies also suggested dividing RTD products into juice-containing and essence-flavored [8].

Although the term 'soft drinks' generally does not include coffee, tea, milk or alcohol [7], recent studies have classified bottled water, bulk/hot water, iced and RTD tea and coffee as soft drinks. Table 1 lists the main types of soft drinks along with brief descriptions [8].

1.1.5 Constituents

Because the main function of soft drinks is hydration, their main ingredient is water [7]. In addition, whether a soft drink is RTD or concentrated, soft drinks are mainly made up of water, sweeteners (natural or artificial), acids, flavorings, colorants, and preservatives. There is also a large number of additional ingredients that can be added to the soft drinks for numerous special effects. Table 2 summarizes the main constituents of soft drinks, along with their general functions, typical use levels and commonly used examples [1].

1.1.6 Flavorings

Flavorings are the main constituents of many types of soft drinks. They are responsible for attracting and pleasing the consumer by providing the soft drinks with their generic identities and unique characters [1].

Flavoring is a mixture of aromatic substances carefully balanced to deliver the correct message to the consumer's sensory receptors. There are two type of flavorings for soft drinks:

water-miscible flavorings (flavoring mixtures and flavoring essences), and water-dispersible flavorings [1].

Water-miscible flavorings are formulated to dissolve easily in water because they typically contain hydrogenated and highly polar compounds. On the contrary, water-dispersible flavorings are insoluble because their constituents contain relatively non-polar oil phase. In order to be added to a soft drink, this type of flavorings has to be in the form of an emulsion, enabling the oil-based flavoring compounds to be introduced in a soluble form [1].

1.1.7 Water-Dispersible Flavorings

Water-dispersible flavorings, also known as beverage emulsions, are the scope of this study. They are designed to introduce oil-soluble flavor substances to final beverages, as well as providing it with cloud effects. They are produced by the mechanical dispersion of an oil phase into an aqueous phase [1].

The oil phase is responsible for carrying the flavor substances. Suitable cloudifying and stabilizing agents are also dissolved in this phase. This includes ester gum, gum damar, gum elemi, sucrose acetate isobutyrate (SAIB), or beeswax [1].

The water phase provides a protective buffer zone around each oil droplet due to its specific hydrocolloidal components that act in this case as emulsifying agents such as the gum acacia (also known as gum Arabic) and the modified starch [1].

Figure 5 shows a typical sequence of beverage emulsions manufacturing process. The mechanical stage in this process, called homogenization, and the uniformity and size of the droplets in the dispersed oil phase are so critical to achieve optimum performance of the finished beverage in terms of flavoring and cloudiness. The ideal diameter of the droplets is 1-2 μm of the emulsion is to be used to provide maximum optical density and to produce a stable cloudy beverage. Choosing the correct mixture of stabilizers is also critical because in order to maintain the emulsions stability, droplets must be kept away from each other, and they also must not interact with the other components of the beverage [1].

1.2Microbial Problems of Soft Drinks

Microbial contamination of soft drinks originates during the manufacturing process. Sources of this contamination may include raw materials, manufacturing environment and equipment, packaging materials (such as cans and bottles), and lack of hygiene [9-11]. Many systems have been developed to ensure food safety in general (also applied to soft drinks manufacturing) by minimizing the potential of microbial contamination. These systems include Hazard Analysis and Critical Control Point (HACCP), Good Manufacturing Practice (GMP), and Good Hygienic Practice (GHP) [12].

Soft drinks are considered to be ideal media for many microorganisms because they contain the nutrients microorganisms need to grow and multiply. This includes water, carbohydrates as a source of carbon, amino acids as a source of nitrogen (important for cell formation during growth), phosphates as a source of phosphorous, minerals such as potassium and calcium (and traces of other minerals such as sulfur, iron, cobalt), and vitamins [1].

Soft drinks are attractive and suitable environments for microbial spoilage because they have high water activity and are rich in vitamins, minerals and other nutrients. Table 3 lists some of the most common bacterial species associated with microbial spoilage of soft drinks, along with their typical effects and quality changes [8].

1.2.1 Types of Microbial Problems

Most of the soft drinks microbial problems are caused by yeast, mold, and bacterial species [8,11]. The latter is the scope of this study.

Microbial issues related to the soft drinks are divided into two main types [11,13]:

1. Spoilage, in which microorganisms grow in and deteriorate the product
2. Poisoning, in which microorganisms grow in and contaminate the product

Spoilage is more likely to be associated with soft drinks, however, several poisoning instants have been reported [11,13].

1.2.2 Spoilage

Microbial spoilage is a metabolic process responsible for developing uncharacteristic sensory attributes in food products including soft drinks, making it unsuitable for human consumption [10,14-17].

Some species of lactic acid bacteria (LAB) belonging to *Lactobacillus* and *Leuconostoc* can grow in soft drinks containing fruit juices (Table 3). They have been isolated from fruits, fruit juices, and packaging materials used in this type of soft drinks, and they have been found to be resistant to some of the most commonly used preservatives in soft drinks manufacturing such as benzoic acid and sorbic acid [14,15].

Acetic acid bacteria (AAB) (Table 3) are aerobic, that is they require some oxygen for growth. Hence, they are less common causes of soft drinks spoilage than LAB [15]. However, AAB can damage the soft drinks packed in oxygen-permeable packages such as some types of PET bottles. They can also tolerate as low pH as 3.0 to 3.8, and show resistance to the most commonly used preservatives in soft drinks manufacturing such as benzoic and sorbic acids, and dimethyldicarbonate [15,18].

Although they are generally acid-intolerance, Coliforms such as *Klebsiella*, *Citrobacter*, and *Enterobacter*, and other members of *Enterobacteriaceae* are found to cause soft drinks spoilage due to their ability to multiply in pH values lower than 4.3[15].

1.2.3 Poisoning

Due to poor hygiene, soft drinks can be contaminated by pathogenic bacteria such as *Escherichia coli* O157:H7 and various serotypes of *Salmonella*. Both were found to cause fruit juice-related foodborne diseases [16,19].

Bacterial pathogens can remain viable in carbonated soft drinks for different periods of time. *Escherichia coli* and *Salmonella,* for example, are capable of surviving up to 48 hours in a cola soft drink, while *Yersinia enterocolitica* has found to survive in a commercial orange soft drink of pH 3.5 for 3 days at 30 °C [19-22].

The formulation of modern beverages uses exotic juices with pH values between 4.8 and 6.2 which proved suitable conditions for the survival and growth of pathogenic bacteria for long periods of time sufficient enough to transit diseases [16].

Another suitable environment for the survival of pathogenic bacteria are the concentrates of soft drinks. *Listeria monocytogenes* and *Y. enterocolitica*, for example, are capable of surviving in freshly pressed orange juice and different other juice concentrates [22,23]. It is worth to mention that incubation at low temperatures (4 °C) has been found to enhance the survival of pathogenic bacteria [24].

1.2.4 Preservation

The most important factor in soft drinks preservation is acidity. It also enhances the heat treatment effects which in turn act as an additional barrier for microbial growth. The low pH values of most of the soft drinks (below 4.0) prevents the growth of the majority of the heterotrophic bacteria. Chilled storage conditions can be used to extend the shelf lives of the open fruit juices. The shelf lives of juices can also be doubled from 35 to 65 days by using oxygen-impermeable packaging materials [8].

Preservation sometimes is not enough to stop the bacterial contamination. For example, the new AAB *Asaia* spp. has been isolated from reclaimed fruit beverages and flavored waters. Also, LAB belonging to *Lactobacillus* and *Leuconostoc* can grow in soft drinks containing fruit juices. *Streptomyces griseus* is another example of bacteria capable of growing in soft drinks even if the temperature is as low as 4 °C, and the oxygen is limited [8].

1.2.5 Pasteurization

Pasteurization is another way used by soft drinks manufacturers to inhibit the microbial contamination in soft drinks made without preservatives such as some of the drinks containing fruit juices and teas. However, similar to preservation, pasteurization can sometimes be not sufficient to stop the bacterial contamination in soft drinks.

Spore-forming bacterial, such as *Propionibacterium* spp., is one of the causes of soft drinks spoilage. *Propionibacterium* spp. can grow even at refrigerated temperatures [25]. Although inhibited in acidic soft drinks, spore-forming bacteria of *Bacillus* and *Clostridium* can cause soft drinks spoilage because their spore can remain viable [14]. Another example of spore-forming bacteria associated with soft drinks spoilage is *Alicyclobacillus* spp. (ACB) (Table 3). Spoilage caused by these bacteria occurs in ice-tea, isotonic water, lemonade and carbonated and noncarbonated fruit juices [26].

It is worth to mention that the importance of spore-forming bacteria as a source of soft drinks spoilage recently increased due to the trending growth in the soft drinks market in the area of functional beverages which are rich in fruit and vegetable juices, and fibers [8].

1.3Microbiological Testing

Consumers expect the quality of the soft drinks they buy to be guaranteed, and that they are safe for consumption [8]. This is an essential customer need that shall be met and maintained at all times by soft drink manufacturers. Many studies have been conducted to show the possible link between the consumption of soft drinks and health issues or hospital admissions.

Because of this, and in light of the previously described potential microbial contaminations of soft drinks, microbiological testing became an essential quality control parameter in the soft drinks industry. It is used to determine the presence or absence of microorganisms in a given sample, reported as a number of microorganisms per sample volume [27].

There are two main conventional microbiology testing methods used in the soft drinks industry: the Membrane Filtration Method, and the Pour Plate Method [27].

1.3.1 Membrane Filtration Method

In this method (Figure 6), a given volume of a liquid sample is filtered through a very thin cellulose membrane filter that includes very fine microscopic pores of diameters much smaller than that of the cells of the tested microorganism. This mechanism will cause the cells

to be trapped over the cellulose membrane filter which is then removed gently from the filtration equipment and placed over an absorbent pad previously soaked with the nutrient medium (also called nutrient broth) and placed in a petri dish. The composition of the nutrient medium is specially formulated to allow the growth of the cells of the concerned microorganism specifically.

The membrane is then gently tapped to stick to the absorbent pad, and the petri dish is then covered and incubated at a temperature favorable for the growth of the concerned microorganism for a certain period of time varies from hours to days depending on the type of the microorganism.

What happens next is that the trapped cells will absorb the nutrients they need from the pad, and start to grow and multiply to form individual visible colonies, the size and shape of which differ from one type of microorganisms to another, but in all cases it will be clearly visible to naked eye and, accordingly, it can be counted.

Every single trapped cell over the cellulose membrane filter that can use the nutrient medium, incubation temperature and time for growth and multiplication, will be able to enter the previously described colony-forming process ending up with a visible colony. The number of colonies will correspond to the number of microorganism's cells initially found in the given volume of the liquid sample.

Nutrient agar in petri dishes can also replace the absorbent pad with the added nutrient medium. The cellulose membrane filter, in this case, is placed over the surface of the solidified nutrient agar. The colony-forming process occurs in this case in the same way as in the absorbent pad case.

It is important to mention that there may have been cells of other microorganisms trapped over the cellulose membrane filter. However, they will not grow either because the nutrient medium composition is not suitable, or the incubation temperature and time are not favorable for their growth. This shows that different microorganisms can be selectively grown by using

specific nutrient media, incubation temperature and time. Moreover, the nutrient medium can be specifically formulated to inhibit the growth of a certain type of microorganisms.

1.3.2 Pour Plate Method

In pour plate method (Figure 7), the nutrient medium used is actually supplied in the form of a mixture of nutrient medium and agar called 'nutrient agar'. This is then mixed with water in a suitable container and sterilized, usually using autoclaves. The container is then placed in a water bath at a temperature of about 45 °C which is suitable for keeping the nutrient agar in the liquid form, and it is not hot enough to kill the microorganisms in the samples.

A measured volume of the sample is then placed in a petri dish, to which around 20 mL of the nutrient agar is added. The petri dish cover is then placed back and the dish is then gently swirled in order to mix the sample thoroughly with the nutrient agar, and to spread the sample in the form of a thin layer all over the petri dish. The nutrient agar including the sample is then allowed to cool down to form a solidified gel layer in the dish. Similar to the membrane filtration method, the dish is then incubated for a certain period of time at a temperature favorable for the growth of the desired microorganism.

What happens next is that the single cells of the tested microorganism will grow and multiply to form visible colonies, mainly on the surface of the nutrient agar, and sometimes embedded in its thin layer (Figure 8a). After the desired incubation period is passed, all visible colonies are counted to obtain final results.

The results of the pour plate method (and the membrane filtration method as well) are reported in the form of colony-forming unit (CFU) per sample volume (e.g. 5 CFU/100 mL). This way of results reporting indicates that the results are based on counting the grown colonies of the microorganism, not on the actual direct microscopic examination of the microorganism's cells.

1.3.3 Advantages and Limitations

The primary advantage of the membrane filtration method as a microbiological testing method in the soft drinks industry is that it allows the testing of large volumes of samples easily because they can be filtered so quickly (sometimes using vacuum pumps) making it the most commonly used microbiological testing method in the soft drinks industry.

On the other hand, and taking into account the wide range of soft drinks types (Table 1), a main limitations of the membrane filtration method is that it is not suitable for all types of soft drinks. The ingredients of some of the products, as shown in table 2, can quickly block the pores of the cellulose membranes used in this method which will either remarkably slow down the filtration process, or will completely stop it. This is usually the case with juices and juice-containing products, and it is the same scenario with the beverage emulsions because of their relatively high density. This makes membrane filtration method unsuitable for the microbiological testing of this type of soft drinks. Alternatively, pour plate method is the one currently used for this purpose, and it is the one used as a gold reference in this study.

Another limitation that applies to both methods is that small-size sample do not give an actual pictures of the microbiological content of the original samples, especially if the latter already contains very low levels of microbial contamination.

In addition, the grown colonies on either the cellulose membrane of the membrane filtration method or the nutrient agar of the pour plate method can be too many to be virtually counted (Figure 8b). They can also be attached to each other forming a single continuous layer of microbiological growth in the petri dish, either over the cellulose membrane filter in the membrane filtration method, or over the nutrient agar if the method used is the pour plate method (Figure 8c).

In these cases, another sample is then taken and diluted with sterile water in a serial manner to get 10, 100, or even 1000-folds dilutions, which are again tested. The resulted microbiological count is then multiplied by the dilution factor to get the final count per the originally given volume of the sample. However, if the microbiological count exceeds a specific

limit, then it can be reported as too numerous to count (TNTC). Whether serial dilutions are used or the results are reported as TNTC, both can just give an idea about how high the count is, or how bad the microbial contamination is, but it cannot help if an exact estimate is to be reported.

Another limitation of the pour plate method is that the growth of the aerobic microorganisms embedded in the nutrient agar layer can be inhibited due to the lack of oxygen leading to false results.

Among the previously mentioned, one of the most important limitations of both methods is the long time required to yield results, making them inappropriate when rapid results are required [28].

1.4Real-Time Polymerase Chain Reaction (Real-Time PCR)

In 1971, Kleppe and colleagues were the first to introduce the concept of making many copies of a DNA molecule by the cycling processes using DNA polymerases and oligonucleotides [29]. This seemed, at that time, to be very remote and challenged by many scientists because of the non-availability of thermostable DNA polymerases, the difficulty and high cost of producing oligonucleotides, and the lack of automated thermo-cycling instruments.

The first demonstration of the PCR process was introduced by Saiki and colleagues in 1985 [30]. By that time, automated oligonucleotide synthesizers were commonly available. This opened the door for a wide range of PCR applications. However, there was still a need to inject fresh thermo-labile polymerases prior to each elongation step, an impractical process that proved that the decisive step in realizing the potential of PCR was the use of thermostable polymerases which was first introduced by Saiki in 1988 [31]. In 1993, the Nobile Prize in chemistry was awarded to Dr. Kary Mullis “for his invention of the polymerase chain reaction (PCR) method” [32-34].

Many refinements have been introduced to the DNA amplification by PCR since its first description. It became an essential instrument now for biologists and biochemists. Its protocols

are very simple and user friendly, and its exponential amplification process provides nanogram quantities of identical DNA starting with only a few copies of the target sequence sufficient for post-amplification processing [35].

1.4.1 What is Polymerase Chain Reaction (PCR)?

PCR is a procedure used primarily to copy and amplify DNA [36]. It uses DNA polymerases to amplify specific DNA pieces using sequence-specific, short oligonucleotides added to the reaction mixture to act as primers. The most commonly used polymerase in PCR reactions is the heat-resistant *Taq* DNA polymerase (from *Thermus aquaticus*) [37].

The importance of being heat resistant comes from the fact that after each cycle of DNA copying, the newly formed double-stranded DNA (dsDNA) molecules must be melted into two single DNA strands by high temperature (~ 95 ^{o}C). The reaction mixture is then cooled to allow the primers to anneal to the new single-stranded DNA (ssDNA) templates, and to allow the polymerase to initiate elongation by adding single complementary nucleotides to create a new DNA strand forming a dsDNA molecule that must then be melted apart before starting the next copying cycle (Figure 9) [37].

Theoretically, if the PCR reaction works with perfect efficiency, the number of DNA copies will increase exponentially, and there will be twice as much dsDNA molecules after each copying cycle. However, this does not happen in reality because PCR reactions do not maintain perfect efficiency because the reactants are consumed after many cycles ending up with a plateau (Figure 10) [37].

One of the main limitation of PCR is that it uses only DNA as a template. It cannot amplify RNA, for example, in the same way as DNA. This was overcome using reverse transcriptase, an enzyme that is capable of generating complementary DNA (cDNA) from an RNA template (Figure 11) [37].

