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Selection of reference genes for gene expression studies in Lactic acid bacteria

Selection of reference genes for gene expression studies in Lactic acid bacteria

J. Huyghe

Lactic acid bacteria (LAB) are used to preserve meat, dairy products and fermented or raw vegetables against food-borne human pathogens. Besides, they have potential as biological control agents of plant pathogenic bacteria and fungi. The success of LAB in preventing the growth and activity of undesirable microorganisms is due to different mechanisms of action including the production of bacteriocins. Bacteriocins of LAB are antimicrobial peptides that have attracted much interest in recent years because of their suitability as food preservatives or plant bio pesticides. One of the prospects on the use of bacteriocins is the possibility of increasing its activity. The synthesis of bacteriocins is controlled by complex regulatory systems, therefore it is of great interest to study the factors affecting the expression of bacteriocin biosynthetic genes.

The aim of this study was to set up a technique select the reference genes to evaluate the expression levels of some selected LAB bacteriocin genes using a biomolecular approach based on reverse transcription quantitative PCR (RT-qPCR). Specifically, the bacteriocins selected for the quantification were nisin synthesized by *Lactococcus lactis* and mesentericin by *Leuconostoc mesenteroides*. RT-qPCR technique allows quantifying the transcript levels of target genes in order to study the influence of environmental conditions in the synthesis of genes. Hence, to set up the technique a reference gene (internal control gene) for the normalization of gene expression values should be selected.

Keywords: RT-qPCR, nisin, mesentericin, bacteriocin, gene expression

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Thank you all!

Jennifer

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
bp	base pairs
cDNA	circular Deoxyribonucleic Acid
cfu	colony forming units
Dha	Dehydroalanine
Dhb	Dehydrobutyrine
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Agency
FDA	Food and Drug Administration
g	gravitational force
GMO	Genetically modified organism
GRAS	Generally Recognized As Safe
HKFs	House Keeping genes
HPK	Histidine Protein Kinase
kDa	kilo Dalton
LAB	Lactic Acid Bacteria
Lan	Lanthionine
LB	Lysogeny Broth
MeLan	Methyllanthionine
mg	milligram
ml	milliliter
mM	millimolar
mRNA	messenger Ribonucleic Acid
NCBI	National Center for Biotechnology Information
ng	nanogram
N-terminal	Nitrogen terminal
OD	Optical Density
ORFs	Open reading frames
PCR	Polymerase Chain Reaction
PVP	Polyvinyl Pyrrolidone
qPCR	quantitative PCR = real-time PCR
QPS	Qualified Presumption as Safe
QS system	Quorum Sensing system
RNA	Ribonucleic Acid
rpm	revolutions per minute
RR	Response Regulator
RT-qPCR	Reverse Transcription quantitative Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
SYBR green	$C_{32}H_{37}N_4S^+$
Taq polymerase	Thermus aquaticus polymerase
V	Volt
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Fermentation of various food stuffs by lactic acid bacteria (LAB) is one of the oldest forms of bio-preservation practiced by mankind. Bacterial antagonism has been recognized for over a century but in recent years this phenomenon has received more scientific attention, particularly in the use of various strains of lactic acid bacteria. One important attribute of many LAB is their ability to produce antimicrobial compounds called bacteriocins. In recent years, interest in these compounds has grown substantially due to their potential usefulness as natural substitute for chemical food preservatives in the production of foods with enhanced shelf life and/or safety. Traditional probiotic dairy strains of LAB have a long history of safe use and most strains are considered commensal microorganisms with no pathogenic potential (Soomro et al., 2002).

The use of LAB and of their metabolic products is generally considered as safe (GRAS) by the Food and Drug Administration (FDA) and is considered with the qualified presumption as safe (QPS) status by the European Food Safety Agency (EFSA). The application of the produced antimicrobial compounds as a natural barrier against pathogens and food spoilage caused by bacterial agents has been proven to be efficient. Nisin is the only bacteriocin that has been officially employed in the food industry and its use has been approved worldwide. Nowadays LAB are mostly applied to dairy products, meat, fermented vegetables and fruit juices (Holzapfel et al., 2001) (Gomez et al.,2002).

One of the prospects on the use of bacteriocins is the possibility of increasing its activity. The synthesis of bacteriocins is controlled by complex regulatory systems, therefore it is of great interest to study the factors affecting the expression of bacteriocin biosynthetic genes.

In this project a method to quantify the expression of bacteriocin genes produced by lactic acid bacteria was set up. Specifically, a biomolecular approach based on reverse transcription quantitative PCR will be used to quantify the expression level of the bacteriocins nisin and mesentericin. The selection of adequate reference genes is essential for qPCR gene expression analysis.

As a starting point, some background information about LAB is given that is necessary to understand this study. This involves general knowledge about the taxonomy and physiology of LAB, their antimicrobial activity, the classification and working mechanism of bacteriocins and some information about the operon regulation in the biosynthesis of both bacteriocins.

In the second chapter the technique of quantitative polymerase chain reaction is explained thoroughly. In the third section of this study, the aim of this project is described more specific. The different goals are summarized before the material and methodology is discussed. In chapter four the different steps of the methodology are summed up and explained thoroughly. The methodology has one main goal: the optimization of the technique by validating reference genes for the normalization of gene expression values. In this way, standard curves can be made of each gene and finally the expression level of bacteriocin genes can be evaluated.

Chapter five contains a discussion of the results and in the last section a conclusion is made that presents the evaluation of the reference gene expression for *L. mesenteroides* CM160.

1 LACTIC ACID BACTERIA (LAB)

1.1 TAXONOMY AND PHYSIOLOGY

Lactic acid bacteria (LAB), also known as *Lactobacillales* are gram-positive, nonsporing, catalase-negative organisms which are not in possession of cytochromes and nonaerobic habit. Still, they are aero tolerant, fastidious, acid-tolerant and strictly fermentative. However, exceptions from this general description do occur because some species can form catalase or cytochromes on media containing hematin or related compounds (Holzapfel et al., 2001). LAB are known to be mesophilic bacteria, but they also have the ability to multiply at temperatures going from 5°C up to 45°C.

The genera that comprise the LAB are at its core *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus* and *Streptococcus*. The two species that are described in this project are *Lactococcus lactis* and *Leuconostoc mesenteroides*. Both these species are considered as probiotics.

The main feature of LAB is their ability to ferment sugar. This fermentation can follow two different pathways (Figure 1). When during the fermentation the glycolysis is applied resulting in lactic acid as the only product, the fermentation is called homo-fermentative. If the fermentation results in many different products, such as ethanol and acetate, besides lactic acid, the fermentation is called hetero-fermentative. *Lactococcus* strains are known to follow the homo-fermentative pathway and *Leuconostoc* strains the hetero-fermentative path (Axelsson et al.,2004).

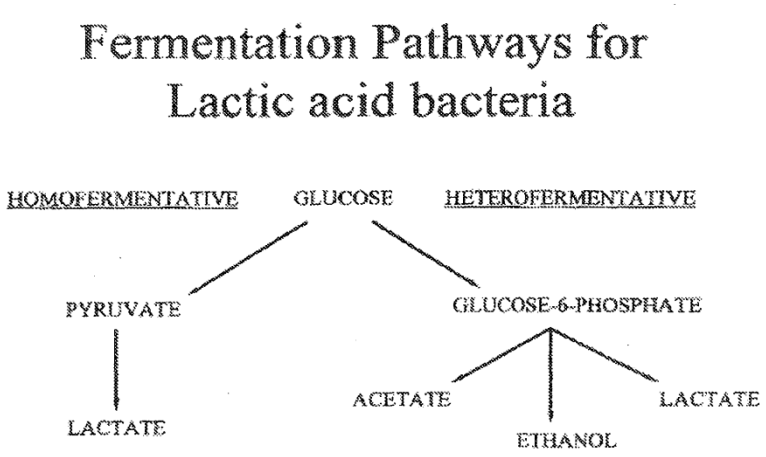


Figure 1 Fermentation of sugar

1.2 ANTIMICROBIAL ACTIVITY: BACTERIOCINS

LAB strains produce numerous kinds of bacteriocins (Reis et al.,2012). Bacteriocins are peptides with antimicrobial activity that generally restrict their activity to strains of species related to the producing species but in some cases they are active against strains across genera including Gram negative bacteria and fungi (Omar et al., 2008) (Diep et al., 2009) (Perez et al., 2014). In addition, bacteriocins are synthesized ribosomally and produced during the primary phase of growth and have low molecular weight. They can be easily degraded by proteolytic enzymes especially by the proteases of the mammalian gastrointestinal tract, which makes them safe for human consumption and lessen the chances of target strains developing any mechanism of resistance. Bacteriocins are in general cationic, amphipathic molecules as they contain an excess of lysyl and arginyl residues. They are usually unstructured when they are incorporated in aqueous solutions but when exposed to structure promoting solvents such as triofluorethanol or mixed with anionic phospholipid membranes they form a helical structure (Zacharof et al., 2012).

1.2.1 CLASSIFICATION OF LAB BACTERIOCINS

Bacteriocins can be divided into four major classes (Table 1). The LAB bacteriocins have greater antibacterial activity at lower pH values (below 5) meaning that their adsorption to the cell surface of Gram positive bacteria including the producing cells is pH dependent. Within any class of bacteriocins there may be amino acid sequence homologies not only within the mature peptide, but also in the N-terminal leader region and the associated proteins in bacteriocin secretion and processing.

a. Class I: Lantibiotics

Lantibiotics are characterized by the presence of the polycyclic thioehter amino acids lanthionine or methyllanthionine. Also, the occurrence of the unsaturated amino acids dehydroalanine and 2-aminoisobutyric acid are demonstrated. They are divided into two types based on structural similarities. Type A embraces relatively elongated, screw shaped, positively charged, amphiphilic, flexible molecules. Their molecular mass alternates between 2 and 4 kDa and they generally act through pore formation or through membrane depolarization. Nisin and lacticin 3147 are the major representatives of this group. Type B lantibiotics, are globular in structure and interfere with cellular enzymatic reactions. Their molecular mass alternates between 2 and 3 kDa. Their net charge is either negative or absent. Class I LAB bacteriocins are small (<5 kDa) heat stable peptides, which undergo major changes after translation resulting in the formation of characteristic thioehter amino acids lanthionine (Lan) and β-methyllanthionine (MeLan). The most famous member of the Lantibiotics group is nisin A. Nisin itself has variants indicated as nisin types A, Z, F, and Q derived from *Lactococci lactis* while types U and U2 are obtained from *Streptococci species* (Potty, 2010).

b. Class II: Non-Lantibiotics

Bacteriocins from class II are a little bit bigger (<10 kDa) and relatively heat stable. They are considered as non-lanthionine containing membrane active peptides. They are divided into two subclasses. Mesentericin belongs to the class IIa bacteriocins which are characterized by the occurrence of a highly conserved hydrophilic and charged N-terminal region that has a disulphide bond linkage. In some bacteriocins, an additional disulphide bond is present. They show their strong inhibitory effect on Listeria sp. as well as other food spoilage and pathogenic bacteria (Yanhua et al., 2012). Subclass IIb refers to two-component (two separate peptides) bacteriocins that require two peptides to work synergistically in order to have an antimicrobial activity. Lactacin F and lactococcin G are members of this group.

c. Class III: Bacteriolysins

This group consists of heat labile proteins which are in general of large molecular weight (>30 kDa). Bacteriocins representing this group are helveticin I by *Lactobacillus helveticus* and Enterolysin produced by *enterococcus faecium.*

d. Class IV: Complex protein mixture

Class IV bacteriocins are defined as complex bacteriocins containing lipid or carbohydrate moieties. It is known that these complex proteins are heat stable.

