UNIVERSITÀ DEGLI STUDI DI PADOVA

Department of Comparative Biomedicine and Nutrition

Master Degree in Biotechnology for Food Science

Developing a qPCR analysis protocol for lactic acid bacteria genera causing meat spoilage

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Abstract

Lactic acid bacteria (LAB) species are widely associated with fresh as well as cooked meat products and are the prevailing spoilage organism in packed meat products. Species composition and metabolic activities of such LAB spoilage communities are determined by the nature of the meat product, storage conditions, and interspecies interactions. The conventional microbiological method used for the detection of the state of spoilage is by total bacterial count. These methods are time-consuming and can be unreliable due to the potential for interference from other microorganisms. In recent years, quantitative polymerase chain reaction (qPCR) has emerged as a rapid and accurate tool for the detection and quantification of LAB. In this thesis work, we present a qPCR analysis protocol for the detection of LAB genera causing meat spoilage. The primers in this study were designed for the genus levels using unique genes in LAB genera commonly associated with meat spoilage and have been optimized for specificity and sensitivity. qPCR analysis of the meat sample was done using the standard curve of the pure culture and its CFU/g was calculated. For the final analysis, the total bacterial count obtained using the plate count method and the qPCR analysis of the same sample were compared. The results showed that the qPCR protocol could detect and quantify LAB genera causing meat spoilage with high specificity and sensitivity. The developed qPCR protocol offers a rapid and reliable tool for the detection and quantification of LAB genera causing meat spoilage, which can be used to prevent spoilage and ensure the safety of meat products. This method can also be used in the food industry for a quick quality check of products and ingredients.

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Contents

Chapter 1

Introduction

Microbial spoilage is the change in composition and sensorial characteristics of the food due to its different chemical, physical, and metabolic processes, which makes it undesirable for consumption. Spoiled food may not cause severe illness as there is the absence of pathogens or toxins, but it is rejected due to the change in smell, texture, taste, and appearance ((Farkas et al., 2007)). Food can be viewed as a dynamic ecosystem, with the microbial community responsible for its spoilage continuously growing and actively altering it. These alterations are due to different factors like chemical changes, physical damage, and due to microbial growth. These changes are driven by the diverse nutrient compositions found in food, which provide favorable conditions for cell proliferation. (Iulietto et al., 2015; Pothakos et al., 2015). Meat is one of the complex foods which allows the growth of various microbes due to its physical and chemical properties. The type of microbial group that is present in the meat is influenced by different factors (Adams, 1998). At the early phage, the initial microbiota is considered to be approximately 10^2-10^3 CFU/g or cm^2 in the meat products (Ray and Bhunia, 2007). During the slaughtering and meat processing, there might be changes in the number and type of microbes that will lead to spoilage when the condition is favorable (Gill, 1998; Luong et al., 2020).

The shelf life of meat is dependent upon the type and number of microbes that are initially present along with their further growth in different storage conditions like pH, gaseous atmosphere, and temperature. The shelf life of the meat is extended by packaging under a CO_2-containing modified atmosphere and maintaining a low temperature during storage. This creates a negative selection pressure for gram-negative spoilage bacteria and also favors the growth of anaerobic microbes such as lactic acid bacteria (LAB) that are associated with fresh meat as well as cooked meats (Dainty and Mackey, 1992; Johansson et al., 2011; Pothakos et al., 2015).

The packaging technique ensures that the redness of meat is preserved and meat remains fresh for a longer time (Doulgeraki et al., 2012; Pothakos et al., 2015).

The ability of microbes to cause food spoilage depends upon the total bacterial number and their ability to produce metabolites that are associated with spoilage. The metabolism of LAB differs from that of gram-negative bacteria. Moreover, their growth rate in packaged meat is slower compared to gram-negative bacteria under aerobic conditions, resulting in a slower spoilage process than that caused by gram-negative bacteria."

These changes help to make the comparison between LAB and gram-negative bacteria more explicit and provide a clearer connection between their metabolism, growth rate, and spoilage effects in packaged meat. (Venkitanarayanan et al., 1997; Casaburi et al., 2015. The commonly used method to determine the state of spoilage and estimate the shelf life of meat is the determination of its total bacterial count. This can be done in various ways and one of them is the traditional plate count method which is time-consuming and does not directly measure the concentration of the specific spoilage causing bacteria (Venkitanarayanan et al., 1997; Kim et al., 2020; Fan et al., 2021. More recently, a widely used molecular method, quantitative Polymerase Chain Reaction (qPCR) has been developed for the detection of different bacterial species, including LAB. This method is culture-independent and quantifies the initial DNA template present in the sample (Kim et al., 2020; Fan et al., 2021. According to Chaillou et al., 2014 qPCR is a promising method as it can successfully quantify the concentration of some LAB associated with spoilage.

This study aimed to develop a new qPCR protocol for the detection of LAB involved in meat spoilage and to compare it with the determination of LAB using specific and selective culturing media.

The background section briefly describes the biological background of LAB, their characteristics during spoilage, and provides an overview of qPCR techniques.

Chapter 2

Background

2.1 Microbial spoilage of meat

The microbiological quality of fresh meat depends on various factors, including the processing conditions and the environment of the slaughterhouse, packaging, and storage conditions, as well as transportation conditions.(Nychas et al., 2008). Storage temperature and packaging atmospheres, such as the availability of O_2, act as selective pressures for the growth of a certain type of microbe, such as psychrotrophic, aerobic, or anaerobic (Nychas et al., 2008; Casaburi et al., 2015).

2.2 Criteria for meat spoilage

Spoilage is usually exhibited by discoloration, a foul smell resulting from the production of volatile compounds and flavors during bacterial metabolism (Russo et al., 2006). Additionally, packages may become bulging due to the formation of CO_2. (Pothakos et al., 2015). Meat spoilage bacteria utilize mainly carbohydrates, amino acids, lipids, and proteins resulting in the production of volatile compounds. When there is about 10^7 CFU of bacteria/cm^2, they produce off smells like sulfides, ammonia, and amines and they can be detected when the population quantity is about 10^8 CFU/cm^2 (Ellis et al., 2006; Doyle, 2007). Along with the volatile compounds, some spoilage bacteria may form slime (Ercolini et al., 2006; Nychas et al., 2008). The availability of different substrates like free amino acids, glucose, nitrogenous compound, and lactic acid are the main precursors of spoilage-causing microbial metabolites (Nychas et al., 2008). Thus, for growth and survival microbes utilize different pathways depending on the availability of substrate and this leads to multiple outcomes, such as slime, off odors, off flavors, and change in texture due to

polymers degradation (Borch et al., 1996; Gram et al., 2002; Nychas et al., 2008).

(a) Bulging of package due to gas production **(b)** Slime Production

Figure 2.1: Showing changes in appearance

2.2.1 Off flavors and odors

Organic acids, ketones, volatile fatty acids, ethyl esters, sulfur compounds, aldehydes, ammonia, and others are the volatile metabolites produced by microbes. The sensory quality of the meat is influenced by the microbial interaction between non-volatile and volatile compounds. When meat is stored aerobically, it can result in the production of undesirable odors such as sulfuric, cheesy, fruity, and putrid smells(Casaburi et al., 2015). A foul smell like acetic acid and acetone is produced by *Brochothrix thermosphacta* due to aerobic metabolism (Koutsoumanis et al., 2006) and homofermentative *lactobacilli* produce a cheesy smell because of 3-methylbutanol and acetone production (Casaburi et al., 2015). In an anoxic modified atmosphere packaging, LAB produces acetic acid and lactic acid, resulting in an acidic and sour aroma. On the other hand, *B. thermosphacta* produces a less intense smell in anaerobic conditions compared to aerobic conditions, as the rate of glucose consumption is affected by CO_2 and O_2 (Pin et al., 2002).

2.2.2 Change in color

A bacterial patina becomes visible on the surface of the meat when a load of microbes reaches around $10^{7.5} - 10^8$ CFU/cm^2 under the aerobic condition. In aerobic environments, the production of hydrogen sulfide by *Latilactobacillus sakei* can lead to the appearance of green sulphomyoglobin on the meat. (Borch et al., 1996). Additionally, certain *Leuconostoc* species may cause the meat to turn green by producing Hydrogen peroxide when meat is exposed to O_2 (Marsden and Henrickson, 1993).

Figure 2.2: Greening of meat

2.3 Meat Packaging

2.3.1 Modified Atmosphere Packaging

Modified atmosphere packaging (MAP) involves replacing the normal atmosphere with a different gas mixture before packaging the product under a barrier material. During the storage, there might be some changes in the product and headspace environment (McMillin et al., 1999; McMillin, 2008). This type of packaging helps to retain freshness and preserves food, especially meat products (Skandamis and Nychas, 2002). Therefore, MAP is used widely to prolongate the shelf life of meat products (Thoden van Velzen and Linnemann, 2008; Schumann and Schmid, 2018). The shelf life of the product refers to the time period from the date of packaging until the properties of the product remain acceptable in the market (McMillin, 2008).

2.3.2 Marinated meat products

The process of marination differs according to place. It can be defined as the process of adding spices, salts, phosphate, acids, sugar, aromatic enhancers, and different sauces like tomato which are usually oil or water-based. This is usually done by adding flavors and tenderizing poor-quality meat, inhibiting microbial growth. In Finland, marinated meats are usually packaged under MAP, and this type of packaging is usually dominated by LAB, due to the low pH and the presence of carbohydrates (Björkroth, 2005).