1.4.2 Why Real-Time PCR?

The application of conventional PCR is primarily the amplification of a target DNA sequence. It is not directed towards the recognition of the PCR amplicon (the PCR amplification

product). For this purpose, post-amplification processing is required such as size analysis, sequencing, and probe hybridization. Some of these techniques are simple and inexpensive, but unfortunately they all are time-consuming and subjected to contamination [37].

Another limitation of conventional PCR that depends on end-point analysis is that it is not quantitative because the final yield of the PCR amplification process does not depend primarily on the concentration of the starting material in the sample [35] and because the reaction is only able to amplify DNA efficiently up to a certain limit before the plateau effect, making it almost impossible to quantify the amount of starting DNA by quantifying the amount of the product [37].

Real-time PCR provides a simple and instant recognition of specific DNA sequences in samples even if the quantity of the starting materials are very small. All real-time PCR machines are designed to measure the progress of amplification by continuously monitoring the changes in fluorescence within the reaction tubes. This technique is time-efficient, and prevents contaminating the work environment because the analysis is performed without opening the reaction tubes [35].

In addition, real-time PCR takes the advantage of the fact that the DNA amplification occurs efficiently early in the reaction process before the plateau effect. It measures the product formation during the "exponential phase" (Figure 10) by correlating the product accumulation to the changes in fluorescence. This provides a means of quantification covering the limitation of conventional PCR [37].

Moreover, the final product can be more characterized by subjecting it to elevated temperatures to determine when it is going to melt. This "melting point", also called "melt temperature" or (T_m) (Figure 12), is a unique characteristic, and it depends on the length of the product and its nucleotide composition [37].

In order to achieve the previously mentioned goals, conventional PCR has been coupled with specific fluorescent chemistries and instrumentation to become real-time PCR.

1.4.3 Chemistries

Chemistries of real-time PCR are specific fluorescent probes. There are many types of probes including DNA-binding dyes like SYBR® green, hydrolysis probes (also known as 5'–nuclease probes because the 5'-exonuclease activity of the DNA polymerase cleaves the probe), hybridization probes, and peptide nucleic acid (PNA) light-up probes [37].

SYBR® green depends on binding its molecules to the minor grooves of the dsDNA emitting one thousand fold greater fluorescence than when it is free in solution [38]. Figure 13 illustrates the mode of action of SYBR® green. This means that the greater the amount of dsDNA produced in the real-time PCR reaction tube, the greater the binding and the fluorescence emitted from the SYBR® green. The main concern about the usage of a DNA-binding dye like SYBR® green is specificity. The dissociation (melt) curve of the amplified product can be analyzed to determine its melting point as shown in figure 12. One peak suggests that one amplified sequence was obtained and the amplification was specific for a single target sequence [37].

Hydrolysis probes, like TaqMan® probes [39], are sequence specific oligonucleotides, labeled dually by two fluorophores; one is called the "quencher" (Q) and the other one is called the "reporter" (R). When both Q and R are attached to the same oligonucleotide, Q absorbs the signal from the R. However, during the amplification, the oligonucleotide is broken by the action of the DNA polymerase separating Q and R and allowing the later to liberate its signal (Figure 14). The hydrolysis of the oligonucleotide is directly proportional to the strength of the signal of R, and is linked to the progress in the DNA amplification. Hydrolysis probes are as precise as DNA-binding dyes, however they offer greater specificity because only sequence-specific amplification is measured [37].

There are several other probes depend on the quencher-reporter theme such as "molecular beacons", "sunrise primers", and "scorpion primers". Other real-time PCR chemistries are called hybridization probes, use what is called "donor" and "acceptor" fluorophores, while PNAs also emit signals upon binding to DNA. Generally, real-time PCR

chemistries are being continuously developed offering increased sensitivity and specificity, reduced cost, and enhanced multiplexing capabilities [37].

Because the aim of this study is to develop a cost-efficient assay, SYBR® green was used because of its low cost compared to other chemistries. Specificity will be ensured by the selection criteria of the primers, as well as analyzing the dissociation curves of the amplified products.

1.4.4 Instrumentation

Detecting the fluorescent signal and recording the progress of the reaction are critical requirements in the real-time PCR technology. Real-time PCR instruments must be able to excite the chemistries by specific inputs of energy. They also must have the ability to detect their emissions. Both excitation and detection have to take place simultaneously and at desired wavelengths (Figure 15) [37].

Real-time PCR instruments can supply the chemistries with the excitation energies by lamps such as tungsten halogen or quartz tungsten halogen. These are classified as broad-spectrum emission devices that may include filters to allow for choosing specific emission wavelengths. Other possible ways to supply the excitation energies are light-emitting diode (LED) or laser. Both are narrow-spectrum mission devices [37].

The chemistries emission energies can be detected by charge-coupled cameras, photomultiplier tubes, or any other types of photodetectors. Narrow filters are generally used to permit only the desired wavelength to pass to the photodetector in order to be measured [37].

An important part of the real-time PCR instrumentation is the thermal cycler to carry out the PCR reaction. It is the part of the PCR responsible for repeating heating and cooling of the reaction mixture. The reaction temperature has to be maintained consistent. Any temperature variations will results in different amplification efficiencies. Temperature can be maintained consistent by using heating blocks, heated air, or a combination of both. Because heating blocks

may take more time to change temperature, heated air is most preferable for fast thermal cycling [37].

Real-time PCR instrumentation cannot be complete without computer hardware and software. Software aims to simplify the reaction data by offering graphical results including amplification curves (Figure 10) that give data regarding the kinetics of the target sequence amplification, and dissociation (melt) curves (Figure 12) that show the characteristics of the final amplified product [37].

1.4.5 Applications

Because it is becoming faster, smaller, cheaper and easier, and because of its powerful ability to distinguish a particular sequence in a sample; real-time PCR has a wide range of applications.

It is used in quality control and quality assurance laboratories of food industry and agriculture for the detection and identification of microbes, genetically modified food and parasites. It is useful in determining the presence and quantity of specific pathogens in samples. It is also used as a precise and of low cost method for the rapid diagnosis of diseases. Forensics makes use also of real-time PCR sensitivity, speed and specificity where samples sizes are relatively small. Generally, real-time PCR is the method of choice for those who are looking for accurate, precise, sensitive, specific, and of low cost way for the detection of nucleic acid sequences [37,40,41].

1.4.6 Advantages

Many methods are used in quantifying nucleic acid sequences such as Northern and Southern Hybridization, HPLC, RNase protection assay, PCR-ELISA, scintillation proximity assay and different gel-electrophoresis PCR end-point systems. However, these methods share one or more of the following disadvantages: they are time consuming, insufficiently sensitive, require the use of radioactivity, and subjected to cross-contamination [42].

On the other hand, real-time PCR has many advantages over the previously mentioned methods. It is relatively faster. It is so sensitive in a way that it can detect less than five copies of a target sequence. It utilizes certain specific chemistries that are non-hazardous, which means no radioactivity is required. Finally, the reaction takes place in closed vessels with no post-PCR manipulations thus minimizing the chances for cross-contamination [37].

1.4.7 Limitations

There are different limitations to PCR. These limitation are applied to different types of PCR including real-time PCR. One of these limitations is that PCR is susceptible to inhibition by certain compounds that may be found in the reaction mixture. Examples are urea and hemoglobin in biological samples, and phenol and organic compounds in food samples [43]. To overcome this problem, certain other polymerases that are specifically resistant to these inhibitors may be used.

The largest limitations to PCR are coupled with human error: improper assay development, incorrect data analysis, unjustifiable conclusions during the experimental design and formation of primer-dimers. Generally, proper designing and validation of the primers is really important to ensure results specificity and accuracy. False positive and negative results must be considered when designing an assay to detect pathogens. Dissociation and amplification curves must be visually inspected, and calculations must be double checked for accuracy [37].

1.5Literature Review

Because of its advantages over other methods, real-time PCR has been used in many studies to quantify total bacteria and specific bacterial species and strains in different matrices, using different chemistries and primers sets, and targeting different sequences.

Real-time PCR was used to investigate the dynamics of bacteria, archaeal, and yeast populations in Kimichi, a Korean traditional fermented food, during its fermentation process, using SYBR® green and broad-range primers, and targeting 16S and 26S rRNA genes [44]. It was also used to quantify bacterial DNA extracted by three different methods from a model soil

system and environmental samples. SYBR® green and 16S rDNA specific universal primers were used in this study [45].

Real-time PCR is a powerful tool to accurately quantify bacterial species in dental plaque, using TaqMan® and SYBR® green, and targeting conserved rejoins in the 16S rRNA genes [46]. A broad-range bacterial quantitative real-time PCR assay, called 'BactQuant' [47], was designed for the quantification of 16S rRNA gene copy number to estimate the bacterial load.

Real-time PCR methods were also developed by many studies that also included the evaluation against culture-based gold standards. One of these methods was developed to detect *Gluconobacter* and *Gluconacetobacter* species in electrolyte replacement drinks [48]. This study was based on the artificial spiking of the samples with the concerned bacterial species, then filtration to collect the cells. The DNA was then extracted from the filters and analyzed by real-time PCR which showed sensitivity similar to the culture-based reference method used in this study.

Another quantitative real-time PCR assay was successfully developed by Rawsthorne & Phister (2006) [49] for the rapid detection of *Zygosaccharomyces bailii* from fruit juices and wine. This study included also the detection of the concerned bacterial species using a culture-based reference method. An excellent correlation was found between the bacterial cells number estimated by real-time PCR and the bacterial count obtained by the culture-based reference method. However, there was an exception where the bacterial cells number detected in one of the juice samples was overestimated by real-time PCR when compared with the reference method. This was found to be due to the less viability of the bacterial cells in that type of juices.

A real-time PCR assay was developed by Furet, Quénée, & Tailliez (2004) [50] for the absolute quantification of lactic acid bacteria in fermented milk products. In this study, there was no significant difference between the real-time PCR method and the culture-based method except for one strain where the results of the reference method was lower than that obtained by real-time PCR. Similar to the previous study of Rawsthorne & Phister (2006) [49] on the fruit

juices and wine, the reason of the low results was thought to be the loss of viability during storage.

Takahashi, Konuma, & Hara-Kudo (2006) [51] developed a real-time PCR assay for the rapid quantification of total bacteria in contaminated ready-to-eat vegetables and fruits. This study also included a comparison of the real-time PCR assay with a standard plate count method. Primers used in this study were targeting the *rpoB* gene responsible for the encoding for the βsubunit of the bacterial RNA polymerase instead of the 16S rRNA gene because the latter has multiple copies and varies among bacterial species. A high correlation between the results of the two methods was found. However, the correlation in this study was made between the C_t values obtained from the real-time PCR (not the bacterial cells calculated), and the plate count obtained from the reference method.

Real-time PCR methods were also developed for matrices other than food and beverages. A real-time PCR method was compared with a conventional agar plate count method by Fu, Carter, Li, Porter, & Kerley (2006) [52] for the enumeration of *Lactobacillus*, *Clostridium perfringens*, and total anaerobic bacteria in dog feces. The comparison showed significant correlation between the results of the two methods for *Lactobacillus* and total anaerobic bacteria. However, no correlation was found between the results of the two methods for *Clostridium Perfringens*.

Another real-time PCR method was developed to estimate bacterial concentrations in fecal samples [53]. This study included spiking the samples with measured quantities of known bacterial strains. The method was valid to estimate the concentrations of the bacterial strains except two strains, of which, one needed a correction factor.

Another real-time PCR method was developed for the quantification of total bacteria, lactobacilli and enterobacteria by Castillo *et al*. (2006) [54]. This study included a comparison between the real-time PCR method and traditional ones including selective culture for lactobacilli and enterobacteria. The real-time PCR method showed higher results than those obtained from the traditional methods. According to this study, the higher values were possibly

because of the overestimation of the real-time PCR method which was caused by the quantification of DNA coming from dead bacteria, or the quantification of free DNA. The higher values were also possibly because of the underestimation of the conventional methods, or because of the differences in the samples pre-treatment processes. Regardless of the higher results, there was a significant correlation between the results obtained from real-time PCR and the conventional methods for total bacteria and lactobacilli.

Chapter Two: Problem and Aims

The rapid growth and expansion of the soft drinks market in different directions, and the necessity to meet and maintain the consumers' expectations of having high quality products that are safe for consumption, both have drawn the attention to the need for rapid and sensitive methods for the detection of potential microbial contaminations, and have made the current conventional culture-based methods inconvenient due to the relatively long periods of time they need to yield results, in addition to their relatively low sensitivity.

In contrast, real-time PCR is a rapid and sensitive molecular detection technique capable of providing quick detection and quantification methods of specific DNA sequences even if the quantity of the starting material is small.

The aim of this work was to develop a real-time PCR assay for the determination of total bacteria in one of the microbiologically sensitive ingredients of soft drinks called beverage emulsions. This included:

1. Developing a DNA extraction protocol from the complex matrix of the beverage emulsions,
2. Selecting a set of universal primers suitable for the broad-range determination of the total bacteria by targeting a specific sequence in the conserved region of the 16S rDNA,
3. Developing an absolute quantification strategy based on a standard curve constructed using *Escherichia coli* genomic DNA standard,
4. Investigating the sensitivity of the real-time PCR reaction using the SYBR® green technology and the selected set of universal primers, and based on the developed quantification strategy in the determination of *Escherichia coli*,
5. Determining the total bacteria in the given beverage emulsion samples, and
6. Making a comparison between the results of both the developed real-time PCR assay and gold standard method.

Chapter Three: Materials and Methods

3.1 Source of Beverage Emulsion Samples and Samples Preparation

Seventeen beverage emulsion samples were obtained from the manufacturing leftovers of 17 different production batches manufactured between July and December 2014, and representing three different soft drinks' commercial brand names. Samples were stored in sterile tubes at 4 to 10°C throughout the period of this study according to the manufacturers' recommendation.

3.2 Bacterial DNA Standard

Escherichia coli DH10B genomic DNA (Ion PGM™ Controls 200 Kit, Ion Torrent™ by Life Technologies, California, USA, reference number: INS1008538) was used in this study as a standard. A dilution of this standard was used as a positive control in the real-time PCR reaction. According to the manufacturer, the concentration of the standard was 100 ng/µL. This was verified by NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, USA). The concentration of the positive control used was 71 pg/µL.

3.3 Nutrient Agar Preparation

Forty grams of the nutrient agar powder (Difco™ Tryptic Soy Agar, BD Diagnostic Systems, North Ryde, Australia, reference number: 236950) were suspended in 1000 mL of purified water, mixed thoroughly, heated with frequent agitation and boiled for 1 minute to be completely dissolved, autoclaved at 121 °C for 15 minutes, then placed in a water bath at 45 °C to remain in the liquid form.

3.4 DNA Extraction Protocol from the Beverage Emulsion Samples

The QIAamp® DNA Mini Kit (Qiagen, reference number: 51304, California, USA) was used to extract the bacterial DNA from the beverage emulsion samples.

One milliliter of each of the already prepared dilutions used in the determination of the total bacterial count in the beverage emulsion samples using the pour plate method was placed

in a 1.5 mL microcentrifuge tube, then centrifuged at 7500 rpm for 10 minutes. The supernatants were then discarded, and the pellets were resuspended in 180 µL of Buffer ATL supplied in the QIAamp® DNA Mini Kit.

Thirty microliters of proteinase K were then added to each of the microcentrifuge tubes, mixed with the suspended pellets by vortexing, then the mixtures were incubated at 56 °C for 3 hours. The samples were dispersed by vortexing the mixtures for approximately 10 seconds, 2 or 3 times per hour during the incubation period. The microcentrifuge tubes were then centrifuged for 10 seconds to remove drops from the inside of the lids. The rest of the steps was completed according to the bacterial DNA extraction protocol in the QIAamp® DNA Mini and Blood Mini Handbook.

3.5 Selection of the Universal Primers

3.5.1 Compliance with the Selection Criteria

Three sets of broad-rage (universal) primers; the forward primer 5'-TCCTACGGGAGGCAGCAGT-3' and the reverse primer, 5'-GGACTACCAGGGTATCTAATCCTGTT-3' [28]; the forward primer 5'-GGATTAGATACCCTGGTAGTC-3' and the reverse primer 5'-TACCTTGTTACGACTT-3' [55]; and the forward primer 5'-AGGAGGTGATCCAACCGAA-3' and the reverse primer ACCTGGAGGAAGAAGGTGGGGAT-3' [56,57] (will be referred to as universal primers sets A, B, and C, respectively); targeting conserved regions in the 16S rDNA of bacteria were selected from the literature and evaluated for their suitability to run this study by testing their compliance with the primers selection criteria outlined in a checklist for optimization and validation of real-time PCR assays developed by Raymaekers *et al*. (2009) which included the T_m of primers to be 58 - 60 °C; the GC content to be 30 - 70 %; not more than two C or G in the last five positions at the 3' end of the primer; the length of the amplicon to be 400 bp as a maximum; no more than four constitutive guanines; primer-dimer to be avoided; and the length of the primer to be 18 - 24 bp [58].

3.5.2 Coverage Ranges

To determine the coverage ranges of the three sets of primers, and to make sure that they cover the bacterial species previously presented in this study, they were tested using the TestPrime 1.0 [59] by running an in silico PCR on the SILVA small subunit (16S/18S) database of *Bacteria*, *Archaea* and *Eukarya* [60]. The search criteria considered a maximum number of mismatches of 3, and a length of the zero-mismatches zone at 3'end of 4 bases.

3.5.3 Annealing Positions

The annealing positions of the three sets of primers on *Escherichia coli* genome, downloaded from GenBank® [61], NCBI Reference Sequence number NC_002695.1 [62], were checked using the primers analysis software Oligo 7 [63] which was also used to check the melting temperatures, primer-dimer configurations, and the GC content of the possible primers.