Table 1 Classification of bacteriocins produced by LAB

Class	Characteristics	Subclass	Bacteriocins
I Lantibiotics	small (<5 kDa), heat stable, contain lanthionine and β-methyllanthionine (post-translational modification)	**Ia:** elongated, positively charged, amphiphilic, flexible molecules	nisin
		Ib: globular molecules	mersacidin
II Non-Lantibiotics	small (<10 kDa), relatively heat stable, do not contain lanthionine	**IIa:** pediocin-like/listeria active molecules	mesentericin, pediocin
		IIb: two-component molecules: require two peptides to work synergistically in order to have antimicrobial activity	lactacin F, lactococcin G
III Bacteriolysins	large (>30 kDa), heat labile		helveticin, enterolysin
IV	complex protein mixture, heat stable		leuconocin S

1.2.2 WORKING MECHANISMS OF BACTERIOCINS

The killing mechanism of bacteriocins is specific to each group of bacteriocins. Moreover, the general cationic nature of bacteriocins plays a very important role in their initial interaction with the cell membrane of their target strains. The negative charge of bacterial cell membranes and the positive charge of bacteriocin molecules create an electrostatic interaction between them, thereby facilitating the approach of the molecules to the membranes. However, this interaction is not responsible for the killing of the target bacterial cells. This interaction is also responsible for the inactivity of most bacteriocins toward Gram-negative bacterial strains. The composition of Gram-negative bacterial membrane differs from that of Gram-positive bacterial membrane in that way that the former contains a lipopolysaccharide outer membrane. Bacteriocins from LAB only become active against Gram-negative bacteria when combined with other agents that compromise the integrity of the outer membrane such as surfactants (Stevens et al., 1991) (Zhang et al., 1999). To apply their killing mechanism, bacteriocins require a receptor molecule or a “docking molecule”

found in their target bacterial cell membrane, which differs among different classes and subclasses. This work will focus on the mode of action of Lantibiotics and class IIa LAB bacteriocins only since this project concerns nisin and mesentericin.

a. Pore-forming Lantibiotics

Lantibiotics, such as nisin, have two known killing mechanisms, although both systems share a common denominator (Breukink et al., 1999) (Wiedemann et al., 2001) (Hsu et al., 2004). It has long been shown that the lantibiotic Nisin disrupts the integrity of the bacterial cell membrane by forming pores (b) that would lead to the waste of the membrane potential and the efflux of small metabolites such as ions, amino acids, nucleotides and other cytoplasmic solutes, resulting in the termination of all biosynthetic processes, leading to cell death (Ruhr et al., 1985) (Sahl et al., 1987). At lower concentrations, nisin has been shown to kill target bacteria through inhibition of the cell wall synthesis (a). The nisin molecule has been shown to bind to lipid II, which is the main transporter of peptidoglycan subunits from the cytoplasm to the cell wall (Figure 2).

Peptidoglycan is the main component of the bacterial cell wall. The binding of nisin to lipid II results in the prevention of proper cell wall synthesis, thereby causing cell death. Furthermore, at higher concentrations, the nisin-lipid II molecule complex initiates membrane insertion that creates pores in the bacterial cell membrane. Thus, the binding of nisin to lipid II facilitates its dual mode of preventive action involving cell wall synthesis and membrane pore formation (Breukink et al., 1999) (Wiedemann et al., 2001) (Perez et al., 2015).

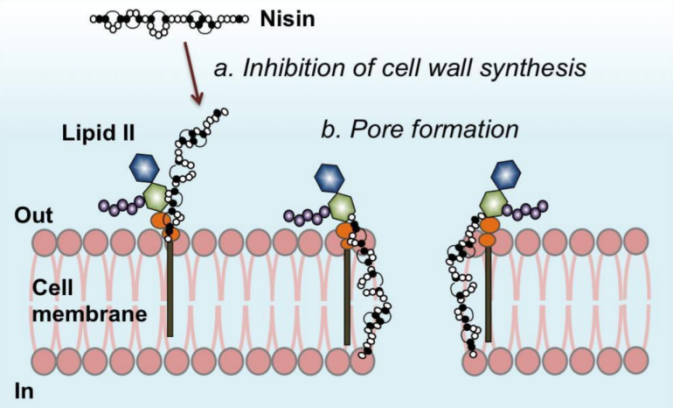


Figure 2 Killing mechanism of class I bacteriocins: (a) inhibition of cell wall synthesis and (b) pore formation (Perez et al., 2015).

b. Membrane permeabilization by Class IIa bacteriocins

Non-lantibiotics are the most commonly occurring bacteriocins. Most members of class II bacteriocins exert their antimicrobial action by inducing membrane permeabilization that subsequently leads to the leakage of cytoplasmic molecules, causing cell death of the target bacteria (Figure 3). The mechanisms of antimicrobial action of class II bacteriocins differ among subclasses (Perez et al., 2014).

Members of class IIa bacteriocins, like mesentericin are known for their high potency against *L. monocytogenes*, a highly pathogenic and robust food-borne bacterium. Class II bacteriocins have been shown to bind to mannose phosphotransferase system proteins, the sugar-uptake system of target bacteria, as docking molecule for their killing mechanism. The conserved amino acid sequence at the N-terminal region of class IIa bacteriocins is responsible for the anti-listerial antimicrobial

activity, whereas the less conserved C-terminal domain is responsible for their antimicrobial activity against other target strains (Filmland et al., 2005) (Johnson et al., 2005).

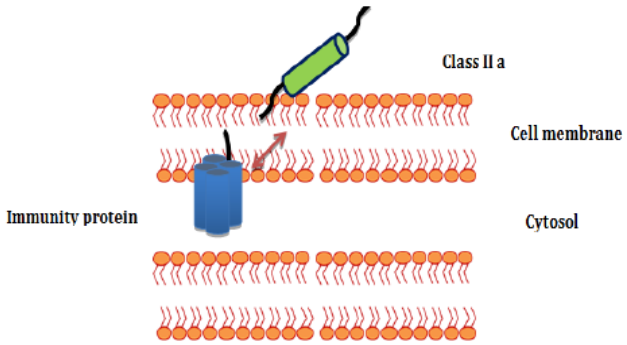


Figure 3 Membrane permeabilization by class IIa bacteriocins (Bharti et al., 2015)

1.3 OPERON REGULATION FOR THE SYNTHESIS OF NISIN AND MESENTERICIN

1.3.1 BIOSYNTHESIS OF NISIN

Analysis of the nisin-producing strain N8 revealed that the *nisZBTCIPRKFEG* gene cluster, responsible for nisin biosynthesis, immunity and regulation, consists of two operons, *nisZBTCIPRK* and *nisFEG*. In Figure 4 the operon regulation for *nisA* is illustrated. Nisin was found to be secreted during the early stages of growth as well as later in the growth cycle. The secreted nisin was adsorbed on the surface of the cells and was released to the medium during mid-exponential growth, when the pH in the medium fell below 5. The operons are coordinately regulated by mature nisin (Runar et al., 1996).

Two naturally occurring nisin variants are nisin A and nisin Z, which differ in a single amino acid residue. *NisB* and *nisC* are believed to be necessary for chemical modification, which involves dehydration and lanthionine formation. *NisT* is an ABC transport protein potentially responsible for the transport of pre-nisin across the cytoplasmic membrane. The lipoprotein *NisI* seems to play a role in nisin immunity, while *nisP* is a protease which separates the leader of the modified nisin precursor peptide to active mature nisin. *NisR* and *nisK* are believed to form a two-component regulatory system necessary for nisin synthesis. The *nisFEG* genes encode an ABC transport system, which has recently been suggested also to be involved in nisin self-protection.

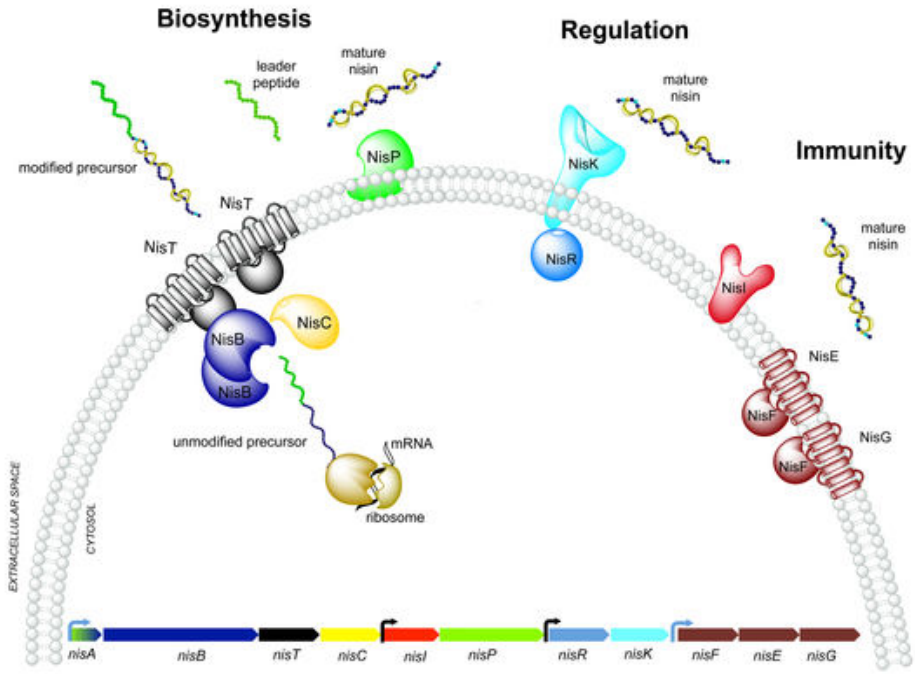


Figure 4 Operon regulation of nisA (Runar et al., 1996)

1.3.2 BIOSYNTHESIS OF MESENTERICIN

At least four genes are required in the production of class IIa bacteriocins, including a bacteriocin structural gene encoding a precursor, an immunity gene encoding an immunity protein, genes encoding an ATP-binding cassette transporter and an accessory protein for extracellular translocation of bacteriocins (Figure 5) (Yanhua et al., 2012).

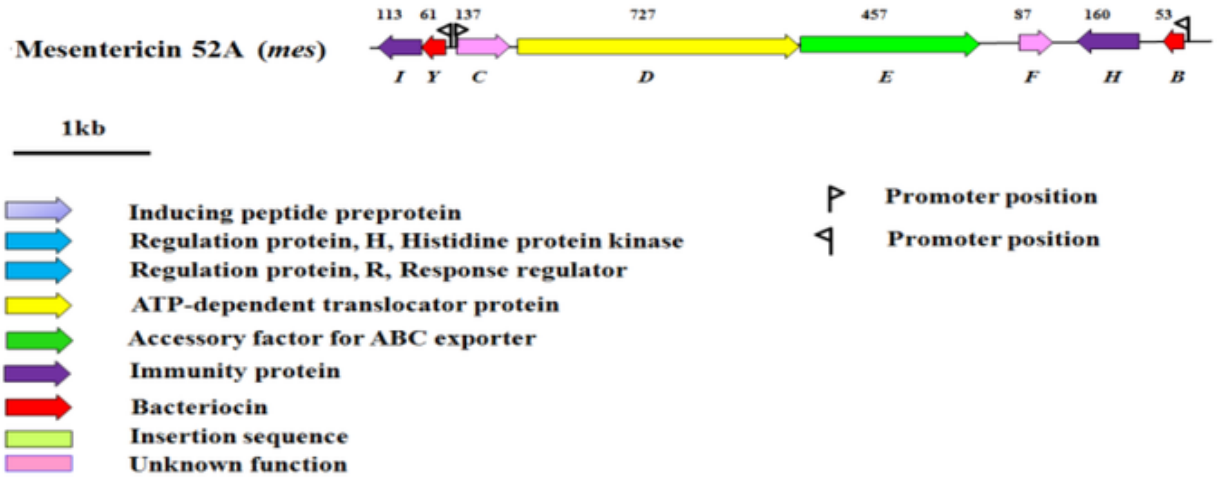


Figure 5 Organization of the gene clusters of class IIa bacteriocins
The figure was involved in the production of mesentericin 52A in Leuconostoc mesenteroides subsp. Mesenteroides FR52. Open reading frames (ORFs) encoding the related proteins are marked with the different color. The number of amino acid residues within each encoded protein is shown below the corresponding ORF (Yanhua et al., 2012).