Meat products that are preserved using different techniques like marination, value-added, non intact meat, and moisture enhanced have a higher risk of contamination/ spoilage as they are prepared using enriched sugar supplements for

tenderizing it (Vihavainen and Björkroth, 2007; Sofos and Geornaras, 2010).

2.4 Lactic Acid Bacteria in meat spoilage

2.4.1 Lactic Acid Bacteria

LAB are gram-positive bacteria that shares, morphological, physiological, and metabolic characteristics (Kandler, 1983; Carr et al., 2002). They are catalase-negative, non-sporulating, acid-tolerant, usually non-respiring, and aerotolerant rods or cocci that produce lactic acid as a fermentation product of carbohydrates (Fischer and Thines, 2017; Vinderola et al., 2019). They are usually found in a nutrient-rich environment like dairy products, meats, sourdough, decomposing plants, and sewage (Fischer and Thines, 2017).

According to recent classification, its family includes *Aerococcaceae, Streptococcaceae, Enterococcaceae, Lactobacillaceae*, and *Carnobacteriacea* (Vinderola et al., 2019; Zheng et al., 2020). They are classified according to their metabolic differences as facultative heterofermentative, obligate heterofermentative, and obligate homofermentative. More than 85% of lactic acid is produced as a sole end product by homofermentative species, whereas heterofermentative species produce CO_2, ethanol, and lactic acid from glucose (Gänzle, 2015; Fischer and Thines, 2017).

2.4.2 Condition leading to LAB spoilage

The mixture of carbon dioxide, nitrogen, and oxygen in different ratios is used in MAP. The ratio of different gases determines the growth of different microbes (Gill, 1996). LAB are the microbes mostly associated with MAP products (Nieminen et al., 2011) along with *B. thermosphacta* which is frequently associated with spoilage of meat stored aerobically or in a vacuum packed (Russo et al., 2006). Some strains of LAB are the main spoilage microbes that are stored under different atmospheres and temperatures (Yost and Nattress, 2002;Chaillou et al., 2014). The growth of LAB is favored by the presence of high carbon dioxide (Gill, 1996) and a high percentage of nitrogen whereas high oxygen concentrations have been reported to promote the cell growth of facultative aerobic bacteria as demonstrated for *B. thermosphacta* (Vihavainen and Björkroth, 2007; Pellissery et al., 2020).

2.4.3 Different LAB species in meat spoilage

Specific LAB species are associated with meats spoiled under certain conditions (Pothakos et al., 2015). The LAB genera that have been involved in meat spoilage under MAP are *Carnobacterium, Leuconostoc,* and *Latilactobacillus* (Tsigarida et al., 2000; Skandamis and Nychas, 2002; Castellano et al., 2004). Among them, the most commonly detected species include *Leuconostoc* spp., *Latilactobacillus. sakei*, and *Latilactobacillus curvatus* and *Latilactobacillus fuchuensis* (Yost and Nattress, 2002; Fontana et al., 2006; Pennacchia et al., 2011; Pothakos et al., 2015). Additionally, *Carnobacterium divergens* and *Carnobacterium maltaromaticum* are also present in meat (Casaburi et al., 2015).

In the marinated meat stored under MAP, *L. sakei, L. curvatus* along with other homofermentative *Latilactobacillus* spp. have been identified as spoilage bacteria (Björkroth, 2005). Even though *Lactococcus* have been commonly associated with dairy fermentation, certain species of *Lactococcus* have been found to cause meat spoilage, particularly in MAP beef(Pothakos et al., 2015). Along with this, *Lactococcus carnosus*, and *Lactococcus garvieae* are found to be a part of spoilage microbes (Sakala et al., 2002; Bromberg et al., 2005; König and Fröhlich, 2017). Their presence may lead to spoilage and a reduction of the shelf life of the product.

Alterations caused by LAB in MAP are discoloration, off-odor, decrease in pH, gas production, and formation of slime (Samelis et al., 2000). These changes become visible when the community has reached the stationary growth phase (Pin et al., 2002). *L. curvatus*, *L. sakei*, *Leuconostoc* spp., and *Carnobacterium* spp. are the LAB that is mainly involved in meat spoilage. Lactic acid is produced by homofermentative LAB whereas ethanol, acetic acid, CO_2, and acetoin causing off smell and slimy rope are produced by heterofermentative LAB (Krockel, 2013). During storage, the increased concentration of CO_2 in packages can result in the production of grey liquid and slime by heterofermentative *lactobacilli* and *Leuconostos* spp. (Borch and Nerbrink, 1989; Björkroth and Korkeala, 1997). Some LAB secretes long-chain, high-molecular-mass, viscosifying, or gelling exocellular polysaccharides into the environment, resulting in slime formation (Ullrich, 2009). According to the study by Lyhs et al., 2004 *Leuconostoc gelidum* and *Leuconostoc gasicomitatum* strains were found to dominate the lactic acid bacterium population associated with strong slime formation in an acetic-acid preserved herring. In the case of MAP of raw beef, green and foul smells were observed (Vihavainen and Björkroth, 2007). The presence of *L. gelidum* and *L. gasicomitatum*, along with the formation of CO_2 can cause packages to bulge and also leads to the production of cheesy-buttery flavor in spoiled meat

stored in a high oxygen atmosphere (Vihavainen and Björkroth, 2007).

2.5 quantitative Polymerase Chain Reaction (qPCR)

Culture-dependent methods like direct plate count can be time-consuming and biased (Ramamurthy et al., 2014). As an alternative, culture-independent methods have been considered as a valuable technique that directly analyses DNA extracted from a food matrix (Rodríguez et al., 2012; Ramamurthy et al., 2014). One such molecular approach is qPCR, which allows for the detection of the targeted bacterium in a food matrix (Rodríguez et al., 2012; Luedtke and Bosilevac, 2015; Ilha et al., 2016).

qPCR is a technique used for quantifying nucleic acid molecules from environmental and biological samples (Taylor et al., 2019). It permits DNA quantification during the amplification process. Standardization is achieved by using a reference sample whose DNA content is known for indirect cell counting (Fischer and Thines, 2017). In qPCR, the amplification is continuously monitored in real time using fluorescence. After each cycle, the fluorescence is measured, and the intensity of the signal reflects the instantaneous amount of DNA amplicons present in the sample at that particular time. During the initial cycle, the fluorescence is too low to be distinguished from the background. The quantification cycle (Cq), which is determined by comparing the unknown sample to a calibration curve built on a serially diluted standard sample, provides the absolute quantification of the targeted DNA. When the fluorescence surpasses the detection threshold, it is considered proportional to the initial number of templates in the DNA sample (Kralik and Ricchi, 2017).

When performing analysis using the qPCR technique, there are two approaches for primer design. One option is to design forward and reverse primers for a genus or subset of species targeting a well-conserved housekeeping gene. In this case, a labeled probe can also be used to increase specificity. Alternatively, primers can be designed in unique genes or a subset of species, using SYBR green as a fluorescent dye. (Cauchie et al., 2017; Jérôme et al., 2022).

Recent studies have employed the use of qPCR method for the detection of LAB. This technique offers several benefits, including high sensitivity, reproducibility, and specificity. Additionally, its detection speed is significantly faster compared to traditional PCR methods (Fan et al., 2021) as well as culture-dependent methods. Within a few hours, the amplification curve can be confirmed, allowing for the analysis of results in a short period of time(Liu et al., 2019).

In comparison to qPCR, droplet digital PCR is another new technique that is reliable for the quantification and detection of LAB. It is the third-generation PCR tool that quantifies without using a calibration curve and the sensitivity of ddPCR is more than qPCR (Porcellato et al., 2016). The main difference between qPCR and ddPCR is that the reactions are divided into thousands of individual reaction vessels before amplification and the data are acquired at the endpoint of response, which allows independent and direct quantification of DNA without a standard curve. This allows more precision and replicable data when compared to qPCR, especially when there is sample contamination which can inhibit primer annealing or Taq polymerase. It can also be used in cases where the sample is contaminated or when the target quantitation is low, making it difficult to detect using qPCR (Taylor et al., 2017).

Chapter 3

Aim of the Study

This study aimed to make a comparison between the selective plate count methods and qPCR analysis. The specific aim was to develop a qPCR protocol for the determination of LAB genera causing meat spoilage.

1. To get a rapid method for estimation of the total number of bacteria on the genus level in comparison to plate count techniques.

2. To design primers on the genus level for qPCR that quantifies LAB species involved in meat spoilage.

Chapter 4

Materials and Methods

4.1 Primer Designing

The genome sequences of different species of *Leuconostoc, Carnobacterium, Brochothrix, Lactococcus*,*Lactilactobacillus*, and *Vagococcus* along with their negative controls for the comparison are listed in Appendix A. The sequences were downloaded from NCBI RefSeq using the NCBI-genome download tool [1], and their amino acid and nucleotide sequences were extracted into fasta format.