3.5.4 Practical Application

To select the most suitable set of primers for this study, the practical application of the three sets of primers was tested by using them to amplify bacterial DNA extracted from eight different beverage emulsion samples by real-time PCR using the reaction conditions detailed in section 3.6. This was followed by melt curve analysis.

3.5.5 Specificity towards Some Bacterial Species Previously Presented in This Study

The specificity of the chosen set of primers towards *Lactobacillus acidophilus* (NCBI Reference Sequence number NC_006814.3) [64] and *Alicyclobacillus acidocaldarius* (NCBI Reference Sequence number NC_013205.1) [65] was investigated by using Oligo 7 [63] to check the annealing positions of the primers and the products sizes. Genomes of the bacterial species were downloaded from GenBank® [61].

3.6 Real-Time PCR Reaction Conditions

The real-time PCR reaction was done by the Applied Biosystems® StepOne™ System (California, USA) using optical grade 48-well plates. Duplicate samples were routinely used. The real-time PCR reaction was performed in a total volume of 20 µL including 10 µL of SYBR® Green PCR Master Mix (Applied Biosystems®, California, USA, reference number:

4344463), 1 µL of each of the forward and reverse primers (10 pmol), 1 µL of DNA, and completed to volume with nuclease-free water. The reaction conditions were 95 °C for 10 minutes and 45 cycles of 95 °C for 15 seconds and 60 °C for 1 minute.

3.7 Standard Curve

A serial dilution from 100 ng/µL to 10 fg/µL of the *Escherichia coli* DH10B genomic DNA standard was prepared in microcentrifuge tubes starting by adding 1 µL of the standard DNA to the first tube that contained 9 µL of nuclease-free water, vortexing for 30 seconds, then moving 1 µL of the first dilution to the second tube that also contained 9 µL and so on as illustrated in figure 16.

The serial dilution was then used to construct a standard curve by plotting the threshold cycle (C_t) values against the logarithm of the DNA concentrations using real-time PCR, the reaction conditions mentioned in section 3.6 of this chapter, and the selected set of primers.

3.8 Quantification Strategy

Using the SYBR® green technology, real-time PCR can determine the amplification cycle at which the increase in the fluorescence reaches a threshold cycle (C_t) which is proportional to log the amount of target DNA in a given sample, hence the number of bacterial cells in this sample, provided that there is only one copy of the target sequence in the genome. The standard curve used in this study was constructed based on *Escherichia coli* DH10B genomic DNA standard where each *Escherichia coli* cell, theoretically, equates to the detection of 4.96 fg DNA [28], on condition that the seven copies of rDNA in each copy of the chromosome are not considered [66].

3.9 Sensitivity of Real-Time PCR in Detecting *Escherichia coli* DH10B Genomic DNA Using Universal Primers and Standard Curve

Based on the constructed standard curve, the sensitivity of real-time PCR in detecting *Escherichia coli* DH10B Genomic DNA using the SYBR® green technology and the selected universal primers was investigated by calculating the lowest and highest numbers of *Escherichia coli* cells determined.

3.10 Determination of Total Bacteria in Beverage Emulsion Samples by Real-Time PCR

Using the same quantification strategy described in the previous section, real-time PCR was used to determine the total bacteria in the 17 beverage emulsion samples. A positive control and no-template-control (NTC) were used.

The amplification was verified by 2% agarose gel electrophoresis (Lonza, Basel, Switzerland, reference number: 50001) of the amplicon followed by visualization of the approximately 466 bp band using ethidium bromide staining (Promega, Wisconsin, USA, reference number: H5041). For band size comparison, GeneRuler 100 bp DNA ladder (Thermo Scientific, California, USA) reference number: SM0243) was used.

3.11 Determination of Total Bacterial Count in Beverage Emulsion Samples Using the Pour Plate Method

One milliliter of each beverage emulsion sample was diluted 10 times by adding it to 9 mL of nuclease-free water in a sterile tube and vortexing for 30 seconds. 1 mL of the dilution was then pipetted in a disposable sterile petri dish. The previous step was performed in duplicate. 20 mL of the nutrient agar previously prepared was then added to each of the petri dishes which were gently swirled. The mixture of the sample and the nutrient agar was then allowed to cool down until a solidified gel layer was formed in each of the petri dishes. The petri dishes were then incubated for 7 days at 22 °C. Finally, all visible colonies were counted, and the average was then calculated and multiplied by the dilution factor 10 to determine the total bacterial count in CFU per 1 mL of the beverage emulsion sample.

3.12 Comparison between the Total Bacterial Determined by Real-Time PCR and the Total Bacterial Count Determined Using the Pour Plate Method

The total bacteria determined by real-time PCR in the beverage emulsion samples using the universal primers set and the standard curve constructed using the *Escherichia coli* DH10B genomic DNA standard was then compared with the total bacteria counts obtained by using the pour plate method.

Chapter Four: Results and Discussion

4.1 DNA Extraction Protocol from the Beverage Emulsion Samples

The bacterial DNA was successfully extracted from the beverage emulsion samples using the modified bacteria DNA extraction protocol. The concentrations of the extracted DNA varied between 2.4 to 130.1 ng/µL of beverage emulsion.

4.2 Selection of the Universal Primers

4.2.1 Compliance with the Selection Criteria

The suitability of the three universal primers sets A, B, and C for this study was evaluated based on the selection criteria developed by Raymaekers *et al*. (2009). Table 5 summarizes the evaluation results.

It was found that the T_m values of the three primers sets were either above or below the recommended range. However, primers of set A were the closest to the recommended range while those of sets B and C were remarkably away from it. All the three primers sets were complying with the recommended GC content. The forward primer of set A and both primers of set C were found to contain three C or G in last five positions at 3' end which made them not complying with the recommended criteria of having less than two C or G in the last five positions at 3' end. The length of the target sequences of the three primers sets were above the recommended length of 400 bp as a maximum. However, the length of the target sequence of the primers set A was the closest to the recommended length, while the lengths of those of the primers sets B and C were impractically longer showing unsuitability for this study. All forward and reverse primers of the three sets contained less than four constitutive guanines except the reverse primer of the primers set C which was not complying with the recommended criteria. Results also showed that the three primers set can form different primer-dimer configurations with ΔG values varied from -1.7 to -4.2 kcal/mol. The lengths of all the primers were complying with the recommended length except the reverse primers of both sets A and B which were longer than 24 bp and shorter than 18 bp, respectively.

4.2.2 Coverage Ranges

The coverage ranges of the three sets of primers were tested using TestPrime 1.0 [59] by running an in silico PCR on the SILVA small subunit (16S/18S) database [60]. Figure 18 shows that the coverage range of the primers set A was 65.7 %, while those of the primers sets B and C were 63.8 % and 0%, respectively. Consequently, the primers set C was excluded. The coverage ranges of both primers sets A and B were also shown to include the bacterial species previously presented in this study.

4.2.3 Annealing Positions

The suitability of the three universal primers sets was further investigated by checking their annealing positions on *Escherichia coli* genome (NCBI Reference Sequence number NC_002695.1) [62] by Oligo 7 [63]. Results (Table 6) (Figure 19) showed the annealing positions of the forward and reverse primers of set A to be 227441 and 227882, respectively, and those of the forward and reverse primers of set B to be 227886 and 228593, respectively. The annealing positions of the forward and reverse primers of set C were 46130 and 125376 which supported excluding the universal primers set C.

4.2.4 Real-time PCR of Bacterial DNA

The three universal primers sets were used to amplify bacterial DNA extracted from eight different beverage emulsion samples by real-time PCR using the reaction conditions detailed in section 3.6 to test their practical application. The amplification using primers set A was successful (Figure 20), and resulted in a uniformed melt curve (Figure 21) with an average T_m value of 83.5 °C confirming the amplification of the target sequence. The primers sets B and C failed to amplify their targets as shown in figure 22, figure 23, and figure 24.

As a results, the selected universal primers set was set A that included the forward primer 5'-TCCTACGGGAGGCAGCAGT-3', and the reverse primer, 5'-GGACTACCAGGGTATCTAATCCTGTT-3'. This was designed by Nadkarni *et al*. (2002) by the alignment of sequences from most of the bacterial groups outlined in Bergey's Manual of Determinative Bacteriology [67], followed by manual assessment of the regions of identity within 16S rDNA [28].

4.2.5 Specificity towards Some Bacterial Species Previously Presented in This Work

Results of the investigation done using Oligo 7 [63] (Table 7) (Figure 25) showed the annealing positions of the forward and reverse primers of set A to be 59615 and 60056 on *Lactobacillus acidophilus* genome (NCBI Reference Sequence number NC_006814.3) [64] and the size of the product to be 467 bp. The annealing positions were also found to be 11113 and 11555 on *Alicyclobacillus acidocaldarius* genome (NCBI Reference Sequence number NC_013205.1) [65] and the size of the product to be 468 bp. This confirmed the suitability of the universal primers set A to conduct this study.

4.3 Standard Curve

The amplification plot of the eight concentrations used to construct the standard curve is shown in figure 26, and the melt curve of the amplification product is shown in figure 27. A uniformed peak with an average T_m value of 84.2 °C can be observed, confirming the amplification of the target sequence.

The standard curve used in this study is shown in figure 28. Each point used to construct this standard curve represented the relation between the logarithms of an *Escherichia coli* DNA concentration and its corresponding C_t (Table 8). The correlation coefficient of the straight line (R^2) was 0.979, the slope was -3.3, the intercept was 24.11, and the efficiency of the curve (E) was 101% (Table 8).

4.4 Sensitivity of Real-Time PCR in Detecting *Escherichia coli* DH10B Genomic DNA Using Universal Primers and Standard Curve

The constructed standard curve showed that it could be used to quantify *Escherichia coli* DH10B genomic DNA concentration as low as 10 fg/µL (corresponding to 2 cells of *Escherichia coli*) and as high as 100 ng/µL (corresponding to 2 x 10^7 cells of *Escherichia coli*), representing C_t values between 9.63 and 32.01, respectively. This was designated as the working range covering between 2 and 2 x 10^7 *Escherichia coli* cells (Figure 28).

The ability to detect as low as 2 cells of *Escherichia coli* showed a very high sensitivity of real-time PCR in detecting bacterial DNA.

A fluorescence signal at a C_t value above 44 was observed. It was corresponding to the NTC to which *Escherichia coli* DH10B genomic DNA standard was not added. This was thought to be bacterial DNA contamination caused by the commercially supplied SYBR® green master mix, primers, or nuclease-free water.

4.5 Determination of Total Bacterial in Beverage Emulsion Samples by Real-Time PCR

4.5.1 Real-Time PCR Reaction

The C_t values of the 17 samples, positive control, and NTC obtained from the real-time PCR reaction using the universal primers are shown in table 9. The amplification of the target was verified by the clear bands appeared between 400 and 500 bp on the agarose gel electrophoresis.

4.5.2 Quantification of the DNA Concentrations

The logarithm of the DNA concentrations and the DNA concentrations of the total bacterial load of the 17 beverage emulsion samples, positive control, and NTC were calculated using the slope, intercept, and the correlation coefficient (R^2) of the constructed standard curve (Table 10).

The calculated DNA concentration of the positive control was shown to be approximately 70.46 pg/µL, confirming the success of the real-time PCR reaction. It was also observed that some DNA was extracted from the NTC (approximately 16 fg/µL) supporting the assumption of reagents contamination.

4.5.3 Determination of the Total Bacteria

The total bacteria determined per each 1 mL of the 17 beverage emulsion samples, as well as the positive control and NTC by real-time PCR are shown in table 11. This was calculated based on the previously described quantification strategy using *Escherichia coli* DNA as a standard, taking a dilution factor of 10 into account, and based on the fact that each *Escherichia coli* cell equates to the detection of 4.96 fg DNA [28], on condition that the seven copies of rDNA in each copy of the chromosome are not considered [66]. The DNA

contamination of the NTC caused by the commercially supplied reagents was shown to be equivalent to 3 bacterial cells per milliliter.

4.6 Comparison between the Total Bacteria Determined by Real-Time PCR and the Total Bacterial Count Determined Using the Pour Plate Method

4.6.1 Determination of Total Bacterial Count in Beverage Emulsion Samples Using the Pour Plate Method

Despite of its limitations, the pour plate method is still widely used as a standard microbiological testing method in the soft drinks industries as well as other microbiology laboratories. It is suitable for many purposes, and its procedure is easy to implement. It also does not require individual equipment sterilization or prolonged filtration steps and it is less expensive because it does not need high initial capital or operating costs.

The total bacterial counts of the 17 beverage emulsion samples were determined using the conventional pour plate method. Results are shown in table 4.

4.6.2 Comparison between the Total Bacteria Determined by Real-Time PCR and the Total Bacterial Count Determined Using the Pour Plate Method

Figure 30 shows the results of the total bacteria determined in the 17 beverage emulsion samples by real-time PCR, against the total bacterial count determined in the same samples using the conventional pour plate method as a reference method (Table 12).

The mean number of the total bacteria determined by real-time PCR was 3117 cells/mL beverage emulsion, ranging from 158 to 9317 cells/mL beverage emulsion, which is relatively higher than the total bacterial count determined by the reference method was 44 CFU/mL beverage emulsion ranging from 0 to 105 CFU/mL beverage emulsion.

The P value calculated using the *t* test to compare the means of the results of the two methods was less than 0.0001, showing that the difference between the means of the results of the two methods was statistically significant.

The ratios between the results of the two methods was also calculated and expressed in cell number/CFU. The ratios varied among the samples from 21.711 to 221.031 cell number/CFU (Table 12) confirming the statistical significant difference between the results of the two methods, and no correlation could be made.

In addition, no clear pattern could be observed between the results obtained by the two methods. For example, the total bacteria determined in three samples (2, 13 and 15) was 1406, 9317, and 4488 bacterial cells/mL, respectively, using the real-time PCR, although it was 50 CFU/mL beverage emulsion in the three of them using the reference method.

Also, the total bacteria determined in four samples (4, 10, 16 and 17) was found to be 3233, 1048, 708, and 158 bacterial cells/mL beverage emulsion, respectively, using the real-time PCR, although the results of the same samples using the reference method showed no bacterial growth in the plates at all.

Many factors were thought to be contributing to the relatively high values obtained by real-time PCR and statistical significant differences between the results of the two methods.

The first was thought to be due to the nature and composition of the beverage emulsion samples which contained preservatives such as sorbate or benzoate, responsible for limiting the microbial growth in the samples. Also, the inability of real-time PCR to differentiate between the DNA of the viable bacterial cells and that of the dead ones, knowing that the reference method used in the beverage emulsions manufacturing was intended for the detection of the viable cells only. This was supported by the findings of [49] and Furet, Quénée, & Tailliez (2004) when they also found that the relatively overestimation of the total bacteria in fruit juices and in fermented milk was possibly due to the less viability of the bacterial cells in their samples. The results of a study done by Castillo *et al*. (2006) also showed relatively higher results obtained by real-time PCR. One of the possible reasons was thought to be the quantification of DNA coming from dead bacteria, or the quantification of free DNA.

The high values obtained by real-time PCR and the statistical significant differences between the results of the two methods could also be due to the low sensitivity of the reference method, also reported by Castillo *et al*. (2006), in which the colonies could be formed by more than one cell, which is the case in some of the bacterial species, leading to underestimation of the bacterial count compared to the sensitive real-time PCR method. A colony, in this case, would be counted as 1 CFU/mL beverage emulsion using the reference method, but would be counted as more than one cell per 1 mL beverage emulsion by real-time PCR.

Another reason could possibly be the presence of more than one copy of the target sequence in the genome of some of the bacterial species which could be counted as more than one cell using real-time PCR.

The effects of the low sensitivity of the reference method and the presence of more than one copy of the target sequence in the genome of some of the bacterial species could be minor when compared to the effects of the preservatives and the inability of real-time PCR to differentiate between the bacterial DNA coming from viable and dead cells. These effects can be included in the calculations of the accepted uncertainty of the difference between the two methods in case the latter factor is eliminated.

It is important to mention that the beverage emulsion samples were stored in sterile tubes at 4 to 10 °C throughout the period of this study to suppress any bacterial growth that might potentially occur. Also, the testing using both methods was performed simultaneously using the same dilutions of the samples to eliminate the error of the possible bacterial growth that could occur due to performing the testing at different times.

Chapter Five: Conclusions and Future Perspectives

In this study, a protocol was successfully developed for the extraction of bacterial DNA from the complex matrix of the beverage emulsion samples. A set of universal primers targeting a conserved region in the 16S rDNA of bacteria was selected, and its specificity towards some of the commonly known bacterial species associated with microbial contamination of soft drinks was confirmed. A standard curve was successfully constructed using *Escherichia coli* DH10B genomic DNA with a correlation coefficient (R^2) of 0.979 and efficiency (*E*) of 101%, and was used to develop a quantification strategy to calculate the bacterial cells numbers.

In conclusion, the developed DNA extraction protocol, the selected set of universal primers, the constructed standard curve and the developed quantification strategy enabled the sensitive determination of the total bacteria in beverage emulsions by real-time PCR, in the range between 10 fg/µL and 100 ng/µL, corresponding to 2 and 2 x 10^7 cells of *Escherichia coli*, respectively. The assay needs 6 – 8 hours instead of 7 days required by the pour plate method.

A comparison was made between the total bacteria determined by the developed real-time PCR assay and the total bacterial count determined by the pour plate method. The result of this comparison showed relatively high values obtained by real-time PCR and statistical significant differences between the results of the two methods. Factors contributing to these observations included the composition of the beverage emulsion samples which contained preservatives responsible for limiting the microbial growth in the samples, the inability of the developed real-time PCR to differentiate between the DNA of viable and dead bacterial cells, the low sensitivity of the reference method, and the possible existence of more than one copy of the target sequence in the genome of some of the bacterial species.