The class IIa bacteriocin production was regulated by quorum sensing (QS) system. QS systems are present in a great amount of bacteria as a primary mechanism for bacteria to monitor the environment for other bacteria. QS systems used for the regulation of class IIa bacteriocin production are composed of three gene products, including an inducer peptide, a membrane-associated histidine protein kinase (HPK), and a cytoplasmic response regulator (RR). The inducer peptide is ribosomally synthesized at low levels as a precursor which appears to be biologically inactive and contains an N-terminal or leader sequence. Sequent cleavage of the precursor at a specific processing site removes the leader sequence from the antimicrobial molecule at the same time. Then inducer peptide is released and exported through the dedicated transport system involving an ABC-type translocator and an accessory protein. The pre-sequence of the bacteriocin plays a dual role in bacteriocin biosynthesis. One is a protective role at the cytosolic side of the cell membrane by keeping the bacteriocin inactive. The other is as a recognition signal during export. At a certain concentration threshold of the inducer peptide, the transmembrane HPK detects a change in environmental signal and is activated, leading to its auto phosphorylation. Then the phosphorylated HPK transfers a phosphate group to its related RR. The phosphorylated RR acts as a transcriptional activator and activates expression of bacteriocin-related genes, including genes encoding bacteriocin, immunity protein, secretory apparatus, and regulatory proteins. Bacteriocin and immunity genes most often stay on the same operon and are expressed simultaneously. The bacteriocin producer cells protect themselves from their own bacteriocin by the immunity protein. At a certain time, all bacteriocin producer cells in the population are believed to secrete bacteriocins which results in a fast activation of the bacteriocin production.

2 QUANTIFICATION OF GENE EXPRESSION

2.1 REVERSE TRANSCRIPTION QUANTITATIVE POLYMERASE CHAIN REACTION: RT-qPCR

Reverse transcription quantitative polymerase chain reaction (RT-qPCR), a variant of polymerase chain reaction (PCR), is a technique commonly used in molecular biology to detect and quantify RNA expression. PCR technique (Figure 6) is used to amplify a single copy or a few copies of a segment of DNA across several orders of magnitude. Only a small amount of this targeted sequence can result in millions of new copies. It is an easy, cheap and reliable way to repeatedly replicate a focused segment of DNA, a concept which is applicable to numerous fields in modern biology and related sciences. The PCR methods rely on thermal cycling that is nothing more than a very accurate heating and cooling device containing the tubes with the DNA samples and other reagents necessary for the amplification reaction. These reagents are:

- Free nucleotides = dNTP's
- Short single stranded DNA fragments = primers
- The enzyme Taq polymerase
  This enzyme originates from the bacterium *Thermus aquaticus* that lives in hot springs and therefore consists of enzymes that can be effective at high temperatures. In this way these enzymes can survive the repeating denaturation steps that occur in the PCR process.

The PCR process consists of three steps:

1. **Denaturation**
   The samples are heated up to a temperature of ca. 90-95°C. This ensures that every trace of double stranded DNA splits into two single strands. The reason for this separation is because of the hydrogen bridges holding the complementary strains together that are much weaker than the covalent bonds between the nucleotides inside a chain. The heating disconnects the hydrogen bonds whereby de double strain falls apart while the covalent bonds are untouched.

2. **Hybridization**
   In a next step the temperature is reduced. Because of this, the DNA-primers are able to bind unto the single strands. The primers can bind due to their complementary sequences with each strand that is located next to the targeted DNA sequence that needs to be replicated.

3. **Extension**
   Once the primer has attached itself to the single strand, the Taq polymerase will extend the DNA whereby free nucleotides or dNTP's are used. The eventual result is a complementary chain of every original single chain. In this first cycle the original present DNA is doubled.

In the next cycle the temperature is increased again followed by another cooling step. Because of this, the double strands are separated and copied individually once more by the Taq polymerase. In each following cycle, the number of strands of the targeted DNA sequence is doubled.

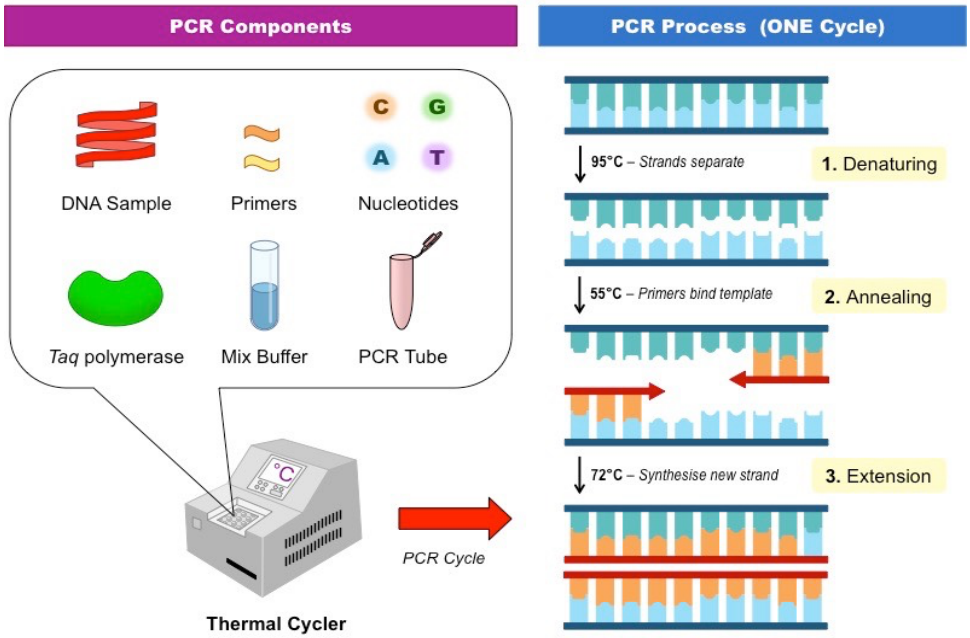


Figure 6 PCR: procedure and reagents (BioNinja, 2017)

There are several variants of PCR. Besides of the traditional PCR to sequence DNA, there is another variant applied for RNA. Since RNA is single stranded, it cannot be multiplied by a normal PCR and it is first transcribed into $_c$DNA. In this case the technique is called RT-PCR. With real-time PCR, also known as qPCR the amplification of a targeted DNA sequence can be monitored during the PCR and not at its end as in conventional PCR. In this project a combination of RT-PCR and qPCR is used, this is called RT-qPCR.

Two common methods for the detection of PCR products in qPCR are:

1. Non-specific fluorescent dyes that intercalate with any double-stranded DNA (e.g. SYBR Green)

2. Sequence-specific DNA probes consisting of oligonucleotides that are labelled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary sequence (e.g. TaqMan probe).

SYBR Green-based detection is the least expensive and easiest method available for real-time PCR. Other methods, such as TaqMan are very specific and require an expensive third primer labeled with a dye and a quencher. SYBR Green specifically binds to double-stranded DNA. The SYBR Green actually penetrates in-between the two strings of the double-stranded DNA. Detection of the fluorescent signal occurs during the PCR cycle at the end of either the annealing or the extension step when the greatest amount of double-stranded DNA product is present. However, SYBR Green detects any double-stranded DNA non-specifically. Therefore, the reaction must contain a combination of primers and master mix that only generates a single gene-specific amplicon without producing any non-specific secondary products. A big advantage of this method is that it can be used in researches concerning multiple genes because the method stays the same whatever the gene may be. In case of the TaqMan method every gene would need its own probe which makes this method very expensive (Sigma-Aldrich, 2017).

2.2 ABSOLUTE AND RELATIVE QUANTIFICATION

Quantitative or real-time PCR can be used to quantify DNA by two common methods: relative quantification and absolute quantification. In the absolute method the determination of the template copy number in the sample is based on a standard curve prepared with serial dilutions of known concentration solution of the test sequence. Therefore, it is necessary that the PCR of the sample and the standard have the same amplification efficiency. Relative quantification on the other hand is based on internal reference genes to determine the change in expression levels between the targeted gene and reference gene. In the relative method, the curve is only used to calculate the reaction efficiency and the result is given in relation to the calibrator. Commonly the number of transcripts is given in relation to the amount of pre-defined reference genes. Such normalization can effectively correct the differences between the compared samples.

One of the most crucial aspects of the process is that the reference gene must be carefully chosen to be stable. Reference genes have sequences different than the target and serve as an internal reaction control. Before a gene can be chosen as a reliable reference, it must satisfy various criteria of high importance. One of the most crucial factors is that its expression level isn't influenced by experimental factors. Also, the reference gene should show minimal variability in its expression between samples. Preferably, the reference shows threshold cycle values that are alike with the gene of interest. In return, reference genes should indicate the variability resulting from imperfections of the applied technology and preparatory work. This ensures that any variation in the amount of genetic material will relate to the same extent as the object of research and control. It seems that basic metabolism genes, called Housekeeping Genes (HKGs) meet these conditions perfectly. They were the first to be examined as reference genes (Thellin et al., 1999) and are involved in essential processes regarding the survival of cells. They should be expressed in a stable and non-regulated constant level. For each experiment an individual and complex approach is needed for this choice along with the validation (Kozera et al., 2013). This project wants to set up a technique in which this selection and validation of reference genes is explained thoroughly step by step. To normalize this protocol and to correct non-specific variation it is preferable to use more than one reference gene. To select the most suitable reference gene, the different efficiencies of the genes are calculated by making standard curves and compared with each other.

3 AIM OF THE PROJECT

The main objective of this project is to set up the RT-qPCR technique to quantify the expression of nisin and mesentericin.

To achieve this main goal some specific objectives are considered:

1. To find suitable reference genes for the normalization of the gene expression values. These genes can be found in previous literature regarding gene expression experiments.

2. To validate expression stability of different putative reference genes.

4 MATERIAL AND METHODOLOGY

In this project the selection of reference genes that serve as an internal control was explained. The focus was to identify stable reference genes which are suitable to normalize real-time RT-qPCR experiments. Therefore, a technique was set up, following several steps. In this chapter, they are explained individually. Figure 7 clarifies the different phases to obtain the standard curves for the relative quantification of the gene expression in a schematic way.