The ortholog prediction tool GET_HOMOLOGUES was used to identify unique genes from amino acid sequences(Contreras-Moreira and Vinuesa, 2013). From the output of ortholog prediction, one of the amino acid sequences was selected, with a preference for enzymes as they tend to be more conserved compared to randomly selected proteins (Peregrín-Alvarez et al., 2009). The nucleotide sequence in Fasta format for the selected amino acid was retrieved from the output of ortholog prediction using EMBOSS seqret. Multiple sequence alignment was then performed using mafft (Katoh and Standley, 2013) and the output file was visualized using UGENE to identify conserved regions(ONonechniNov et al., 2012). Primers were designed in these unique genes using an online software primer3 based on the alignment results from UGENE (Untergasser et al., 2012).

If the extracted amino acid sequence was not well conserved, two different strains were selected, and their nucleotide sequence was extracted. A blast analysis was performed between the sequences, and the steps were repeated if more than two strains were involved. The output file was evaluated and the strains with the highest percentage of similarity were noted. The amino acid sequence that was well conserved among all the strains was listed, and the nucleotide sequence for the listed amino

[1] https://github.com/kblin/ncbi-genome-download/

acid was extracted. Multiple alignments were performed using mafft, following the same steps as mentioned above.

After designing primers for each bacterium, the specificity was checked by blasting it in NCBI. The primers with high specificity were selected and their variation in each strain was checked in UGENE. If there was variability in just one base in strains it was ignored, if there were variability in more bases in different strains the bases were replaced with a degenerate base pair. The primers designed on unique genes in this study are listed in Appendix B.

4.2 Isolation of DNA from pure cultures

Lysis solution was prepared for six strains of LAB by mixing the stock solution (Appendix C). All the steps below were performed in a safety cabinet. 100 μl of stock solution was added to the sample and vortexed to dissolve the pellet until clumps were not visible. The sample was then incubated at 37°C for 45 minutes. 500 μl of GES reagent was added and it was gently mixed by inverting the tube for 10 minutes, then the sample was kept on ice for 5 minutes. After this 250 μl of ice cold NH_4Ac was added and mixed by turning it upside down. The sample was then placed on ice for another 20 minutes. 500 μl of Chlorophorm-2-Pentanol (24:1) was added and mixed by shaking it. It was then centrifuged for 10 minutes at 13,000 RPM. After the centrifugation, the upper phage was pipetted carefully and transferred into new Eppendorf tubes. 380 μl of freezer-cold 2-Propanol was added and mixed well by inverting it until white DNA precipitation was seen. It was again centrifuged for 4 minutes at 13, 000 rpm and the liquid was discarded carefully without losing the DNA pellet. Around 500 μl of 70 % (RT) ethanol was added and centrifuged for 2 minutes and the ethanol was poured away. This step was repeated three times in total. After the last wash remaining drops of ethanol were pipetted out and the pellets were left to dry for 1 hour. After this, the samples were stored in the freezer (Pitcher et al., 1989; Björkroth and Korkeala, 1996).

4.3 Primer Specificity

Strains of *L. gasicomitatum* (LMG 18811), *C. maltaromaticum* (ATCC 35586), *B. thermosphacta* (CCUG 35132), and *L. sakei* (CCUG 31331) were taken for checking the specificity of the designed primer. Serial dilution of all four strains over the range of $10^2 - 10^8$ was done and used for the qPCR analysis.

The qPCR for the quantification of the targeted bacterial strain was performed in a 96-well plate with a final volume of 20 μl of the reaction mixture in each well. The reaction mixture contained 10 μl of master mix, 0.5 μl of each forward and reverse primer for specific bacteria, 7 μl of water, and 2 μl of templet DNA which was loaded at the end in an individual well. Amplification was performed using the following steps. The activation of the enzyme was done at 95°C for 120 s followed by the denaturation and annealing step done at 95°C for 5 s and 60°C for the 30 s respectively. The second cycle was repeated 40 times. Amplification was followed by the analysis of the melting curve where there was a gradual increment of the temperature from 65°C to 95°C with 0.5°C increments, during each temperature increment there was a hold for 15 s to allow the temperature to equilibrate. The analysis was done in the BioRad machine, and the experimental data were analyzed with CFX Maestro Software.

4.4 Meat Samples

Meat samples for this study were taken from chicken breast fillets, marinated chicken breast fillets, chicken thigh fillets, Beef, minced beef, Pork, Marinated and Unmarinated Rainbow trout (*Oncorhynchus mykiss*), Baltic herring fillet (*Clupea harengus membras*) from different stores in Finland at the end of its shelf life. All the above meat samples were packed in a modified atmosphere except for the Baltic herring fillet. The above-mentioned meat sample was taken because these are high-value foods, they have short shelf lives and they are known to contain LAB species that can be detected using the primers designed here.

4.5 Bacterial strains, media, and culture conditions

For the sample preparation, 25 g of meat was homogenized with 225 ml of peptone solution with 0.85% NaCl and blended in a Lab Blender 400, Seward, Worthing, UK for 60 seconds. Serial dilution was performed on the peptone solution with 0.85% NaCl from the homogenate meat sample and appropriate dilution was spread on the selective media for the isolation of different bacterial species.

Different media were tested for the selective isolation of different bacterial species. The media that performed well was used for the isolation of different bacterial species. Even though the media were not 100 % selective, colony morphologies were considered to differentiate it among different species.

4.5.1 Selective media for *Leuconostoc*

For the isolation of *Leusonostoc* Phenylethyl Alcohol Sucrose Agar Media (PES) was used as a specific and selective medium (MIYAO and OGAWA, 1988). The composition of PES media was modified with the addition of 0.01 g/l of sterile Triphenyltetrazolium chloride (TTC) after autoclave treatment (Wasney et al., 2001). Meat samples from different dilutions were plated on the PES plates and incubated at 25°C in an anaerobic condition for 72 hours.

4.5.2 Selective media for *Carnobacterium*

For the isolation of Carnobacterium, initially Cresol Red Thallium Acetate Sucrose Inulin (CTSI) according to Wasney et al., 2001 was used but no growth was seen in the medium so, later Elliker agar, modified to pH 9 (Elliker et al., 1956) was used as a selective media. Meat samples from different dilutions were plated on the Elliker pH 9 plates and incubated at 37°C in an aerobic conditions for 48 hours.

4.5.3 Selective media for *Brochothrix*

For the isolation of Brochothrix STAA media was used as a selective media. Meat samples from different dilutions were plated on the STAA plates and incubated at 25°C in aerobic conditions for 48 hours.

4.5.4 Selective media for *Latilactobacillus*

For the isolation of Latilactobacillus Rogosa agar was used. Meat samples from different dilutions were plated on the Rogosa plates and incubated at 25°C in anaerobic conditions for 72 hours. This medium can be considered as a selective medium for lactobacilli.

There is no specific media for the isolation of *Vagococcus* and *Lactococcus* species. Elliker pH 9 media can be used for the isolation of *Vagococcus* but it will not work as a differential media.

The bacterial colonies from different media were counted and CFU/g of different species was calculated for the meat sample.

4.5.5 Bacterial Strains

The type strains that were used in this study are, *L. gasicomitatum* LMG 18811, *C. maltaromaticum* ATCC 35586, *C. divergens* ATCC 35677, *B. thermosphacta* CCUG

35132, *Lactococcus carnosus* MKFS47, *L. sakei subsp. carnosus* CCUG 31331, *L. fuchuensis* DSM 14340 from the local strain collection in the Department of Food Hygiene and Environmental Health, Faculty of Veterinary Medicine, University of Helsinki.

Leuconostoc and *Latilactobacillus* were cultured in MRS at 25°C in anaerobic conditions, and *Carnobacterium, Brochothrix,* and *Lactococcus* were cultured in Tryptone Soy Yeast extract (TSYE) at 25°C in aerobic condition for 24 hours

4.6 Isolation of DNA from meat sample and pure culture

4.6.1 Sample preparation

From the homogenate meat sample, 2 ml of microbial food culture was added to a 2 ml Collection tube, and it was centrifuged at 13,000 RPM × g for 1 minute. The supernatant was decanted, and the tube was spined again at 13,000 RPM × g for 1 minute. Excess supernatant was pipetted out and the cell pellets were stored in the freezer for further use.
For the pure culture, the same procedure was followed.

4.6.2 DNA isolation

The isolation of DNA from the meat sample and pure culture was done by using a DNeasy PowerFood Microbial Kit (Qiagen, Hilden, Germany). For this, the cell pellet was resuspended in 450 μl of MBL solution which was warmed at 55°C for about 5-10 minutes. The resuspended cell was transferred to the PowerBead Tube provided in the kit and Fastprep (MP Biomedicals, Santa Ana, CA, USA) was used at 5.5 m/s for 40 seconds. After this, the tubes were centrifugated at 13,000 RPM × g for 1 minute at room temperature and the supernatant was transferred to a clean 2ml Collection tube. 100 μl of IRS solution was added, vortexed briefly, and incubated at 2-8°C for 5 minutes. After this, the tubes were centrifuged at 13,000 RPM × g for 1 minute at room temperature and the supernatant was transferred to a 2 ml Collection tube by avoiding the pellet. 900 μl of MR solution was added, vortexed, and all the samples were loaded onto the MB Spin column (two loads). The spin column was centrifuged at 13,000 RPM × g. The spin column was transferred to a clean collection tube provided and 650 μl of PW solution was added and

centrifuged at 13,000 RPM × g for 1 minute. The flow through was discarded and 650 μl of ethanol was added and again centrifuged at 13,000 RPM × g. Again, the flow through was discarded and centrifuged at 13,000 RPM × g for 2 minutes. The MB spin column was placed in the clean collection tube and 100 μl of EB solution was added in the middle of the spin column and centrifuged at 13,000 RPM × g for 1 minute for the release of DNA. The spin column was discarded, and the extracted DNA was stored in the freezer for further analysis.