Future possible modifications to the developed assay to overcome the effect of the preservatives and the inability of real-time PCR to differentiate between the DNA of viable and dead bacterial cells can be by the artificial spiking of the samples with the measured quantities of certain bacterial species, filtration to collect the cells, then DNA extraction from the filters

before running the real-time PCR reaction as previously reported by Saint-Cyr *et al*. (2014) [53] and Gammon *et al*. (2007) [48] showing relatively similar results by moth methods.

The effect of the preservatives and the inability of real-time PCR to differentiate between the DNA of viable and dead bacterial cells can also be eliminated in the future by the treatment of the bacterial cells with ethidium bromide monoazide (EMA) which is a DNA-intercalating dyes that can selectively permeate the membranes of the dead bacterial cells and cleave DNA. This method has been used in several studies [68-72] where the differentiation between the DNA coming from the viable and dead bacterial cells was required.

Targeting specific RNA sequences that exist only in viable bacterial cells, instead of DNA, can also be considered as a possible alternatives to overcome the effect of the preservatives and the inability of real-time PCR to differentiate between the DNA of viable and dead bacterial cells can be by

To eliminate the possible factor of the existence of more than one copy of the target sequence in the genome of some of the bacterial species which lead to overestimation of the bacterial cells number, targets other than the conserved regions in the 16S rDNA of the bacterial cells can be considered as previously addressed in a study by Takahashi, Konuma, & Hara-Kudo (2006) [51] who reported using the *rpoB* gene responsible for the encoding for the βsubunit of the bacterial RNA polymerase instead of the 16S rRNA gene.

Because of its high sensitivity, using real-time PCR for the detection of total bacteria in beverage emulsions has many possible advantages. It can provide several benefits to the beverage emulsion manufacturers and can be used for many purposes in the future.

The developed real-time PCR method for the detection of total bacteria in beverage emulsions can give a full history of the bacterial growth in a given sample. It can be used in the investigations of customers' complaints when fast responses are always required. It can also be used to extend the shelf lives of the products, or to help in the identification of the root causes of sensory off notes (uncharacteristic taste, odor, or appearance). It can be used to confirm the

absence of certain specific bacterial strains, to investigate the potential cross-contamination between beverage emulsion batches manufactured in the same process equipment, or as a screening tool for the bacterial contamination of the process equipment itself. It can also be used in the evaluation and monitoring of the efficiency of the added preservatives.

Tables

Table 1. Types of soft drinks and their descriptions [8].

Types of Soft Drink	Descriptions
Bottled Water	Drinking water. It constitutes water with added flavorings and minerals/vitamins. Can be further classified into: 1. Still Water (noncarbonated, mineral, spring or table water): Drinking water with/without flavorings and vitamins/minerals 2. Carbonated Water (mineral, spring or table water): Drinking water with low levels of carbonation. It is either naturally sparkling or sparkling by carbon dioxide injection. 3. Flavored Water: Unsweetened water, with added essences and/or some aromatic substances as flavorings.
Bulk/Hot Water	Drinking water sold in packs (10 L or more) for use in dispensers.
Carbonates	Sweetened soft drinks with added carbon dioxide. It can be RTD or dilutable.
Juice	100% pure fruit or vegetable juice, with sweetening agents. No ingredients are added except permitted vitamins and minerals.
Nectars	Diluted fruit or vegetable juice and pulp, with sweetening agents. Vitamins and minerals are added.
Still Drinks	RTD flavored, noncarbonated beverages. They contain fruit, non-fruit flavors, or juice content.
Squash/Syrups	Concentrates (not RTD). This includes fruit and non-fruit flavors products.
Fruit Powders	Non-RTD products in the form of powder.
Iced/RTD Tea/Coffee Drinks	These are tea-based or coffee-based RTD products. They can also be in the form of non-RTD powders or liquid concentrates.
Sports Drinks	Also be described as “isotonic”, “hypertonic”, or “hypotonic”. The can be still or carbonated, RTD, non-RTD powders, or concentrates. They can also be fruit or non-fruit flavored.
Energy Drinks	Energy-enhancing drinks. They are mainly carbonated. Contain glucose, caffeine, taurine, guarana, exotic herbs and substances, vitamins and minerals.

RTD: Ready-to-drink

Table 2. Soft drinks main constituents, general functions, typical use levels and commonly used examples [1].

Constituent	Function	Typical Use Level	Commonly Used Examples
Water (rigid requirement must be met)	Enables body metabolism by providing the needed hydration. Also, carries other ingredients.	Up to 98% v/v (when high-intensity sweeteners are used)	-
Sugars	Give sweetness and body to the soft drink. Also, act as a synergist and give balance to the flavors.	7-12% (if used as a sole source of sweetener)	Carbohydrates (e.g. Sucrose, Glucose Syrup, and Fructose)
Fruit Juice	Gives a sources of fruit identity, flavor, and mouthfeel. Also contributes to sweetness and acidity.	Usually up to 10%	-
High-Intensity Sweeteners	Give sweetness. Reduces the soft drinks calories, and act as a synergists. They are used usually in combination, e.g. aspartame with acesulfame K.	Used based on sucrose equivalence (e.g. aspartame can be used at 0.40-6% m/v if used as a sole source of sweetener)	Acesulfame K, Aspartame, Alitame, Cyclamate, Neohesperidin Dihydrochalcone, Sucralose, Neotame, Saccharine, and Stevioside
Carbon Dioxide	Used in carbonated beverages only. Gives mouthfeel and sparkle to the beverage.	0.30-6% m/v	-
Acids	Give sharpness, sourness, and background to the flavor, and increase the effect of thirst-quenching.	0.05-0.03% m/v	Citric Acid, Tartaric Acid, Phosphoric Acid, Lactic Acid, Acetic Acid, Malic Acid, Fumaric Acid, Ascorbic Acid
Flavorings	Give flavor, character, and identity to the soft drink.	0.10-28% m/m (in case of natural-identical or artificial flavorings) Up to 0.5% mm (in case of natural flavorings)	-
Water-Dispersible Flavorings (Beverage Emulsions)	Act as carriers for the oil-based flavors or clouds. Provide cloudy effects to the soft drinks to enhance or replace the cloud from natural juices.	0.1% m/v	-
Colors	Give standard and identified color tones to the soft drinks.	0-70 ppm	B-Carotene and Paprika (natural) Sunset Yellow and Brilliant Blue (synthetic)

Preservatives	Limit the microbial attack and prevent destabilization of the soft drinks.	Statutory limits apply, e.g. up to 250 ppm sorbic acid in EU	Sulfur Dioxide, Benzoic Acid, and Sorbic Acid
Antioxidants	Limit the deterioration of the flavor and cloud, and prevent oxidation.	Less than 100 ppm (also subject to the legislation of the user-country)	Butylated hydroxyl anisole (BHA), Butylated hydroxyl toluene (BHT), Ascorbyl palmitate, natural extracts rich in tocopherol, and synthetic α-, γ-, and δ-tocopherols
Saponins	Used mainly in carbonated beverages to give heading foam.	Up to 200 mg/l (EU) Up to 95 mg/l (USA)	Quillaia Extract
Hydrocolloids	Give viscosity, shelf life stability, and mouthfeel.	0.1-0.2% per GMP (minimum amount required to give the desired effect)	Mucilaginous Gums
Vitamins/Minerals	Used mainly in healthy-living drinks to provide nutritional requirements.	ADI applies	-

Table 3. Most common bacterial species associated with microbial spoilage of soft drinks, and their typical effects and quality changes [8].

Group	Genera/Species	Metabolites	Quality Changes in the Sensory Characteristic	
			Visual	Odors
LAB	***Lactobacillus*** *acidophilus, L. brevis, L. buchneri, L. paracasei, L. perolens, L. plantarum* ***Leuconostoc*** *mesenteroides* ***Weissella*** *confuse*	Lactic acid, carbon dioxide, ethanol, acetate, succinate, diacetyl, and formate (formic acid is used as an apple juice spoilage indicator (add reference)) depending on the species and growth conditions	Loss of carbonation, astringency, turbidity, ropiness, and forming biofilms over production surfaces and packaging materials	Buttery, sour, cheesy, green apple
AAB	***Acetobacter*** *suboxydans;* ***Gluconobacter*** *oxydans;* ***Gluconacetobacter*** *sacchari;* ***Asaia*** *lannensis, A. bogorensis*	Acetic acid, gluconic acid, lactic acid, succinic acid, carbon dioxide, acetaldehyde, and ketones	Package swelling, haze, ropiness, sediments, biofilms over production surfaces and packaging materials	Flavor changes, sour, vinegar
ACB	***Alicyclobacillus*** *acidoterrestris, A. acidophilus, A. acidocaldarius, A. cycloheptanicus, A. hesperidium, A. herbarius, A. pomorum*	2,6-Dibromophenol, guaiacol	Sediment, haze, and discoloration may occur rarely	Musty, stale

Table 4. Determination of total bacterial count in beverage emulsion samples using the pour plate method.

Sample ID	Total Bacterial Count (CFU/mL Dilution)			Total Bacterial Count (CFU/mL Beverage Emulsion)
	1	2	Mean	
1	6	5	5.5	55
2	5	5	5	50
3	2	1	1.5	15
4	0	0	0	0
5	6	11	8.5	85
6	7	4	5.5	55
7	8	4	6	60
8	8	8	8	80
9	4	4	4	40
10	0	0	0	0
11	3	2	2.5	25
12	8	6	7	70
13	6	4	5	50
14	10	11	10.5	105
15	2	8	5	50
16	0	0	0	0
17	0	0	0	0
Negative Control	0	0	0	0

Table 5. Evaluation of three sets of universal primers based on the selection criteria developed by Raymaekers *et al*. (2009).

Primers Set	Primers Forward (5'→3') Reverse (5'→3')	Selection Criteria							References
		T_m of Primers: 58 - 60 ^{O}C	**GC Content: 30 - 70%**	**Not more than two C or G in last five positions at 3' end**	**Length of Amplicon: max 400 bp**	**No more than four constitutive guanines**	**Avoid primer–dimer**	**Length of Primer: 18 – 24 bp**	
A	TCCTACGGGAGGC AGCAGT	62.6*	63.2	3*	467*	Less than four	Can make two forms of primer-dimer, ΔG= -1.7 and -4.2 kcal/mol	19	[28]
	GGACTACCAGGGT ATCTAATCCTGTT	61.4*	46.2	2		Less than four		26*	
B	GGATTAGATACCC TGGTAGTC	53.8*	47.6	2	723**	Less than four	Can make two forms of primer-dimer, ΔG= -3.7 kcal/mol for both	21	[55]
	TACCTTGTTACGA CTT	46.7*	37.5	2		Less than four		16*	
C	AGGAGGTGATCCA ACCGAA	26.3*	52.6	3*	79269**	Less than four	Can make two forms of primer-dimer, ΔG= -3.7 and -3.4 kcal/mol	19	[56,57]
	ACCTGGAGGAAGA AGGTGGGGAT	30.4*	56.5	3*		Four constitutive guanines*		23	

(*) not complying with the selection criteria

(**) impractically long suggesting unsuitability for this study

Table 6. The annealing positions of the three universal primers set on *Escherichia coli* genome (NCBI Reference Sequence number NC_002695.1) [62] by Oligo 7 [63].

Primers Set	**Primers** Forward (5'→3') Reverse (5'→3')	**Annealing Position**
A	TCCTACGGGAGGCAGCAGT	227441
	GGACTACCAGGGTATCTAATCCTGTT	227882
B	GGATTAGATACCCTGGTAGTC	227886
	TACCTTGTTACGACTT	228593
C	AGGAGGTGATCCAACCGAA	46130
	ACCTGGAGGAAGAAGGTGGGGAT	125376

Table 7. The annealing positions of the universal primers set A on both *Lactobacillus acidophilus* and *Alicyclobacillus acidocaldarius*, and their products sizes.

Genera/Species	**NCBI Reference Sequence Number**	**Reference**	**Annealing Position of the Forward Primer** (5'→3')	**Annealing Position of the Reverse Primer** (5'→3')	**Product Size (bp)**
Lactobacillus acidophilus	NC_006814.3	[64]	59615	60056	467
Alicyclobacillus acidocaldarius	NC_013205.1	[65]	11113	11555	468

Table 8. The relation between the threshold cycles (C_t) and the logarithms of *Escherichia coli* DNA concentrations used to construct the standard curve.

C_T	DNA Conc. (pg/µL)	Log DNA Conc.
9.6277	100000	5
10.5927	10000	4
13.5406	1000	3
15.9598	100	2
19.9753	10	1
23.6546	1	0
27.8073	0.1	-1
32.0063	0.01	-2

Slope = -3.3, Intercept = 24.09, R^2 = 0.979, E (%) = 101

Table 9. The mean of the C_t values of the 17 samples, positive control and NTC used in the estimation of bacterial cells number in beverage emulsion samples by real-time PCR.

Sample ID	C_T		
	1	**2**	**Mean**
1	23.9985	24.681	24.33975
2	24.2443	23.9664	24.10535
3	23.6229	23.6868	23.65485
4	22.4795	23.3461	22.9128
5	22.602	22.9987	22.80035
6	22.9191	23.4559	23.1875
7	22.6037	22.9084	22.75605
8	22.8918	23.9332	23.4125
9	22.3091	25.474	23.89155
10	22.6971	26.3567	24.5269
11	22.1431	22.147	22.14505
12	22.8653	22.4998	22.68255
13	21.4351	21.3583	21.3967
14	21.6426	22.1813	21.91195
15	22.2405	22.6458	22.44315
16	24.6329	25.5432	25.08805
17	26.6263	27.8397	27.233
Positive Control (71 pg/µL)	17.6032	17.3844	17.4938
NTC	29.2746	29.7521	29.51335

NTC: No Template Control

Table 10. The logarithm of the DNA concentrations and the DNA concentrations of the total bacterial load of the 17 beverage emulsion samples, positive control, and NTC.

Sample ID	Average C_T Values	Log DNA Concentration	DNA Concentration (pg/µL)
1	24.33975	-0.227472259	0.592280918
2	24.10535	-0.156411373	0.697571336
3	23.65485	-0.019837444	0.955350106
4	22.9128	0.20512301	1.60369956
5	22.80035	0.239213439	1.734656306
6	23.1875	0.121844745	1.323868184
7	22.75605	0.252643462	1.789136442
8	23.4125	0.05363357	1.13144532
9	23.89155	-0.091595599	0.809849653
10	24.5269	-0.284208798	0.519746055
11	22.14505	0.437874696	2.740783277
12	22.68255	0.274925779	1.8833272
13	21.3967	0.664745062	4.621096762
14	21.91195	0.508541473	3.225087282
15	22.44315	0.347502468	2.225883696
16	25.08805	-0.454327468	0.351295456
17	27.233	-1.104592174	0.078597336
Positive Control (71 pg/µL)	17.4938	1.847951255	70.46139785
NTC	29.51335	-1.795904849	0.015999085

NTC: No Template Control

Table 11. Determination of total bacteria per each 1 mL of the 17 beverage emulsion samples, as well as the positive control and NTC by real-time PCR.

Sample ID	DNA Concentration (pg/µL)	Total Bacteria by Real-Time PCR (Cells/mL Dilution)*	Total Bacteria by Real-Time PCR (Cells/mL Beverage Emulsion)
1	0.592280918	119	1194
2	0.697571336	141	1406
3	0.955350106	193	1926
4	1.60369956	323	3233
5	1.734656306	350	3497
6	1.323868184	267	2669
7	1.789136442	361	3607
8	1.13144532	228	2281
9	0.809849653	163	1633
10	0.519746055	105	1048
11	2.740783277	553	5526
12	1.8833272	380	3797
13	4.621096762	932	9317
14	3.225087282	650	6502
15	2.225883696	449	4488
16	0.351295456	71	708
17	0.078597336	16	158
Positive Control (71 pg/µL)	70.46139785	14206	-
NTC	0.015999085	3	-

(*) Dilution Factor= 10, applied on the samples only

Table 12. Comparison between the total bacterial determined by real-time PCR and the total bacterial count determined using the pour plate method.

Sample ID	Total Bacteria by Real-Time PCR (Cells/mL Beverage Emulsion)	Total Bacterial Count (CFU/mL Beverage Emulsion)	Ratio (Cell Number/CFU)
1	1194	55	21.711
2	1406	50	28.128
3	1926	15	128.407
4	3233	0	-
5	3497	85	41.145
6	2669	55	48.529
7	3607	60	60.119
8	2281	80	28.514
9	1633	40	40.819
10	1048	0	-
11	5526	25	221.031
12	3797	70	54.243
13	9317	50	186.335
14	6502	105	61.926
15	4488	50	89.753
16	708	0	-
17	158	0	-
Mean	3117	44	71.61

Figures

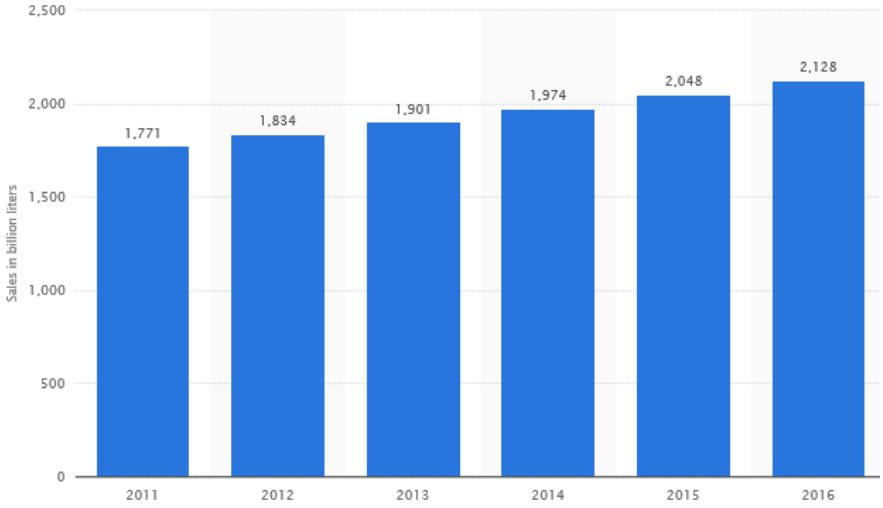

Figure 1: The global soft drinks volume sales (in billion liters) from 2011 to 2016. Reprinted from [2].