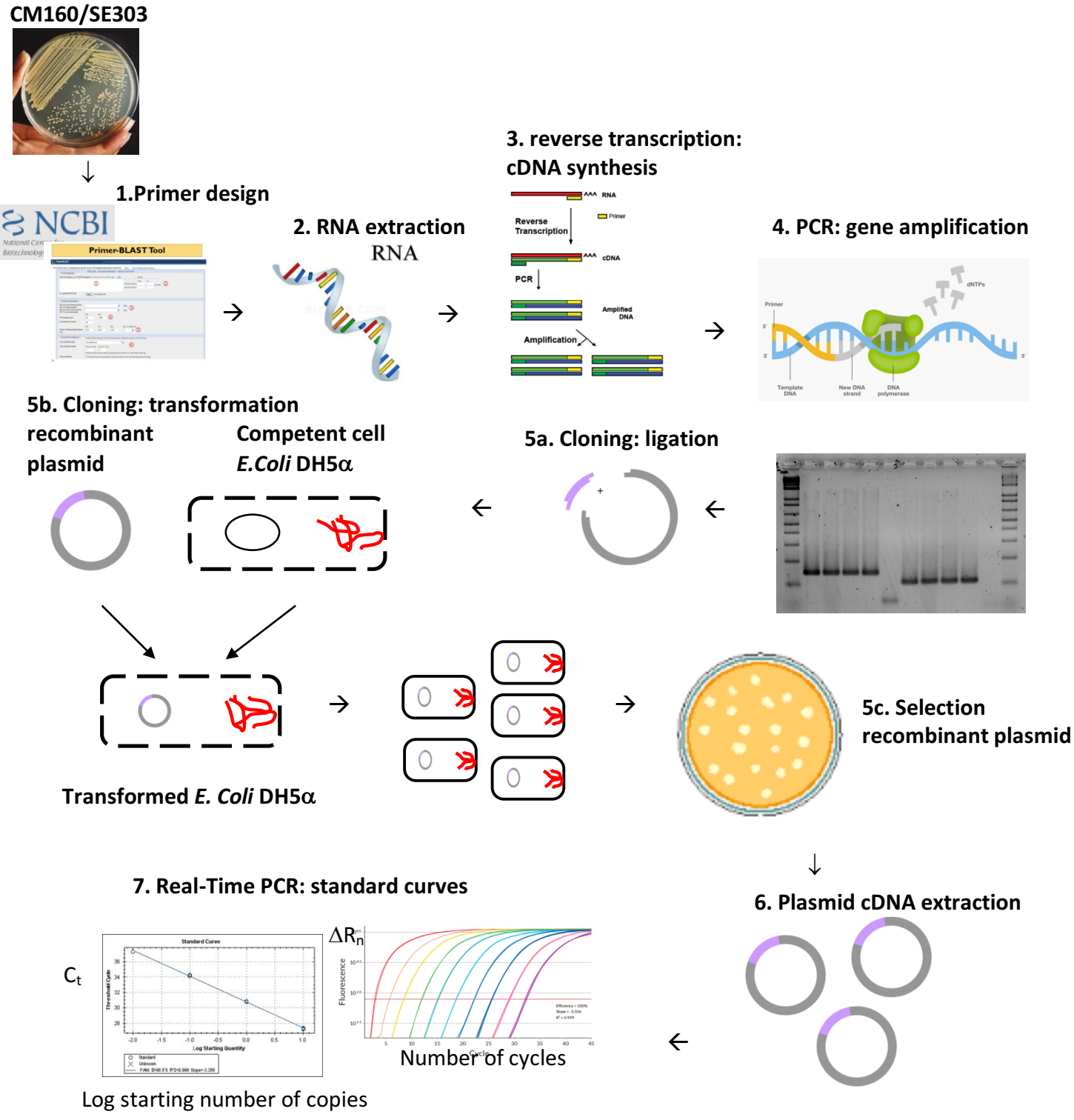


Figure 7 Schematic of the different steps to quantify gene expression

4.1 BACTERIAL STRAINS AND CULTURE CONDITIONS

The bacterial strains used in this project are *Leuconostoc mesenteroides* CM160, *Lactococcus lactis* SE303 and *Escherichia coli* DH5α.

Both the strains of *L. mesenteroides* and *L. lactis* were respectively isolated from cherry and soya beans as described in previous papers (Trias et al., 2008b) (Roselló et al., 2013). They were chosen because it is known that they are bacteriocin producer strains of mesentericin and nisin and these are the two genes of interest for this project. To obtain the cultures, these LAB were grown in MRS agar and incubated for 48 hours at 23°C (Oxoid; Unipath Ltd., Basingstoke, Hampshire, England).

Escherichia coli DH5α was used as a host for the recombinant vectors. To obtain cultures of this *E. coli* strain, Luria-Bertani (LB) agar is used as the growth media and an incubation of 24 hours at 37°C is applied.

All three strains were stored at -80°C in LB for *E. coli* or MRS for *L. mesenteroides* and *L. lactis* provided with 20% of glycerol to protect the cells.

4.2 PRIMER DESIGNS

The reference genes were selected from previous papers in which gene expression was studied (Rocha et al., 2015) (Takle et al., 2007) (Zhao et al., 2011) (Florindo et al., 2012).
These reference genes are needed for the normalization of the expression of the genes of interest. The primer sequences couldn't be found in the literature and needed to be designed manually. First, the sequence of the gene was searched in the GenBank database. Then, the sequence of each gene was compared for both strains using the Multalin software. Since the sequences aren't similar, the primers were designed for each species independently. The primer blast was used to find the forward and reverse sequence of the primer. When selecting the primer sequences, some parameters must be considered to choose the best one for a qPCR amplification. The length of the primers needs to be taken into account and has to be within 18 to 24 nucleotides. For the qPCR, only a product length between 100 bp and 200 bp can be used so this is another parameter that should be considered. An overview of the sequences for each gene can be found in Table 2.

Table 2 Overview of all the reference genes with their product length, primer sequences 5' - 3' and annealing temperature

Gene	Product length	Primer sequences 5’ - 3‘	Annealing temperature
gyrA Lm	114 bp	f-ACCGGAATTGCTGTTGGAATG	67,9°C
		r-CATCAGGTCGGCTGTGGTAG	65,7°C
gyrB Lm	166 bp	f-TCTACCCGGAAAGTTGGCAG	66,2°C
		r-TGAAGCTTTCCCCACGTTCA	67,8°C
GAPD Lm	136 bp	f-AGTCCACGCTTACACAGCTT	61,5°C
		r-CATTCCGATTGCCTTTGCGG	71,5°C
ldh Lm	151 bp	f-TCGTCCTACACCGACAATGC	66,1°C
		r-AGTAGCCGAAATGGCGCTTA	65,6°C
recA Lm	114 bp	f-GGTGTTGGTGGCTATCCCAA	66,9°C
		r-GGTGTTGGTGGCTATCCCAA	69,2°C
rpoD Lm	120 bp	f-GGTGTTGGTGGCTATCCCAA	67,3°C
		r-GGTGTTGGTGGCTATCCCAA	66,3°C
tufA Lm	166 bp	f-GAGAAGCGTCACTATGCCCA	65,3°C
		r-CACCAACTTGACGTGCCAAC	66,6°C
gyrA Ll	177 bp	f-TGGCAATCCTTTTGCTGTCC	67,2°C
		r-TGGCAATCCTTTTGCTGTCC	68,5°C
gyrB Ll	92 bp	f-CCGGAATTCCACGTCCATCA	70,2°C
		r-TGAAGCACTTGCAGGATTTGC	67,4°C
GAPD Ll	153 bp	f-ACCAACTTCAGCCCAGTTGA	64,7°C
		r-GCAATGCTTGCTCACTTGCT	65,6°C
ldh Ll	172 bp	f-TGAGTCACGATTGAGCGCAT	67,0°C
		r-TAGATGGCGTTTCTTGGGGG	68,0°C
recA Ll	165 bp	f-AGCCATGCGTAAACTTGCAG	65,1°C
		r-GAACCACGGACATCAAGACG	65,6°C
rpoD Ll	165 bp	f-TCTCGACTTATGCCACATGGT	64,4°C
		r-CTTCTGGTGAAGGATCGCGT	66,5°C
tufA Ll	165 bp	f-TGATACCACGTTCGCGTTCT	65,5°C
		r-ACCGTAGCAAACCACACGTT	64,3°C
mesY	148 bp	f- GCCAAACGATGTATGCCAGC	67,6°C
		r- AGTCTGTGGAAGCATATCAGCAA	64,8°C
nisA	112 bp	f- CGAAGAAAGATTCAGGTGCATCA	67,3°C
		r- CAATGACAAGTTGCTGTTTTCATG	65,3°C

4.3 RNA ISOLATION AND SYNTHESIS OF cDNA

For this project RNA was isolated from two strains: CM160 and SE303.

Some precautions needed to be followed when working with RNA. All techniques were performed aseptically wearing gloves and using RNase free tubes. Other contamination with RNase from plastic elements was avoided by washing them with a solution of 0,1 M NaOH and 1 mM EDTA and rinsing with RNase-free water. Elements of glass were washed with detergent and were made RNase free by a heating process at 240°C for 4 hours. Finally, water and other solutions were treated with 0,1% DEPC. This cannot be used for TRIS buffers. In this case, the water was primarily treated with DEPC and then the buffer was added.

First, the cells were disrupted in three stages. This was accomplished by an enzymatic lysis, using lisozim and mutanolisin, followed by a mechanical disruption of the bacterial cellular wall using the Tissuelyser and a digestion procedure using proteinase K.

For the extraction of RNA 500 µl of culture of each strain was transferred into an Eppendorf tube that was RNase free. Next, 1000 µl of RNAprotect was added and the tube was vortexed. This RNAprotect is necessary to prevent degradation of the fragile RNA and to immediately stabilize the RNA for the expression study. After a centrifugation step of 10 minutes at 8000 rpm the supernatant was removed and the obtained pellet dried. In the following step the pellet was solved in 200 µl of TE buffer with 15 mg/ml lysozyme and 20 µl of proteinase K. This TE buffer was prepared using DEPC treated water. Next, 6 µl of mutanolisin (5000 U/ml) was added and after an incubation of 45 minutes at 37°C while shaking, 700 µl of RLT buffer that includes 10 µl/ml β-mercaptoethanol was added. For the second stage of the disruption, the suspension was transferred into a 2 ml Eppendorf tube with 50 mg of acid washed glass beads. The mechanical disruption was fulfilled when this solution was put in the Tissuelyser (at 30 s^{-1} for 5 min). Finally, 760 µl of supernatant was collected and 590 µl of ethanol (80%) was added.

To purify the RNA, 700 µl of lysate was poured into the column and then centrifuged for 15 seconds at >10000 rpm. The supernatant was removed, but when more volume was detected, successive centrifugation steps were necessary. Next, 700 µl of RW1 buffer was added to the column and centrifuged again. The collector tube with the eluent could be discarded since the column contained the sample. In the following step, 500 µl of RPE buffer was added to the column and centrifuged. The function of the RPE buffer was to clean the membrane. The samples were then centrifuged for 2 minutes at 15500 rpm and in this step the ethanol was removed. Next, the column was replaced in a new collector tube. Now the column was centrifuged for 1 minute without adding anything. This step was executed twice. When all the ethanol was removed, 50 µl of RNase free water was added and the column was left on ice for 15 minutes followed by a final centrifugation step of 1 minute. From here on the sample was not anymore present in the column, but in the tube. Finally, the amount of RNA was quantified by Nanodrop.