4.7 qPCR Analysis

The qPCR for the quantification of the targeted bacteria was performed in a 96-well plate with a final volume of 20 μl of the reaction mixture in each well. The reaction mixture contains 10 μl of master mix, 0.5 μl of each forward and reverse primer for specific bacteria, 7 μl of water, and 2 μl of templet DNA from a pure culture which was loaded at the end in an individual well. Amplification was performed using the following steps. The activation of the enzyme was done at 95°C for 120 s followed by the denaturation and annealing step done at 95°C for 5 s and 60°C for the 30 s respectively. The second cycle was repeated 40 times. Amplification was followed by the analysis of the melting curve where there was a gradual increment of the temperature from 65°C to 95°C with 0.5°C increments, during each temperature increment there is a hold for 15s to allow the temperature to equilibrate. The analysis was done in the BioRad machine, and the experimental data were analyzed with CFX Maestro Software.

The standard curve was analyzed with three biological replicates and two technical replicates. Four different dilutions (10^6 to 10^4) of the DNA template were used, whose concentrations were already calculated from the plating method. The mean Cq value and Log starting value of the standard reference sample of the technical replicate were calculated and the average of the mean value of each biological replicate was determined. Slope, R^2, and efficiency were calculated by plotting the Cq value against Log starting value of the reference or standard sample (pure culture).

For the meat samples, the same protocols were used without any replicates. The data from the meat sample were compared with the standard curve to calculate the total bacterial count of different species.

4.8 Comparison between plating data and qPCR analysis

The total CFU/g obtained from the food samples using the plating method for each species was compared with the CFU/g obtained from the same food samples during qPCR analysis. The results were recorded and subsequently analyzed.

Chapter 5

Results and Discussion

5.1 Specificity and accuracy of Primer pairs

The strains of *L. gasicomitatum* (LMG 18811), *C. maltaromaticum* (ATCC 35586), *B. thermosphacta* (CCUG 35132), and *L. sakei* (CCUG 31331) were used to check the specificity of the designed primer pair. A standard curve was produced, to establish quantification criteria, using serial dilutions of all four strains over the range of 10^2-10^8 CFU/ml. For high-efficiency primers, the standard curve should have an R^2 value $\geq$ 0.98 and a slope between -3.1 to 3.6 (Broeders et al., 2014). The amplification curve obtained from the qPCR reaction for *B. thermosphacta, L. gasicomitatum,* and *L. sakei* were good whereas the curve for *C. maltaromaticum* was not as expected. Standard curve of *B. thermosphacta, L. gasicomitatum, C. maltaromaticum , L. sakei* had R^2 value of 0.998, 0.953, 0.488, 0.967, showed slope of -3.481, -3.674, -2.090, 4.312 and showed amplification efficiency of 93.8%, 87.1%, 200.9% and 70.6%, respectively. All the R^2 values were around 0.98 except for *C. maltaromaticum*. This indicates that the primer pairs exhibited high efficiency and specificity for distinguishing the target species in a sample, except for *C. maltaromaticum*. A single peak of the melting curve was observed which indicated the absence of primers dimers and nonspecific amplicon (Kim et al., 2011).

The reason for the primer pairs for *C. maltaromaticum* not having high efficiency, may be due to the degenerate base pair bases of the primers or due to the reaction conditions like melting temperature that can influence annealing of the primer templet and result in poor amplification (Taylor et al., 2010).

The primers in this study were designed for the genus levels using unique genes as the targets. The advantage of this is that we do not have to run the reactions separately using different primers, as needed for the species-level approach, and we

were able to cover several species in the single reaction. However, the disadvantage of this method is that we might have to introduce degenerate base pairs in the primers as we assumed that the unique gene, we selected were available in all strains. As there is strain variation, a risk that these unique genes are not available in all strains of a species exists. As an alternative to this method, primers could have been designed for a less conserved housekeeping gene which is present in all strains as done in the study of Taylor et al., 2017.

5.2 Plate Count Method

Homogenated meat samples were cultured using selective media and different 10-fold dilutions of the samples shown in Table 5.1. STAA was used for the selective isolation of *Brochothrix*, CTSI, and Elliker pH 9 were used for, *Carnobacterium*, Rogosa was used for *Latilactobacillus*, and PES was used for *Leuconostoc*. These selective media were carefully chosen according to the meat sample and the growth of the bacterial strains used in this study we tested in these media.

Table 5.1: Media used for the selective isolation

Sample	Selective Media
Chicken	STAA, CTSI, Rogosa, PES
Chicken 2	Elliker pH 9, STAA, Rogosa, PES
Chicken 3	Elliker pH 9, Rogosa, STAA
Pork	PES, Rogosa, STAA, CTSI
Minced beef	PES, Rogosa
Marinated rainbow trout 2	Rogosa
Unmarinated and marinated rainbow trout	Elliker pH 9, STAA, Rogosa, PES
Unmarinated rainbow trout inoculated with *Carnobacterium*	Elliker pH 9, STAA, Rogosa, PES
Beef	PES, Rogosa
Beef 2	Elliker pH 9, PES, Rogosa

The PES media was modified by adding TTC, resulting in improved growth of *Leuconostoc* compared to the original PES media. However, in other media such as CTSI, which was used for *Carnobacterium*, no growth was observed when the type

strains of *C. maltramaticum* and *C. divergens* were used. As a result, Ellike agar media with a pH of 9 was chosen for the isolation of *Carnobacteria*. The recovery rate for Elliker pH 9 media was close to 100%. In STAA used for *Brochothrix*, we did not get many colonies which might be due to the absence of *Brochothrix* or they might be present in very low numbers. The Rogosa media for *Latilactobacillus* was selective in our experiments. For the isolation of *Lactococcus*, no selective media were available. In the PES medium, two types of colonies were observed, slimy shiny colonies and small red/white colonies. The presence of slimy shiny colonies indicated the presence of *Leuconostoc*, and the production of slime or blob in the colonies is due to the production of dextran. The other red or white colonies were not identified. *Carnobacterium* formed creamy, round colonies in Elliker pH 9 media whereas *Brochothrix* formed yellowish, round colonies in STAA medium. *Latilactobacillus* produced white, round, and smooth colonies in Rogosa.

The colonies obtained from different dilutions were counted, and their average count was calculated for each selective medium. The average count was then used to calculate the total bacterial count in the meat sample. The total CFU/g for each sample is shown in Table5.2

Table 5.2: Total CFU/g calculated from plate counting

Sample	*Leuconostoc*	*Carnobacterium*	*Brochothrix*	*Latilactobacillus*
Chicken	-	-	-	6.2×10^6
Chicken2	<1000	2.4×10^7	1.0×10^4	<1000
Chicken 3	-	4.4×10^6	-	3.1×10^6
Marinated Chicken Breast Fillet	2.1×10^4	<1000	-	5.0×10^4
Beef	8.9×10^6	-	-	<1000
Beef 2	8.9×10^4	<1000	-	4.0×10^5
Minced Beef	<1000	-	-	<1000
Pork	<1000	-	2.2×10^4	<1000
Marinated Rainbow Trout	<1000	3.0×10^4	-	0
Marinated Rainbow Trout 2	-	<1000	-	-
Unmarinated Rainbow Trout	<1000	3.4×10^5	3.8×10^5	0
Unmarinated Rainbow Trout Inoculated with *Carnobacterium*	-	1.9×10^7	-	-

5.3 qPCR Analysis

A standard curve for a reference sample or a standard sample was created using different dilutions of the pure culture of a single strain of *Leuconostoc, Carnobacterium, Brochothrix, Latilactobacillus,* and *Lactococcus*. The samples from the pure cultures were run with three biological and two technical replicates. The average Cq value was determined by taking the mean value of the technical replicates for each dilution. From these average Cq values, the average Cq value of the biological replicates for each dilution was calculated. Similarly, the average Log starting value of the sample was estimated using a similar approach. A graph was then created, plotting the average Cq value against the Log starting value of the sample. By analyzing this graphs (as shown in Figure5.1) the slope, intercept, and R_2 values were calculated. The presence of a straight line in the standard curve indicated a favorable outcome, suggesting a reliable result.

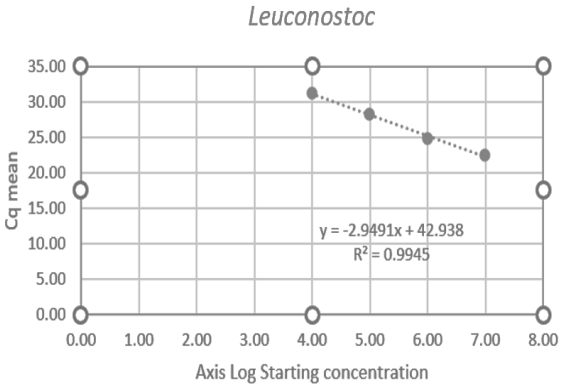
For the meat samples, qPCR analysis was done and the corresponding Cq values were recorded. Using the slope and intercept values obtained from the standard curve of the pure culture, the CFU/g was calculated for each sample 5.3. To account for any potential errors, the qPCR analysis was also conducted without the standard or reference sample, and the resulting error was calculated. To correct for this error, a single dilution of a standard or reference sample was included in the qPCR analysis alongside the meat sample. By comparing the results to the previous values, the extent of the difference between them was calculated and adjusted accordingly.