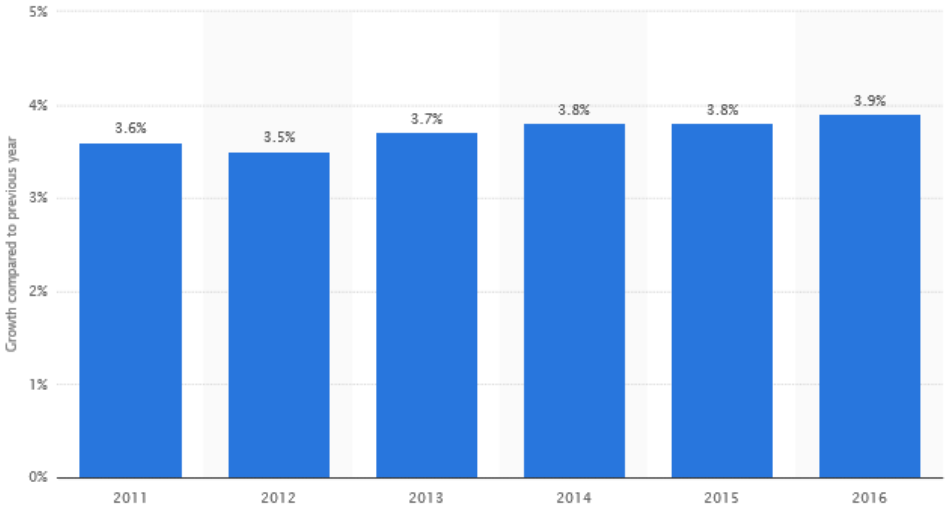

Figure 2: The projected growth of the soft drinks sales (%) from 2011 to 2016. Reprinted from [3].

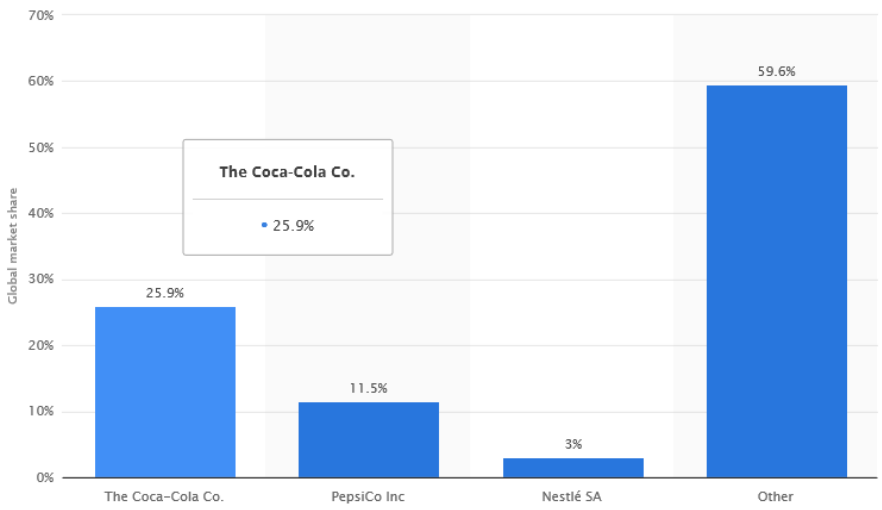

Figure 3: The global market shares (%) of the soft drink companies in 2011 based on sales value. Reprinted from [73].

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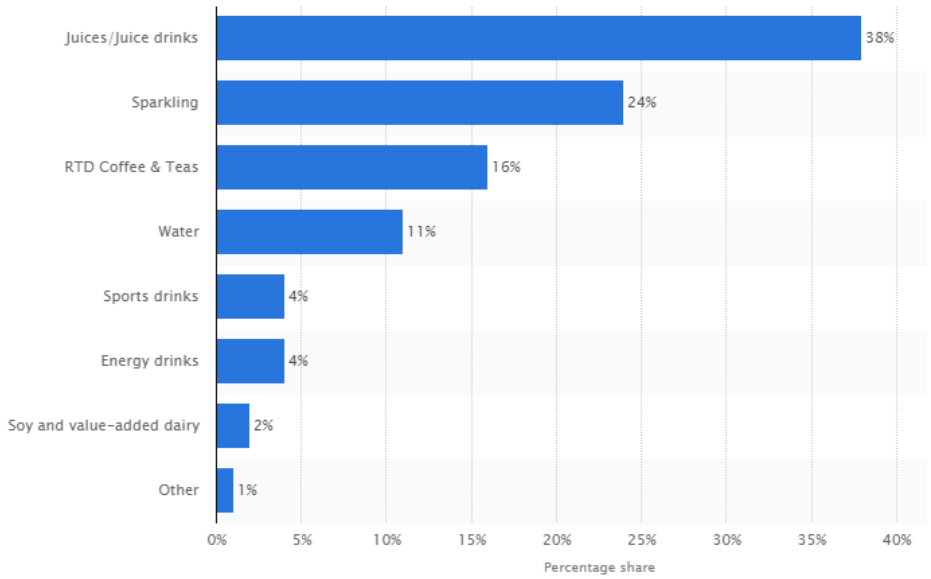


Figure 4: The product portfolio distribution share (%) of The Coca Cola Company worldwide in 2011 by category. Reprinted from [6].

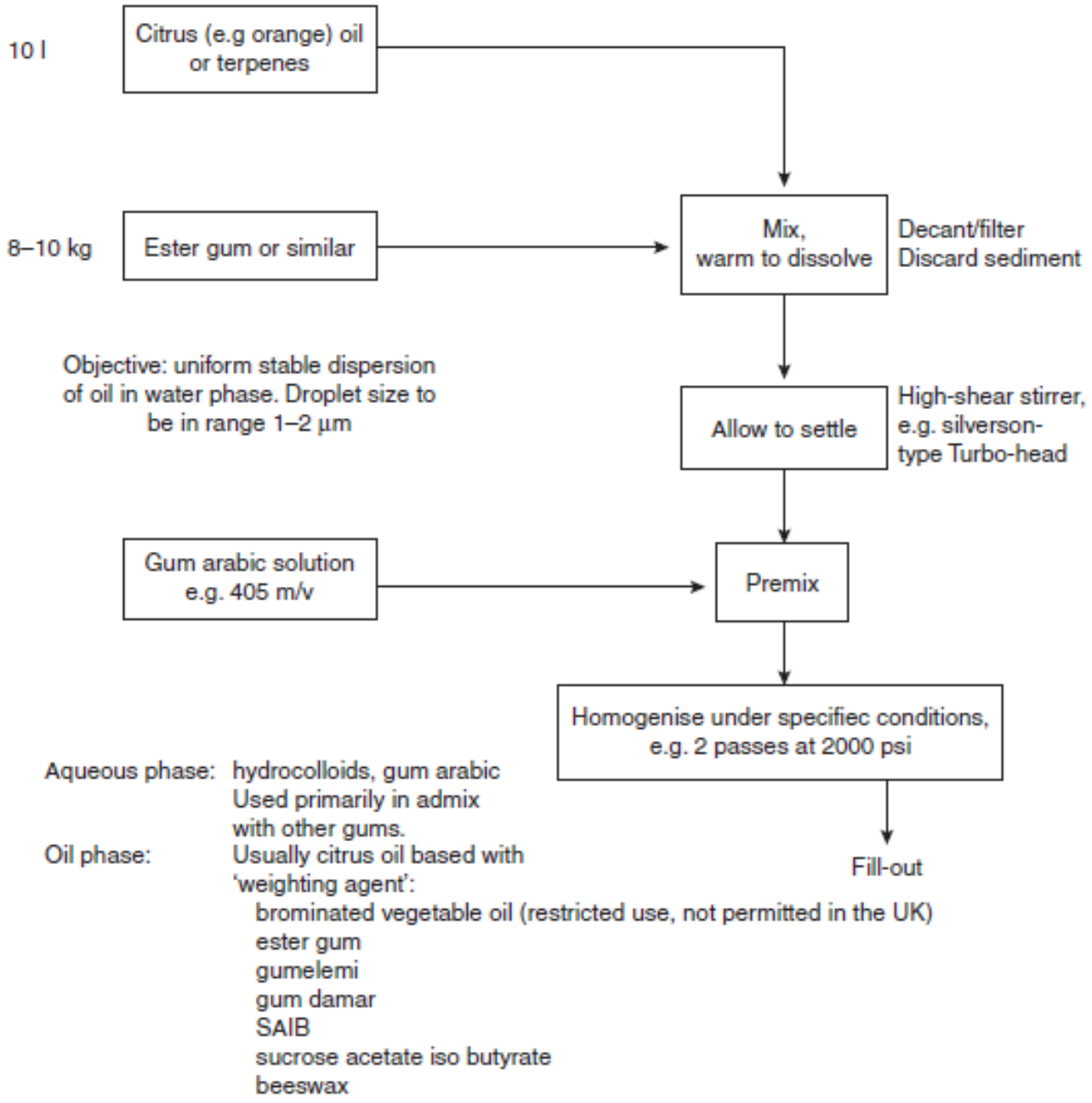


Figure 5: A typical sequence of beverage emulsions manufacturing process. Reprinted from [1].

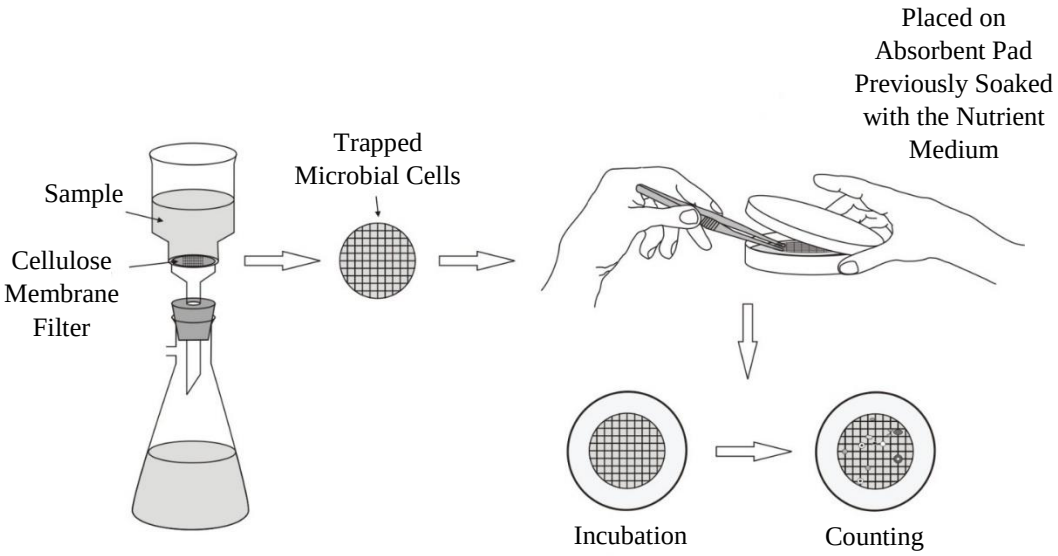


Figure 6: Membrane filtration method [27]. Reprinted from [74].

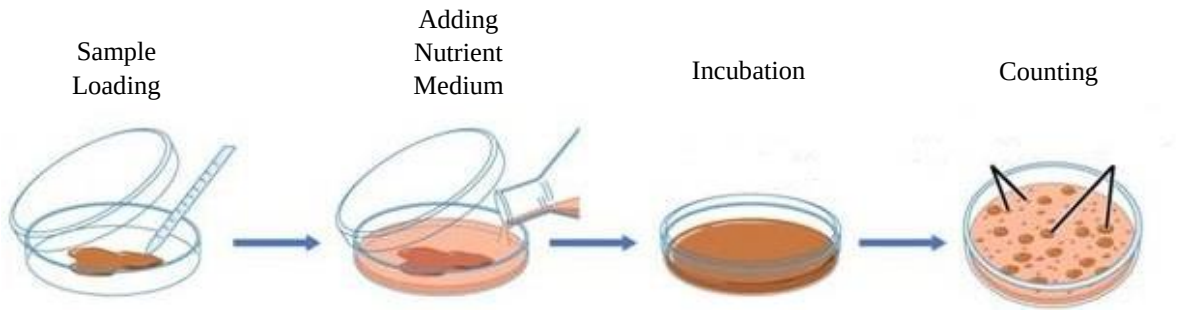
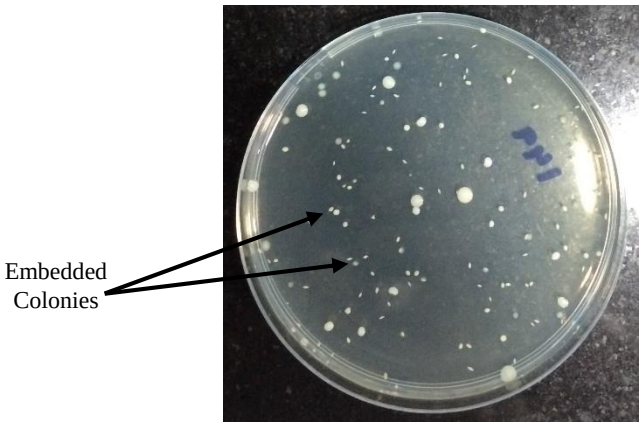
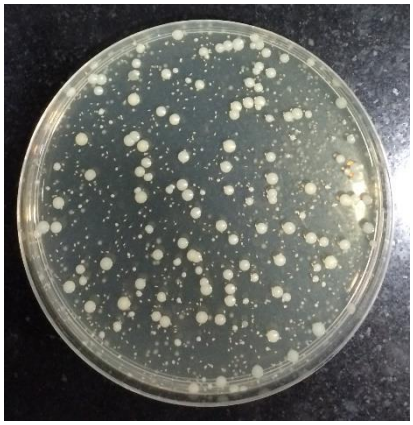


Figure 7: Pour plate method [27]. Reprinted from [75]

a) Countable bacterial growth



b) Too numerous to count (TNTC) bacterial growth



c) A continuous layer of bacterial growth

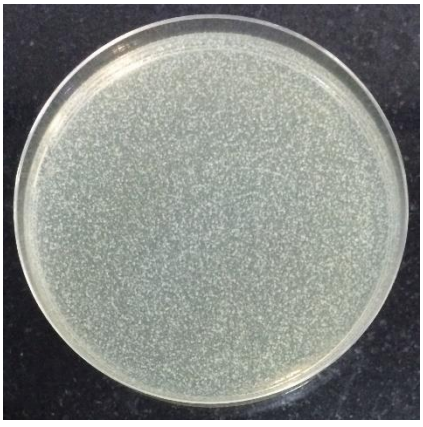


Figure 8: Bacterial growth by pour plate method.

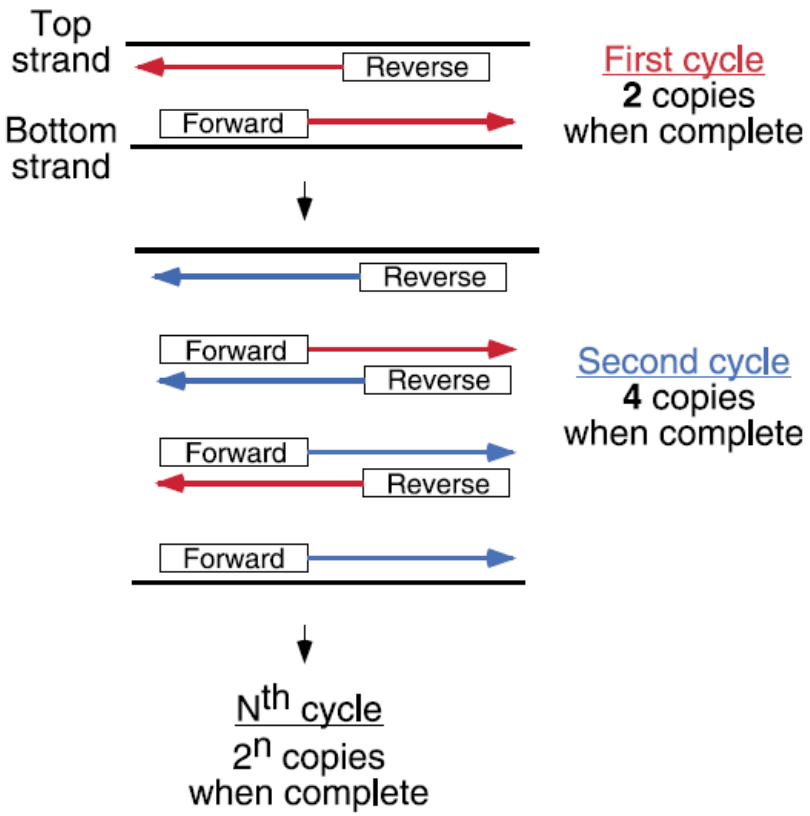


Figure 9: Polymerase Chain Reaction (PCR). Reprinted from [37].