RNA samples were treated with a turbo DNA-free kit to remove DNA. Depending on the concentration of the RNA measured by Nanodrop, samples were treated using routine treatment (<200 ng RNA/µl) or rigorous treatment (>200 ng RNA/µl). 5 µl of 10x turbo DNase buffer was added to 50 µl of RNA sample and mixed thoroughly. 1 µl of TURBO DNase was added. This enzyme degrades all the DNA that is left in the RNA sample. Next, the tube was vortexed at low speed. After the samples were homogenized while pipetting, the tubes were incubated for 30 minutes at 37°C. For samples that need a rigorous treatment, 1 µl of TURBO DNase was added twice. The same incubation was followed. Then, 0,1 (routine) or 0,2 (rigorous) volumes of inactivation reagent were added. The tube was homogenized by vortexing and incubated for 2 minutes at room temperature whereby the tube was shaken two or three times. This step was followed by a centrifugation of two

minutes at 10000 rpm. This step was necessary to precipitate the inactivation solution. The supernatant that contains the RNA was transferred to a siliconized tube with a Pasteur pipette. Finally, the tube was set on ice and the sample was quantified with the Nanodrop.

To obtain the $_c$DNA a reverse transcription was executed to convert the RNA into $_c$DNA. This protocol was performed using a High Capacity $_c$DNA Reverse Transcription kit (Applied Biosystem). To get a good result, it is recommended to work with a RNA concentration of 200 ng/µl. The volumes of the mastermix can be found in Table 3.

Table 3 Mastermix reverse transcription

Reagents for mastermix	**Volume for 1 reaction (µl)**
10x RT buffer	2
25x dNTP mix (100 mM)	0,8
10x RT Rrandom Primers	2
MultiScribe RT (50 U/µl)	1
Nuclease free H_2O	4,2
RNA sample	10
Total volume per tube	10

When each tube was filled with 10 µl of mastermix, 10 µl of RNA sample was added and the tubes were placed in the thermocycler (GeneAmp PCR System 9700). The conditions for the thermocycler were: 10 minutes at 25°C, 2 hours at 37°C, 5 minutes at 85°C and finally the samples were cooled until 4°C. When the reverse transcription was finished the concentration of $_c$DNA was measured with the Nanodrop again. The samples were stored in siliconized tubes at -80°C.

4.4 STANDARD CURVES

To set up the RT-qPCR technique, standard curves of each reference gene were obtained. Depending on the obtained curves, the best reference gene was chosen.

To set up this technique several steps needed to be fulfilled. First, the genes were amplified by PCR. These genes were then cloned into a vector to make the stock solutions of $_c$DNA for the preparation of the standard curves more stable. This was necessary because different dilutions should be made to create the standard curves that will allow the quantification of the gene expression. In a next step, the plasmids, containing each a specific gene, were extracted. Finally, these samples were used to prepare the standard curve.

4.4.1 GENE AMPLIFICATION

To amplify the *mesY* gene of *L. mesenteroides* CM160, the *nisA* gene of *L. lactis S*E303 and the reference genes for both strains, a PCR was performed of the earlier obtained $_c$DNA samples. To start the PCR a first step was to prepare the mix with the correct primers (Table 4).

Table 4 PCR mix reverse transcription

Reagents for PCR mix	**Volume for 1 reaction (µl)**
H_2O	18,13
10x Buffer	2,5
$MgCl_2$ (50 mM)	0,75
dNTP's (10 mM)	0,5
Primer Forward (10 µM)	0,5
Primer Reverse (10 µM)	0,5
Taq polymerase (5 U/µl)	0,25
cDNA sample	2
Total volume per tube	25

When each tube was filled with 23 µl of mastermix, 2 µl of $_c$DNA sample was added and the tubes were placed in the thermocycler (Table 5).

Table 5 Conditions PCR reverse transcription

	Temperature	**Time**
	95°C	10 minutes
40 cycles	95°C	20 seconds
	Annealing Tª	30 seconds
	72°C	30 seconds
	72°C	5 minutes
	4°C	∞

The annealing temperature depends on the melting temperature of each primer. These melting temperatures can be found in Table 2. Both the melting temperature for the forward and reverse sequence of each primer were compared. The lowest value was subtracted with 5 and the obtained value was the optimal temperature to perform the PCR. The primers were divided in different groups

for the PCR with primers that have similar melting temperatures. Fourteen reference genes were divided into 5 groups based on the applied annealing temperature (Table 6).

Table 6 PCR groups

Group	Annealing temperature	Genes
1	56,5°C	*GAPD* Lm
2	60,1°C	*gyrA* Lm, *ldh* Lm, *tufA* Lm, *recA* Ll
3	61,2°C	*gyrB* Lm, *rpoD* Lm
4	61,9°C	*recA* Lm, *gyrA* Ll, *gyrB* Ll, *ldh* Ll
5	59,3°C	*rpoD* Ll, *GAPD* Ll, *tufA* Ll

To check if *mesY*, *nisA*, and the reference genes have been amplified, a gel electrophoresis of one hour at 90 V was performed in 3% agarose. When the electrophoresis was finished, an image was obtained with the different bands and the product length was read. To take the image, the gel was kept in a SYBR safe solution for 15 minutes and then was put in a UV chamber that is connected with a computer. Using the UVIpro software, the image was taken of the gel.

4.4.2 CLONING

During the cloning protocol mesentericin, nisin and the reference genes were inserted individually in the pSpark-TA DNA vector. Then, the obtained plasmids were incorporated into the *E. coli* strain to make the samples of the recombinant vectors more stable. After the multiplication, the vector, in formation of a plasmid, was extracted from the competent cells.

4.4.2.1 Preparation of the competent cells

To prepare these competent cells, colonies of *Escherichia coli* DH5α were used. An overnight culture was prepared and 2 ml of this culture was added into 100 ml of LB broth. The culture was incubated at 37°C in agitation at 120 rpm. Since the cells must be in their exponential phase for an easier rupture, the absorbance needs to reach a value around 0,5 for OD_{600nm}. Depending on the sample, it is known that this takes about 4 hours. When the correct absorbance was reached, 40 ml of the culture required a centrifugation at 4000 rpm at 4°C for 10 minutes.

The obtained supernatant was discarded and 20 ml sterile $CaCl_2$ 50 mM was added. After homogenization, the suspensions were kept on ice for 30-45 minutes. In the following similar centrifugation step, the supernatant was again discarded. The cells were homogenized into 2 ml of sterile 50 mM $CaCl_2$ provided of 15% glycerol. Anew the suspension was kept on ice for 30-45 minutes and finally distributed in Eppendorf tubes of 1,5 ml that contain 50 µl of the glycerol solution. The competent cells were preserved at -80°C.

4.4.2.2 Ligation of the plasmid and transformation of the host cell

It is chosen to amplify the genes in a vector to make the $_c$DNA samples more stable. In this procedure, the genes were inserted in the pSpark®- TA vector (Figure 8). This step is also known as the ligation reaction. After the ligation reaction followed the incorporation of the plasmid in the competent cells of *E. coli* DH5α. This is also known as the transformation of the host cell. To check if the cloning was successful a PCR was done. Finally, if the PCR was positive, the transformed *E. coli* DH5α cells were stored at -80°C.

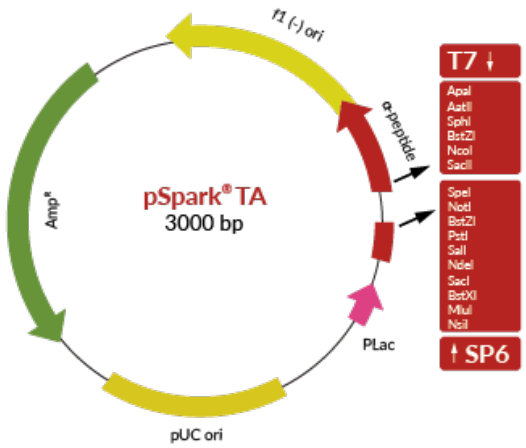


Figure 8 pSpark-TA cloning vector

1. Ligation reaction

    a. Prepare the mix for the ligation reactions in Eppendorf tubes of 1,5 ml (Table 7).

    Table 7 Ligation mix cloning protocol

Reagents	**Volume for 1 reaction (µl)**
pSpark-TA DNA cloning vector (50 ng/µl) (Canvax, Spain)	1
5x T4 DNA ligase buffer	2
T4 DNA ligase (5 Weiss units/µl)	1
Sterile milli-Q water	4
PCR product	2
Total volume per tube	10

    When the mix was ready, it was distributed in different reaction tubes. (8 µl/tube)

    b. Add 2 µl of PCR product in each tube. In case of the control positive, add 1 µl of control insert DNA and 1 µl of sterile milli-Q water.

    c. Leave the tubes for one hour at 22°C.

2. Transformation

    a. Unfreeze on ice the tubes of 1,5 ml that contain 50 µl of competent cells.

    b. Add 10 µl of ligation reaction mix. Mix the tubes and leave them 30 minutes on ice.

    c. Thermal shock in thermoblock: 45 seconds at 42°C

    d. Keep the tubes 2 minutes on ice.

    e. Add 600 µl of LB liquid in the tubes. Incubate them for 45 minutes at 37°C while shaking at 300 rpm.

    f. Vortex the tubes. Foresee two LB + ampicillin plates for each tube and add respectively 100 µl and 200 µl of culture on each plate. Homogenize the volume over the entire plate with a Digralsky spreader and incubate the plates at 37°C for 24 hours.

To check if the gene was ligated into the vector, a PCR was performed on the colonies that have grown on the LB + ampicillin plates. Since the colonies have grown despite the presence of ampicillin, it is known that these colonies contain the vector. It is to say that colonies in which the vector was implemented correctly become resistant for ampicillin.

g. To make sure that the PCR products are pure, four transformed colonies were chosen of each gene, including the positive and negative control. These colonies were then transferred on a new LB + ampicillin plate and incubated again at 37°C for 24 hours.
h. Of the plates with the grown pure colonies, one colony was taken and suspended in an Eppendorf tube of 1,5 ml with 100 µl of Milli-Q water.
i. The plates with the grown transformed cells were kept in the fridge.
j. The tubes were boiled at 95°C for 10 minutes. Next, the PCR mix was prepared with the generated primers: T7 5'-TAATACGACTCACTATAGGC-3' and Sp6 5'-AGGTGACACTATAGAATAC-3' (Table 8).

Table 8 PCR mix cloning protocol

Reagents	**Volume for 1 reaction (µl)**
Sterile Milli-Q water	17,875
Buffer (10x)	2,5
$MgCl_2$	0,75
DNTP's (10 mM)	0,5
Primer T7 (10 µM)	0,5
Primer Sp6 (10 µM)	0,5
Taq polymerase biotools (5 u/µl)	0,375
DNA sample	2
Total volume per tube	25

When the mix was ready, it was distributed in the different reaction tubes (23 µl per tube).

k. For the PCR conditions, first a temperature of 98°C was kept for 2 minutes. Next, 98°C was maintained for 10 seconds, followed by 45°C for 30 seconds and 72°C for 35 seconds. These three temperatures are considered as one cycle and are repeated 35 times. Then, a temperature of 72°C is kept for 12 minutes and finally the samples were cooled down to 4°C.
l. To check if the PCR was successful an electrophoresis with an agarose gel of 1,5% agarose was done.
wanted amount of bp = bp inserted + 153 bp of the vector
Since the number of base pairs of the control DNA insert is 600 bp, the total product length after electrophoresis will be 753 bp.
m. One colony per gene that had a positive PCR was chosen and spread again on a new LB + ampicillin plate that was incubated for 24 hours at 37°C.
n. The genes were stored at -80°C in LB broth with 20% glycerol.