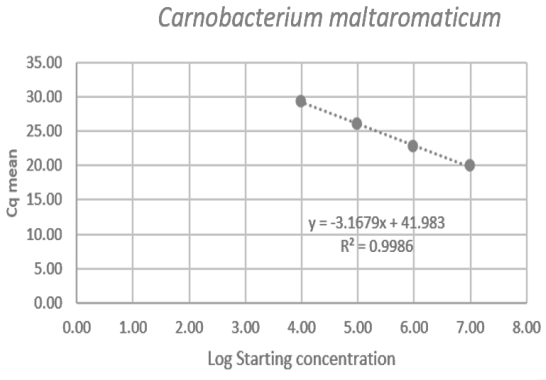
When comparing the Cq values of the standards from different runs, there was minimal difference observed compared to the previous run. However, when analyzing the samples from all runs, a larger difference was noticed. Even when running the samples in a technical replicate, variations were observed in their Cq values. These variations could potentially be attributed to differences between the samples themselves, the use of SYBR green, variations in the performance of the qPCR machine, or the presence of other fluorescent substances in the food samples. To avoid this variation, one possible solution is to replace SYBR green with a more sensitive fluorescent probe. Additionally, we can use two primers and probes enhancing the sensitivity of the reaction.

Table 5.3: Total CFU/g calculated from qPCR analysis

Sample	*Leuconostoc*	*Carnobacterium*	*Brochothrix*	*Lactococcus*	*Latilactobacillus*
Chicken	4.25 x 10^7	3.9 x 10^6	3.1 x 10^2	1.0 x 10^3	1.1 x 10^6
Chicken2	1.1 x 10^6	5.6 x 10^7	<1000	6.7 x 10^4	2.7 x 10^6
Chicken 3	-	6.3 x 10^6	-	3.1 x 10^5	5.8 x 10^5
Marinated Chicken Breast Fillet	8.0 x 10^5	3.3 x 10^4	-	6.3 x 10^2	2.1 x 10^3
Beef	3.09 x 10^8	3.6 x 10^6	4.3 x 10^5	1.0 x 10^5	3.9 x 10^4
Beef 2	3.5 x 10^6	-	-	-	4.8 x 10^4
Minced Beef	3.16 x 10^6	1.0 x 10^4	5.0 x 10^2	-	2.5 x 10^4
Pork	1.02 x 10^6	9.9 x 10^3	7.4 x 10^2	-	7.0 x 10^3
Marinated Rainbow Trout	1.1 x 10^3	1.0 x 10^4	1.0 x 10^3	-	-
Marinated Rainbow Trout 2	-	-	9.1 x 10^3	-	-
Unmarinated Rainbow Trout	8.6 x 10^3	9.5 x 10^4	5.7 x 10^3	-	5.5 x 10^2
Unmarinated Rainbow Trout Inoculated with *Carnobacterium*	-	3.1 x 10^6	-	-	-

5.3.1 Graphs of standard curves





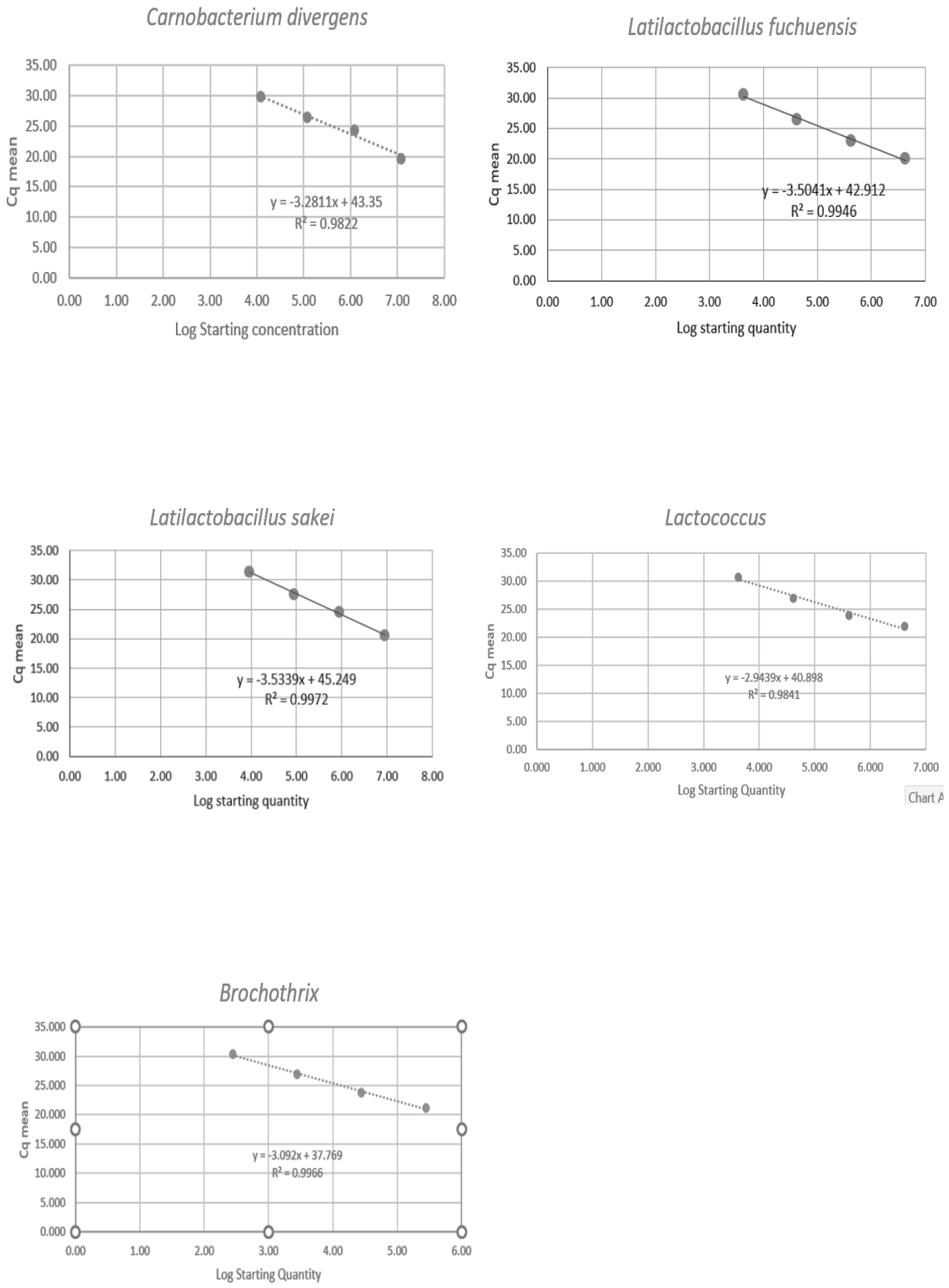


Figure 5.1: Standard curve of different LAB species

5.4 Comparison of plating and qPCR analysis

Table 5.4 shows comparison between the total bacterial count obtained using the plate count method and the qPCR analysis of the same sample.

Table 5.4: Comparison of total CFU/g from plate counting and qPCR analysis

Sample	*Leuconostoc*		*Carnobacterium*		*Brochothrix*		*Latilactobacillus*	
	Plate Count	qPCR Analysis	Plate Count	qPCR Analysis	Plate Count	qPCR Analysis	Plate Count	qPCR Analysis
Chicken	-	4.25×10^7	-	3.9×10^6	-	3.1×10^2	6.2×10^6	1.1×10^6
Chicken2	<1000	1.1×10^6	2.4×10^7	5.6×10^7	1.0×10^4	<1000	<1000	2.7×10^6
Chicken 3	-	-	4.4×10^6	6.3×10^6	-	-	3.1×10^6	5.8×10^5
Marinated Chicken Breast Fillet	2.1×10^4	8.0×10^5	<1000	3.3×10^4	-	-	5.0×10^4	2.1×10^3
Beef	8.9×10^6	3.09×10^8	-	3.6×10^6	-	4.3×10^5	<1000	3.9×10^4
Beef 2	8.9×10^4	3.5×10^6	<1000	-	-	-	4.0×10^5	4.8×10^4
Minced Beef	<1000	3.16×10^6	-	1.0×10^4	-	5.0×10^2	<1000	2.5×10^4
Pork	<1000	1.0×10^6	-	9.9×10^3	2.2×10^4	7.4×10^2	<1000	7.0×10^3
Marinated Rainbow Trout	<1000	1.1×10^3	3.0×10^4	1.0×10^4	-	1.0×10^3	0	-
Marinated Rainbow Trout 2	-	-	<1000	-	-	9.1×10^3	-	-
Unmarinated Rainbow Trout	<1000	8.6×10^3	3.4×10^5	9.5×10^4	3.8×10^5	5.7×10^3	0	5.5×10^2
Unmarinated Rainbow Trout Inoculated with *Carnobacterium*	-	-	1.9×10^7	3.1×10^6	-	-	-	-

The selection of plating and qPCR analysis for the samples was based on information obtained from relevant literature, which described the usual groups of bacteria found in similar samples.