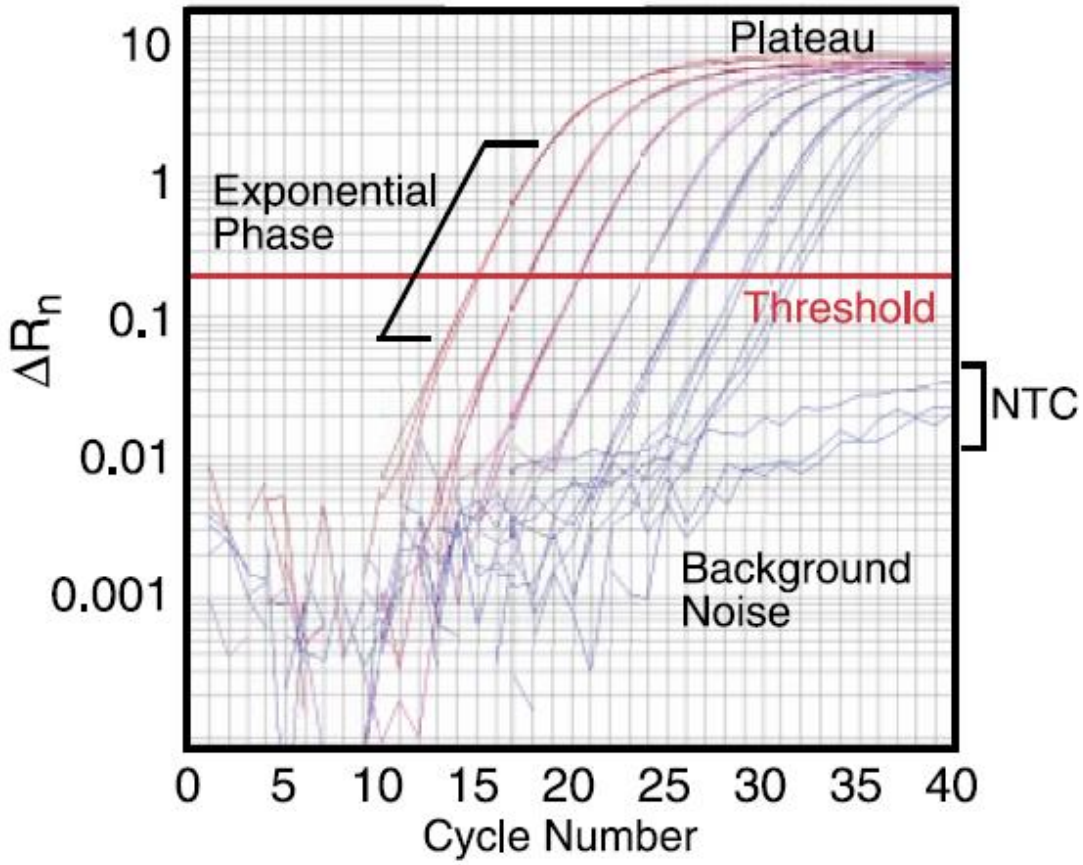


Figure 10: A typical amplification plot of PCR showing the background noise, threshold, exponential phase, plateau, and a typical signal of no-template-control (NTC). Reprinted from [37].

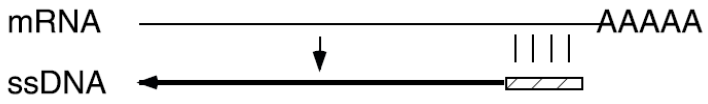


Figure 11: Reverse transcription converting RNA to cDNA using reverse transcriptase. Reprinted from [37].

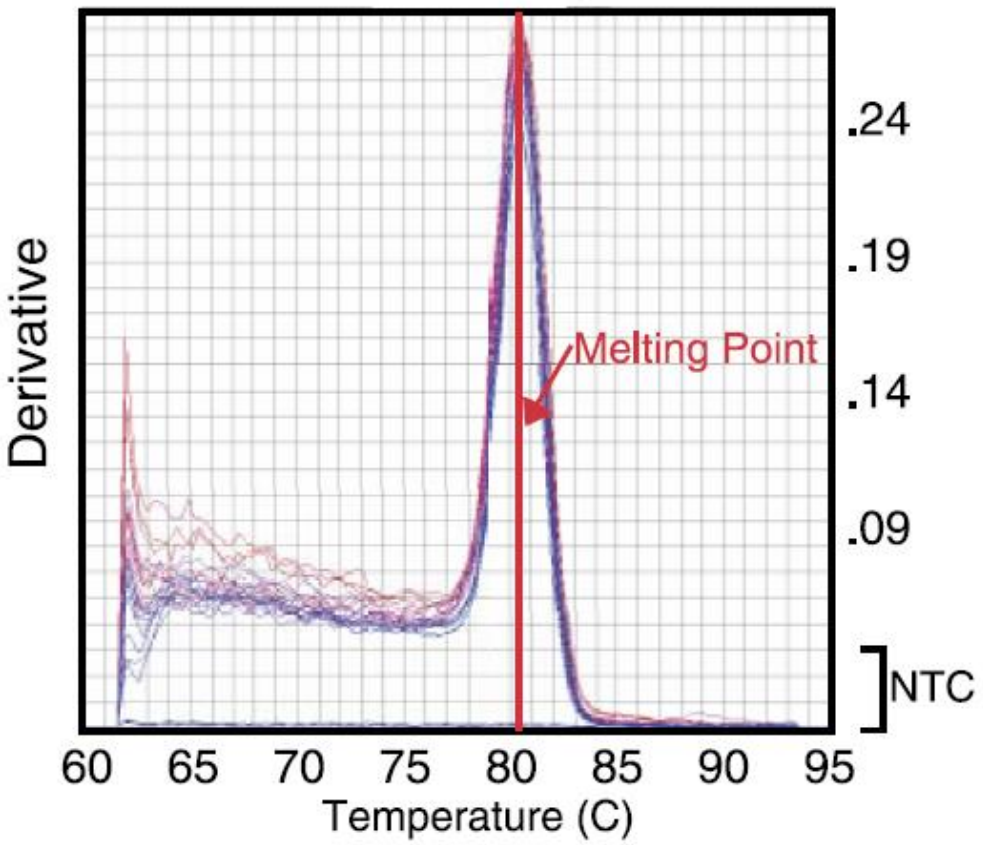


Figure 12: Dissociation (melting) curve showing a typical melting point. Reprinted from [37].

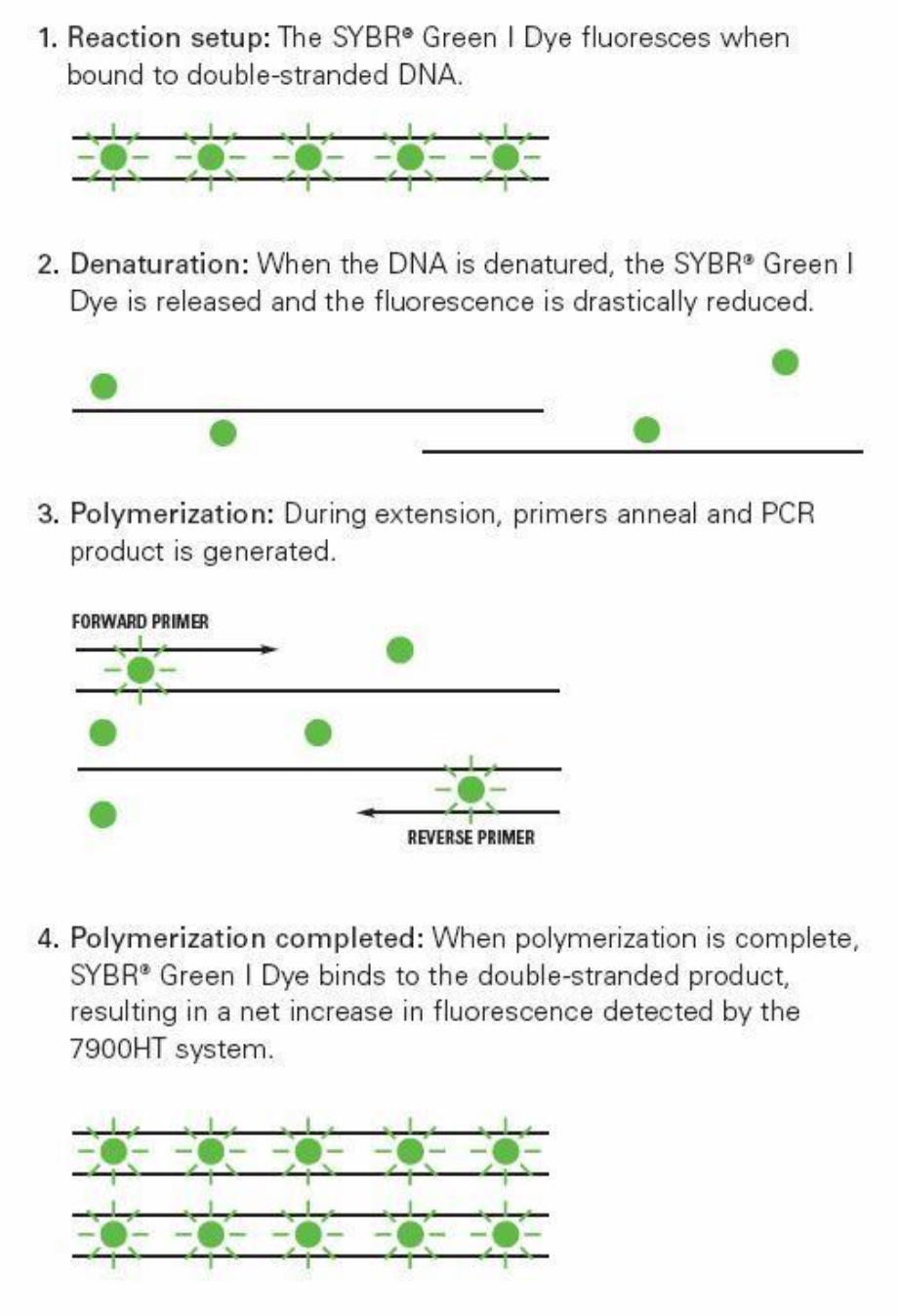


Figure 13: Mode of action of SYBR® green. Reprinted from [76].

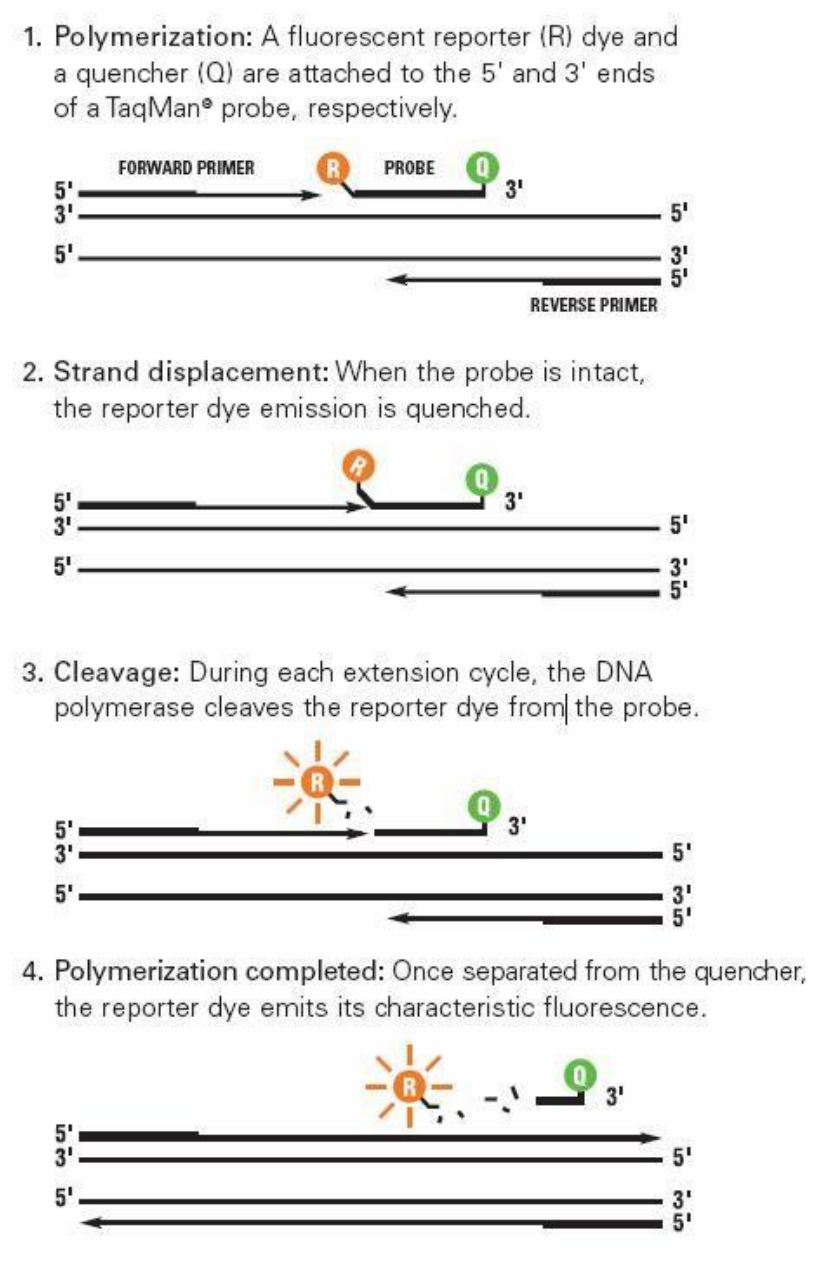


Figure 14: Mode of action of TaqMan®. Reprinted from [76].

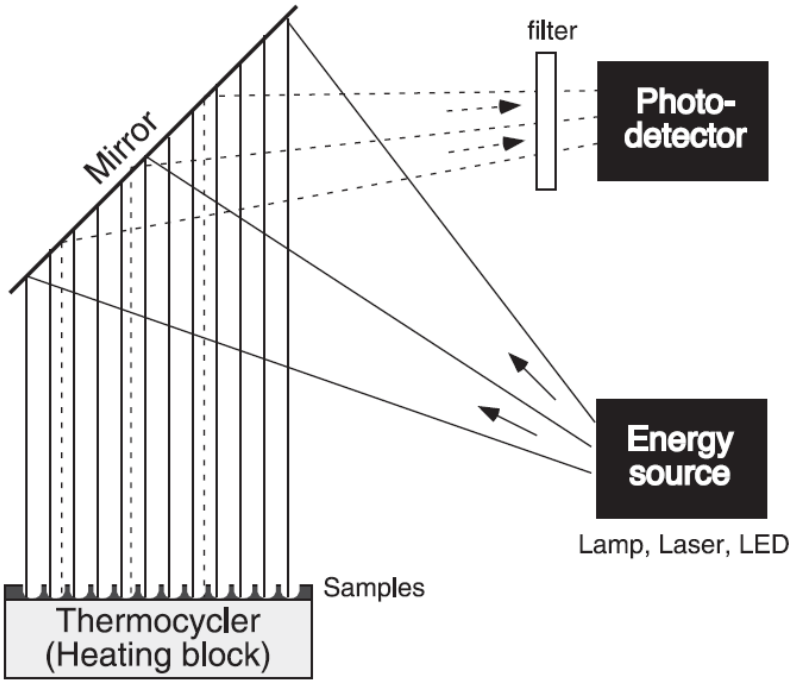


Figure 15: Instrumentation of real-time PCR showing both excitation and detection taking place simultaneously. Reprinted from [37].

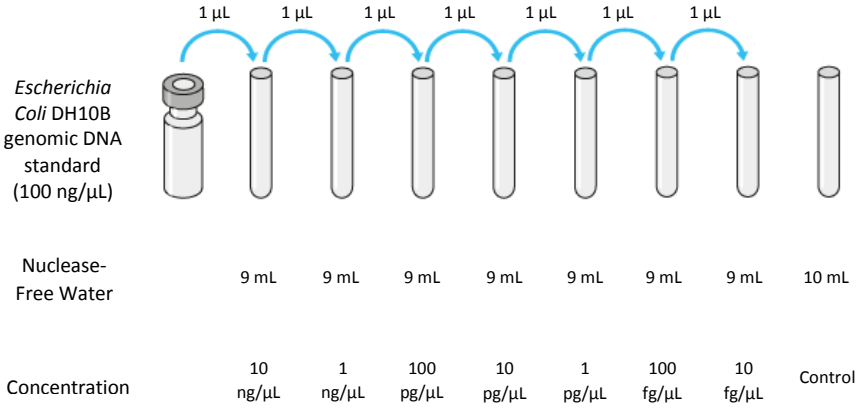


Figure 16: Serial dilution used to construct a standard curve by real-time PCR and the selected set of primers using *Escherichia coli* DH10B genomic DNA (100 ng/µL) as a standard.