4.4.3 PLASMID ISOLATION

Now, the plasmids have been extracted to prepare the standard curves.
The protocol used is designed for a purification of up to 20 µg of high-copy plasmid DNA from 1–5 ml

overnight cultures of *E. coli* in LB medium. All centrifugation steps were executed at 13000 rpm for 1 minute when not mentioned and the whole protocol was carried out at room temperature. Only for the preparation of the overnight culture aseptic conditions were acquired.

1. Pelleted bacterial cells, obtained by a centrifugation of 3 minutes at 8000 rpm of 1,5 ml overnight culture (37ºC 200 rpm LB+amp), were solved into 250 µl Buffer P1. Ensure that RNase A has been added to Buffer P1. RNase A is required to prevent RNA contamination of the purified plasmid DNA. The solution with the bacterial pellet was vortexed until no cell clumps remain.
2. 250 µl Buffer P2 was added and mixed thoroughly, but gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. Do not allow the lysis reaction to proceed for more than 5 minutes. Continue mixing the solution until a homogeneously colored suspension is achieved.
3. 350 µl Buffer N3 was added and mixed immediately by inverting the tube to avoid localized precipitation. The solution should be cloudy; a homogeneous colorless suspension indicates that the SDS has been effectively precipitated.
4. Centrifuge for 10 minutes until a compact white pellet is formed.
5. Apply 800 µl of the supernatant from step 4 to the column by pipetting.
6. Centrifuge for 1 minute and discard the flow-through.
7. Wash the column by adding 0,75 ml Buffer PE and centrifuging for 30-60 seconds.
8. Discard the flow-through and centrifuge for an additional minute to remove residual wash buffer. Important: residual wash buffer will not be completely removed unless the flow-through was discarded before this additional centrifugation. Residual ethanol from buffer PE may inhibit subsequent enzymatic reactions.
9. Place the column in a clean micro centrifuge tube. To elute DNA, add 50 µl Buffer EB to the center of each column, leave it for 15 minutes and centrifuge for 1 minute. The columns were removed and the tubes with the DNA samples were kept on ice. Finally, the concentration of DNA was measured with the Nanodrop.
10. Calculation of the number of copies of the extracted plasmids.
    This project sets off from a stock solution of 100 µl with 5 x 10^8 copies/µl of each gene. To prepare this stock, the volumes of water and raw extracted plasmid sample were calculated with a formula. In this formula, the concentration of the obtained sample, the number of base pairs of the product and the number of base pairs of the vector were filled in.

$$number\ of\ copies\ of\ the\ gene/\mu l = \frac{concentration\ of\ DNA\ \frac{g}{\mu l} * 6{,}023.10^{23} (number\ of\ Avogadro)}{total\ productlength\ plasmid + insert\ in\ bp * 650\ (MW\ of\ one\ bp)}$$

When the number of copies of the extracted plasmids was known, the volumes of water and sample to obtain 100 µl with 5 x 10^8 copies/µl were calculated easily using the dilution formula: $C_1 * V_1 = C_2 * V_2$.

4.4.3.1 Plasmid DNA purification: Qiaquick PCR purification kit

After cloning, a cleaning step was performed for the sequencing since a lot of unwanted reagents were still present in the samples. These reagents needed to be removed because they could impede the sequencing process of the wanted gene. In general, it was wanted to eliminate as much as possible of the cells except for the plasmid that contains the sequence of the reference gene.

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix.
2. If pH indicator I has been added to Buffer PB, check that the color of the mixture is yellow. If the color of the mixture is orange or violet, add 10 μl of 3 M sodium acetate, pH 5 and mix. The color of the mixture will turn to yellow.
3. Place the column in a provided collector tube.
4. To bind DNA, apply the sample into the column and centrifuge.
5. Discard flow-through. Place the column back into the tube.
6. To wash, add 0,75 ml Buffer PE to the column and centrifuge.
7. Discard flow-through and put the column back in the tube. Centrifuge the column for an additional minute.
   IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
8. Place the column in a clean micro centrifuge tube.
9. To elute DNA, add 30 μl Buffer EB to the center of the membrane and wait 5 minutes before the centrifugation of the column. Alternatively, for increased DNA concentration, add 30 μl elution buffer to the center of the membrane, let the column stand for 1 minute and then centrifuge.
   IMPORTANT: Ensure that the elution buffer is dispensed directly onto the membrane for complete elution of bound DNA.
10. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

4.4.3.2 Sequencing

After plasmids with the correct insert size, orientation and restriction pattern were identified, the clones were sequenced to confirm that the sequence was correct. To prepare the sequencing reaction, a solution of 5 μl of sample was mixed with 5 μl of 5 μM primer. For the primer either the forward or the reverse could be used. This solution was then send to the Macrogen lab in The Netherlands for analysis and the registered sequences were send by mail. The alignment was made of the received sequence with the sequence of the gene found in the Genbank. When this alignment doesn't show any similarity, the alignment was done with the reverse and complementary strain of the analyzed sequences. When a complementary part was found in the alignment, we were sure that the correct gene was amplified.

4.4.4 SELECTION OF THE OPTIMAL PRIMER CONCENTRATION

First, the optimal concentration of the primers for each reference gene was determined using qPCR. The four primer concentrations that were tested in this project were: 2 μM, 6 μM, 10 μM and 18 μM. These concentrations respectively corresponded to a final concentration of 100 nM, 300 nM, 500 nM and 900 nM when added in the mix. The optimal primer concentration was determined for a concentration of 10^6 copies/μl of plasmid. This concentration was the same for all the genes and was chosen because it lies in the middle of the standard curves. Only the concentration of the primers changes. Each primer concentration was tested two times and a third well was prepared for a negative control. For the mix, 6 μl of sterile Milli-Q water, 10 μl of SYBR Green mix and 1 μl of each primer at the optimal concentration was needed for one reaction. 2 μl of plasmid sample was added so that each well contained 20 μl.

When the mix was ready, 18 μl was distributed in the three wells provided for each gene. Finally, 2 μl of plasmid sample was added in the first two wells. This resulted in 10^6 copies per well when 2 μl of 5 x 10^5 copies/μl was added. The third well should be left blank for the control negative. For this project, SYBR Green was utilized as a non-specific fluorescent dye. The conditions for the qPCR were

analogue during the whole project. A PCR device 7500 Fast Real-Time PCR System (Applied Biosystems) was used (Table 9).

Table 9 Conditions qPCR

	Temperature	Time
50 cycles	95°C	10 minutes
	95°C	15 seconds
	60°C	1 minute
	95°C	15 seconds
	60°C	1 minute
	95°C	15 seconds
	60°C	15 seconds

At the end of the last cycle, a melt curve analysis (dissociation curve) was performed to assess the specificity of the amplification of each pair of primers.
When the qPCR was finished, the Ct value of the DNA samples (10^6 copies) of each amplified gene using different primer concentrations was determined to select the optimal primer concentration using the software 7500 Fast System SDS (version 1.3.1).

4.4.5 PREPARATION OF THE STANDARD CURVES

Once the optimal concentration of primers was established, the amplification efficiency of each gene was determined by standard curves. From the plasmid stock solution of 5 x 10^8 copies/µl, different serial dilutions were prepared to construct the standard curve (Figure 9). Serial decimal dilutions from 5 x 10^8 copies/µl until 5 x 10^2 copies/µl were made.

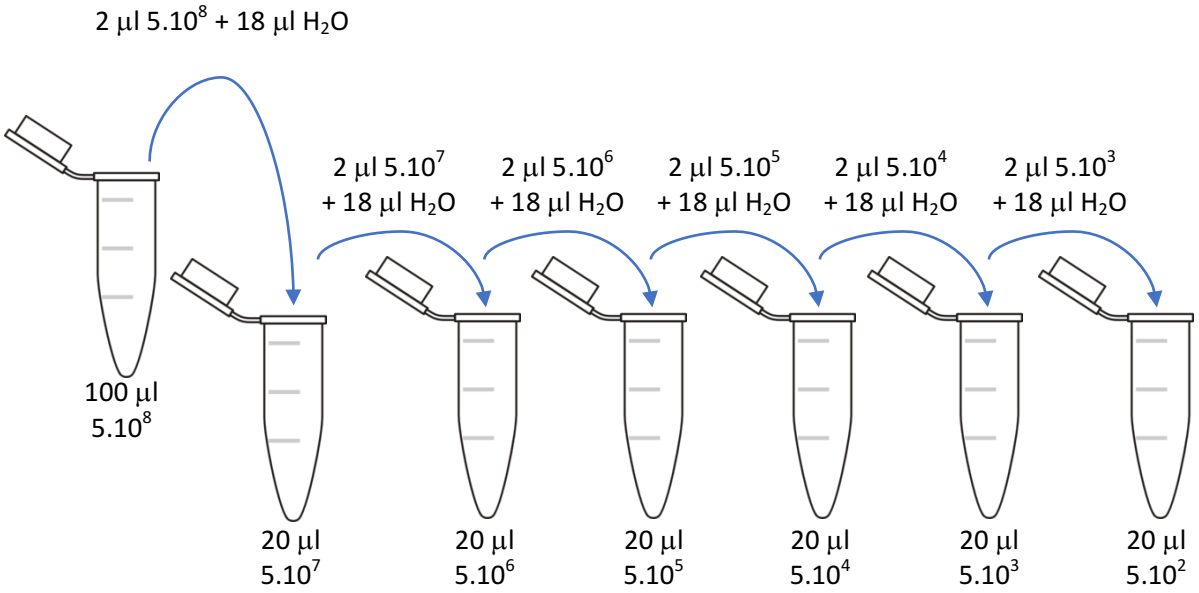


Figure 9 Preparation plasmid dilutions

The amplification was performed as explained before and again, the Ct values were determined and used to draft the standard curves.

The standard curves were obtained using the software attached to the qPCR device. To acquire a representative calibration curve, some parameters needed to be considered. A first checkpoint was the correction according the baseline. The next parameter was the formation of the curves. Next, the melting temperature of the amplified gene needed to be checked. All the temperatures that deviated from this value represented other sequences that were amplified and needed to be left out. Finally, some values needed to be respected as well. The threshold value had to be between 0,1 and 0,2.

Each curve represented a linear equation with a slope and an intercept. Using the slope, the efficiency of the amplification of each gene could be calculated with a simple formula:

$$Efficiency\ (\%) = \ (10^{(-\frac{1}{slope})} - 1) * 100$$

The value for the efficiency lies between 0 and 100.

4.5 SELECTION OF REFERENCE GENES

Before a gene can be chosen as a reliable reference, it must satisfy various criteria of high importance. One of the most crucial factors is that its expression level isn't influenced by experimental factors. Therefore, the objective was to use different growth conditions to culture target bacteria in order to check the influence of this factors in the expression level of the reference gene.

4.5.1 SELECTION OF CULTURE MEDIA

Strains CM160 and SE303 were grown in five different media: LBP (Lactose-Bromcresol-Purple), MRS (De Man-Rogosa-Sharpe), a tenth dilution of MRS, MRS0.2c containing the same components as MRS except containing ten times less glucose and a modified receipt of MRS0.2c (Roselló, 2016).