When comparing the values from the plate count and qPCR analysis in *Leuconostoc*, the values were quite similar in Beef 1, unmarinated and marinated rainbow trout, marinated chicken breast fillet, and Beef 2. However, there were noticeable differences in minced beef meat, pork, and Chicken 2. On the other hand, all samples showed similar values for *Carnobacterium*, including unmarinated and marinated rainbow trout, unmarinated rainbow trout inoculated with *Carnobacterium*, marinated chicken breast fillet, Chicken 2, and Chicken 3. The plating data for *Brochothrix* were less consistent, with variations observed in unmarinated rainbow trout, pork, and Chicken 2. For *Latilactobacillus*, similar values were obtained for Chicken 1, pork, unmarinated and marinated rainbow trout, marinated chicken breast fillet, Beef 2, and Chicken 3, while differences were seen in beef, minced beef meat, and Chicken 2. *Lactococcus* was analyzed using qPCR as no selective media were available for plating.

The variation between the results obtained using the plating method and qPCR analysis can rise from many reasons. The recovery rate of the media used for the isolation is not 100%, and they are not completely selective, which means that other bacteria may grow alongside the targeted ones. Additionally, differences may arise

due to the complexity of the sample when isolating DNA. During qPCR analysis, DNA is obtained from both living and dead bacteria cells, so the resulting values may represent all cells present, not just the living ones.

While spoilage bacteria in this study are relatively viable during the analysis, the potential issue becomes more significant when applying this method to fermented foods, where the proportion of viable bacteria may be considerably higher. In these situations, one approach to mitigate interference from dead cells in the analysis is the utilization of specific chemicals that selectively exclude them. Alternatively, the analyses can be directed towards targeting RNA, thereby minimizing potential disruptions caused by the presence of dead cells (Kralik and Ricchi, 2017).

The main reason for using qPCR over other techniques is that the time required for the analysis of the sample is much shorter than in the case of the traditional plating techniques as well as 16S rRNA gene amplicon sequencing. The plating method typically requires more than 3 days to complete, while 16S rRNA gene amplicon sequencing involves a full day for DNA preparation and additional days for PCR and sequencing. In contrast, qPCR analysis can be completed within 5-6 hours (although it may take slightly longer if there are more samples). qPCR offers several advantages as it can be utilized at various levels including genus, species, and strain. Additionally, it can be applied at the group level, such as LAB, by using probes. At the strain level, qPCR methods can be developed by targeting specific genes unique to troublesome strains encountered in the food industry. This allows for the precise identification and monitoring of the strains causing problems.

When it comes to obtaining information at the species level, both 16S rRNA gene amplicon sequencing and plating methods have limitations. However, other alternative methods than qPCR exist, although they tend to be time-consuming and costly. Moreover, there are cases where selective media are not available like for *Lactococcus* as mentioned before. In such cases, qPCR serves as a viable alternative, offering a practical and efficient solution for species-level identification.

The traditional methods used for the detection of pathogenic bacteria take a longer time, qPCR is currently used to complement the traditional method for the detection of pathogens like *Salmonella*, and *Escherichia coli* to confirm the presence of the pathogen in the sample, so the detection and isolation method will be less work since only those samples detected positive in the qPCR are cultured (Derzelle et al., 2011; Kasturi and Drgon, 2017). A similar approach can be applied for the analysis of lactic acid bacteria (LAB), where qPCR can be utilized to determine the appropriate media for plating and the optimal dilution for LAB isolation. This

method can also be used in the food industry for a quick quality check of the products and ingredients.

However, there are certain limitations associated with qPCR. It is a targeted method, meaning it provides information specifically on the targets of interest, unlike 16S gene amplicon sequencing, which offers a broader overview of the entire sample composition. Thus, qPCR is not entirely comprehensive or independent. In cases where comprehensive information is required, sequencing methods, such as 16S rRNA gene amplicon sequencing, offer better insights, although limited to the genus level.

Other alternatives include using MALDI-TOF but for this, a large set of bacteria should be isolated and grown in the same media under the same conditions, which may not always be feasible due to varying growth requirements among different bacterial species. Similarly, ribotyping suffers from the same limitation as MALDI-TOF. While in the case of qPCR, the proportion of the targeted bacteria is obtained. (Soro-Yao et al., 2014). Another alternative is long-range sequencing, although it tends to be significantly more expensive compared to qPCR.

Overall, while qPCR offers advantages such as targeted detection and quantification, the choice of method will depend on the specific requirements, considering factors such as comprehensiveness, cost, and feasibility.

Chapter 6

Conclusion and Further Research Perspectives

The qPCR technique was a significantly faster approach compared to the plating method and the primer pairs designed for different species of Lactic Acid Bacteria yielded satisfactory results in the selected food samples.

In this study, the primer pairs were designed at the genus level for the detection of LAB in food samples. To obtain greater sensitivity and accuracy, incorporating probes along with primers can enhance the analysis. Since we designed the primers on unique genes, we had to introduce degenerate base pairs in them. To avoid this, we could design primers on less conserved housekeeping genes.

This study provides evidence that the qPCR method is a rapid and more reliable method for identifying LAB groups responsible for meat spoilage. Even though this technique was designed at the genus level, it can be adapted and altered to detect species, strains, or a higher taxonomic group like Lactic Acid Bacteria.

In conclusion, this research highlights the alternative approach to the traditional plating method for the detection of LAB causing meat spoilage. Other techniques like MALDI-TOF, ribotyping, ddPCR can be employed for rapid detection of pure colonies of meat spoilage LAB. However, they have their limitation if you want to avoid culturing.

In comparison to the traditional plating methods, qPCR is a promising alternative for the quantification of LAB responsible for food spoilage.

Appendix A

A.1 List of LAB used in this study

Species	***Strain***	***Accession***
Leuconostoc citreum	CBA3621	NZ_CP042410
Leuconostoc gasicomitatum	10.16.3	NZ_JAHBFK010000013
Leuconostoc gasicomitatum	6.2.3	NZ_JAHBFJ010000008
Leuconostoc gasicomitatum	A.21.4	NZ_JAHBFI010000009
Leuconostoc gasicomitatum	A.5.3	NZ_JAHBFH010000017
Leuconostoc gasicomitatum	A.8.4	NZ_JAHBFG010000012
Leuconostoc gasicomitatum	ab.2	NZ_JAHBFF010000010
Leuconostoc gasicomitatum	C120c	NZ_FBTC01000030
Leuconostoc gasicomitatum	C122c	NZ_FBSY01000020
Leuconostoc gasicomitatum	CBA3613	NZ_CP058617
Leuconostoc gasicomitatum	EPV3	NZ_JAHBEY010000003
Leuconostoc gasicomitatum	HS10	NZ_JAHBFD010000018
Leuconostoc gasicomitatum	HS1	NZ_JAHBFE010000031
Leuconostoc gasicomitatum	JL3-1	NZ_JAHBFN010000033
Leuconostoc gasicomitatum	Jla4-8	NZ_JAHBEX010000016
Leuconostoc gasicomitatum	JP13-3	NZ_JAHBEW010000012
Leuconostoc gasicomitatum	KG16-1	NZ_LN890331
Leuconostoc gasicomitatum	KSL4-2	NZ_FBTB01000023
Leuconostoc gasicomitatum	LMG 18811	NC_014319
Leuconostoc gasicomitatum	MFPA44A1401	NZ_OBMW01000048
Leuconostoc gasicomitatum	Mk11-2	NZ_JAHBEV010000002
Leuconostoc gasicomitatum	Mk12-18	NZ_JAHBEU010000018
Leuconostoc gasicomitatum	Ms25-3	NZ_JAHBET010000020

Species	***Strain***	***Accession***
Leuconostoc gasicomitatum	NAFIM5a-6	NZ_JAHBES010000005
Leuconostoc gasicomitatum	NBRC 113245	NZ_BPKT01000001
Leuconostoc gasicomitatum	PB1a	NZ_FBSX01000029
Leuconostoc gasicomitatum	PB1e	NZ_FBTD01000018
Leuconostoc gasicomitatum	PL111	NZ_FBTU01000029
Leuconostoc gasicomitatum	POHU19	NZ_JAHBFM010000012
Leuconostoc gasicomitatum	POULM2-8	NZ_JAHBER010000033
Leuconostoc gasicomitatum	R-46608	NZ_JAHBFC010000029
Leuconostoc gasicomitatum	R-46710	NZ_JAHBFB010000023
Leuconostoc gasicomitatum	R-46850	NZ_JAHBFA010000003
Leuconostoc gasicomitatum	R-46920	NZ_JAHBEZ010000018
Leuconostoc gasicomitatum	RSNU1f	NZ_JAHBFL010000016
Leuconostoc gasicomitatum	TMV 2.1619	NZ_CP017197
Leuconostoc gasicomitatum	Vvan8	NZ_JAHBEQ010000013
Leuconostoc gelidum	AMKR21	NZ_JAHBFX010000007
Leuconostoc gelidum	C220d	NZ_JAHBFV010000023
Leuconostoc gelidum	DSM 19374	NZ_JAHBFZ010000003
Leuconostoc gelidum	DSM 19375	NZ_JAHBGA010000003
Leuconostoc gelidum	Ebr1-8	NZ_JAHBFU010000005
Leuconostoc gelidum	HS9	NZ_JAHBFT010000003
Leuconostoc gelidum	JPBL22	NZ_JAHBFS010000010
Leuconostoc gelidum	KAPA3-9	NZ_JAHBFR010000004
Leuconostoc gelidum	KCTC 3527	NZ_AEMI01000043
Leuconostoc gelidum	Kg1-2	NZ_JAHBFQ010000004
Leuconostoc gelidum	NBRC 113246	NZ_BPKU01000001
Leuconostoc gelidum	Vvan9	NZ_JAHBFO010000029
Leuconostoc gelidum	PB4d	NZ_JAHBFP010000011
Leuconostoc gelidum	PLK1c	NZ_JAHBFW010000007
Leuconostoc gelidum	POKY4-4	NZ_JAHBFY010000007
Leuconostoc gelidum	TMW 2.1618	NZ_CP017196
Leuconostoc inhae	DSM 15101	NZ_BPKW01000001