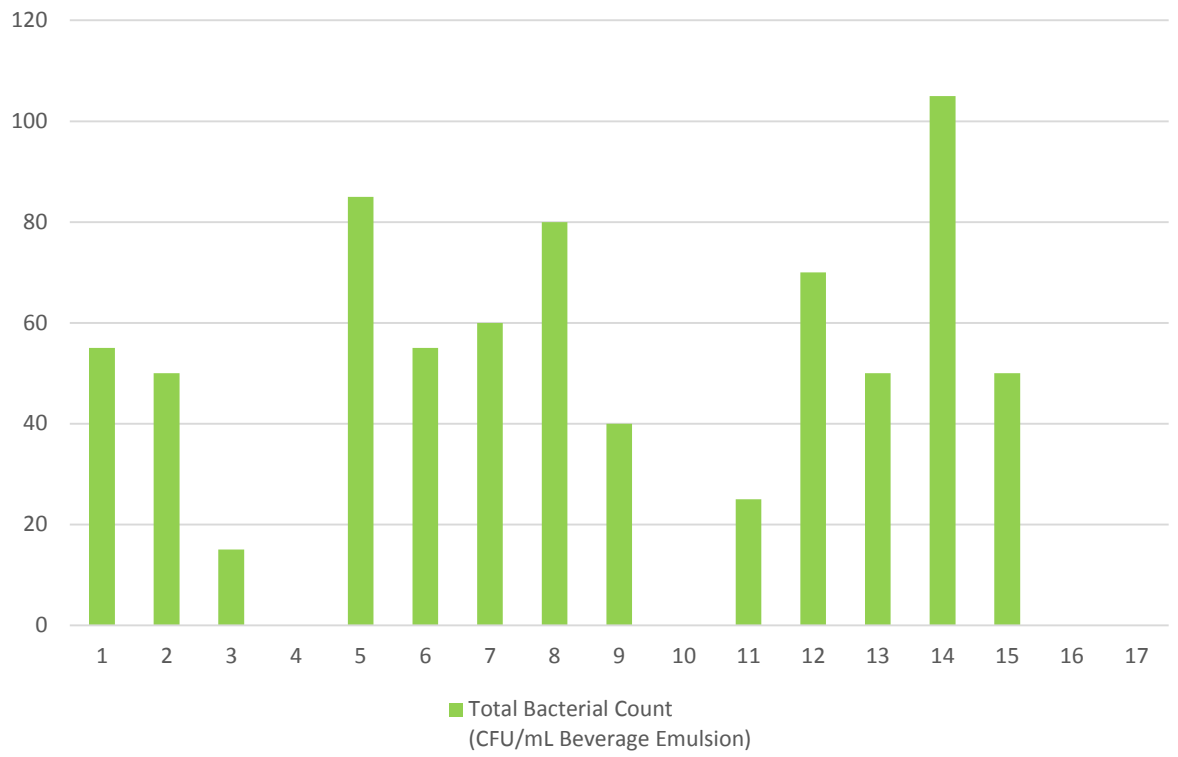
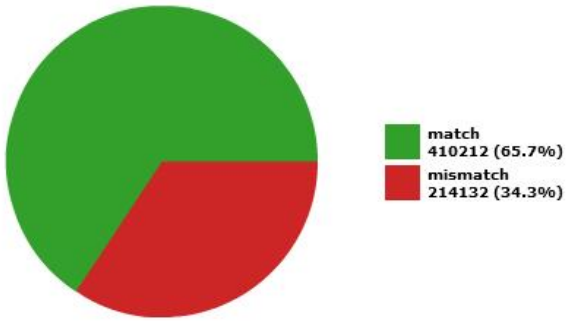
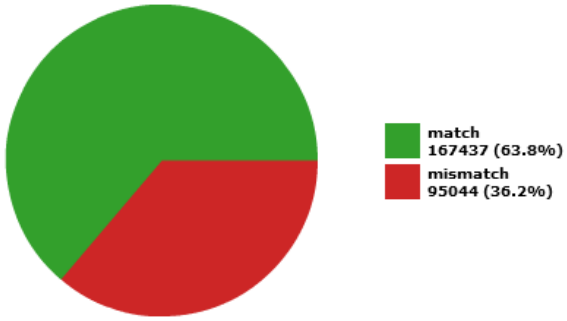


Figure 17: Determination of total bacterial count in beverage emulsion samples using the pour plate method.

Primers Set A



Primers Set B



Primers Set C

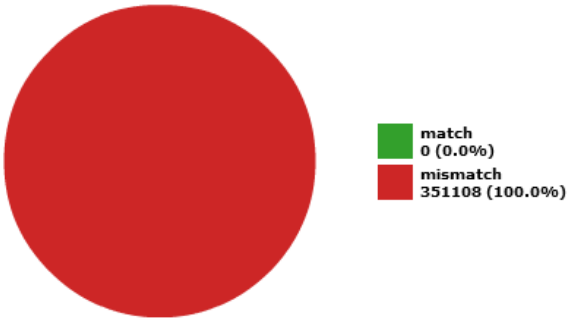


Figure 18: The coverage ranges of the three universal primers sets tested by TestPrime 1.0 [59].

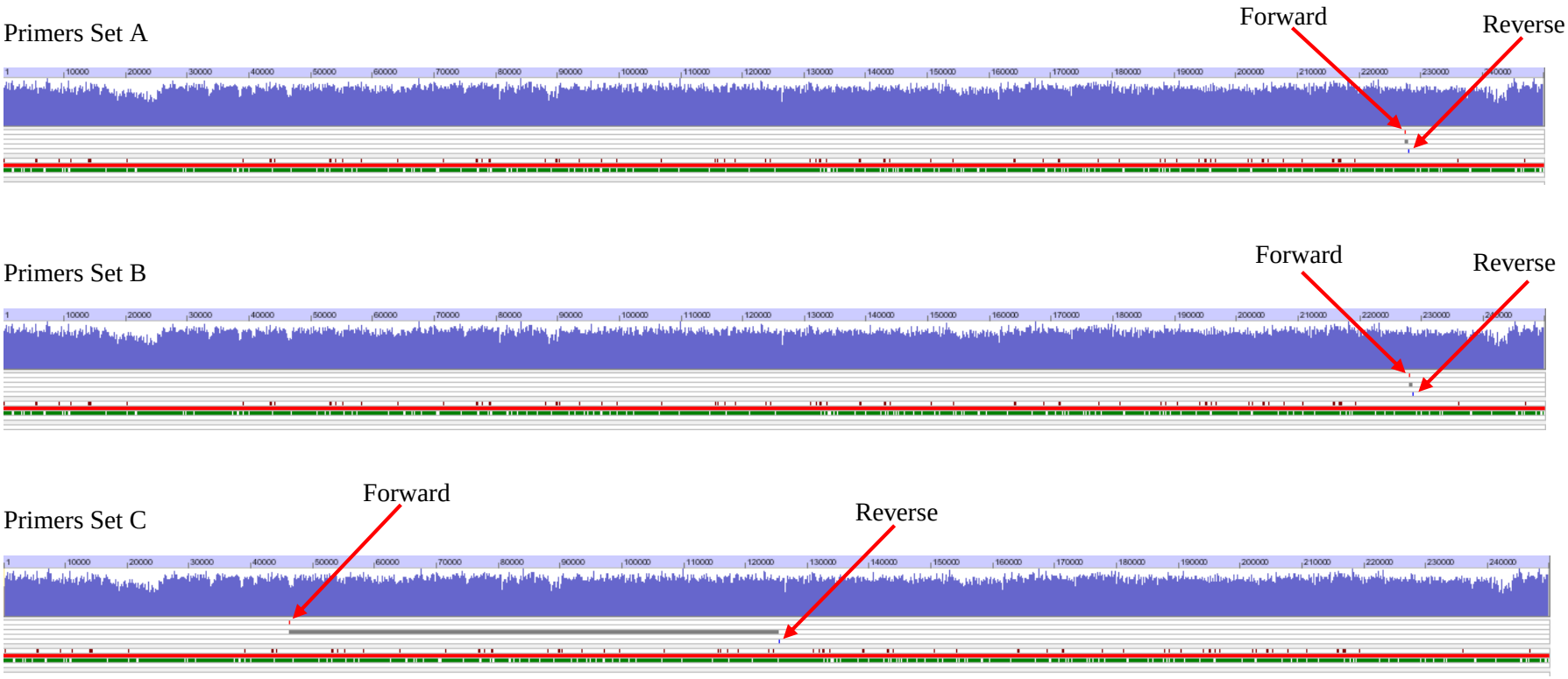


Figure 19: Annealing positions of the three universal primers set on *Escherichia coli* genome (NCBI Reference Sequence number NC_002695.1) [62] by Oligo 7 [63].

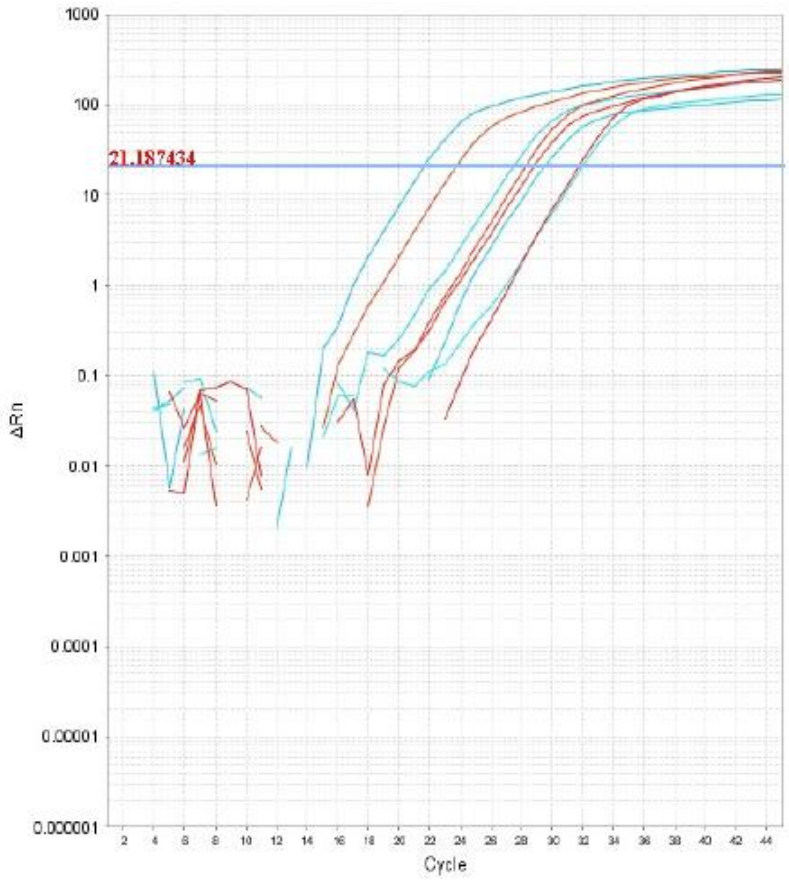


Figure 20: Amplification plot of bacterial DNA extracted from eight different beverage emulsion samples by real-time PCR using the universal primers set A.

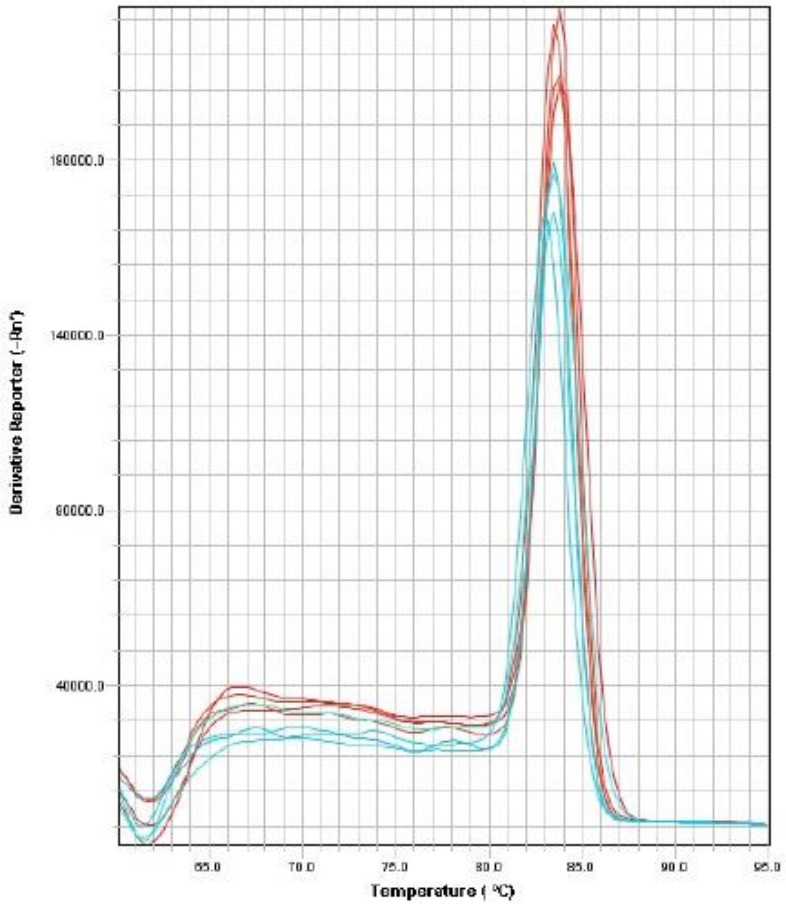


Figure 21: Melt curve of the amplification product of bacterial DNA extracted from eight different beverage emulsion samples by real-time PCR using the universal primers set A showing a uniformed peak with an average T_m value of 83.5 °C confirming the amplification of the target sequence

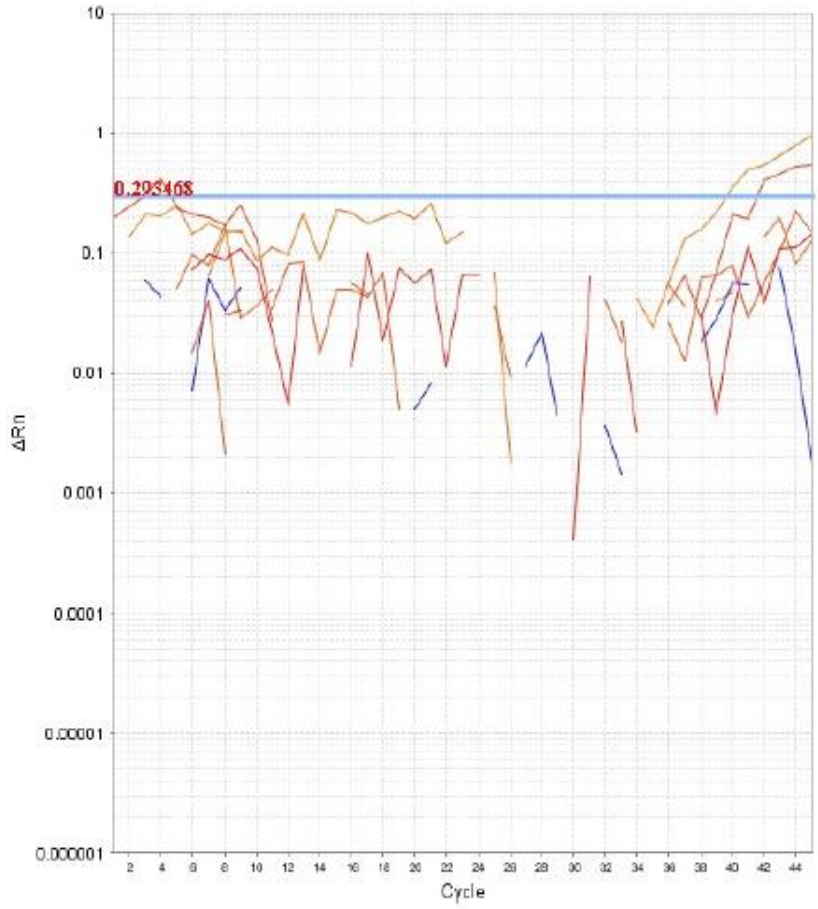


Figure 22: Amplification plot of bacterial DNA extracted from eight different beverage emulsion samples by real-time PCR using the universal primers set B.

Figure 23: Amplification plot of bacterial DNA extracted from eight different beverage emulsion samples by real-time PCR using the universal primers set C.

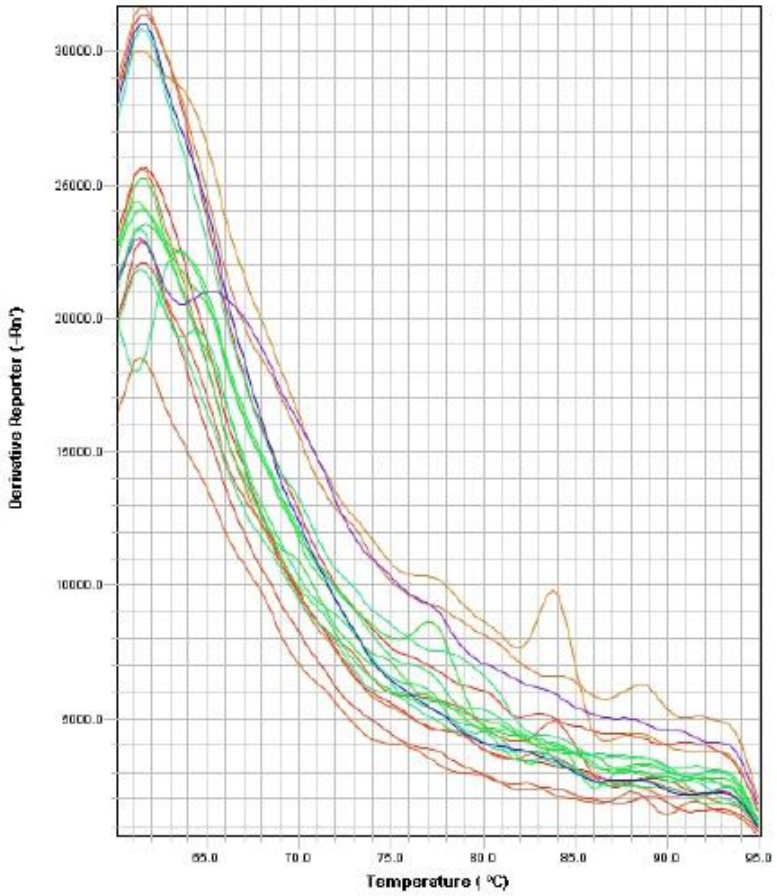


Figure 24: Melt curve of the amplification product of bacterial DNA extracted from eight different beverage emulsion samples by real-time PCR using the universal primers sets B and C.