An overnight culture for each strain in each media was prepared. After an incubation of 24 hours at 30°C in agitiation of 100 rpm, the absorbance of each overnight culture was adjusted to an OD_{600nm} of 0,150. It is known that when the absorbance reaches 0,150, the culture contains 10^8 cfu/ml. Each media was inoculated with a 10% (v/v) preculture. Wells of microplates were filled with 180 µl of the corresponding media and 20 µl of preculture at an absorbance of 0,150. Growth was monitored at 30°C for 48 hours using a Bioscreen C microplate reader (Labsystems, Helsinki, Finland) by means of measurements of OD_{600nm}. A vibrational shaking of 20 seconds was programmed before OD reading to prevent cell sedimentation. OD was measured with intervals of 15 minutes for each inoculated medium. Maximum OD were determined from OD measurements. Five replicates of each media were performed. For each strain the first five wells were filled with culture in MRS, followed by each five wells for LBP, MRS0.2, MRS tenth dilution and MRS0.2c. Also, a negative control which only contained media was included.

4.5.2 QUANTIFICATION OF THE EXPRESSION OF THE REFERENCE GENES

Two media and two temperatures were chosen for the evaluation of the expression of the reference genes for CM160 strain. In Figure 10 the different steps performed are schematized. Further, every step will be explained individually.

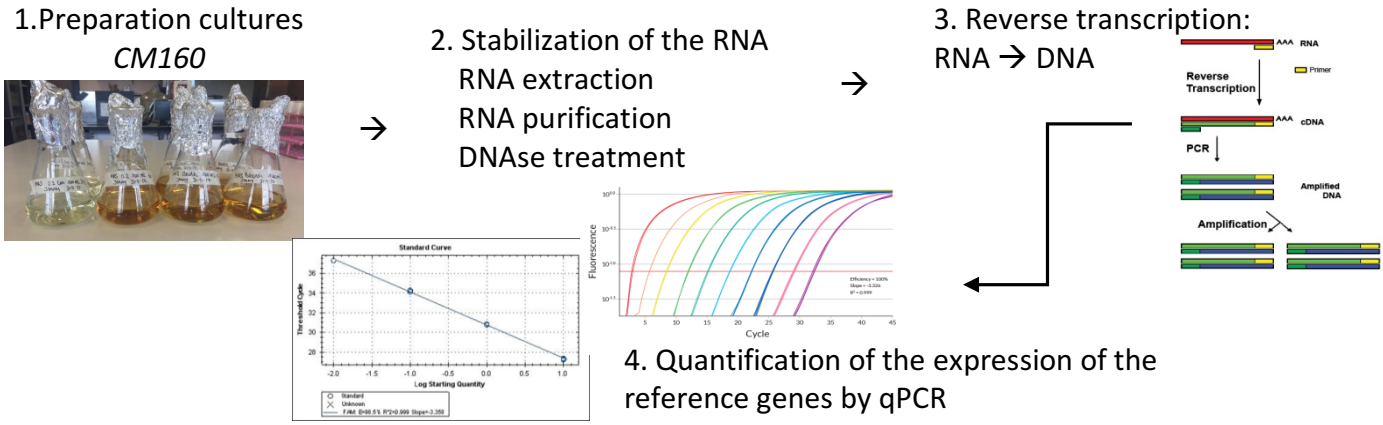


Figure 10 Schematic for the quantification of reference genes in different conditions

4.5.2.1 Preparation of the cultures and stabilization of the RNA

The two media that were chosen for the preparation of the cultures of *L. mesenteroides* CM160 are MRS and MRS0.2. Also, two temperatures, 20°C and 37°C were tested. This results in a total of four conditions.

First, the strain was grown on MRS plates and a preculture was made of 20 ml for each corresponding media. Culture from the plates was added with a loop and homogenized by vortexing until the value for OD_{600nm} reached 0,2. Then, the rest of each preculture was distributed equally in the two autoclaved Erlenmeyer flasks that each contained 100 ml of the complementary media. For each media one Erlenmeyer was incubated at 20°C and one at 37°C while shaking at 100 rpm. The absorbances for 600 nm were measured every half hour. In this way, a growth curve was drawn for each condition and the growth rate (μ) was calculated. At the beginning the absorbance and the cfu/ml at time zero were measured for each media, the samples were serially diluted and appropriate dilutions were seeded on MRS plates. These plates were incubated at 30°C for 24 hours. Colony forming units were counted and cfu/ml was calculated.

During the experiment, samples of 500 μl of culture were taken for RNA extraction when an absorbance (OD_{600nm}) around 0,2 was attained. This was done for two following measurements to obtain two RNA samples for each condition. Also, when samples were taken for RNA, cell counting was performed. This was repeated at the end of the experiment, when the highest optical density for each culture was measured.

To stabilize the RNA samples, 1000 μl of RNA protect was added into the RNase free tube with 500 μl of culture immediately after taking the sample. Next, the tubes were vortexed for 5 seconds followed by an incubation of 5 minutes at room temperature. After a centrifugation step of 10 minutes at 8000 rpm, the supernatant was decanted, the tubes left to dry and stored at -20°C.

4.5.2.2 Extraction of RNA and synthesis of DNA

This protocol is analogue to the one explained in 4.3.

4.5.2.3 Quantification of the expression of the reference genes by qPCR

This protocol is analogue to the one explained in 4.4.4. The same mix was prepared using the corresponding primers with the optimal concentration for each gene. This means that for each gene the mix was prepared separately. As described in 4.4.4.1, 18 μl of mix is added in each well together with 2 μl of sample. Here, the samples are the $_c$DNA samples for each condition.

Since the sequence of all the reference genes are inside the genome of the strain, CM160, the corresponding gene will be amplified when the correct primers are added into the mix. In this way, the expression of the reference gene can be evaluated for each condition.

4.6 PREPARATION OF THE DIFFERENT CULTURE MEDIA, BUFFERS AND OTHER USED REAGENTS

4.6.1 CULTURE MEDIA

De Man – Rogosa – Sharpe (MRS agar) (Panreac, Barcelona, Spain)
10 g/l peptone, 8 g/l beef extract, 4 g/l yeast extract, 20 g/l glucose, 1 ml polysorbate 80, 2 g/l K_2HPO_4, 5 g/l $C_2H_3NaO_2 \cdot 3H_2O$, 2 g/l $C_6H_{17}N_3O_7$, 0,2 g/l $MgSO_4 \cdot 7H_2O$, 0,05 g/l $MnSO_4 \cdot 4H_2O$ and 10 g/l agar. Sterilization of the media in the autoclave during 20 minutes at 121°C.

De Man – Rogosa – Sharpe 0.2c (MRS0.2)
2 g/l glucose, 2 g/l K_2HPO_4, 5 g/l $C_2H_3NaO_2 \cdot 3H_2O$, 2 g/l $C_6H_{17}N_3O_7$, 0,2 g/l $MgSO_4 \cdot 7H_2O$, 0,05 g/l $MnSO_4 \cdot H_2O$, 3 g/l casamino acids.

De Man – Rogosa – Sharpe 0.2c modified (MRS0.2cm) (G. Roselló Prados, 2016)
5 g/l glucose, 2 g/l K_2HPO_4, 5 g/l sodium acetate, 2 g/l $C_6H_{17}N_3O_7$, 0,2 g/l $MgSO_4 \cdot 7H_2O$, 0,05 g/l $MnSO_4 \cdot H_2O$, 12 g/l casamino acids.

LBP (Lactose-Bromcresol-Purple)
20 g/l tryptone, 5 g/l yeast extract, 10 g/l lactose, 2,5 g/l gelatin, 0,4 g/l NaCl, 2,5 g/l sodium acetate. Ajust pH to 7.

Luria Bertani agar (LB agar)
10 g/l tryptone (Oxoid, Hampshire, UK), 5 g/l yeast extract (Oxoid), 10 g/l NaCl (Merck, Darmstadt, Germany) and 15 g/l agar (Oxoid). Sterilization of the media in the autoclave during 20 minutes at 121°C.

Luria Bertani agar with ampicillin (LB agar + ampicillin)
10 g/l tryptone (Oxoid), 5 g/l yeast extract (Oxoid), 10 g/l NaCl (Merck) and 15 g/l agar (Oxoid). Sterilization of the media in the autoclave during 20 minutes at 121°C. Add 100 mg/l of the antibiotic ampicillin dissolved in distilled sterile water (Sigma-aldrich, Schnelldorf, Germany).

Luria Bertani broth (LB broth)
Same ingredients like LB agar, but without agar.

Luria Bertani + 20% glycerol (LB broth + 20% glycerol)
Same ingredients like LB agar, but before adding distilled water until 100 ml, 25,18 g of glycerol is added.

4.6.2 BUFFERS

DNA extraction buffer (Llop et al., 1999)
200 mM Tris HCl, 250 mM NaCl, 25 mM EDTA, 0,5% Sodium dodecyl sulphate (SDS), 2% Polyvinylpyrrolidone (PVP), PH 7,5. Sterilize by filtration.

DNA loading buffer (Glycerol 30%, bromophenol blue 0,25% and xylene cyanol 0,25%)
30 ml glycerol, 0,25 ml bromophenol blue, 0,25 ml xylene cyanol in 100 ml of Milli-Q water.

TE buffer 50X
121 g Tris, 28,55 ml glacial acetic acid and 50 ml EDTA 0,5 M pH 8.0 in 350 ml of Milli-Q water.

TE buffer
30 mM Tris·C, 1 mM EDTA, pH 8.

4.6.3 OTHER REAGENTS

1KB DNA LADDER (50 ng/μl)
10 μl of 1KB plus Ladder, 40 μl loading buffer and 150 μl of water

SYBR® Safe DNA gel stain (Invitrogen™, Applied Biosystem, Foster City, EUA).

β-mercaptoethanol (Sigma-aldrich, St. Louis, USA).

5 RESULTS AND DISCUSSION

5.1 STANDARD CURVES OF REFERENCE GENES

E. coli cells were transformed with *pSpark TA* vector to obtain enough DNA of selected reference genes to construct the standard curves. To check if the gene was ligated into the vector a PCR was performed on the colonies that have grown on the LB + ampicillin plates. In the case of *mesY* gene, 4 colonies were selected to be ampicillin resistant but only three (colonies 2, 3 and 4) of them contained the gene in the plasmid (Figure 11 above). In the case of *nisA* gene also three out of four colonies contained the gene in the plasmid (colonies 1,2 and 3) (Figure 12 above). Finally, also all colonies of *E. coli* transformed with the vector ligated with the reference genes had positive amplification (Figure 11 bottom).

Indeed, extracts of plasmidic DNA of the different target genes were obtained from *E. coli* cells to construct the standard curves.