Negative control for Leuconostoc	***Strain***	***Accession***
Fructobacillus durionis	DSM 19113	NZ_FOLI01000017
Fructobacillus fructosus	NRIC 1058	NZ_DF968006
Oenococcus oeni	AWRIB429	NZ_CP084701
Weissella cibaria	CMS3	NZ_CP013934
Weissella confusa	LM1	NZ_CP080582
Weissella viridescens	NJ100	NZ_CP061835

Species	***Strain***	***Accession***
Carnobacterium divergens	A10	NZ_NRPZ01000001
Carnobacterium divergens	A11	NZ_NRPY01000008
Carnobacterium divergens	A12	NZ_NRPX01000002
Carnobacterium divergens	A13	NZ_NRPW01000016
Carnobacterium divergens	A2	NZ_NRQH01000002
Carnobacterium divergens	A4	NZ_NRQF01000005
Carnobacterium divergens	A8	NZ_NRQB01000017
Carnobacterium divergens	A9	NZ_NRQA01000015
Carnobacterium divergens	B1	NZ_NRPQ01000003
Carnobacterium divergens	B2	NZ_NRPP01000003
Carnobacterium divergens	B3	NZ_NRPO01000041
Carnobacterium divergens	B4	NZ_NRPN01000003
Carnobacterium divergens	B5	NZ_NRPM01000034
Carnobacterium divergens	B6	NZ_NRPL01000002
Carnobacterium divergens	B7	NZ_NRPK01000013
Carnobacterium divergens	B8	NZ_NRPJ01000012
Carnobacterium divergens	C10	NZ_NROZ01000003
Carnobacterium divergens	C11	NZ_NROY01000033
Carnobacterium divergens	C12	NZ_NROX01000041
Carnobacterium divergens	C13	NZ_NROW01000004
Carnobacterium divergens	C14	NZ_NROV01000003
Carnobacterium divergens	C15	NZ_NROU01000034
Carnobacterium divergens	C16	NZ_NROT01000013
Carnobacterium divergens	C17	NZ_NROS01000043

Species	***Strain***	***Accession***
Carnobacterium divergens	C18	NZ_NROR01000041
Carnobacterium divergens	C1	NZ_NRPI01000040
Carnobacterium divergens	C2	NZ_NRPH01000041
Carnobacterium divergens	C3	NZ_NRPG01000033
Carnobacterium divergens	C4	NZ_NRPF01000033
Carnobacterium divergens	C5	NZ_NRPE01000003
Carnobacterium divergens	C6	NZ_NRPD01000012
Carnobacterium divergens	C7	NZ_NRPC01000003
Carnobacterium divergens	C8	NZ_NRPB01000002
Carnobacterium divergens	C9	NZ_NRPA01000035
Carnobacterium divergens	CDIV41	NZ_FLLU01000001
Carnobacterium divergens	DSM 20623	NZ_JQBS01000005
Carnobacterium divergens	MFPA43A1505	NZ_LT992558
Carnobacterium divergens	NCTC13772	NZ_UFVP01000002
Carnobacterium divergens	TMW 2.1577	NZ_RSDV01000001
Carnobacterium divergens	TMW 2.1579	NZ_CP016843
Carnobacterium maltaromaticum	10040100629	NZ_CAJGUR010000001
Carnobacterium maltaromaticum	18ISCm	NZ_CP045040
Carnobacterium maltaromaticum	3-18	NZ_CVMZ01000159
Carnobacterium maltaromaticum	A14	NZ_NRPV01000007
Carnobacterium maltaromaticum	A15	NZ_NRPU01000003
Carnobacterium maltaromaticum	A16	NZ_NRPT01000003
Carnobacterium maltaromaticum	A17	NZ_NRPS01000003
Carnobacterium maltaromaticum	A18	NZ_NRPR01000003
Carnobacterium maltaromaticum	A1	NZ_NRQI01000013
Carnobacterium maltaromaticum	A3	NZ_NRQG01000014
Carnobacterium maltaromaticum	A5	NZ_NRQE01000013
Carnobacterium maltaromaticum	A6	NZ_NRQD01000014
Carnobacterium maltaromaticum	A7	NZ_NRQC01000012
Carnobacterium maltaromaticum	ATCC 35586	NZ_AGNS01000074
Carnobacterium maltaromaticum	BF1	NZ_JAGYWR010000010
Carnobacterium maltaromaticum	DSM 20342	NZ_JQBG01000005
Carnobacterium maltaromaticum	DSM 20342	NZ_JQMX01000001
Carnobacterium maltaromaticum	DSM 20722	NZ_JQBU01000004

Species	***Strain***	***Accession***
Carnobacterium maltaromaticum	DSM 20730	NZ_JQBV01000001
Carnobacterium maltaromaticum	EBP3019	NZ_WNJS01000121
Carnobacterium maltaromaticum	JIP 2891	NZ_CAJGUS010000001
Carnobacterium maltaromaticum	LMA28	NC_019425
Carnobacterium maltaromaticum	ML_1_97	NZ_CVNA01000228
Carnobacterium maltaromaticum	NBRC 15685	NZ_BJOJ01000001
Carnobacterium maltaromaticum	SF668	NZ_WNJR01000011
Carnobacterium maltaromaticum	SK_AV1	NZ_PKFM01000001
Carnobacterium maltaromaticum	SK_AV2	NZ_PKFL01000001
Carnobacterium maltaromaticum	SK_AV3	NZ_PKFK01000001
Carnobacterium maltaromaticum	SK_AV4	NZ_PKFJ01000001
Carnobacterium maltaromaticum	SK_AV5	NZ_PKFI01000001
Carnobacterium maltaromaticum	SK_AV6	NZ_PKFH01000001
Carnobacterium maltaromaticum	SK_LD1	NZ_PKFG01000001
Carnobacterium maltaromaticum	SK_LD2	NZ_PKFF01000001
Carnobacterium maltaromaticum	SK_LD3	NZ_PKFE01000001
Carnobacterium maltaromaticum	TMW 2.1581	NZ_CP016844
Carnobacterium maltaromaticum	UAL307	NZ_LHUF01000001

Negative control for Carnobacterium	***Strain***	***Accession***
Enterococcus avium	352	NZ_CP034169
Enterococcus gallinarum	EGM181	NZ_CP050485
Vagococcus coleopterorum	HDW17A	NZ_CP049886
Vagococcus fluvialis	35B2	NZ_CP081466
Enterococcus avium	352	NZ_CP034169
Enterococcus gallinarum	EGM181	NZ_CP050485
Vagococcus coleopterorum	HDW17A	NZ_CP049886
Vagococcus fluvialis	35B2	NZ_CP081466

Species	***Strain***	***Accession***
Brochothrix thermosphacta	BF1	NZ_JAGYWQ010000010
Brochothrix thermosphacta	BI	NZ_CP023483
Brochothrix thermosphacta	BII	NZ_CP023643
Brochothrix thermosphacta	BSAS1 3	NZ_OUNC01000083
Brochothrix thermosphacta	Bth-7803	NZ_MDLL01000001
Brochothrix thermosphacta	Bth-7804	NZ_MDLU01000001
Brochothrix thermosphacta	Bth-7806	NZ_MDLM01000001
Brochothrix thermosphacta	Bth-7807	NZ_MDLN01000001
Brochothrix thermosphacta	Bth-7808	NZ_MDLO01000001
Brochothrix thermosphacta	Bth-7809	NZ_MDLV01000001
Brochothrix thermosphacta	Bth-7810	NZ_MDLP01000001
Brochothrix thermosphacta	Bth-7811	NZ_MDLT01000001
Brochothrix thermosphacta	Bth-7813	NZ_MDLQ01000001
Brochothrix thermosphacta	Bth-7816	NZ_MDLR01000001
Brochothrix thermosphacta	Bth-7818	NZ_MDLS01000001
Brochothrix thermosphacta	CD 337	NZ_LT993737
Brochothrix thermosphacta	DSM 20171	NZ_MDLK01000001
Brochothrix thermosphacta	EBP 3070	NZ_OOIK01000071
Brochothrix thermosphacta	HO01	NZ_OBMV01000032
Brochothrix thermosphacta	TAP 175	NZ_OUNB01000056
Brochothrix thermosphacta	TMW 2.1564	NZ_CP016839
Brochothrix thermosphacta	TMW 2.1572	NZ_CP016841
Brochothrix thermosphacta	TMW 2.2101	NZ_RSDU01000001