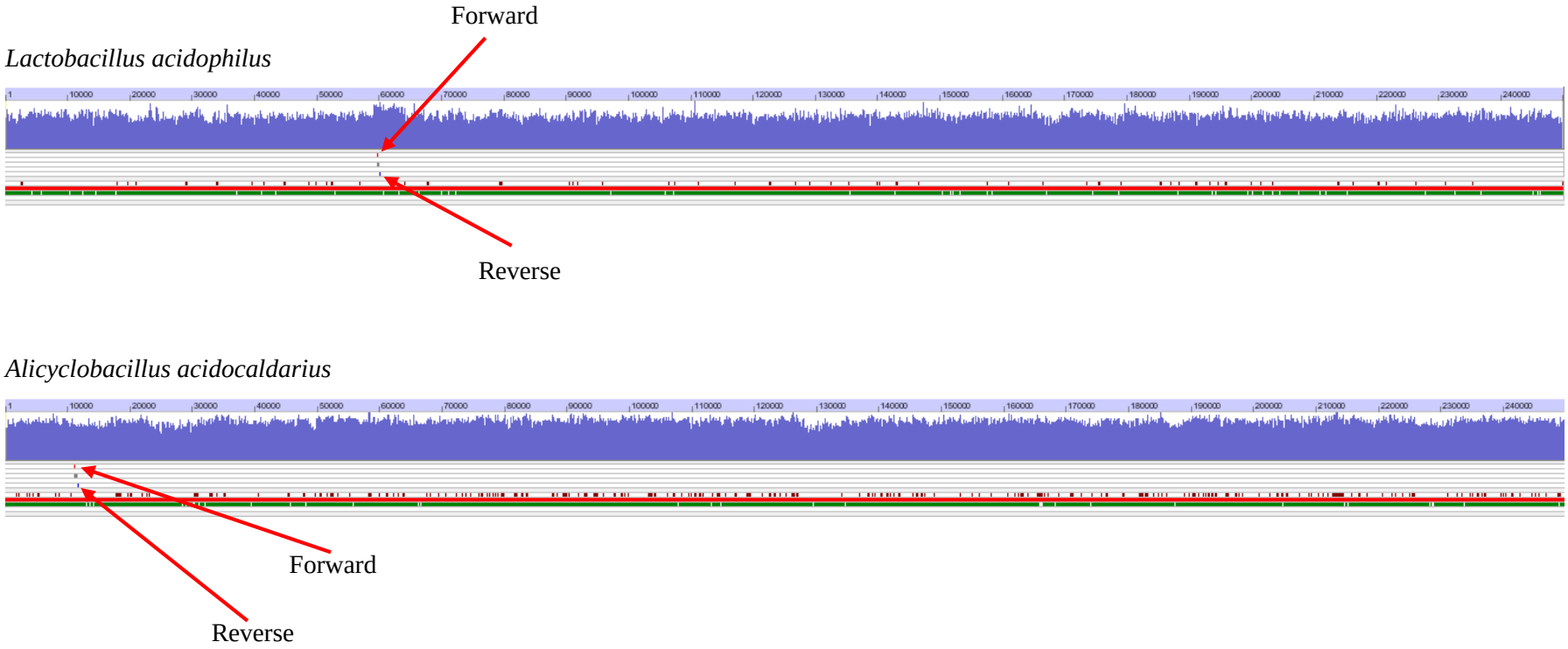


Figure 25: Annealing positions of the universal primers set A on *Lactobacillus acidophilus* genome (NCBI Reference Sequence number NC_006814.3) [64] and *Alicyclobacillus acidocaldarius* genome (NCBI Reference Sequence number NC_013205.1) [65] by Oligo 7 [63].

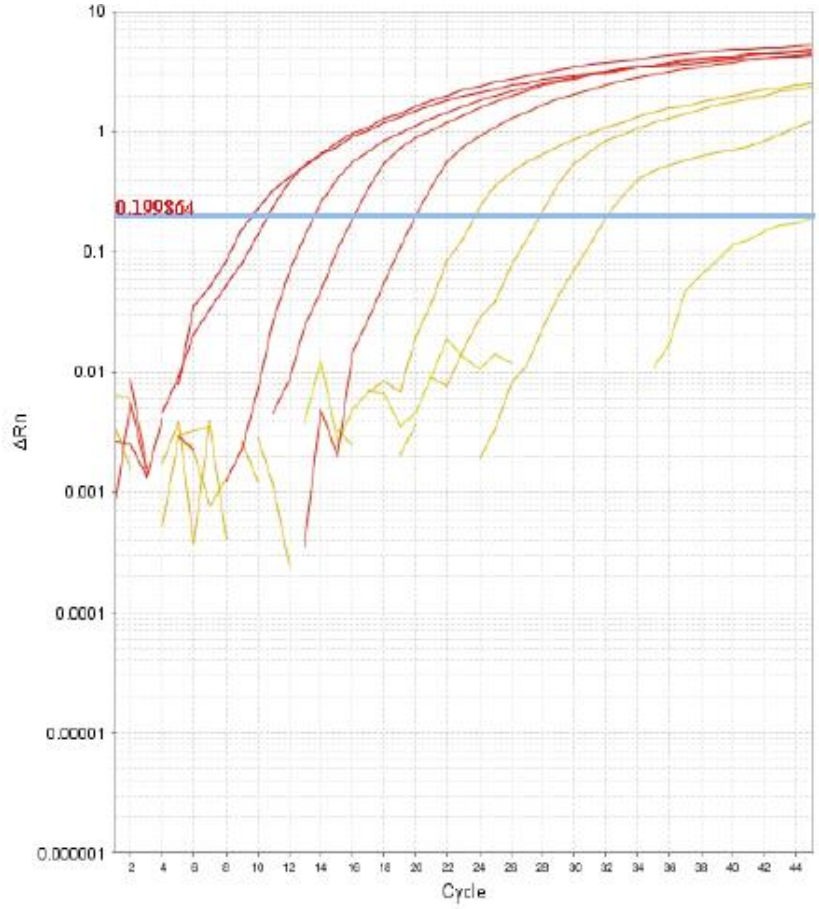


Figure 26: Amplification plot of the eight concentrations of *Escherichia coli* DH10B genomic DNA standard between 100 ng/µL and 10 fg/µL used to construct a standard curve by real-time PCR and the universal primers.

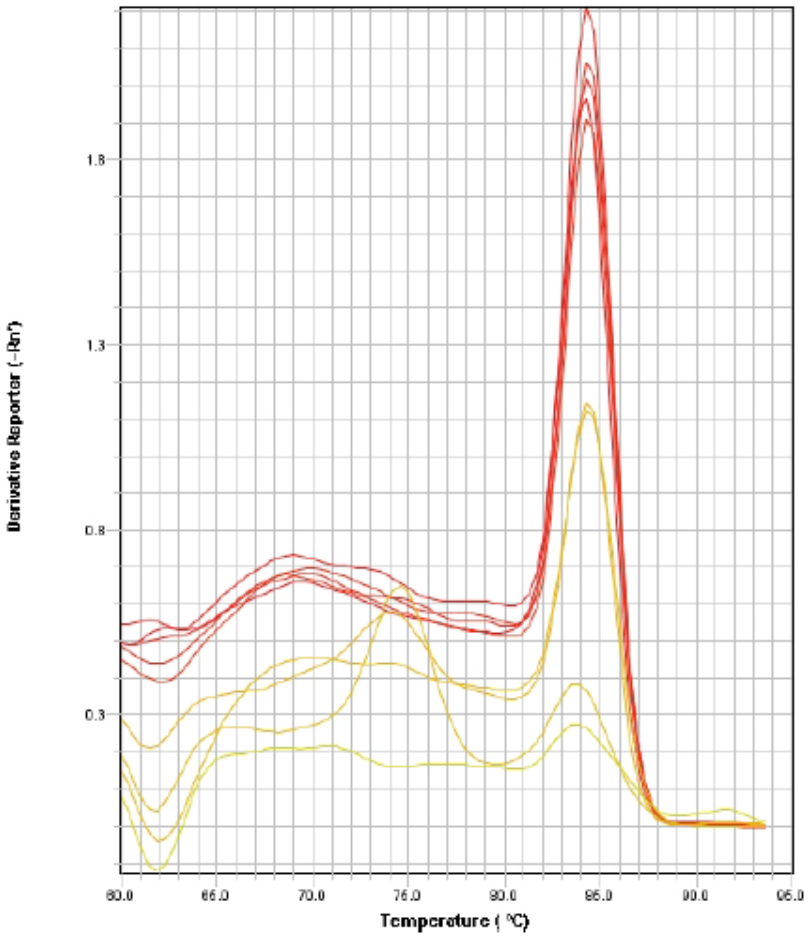


Figure 27: Melt curve of the amplification product of *Escherichia coli* DH10B genomic DNA standard used to construct the standard curve by real-time PCR and the universal primers showing a uniformed peak with an average T_m value of 84.2 °C confirming the amplification of the target sequence.

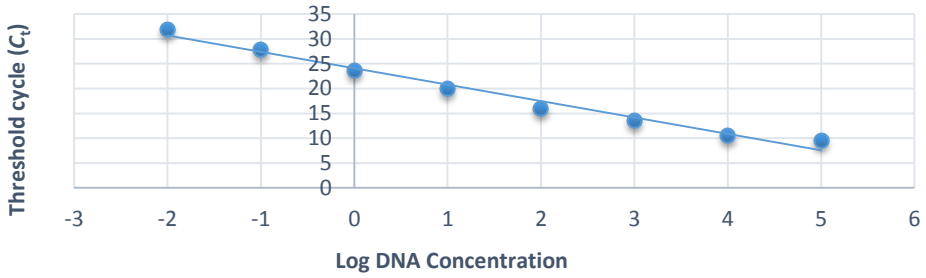


Figure 28: Standard curved constructed using *Escherichia coli* DH10B genomic DNA standard. This is used in the quantification of the total bacterial count using real-time PCR and universal primers. The correlation coefficient of the straight line (R^2) was 0.979, the slope was -3.3, the intercept was 24.11, and the efficiency of the curve (E) was 101%.

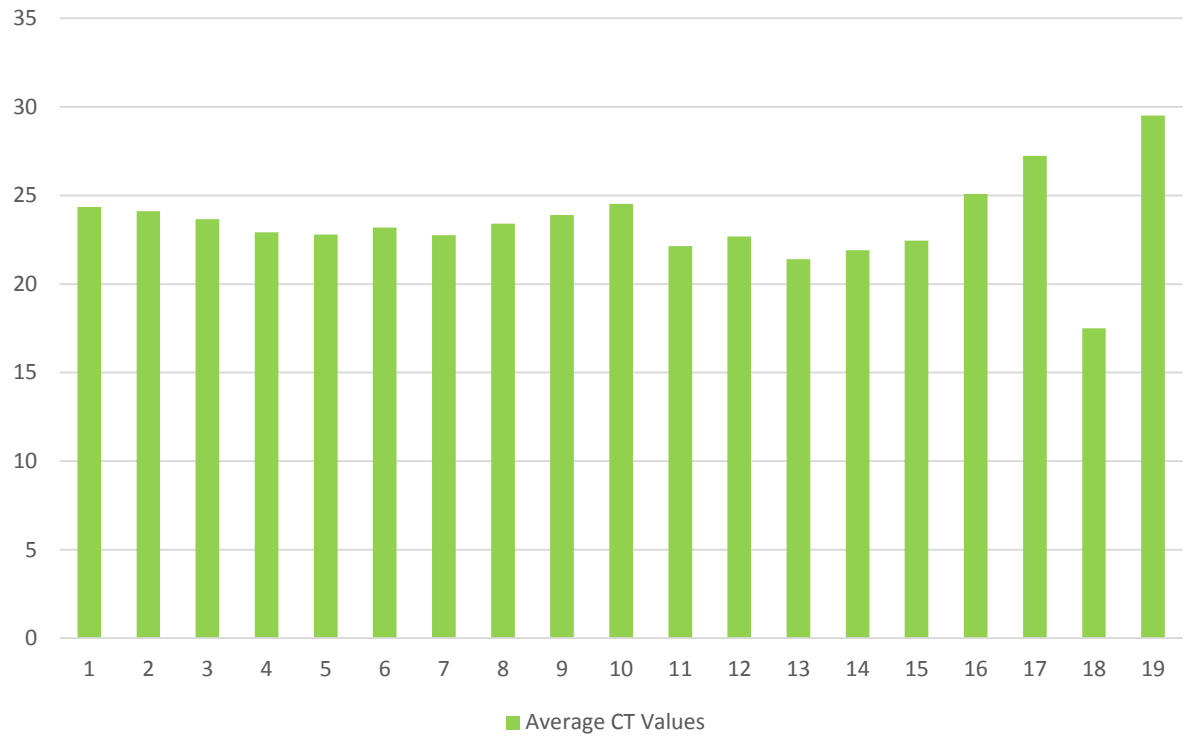


Figure 29: The average C_t values of the 17 samples, positive control and NTC used in the estimation of the total bacteria in beverage emulsion samples by real-time PCR. Sample number 18 represents the positive control, while sample number 19 represents the NTC.

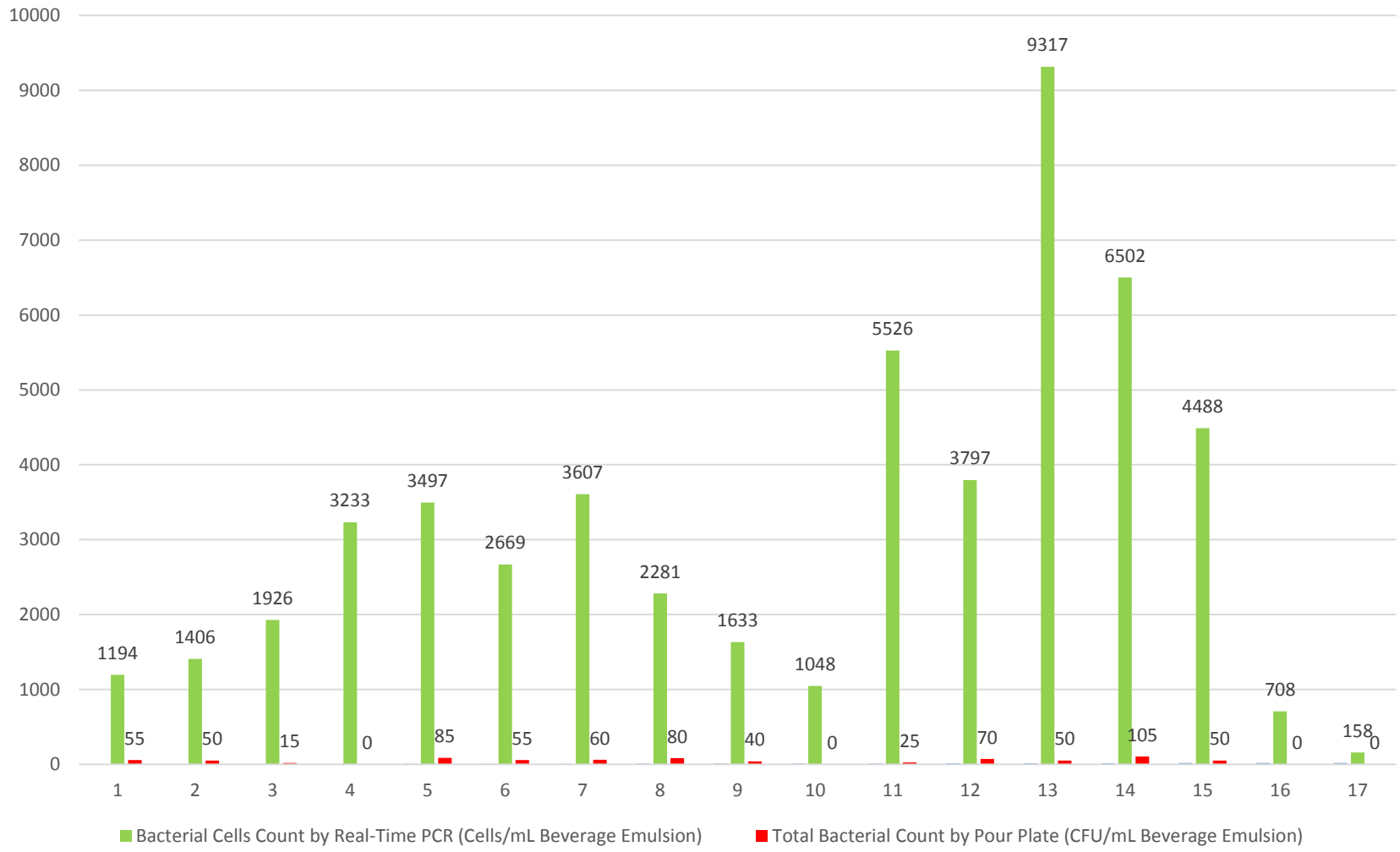


Figure 30: Total bacteria determined by both real-time PCR and pour plate method.

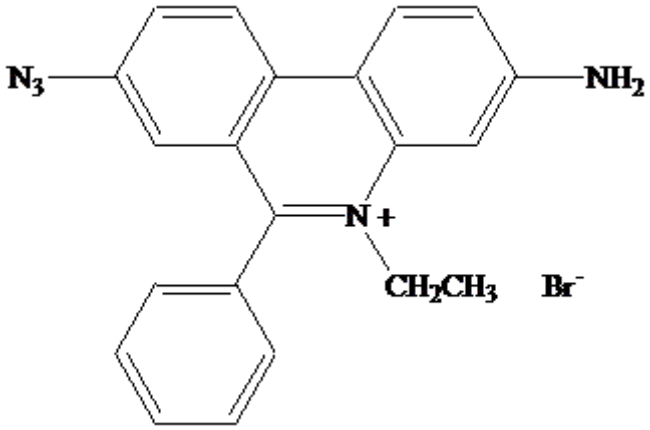


Figure 31: Chemical structure of ethidium bromide monoazide (EMA). Reprinted from [72].

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