Negative control for Brochothrix	***Strain***	***Accession***
Listeria monocytogenes	AT3E	NZ_CP023752
Listeria monocytogenes	C7	NZ_CP075872
Listeria monocytogenes	HPB5622	NZ_CP019167
Listeria monocytogenes	NCTC10357	NZ_LT906436

Species	***Strain***	***Accession***
Lactococcus carnosus	TMW 2.1612	NZ_CP017194
Lactococcus carnosus	TMW 2 1613	SRR17843212
Lactococcus carnosus	TMW 2.1894	SRR17843211
Lactococcus carnosus	TMW 2.1895	SRR17843202
Lactococcus carnosus	TMW 2.1896	SRR17843201
Lactococcus carnosus	TMW 2.1902	SRR17843197
Lactococcus carnosus	TMW 2.1903	SRR17843196
Lactococcus carnosus	BF1	NZ_JAGYWS010000010
Lactococcus carnosus	CMTALT17	NZ_OBKP01000068
Lactococcus carnosus	CNCM I-4031	NZ_LT603685
Lactococcus carnosus	MKFS47	NZ_LN774769
Lactococcus paracarnosus	TMW 2.1893	SRR17843205
Lactococcus paracarnosus	TMW 2.1897	SRR17843204
Lactococcus paracarnosus	CMTALT02	NZ_LT992557
Lactococcus piscium	DSM 6634	NZ_JXJW01000001

Negative control for Lactococcu	***Strain***	***Accession***
Lactococcus garvieae	IBB3403	NZ_CP028386
Lactococcus lactis	LAC460	NZ_CP059048
Lactococcus plantarum	NBRC 100936	NZ_BCVM01000001
Lactococcus raffinolactis	WiKim0068	NZ_CP023392

Species	***Strain***	***Accession***
Latilactobacillus curvatus	CBA3617	NZ_CP042389
Latilactobacillus curvatus	DSM 20019	NZ_CP026116
Latilactobacillus curvatus	FBA2	NZ_CP016028
Latilactobacillus curvatus	FLEC03	NZ_LT841333
Latilactobacillus curvatus	IRG2	NZ_CP025476
Latilactobacillus curvatus	KG6	NZ_CP022475
Latilactobacillus curvatus	MRS6	NZ_CP022474
Latilactobacillus curvatus	NBRC 15884	NZ_BJOQ01000001
Latilactobacillus curvatus	RI-124	NZ_MKDR01000001
Latilactobacillus curvatus	RI-193	NZ_MKGD01000001
Latilactobacillus curvatus	RI-198	NZ_MKGC01000001
Latilactobacillus curvatus	RI-406	NZ_MKDG01000001
Latilactobacillus curvatus	S46	NZ_SUMW01000010
Latilactobacillus curvatus	SRCM103465	NZ_CP035110
Latilactobacillus curvatus	TMW 1.1928	NZ_CP031003
Latilactobacillus curvatus	VRA_2sq_n	NZ_WKKT01000028
Latilactobacillus curvatus	WDN19	NZ_AP024685
Latilactobacillus curvatus	WiKim38	NZ_CP017124
Latilactobacillus curvatus	WiKim52	NZ_CP016602
Latilactobacillus curvatus	ZJUNIT8	NZ_CP029966
Latilactobacillus fuchuensis	DSM 14340	NZ_BAMJ01000071
Latilactobacillus fuchuensis	MFPC41A2801	NZ_LT984417
Latilactobacillus fuchuensis	MKJ35	Proprietary
Latilactobacillus fuchuensis	MKL13	Proprietary
Latilactobacillus fuchuensis	MKL73	Proprietary
Latilactobacillus sakei	23K	NC_007576
Latilactobacillus sakei	CBA3614	NZ_CP046037
Latilactobacillus sakei	CBA3635	NZ_CP059697
Latilactobacillus sakei	DS4	NZ_CP025839
Latilactobacillus sakei	FAM18311	NZ_CP020459
Latilactobacillus sakei	J160x1	NZ_LT907931
Latilactobacillus sakei	J18	NZ_LT907930
Latilactobacillus sakei	J54	NZ_LT960790
Latilactobacillus sakei	J64	NZ_LT960781

Species	***Strain***	***Accession***
Latilactobacillus sakei	DSM 20017	NZ_AP017929
Latilactobacillus sakei	LZ217	NZ_CP032652
Latilactobacillus sakei	MBEL1397	NZ_CP048116
Latilactobacillus sakei	MFPB16A1401	NZ_LT960788
Latilactobacillus sakei	ob4.1	NZ_CP075489
Latilactobacillus sakei	Probio65	NZ_CP020806
Latilactobacillus sakei	WiKim0063	NZ_CP022709
Latilactobacillus sakei	WiKim0072	NZ_CP025136
Latilactobacillus sakei	ZFM220	NZ_CP032633
Latilactobacillus sakei	ZFM225	NZ_CP032635
Latilactobacillus sakei	ZFM229	NZ_CP032640

Negative control for Latilactobacillus	***Strain***	***Accession***
Dellaglioa algida	CMTALT10	NZ_OBKY01000024
Dellaglioa algida	DSM 15638	NZ_AZDI01000001
Dellaglioa algida	LTS37-1	NZ_SRRQ01000001
Dellaglioa algida	NAGRM3a-7	NZ_SRSA01000001
Lactobacillus oligofermentans	DSM 15707	NZ_LN898144

Species	***Strain***	***Accession***
Vagococcus coleopterorum	HDW17A	NZ_CP049886
Vagococcus fessus	CCUG 41755	NZ_NGJY01000001
Vagococcus fluvialis	110B2	NZ_CP081459
Vagococcus fluvialis	12B2	NZ_CP081472
Vagococcus fluvialis	25B2	NZ_CP081470
Vagococcus fluvialis	35B2	NZ_CP081466
Vagococcus fluvialis	36B2	NZ_CP081461
Vagococcus fluvialis	bH819	NZ_FWFD01000021
Vagococcus fluvialis	DIV0015	NZ_JAFLWJ010000001
Vagococcus fluvialis	DIV0038b	NZ_JAFLWK010000001
Vagococcus fluvialis	DIV0068	NZ_JAFLWL010000001
Vagococcus fluvialis	DIV0098	NZ_JAFLWM010000001
Vagococcus fluvialis	DIV0648b	NZ_JAFLWN010000001
Vagococcus fluvialis	DIV0657d	NZ_JAFLWO010000001
Vagococcus fluvialis	DSM 5731	NZ_QPJV01000001
Vagococcus fluvialis	MSG3302	NZ_JAFLWP010000001
Vagococcus fluvialis	NCDO 2497	NZ_NGJX01000001
Vagococcus fluvialis	UFMG-H6	NZ_JAAVMC010000010
Vagococcus fluvialis	UFMG-H7	NZ_JAAVMB010000010

Negative control for Vagococcus	***Strain***	***Accession***
Enterococcus avium	352	NZ_CP034169
Enterococcus gallinarum	EGM181	NZ_CP050485

Appendix B

B.1 List of Primers designed in this study

Bacterial species	*Primer name*	*Primer*
Leuconostoc	Leuconostoc_01_F	TGCACCHGGTATTCCACAAA
	Leuconostoc_01_R	AACTGGTCGCTTTGTTGTTT
	Leuconostoc_02_F	TTTGCACCHGGTATTCCACA
	Leuconostoc_02_R	TCAAACAAGTTTGAAACAAC
Brochothrix	Brochothrix_01_F	AGATGATCGAACTGCTGGGA
	Brochothrix_01_R	AGTCCCCAATCATTGACAGGA
	Brochothrix_02_F	TGGGGACTTGATAACGTTGAT
	Brochothrix_02_R	AGCCTACTTCTTGCCCTTGT
Carnobacterium	Carnobacterium_01_F	TGGGTAGATCGYTTCGGTAC
	Carnobacterium_01_R	TTAGCKGTTGTWCCTGGYAC
	Carnobacterium_02_F	ACTTGGGTAGATCGYTTCGG
	Carnobacterium_02_R	TGCATCTTTAGCKGTTGTWCCT
Lactococcus	Lactococcus_01_F	AGGCTATACTGGTGGGCATG
	Lactococcus_01_R	TCAGCCAAAATARATCGAGCA
	Lactococcus_02_F	GCCTTACGVCCATTAGCAC
	Lactococcus_02_R	CCACCAGCAAAKAYAGCACG
Latilactobacillus	Latilactobacillus_01_F	CAAGGKGTTTTYGATGCYTA
	Latilactobacillus_01_R	GCAATCCGWGGTAARTCAGG
	Latilactobacillus_02_F	GTGTGGGATTGGBATGGATGG
	Latilactobacillus_02_R	CCKTCTGGTAAGCGATCTGAC
Vagococcus	Vagococcus_01_F	CCWGACCGTGCBATYCAATT
	Vagococcus_01_R	GGWGGGTAAGCAACTTCWGC
	Vagococcus_02_F	GTCCWGACCGTGCBATYCA
	Vagococcus_02_R	TCATTTCMATTGGWGGGTAAGCA

Appendix C

The concentration of the stock solution	**Volume** μl
50 mg/ml Mutanolysin (Mu)	136.4 μl
1000 IU/ml Lysozyme (Lz)	272.72 μl
10:1 TE	136.4
10mg/ml Rnase	54.5

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