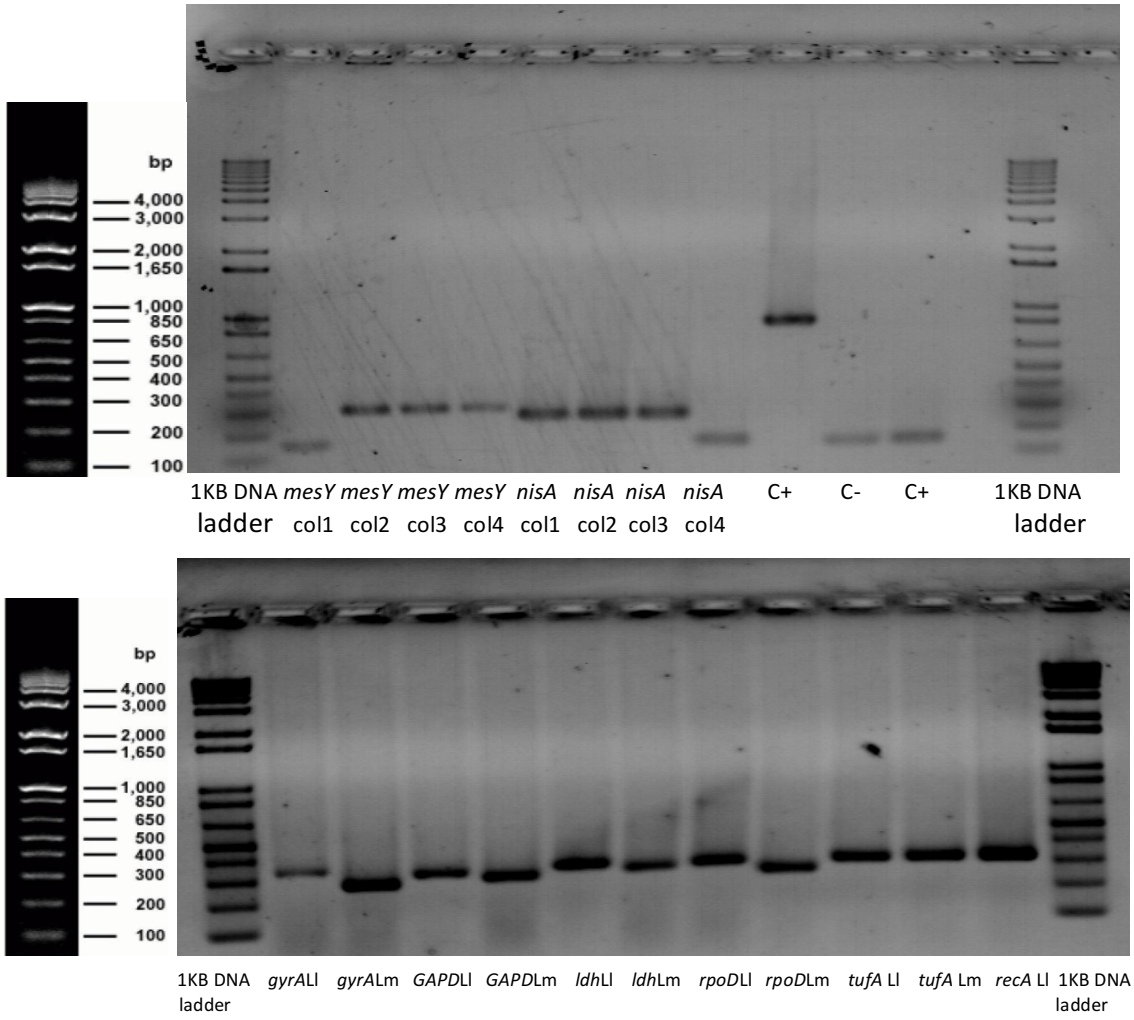


Figure 11 PCR analysis of E. coli cells transformed with pSpark TA vector including mesY, nisA genes (above) and reference genes (bottom)

The optimal primer concentration has been determined for each gene. It is intended to find the concentration that results in the lowest Ct. However, it is also preferred to use the lowest concentration of primer possible. For this, the difference in Ct should be high enough before a higher concentration is chosen. Table 10 and Table 11 give an overview of the different Ct values and melting temperatures for each primer concentration and each gene in SE303 and CM160 respectively. In Figure 12, Figure 13 and Figure 14 the different amplification plots with their corresponding dissociation curves are illustrated for each gene.

Besides the amplification curves, the dissociation curves need to be considered as well to verify the specificity of the technique. In the dissociation curves the melting temperature of the genes can is displayed. For all the samples of one gene the peak for the melting temperature should be alike. When different peaks are noticed, this indicates the amplification of other sequences then the target gene.

Based on the analysis of these curves the optimal primer concentrations are chosen for each gene. For *gyrA* Ll, *GAPD* Ll, *ldh* Ll, *tufA* Ll, *tufA* Lm and *recA* Ll the optimal concentration is 100 nM. *GAPD* Lm, *ldh* Lm, *rpoD* Lm and *rpoD* Ll have an optimal concentration of 300 nM and *gyrA* Lm amplifies optimal with a primer concentration of 500 nM.

Table 10 Ct values obtained from the amplification reaction of the reference genes for SE303 with DNA samples of 10^6 copies of the target gene (n=2). Melting temperatures of each reaction are in parentheses.

Primer concentration (nM)	***gyrA* Ll**	***GAPD* Ll**	***ldh* Ll**	***rpoD* Ll**	***recA* Ll**	***tufA* Ll**
100 nM	15,10 (77,5)	16,28 (76,5)	15,75 (77,7)	-	15,38 (79,2)	16,90 (79,7)
300 nM	-	15,29 (76,8)	15,77 (77,7)	16,72 (80,7)	14,87 (79,2)	16,72 (79,7)
500 nM	14,79 (77,5)	15,09 (76,8)	15,57 (77,6)	17,43 (80,6)	14,97 (79,1)	16,95 (79,5)
900 nM	14,15 (78,0)	14,90 (77,1)	15,83 (77,6)	16,89 (80,5)	15,13 (79,1)	16,94 (79,5)

Table 11 Ct values obtained from the amplification reaction of the reference genes for CM160 with DNA samples of 10^6 copies of the target gene (n=2). Melting temperatures of each reaction are in parentheses.

Primer concentration (nM)	***gyrA* Lm**	***GAPD* Lm**	***ldh* Lm**	***rpoD* Lm**	***tufA* Lm**
100 nM	21,50 (78,1)	17,14 (81,1)	16,61 (79,3)	18,15 (78,5)	16,27 (82,3)
300 nM	18,42 (78,1)	15,29 (81,2)	15,10 (79,2)	15,53 (78,5)	15,43 (81,8)
500 nM	17,36 (78,5)	14,45 (81,6)	15,11 (79,2)	15,66 (78,5)	15,31 (82,0)
900 nM	16,77 (78,7)	14,51 (81,8)	-	15,55 (78,5)	15,46 (82,0)

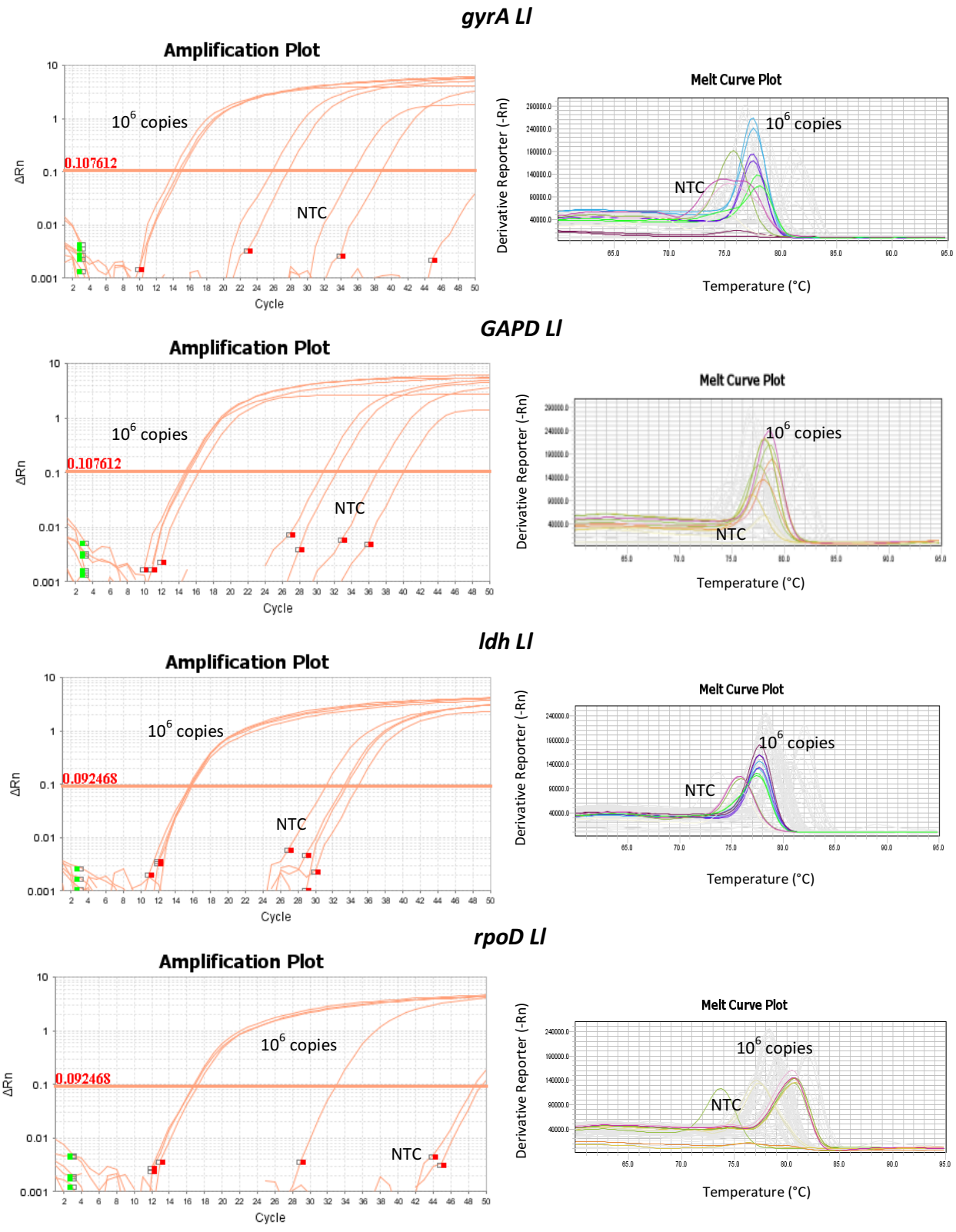
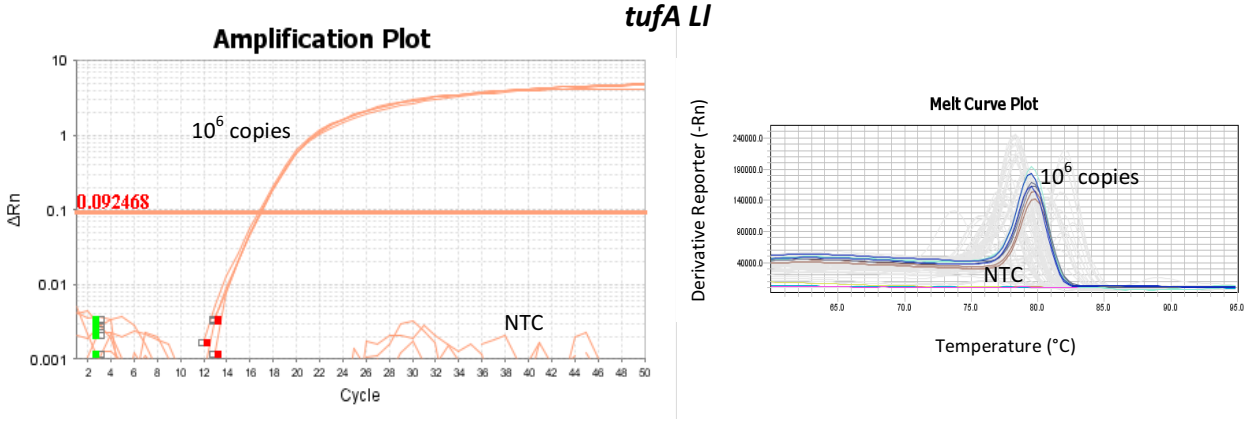
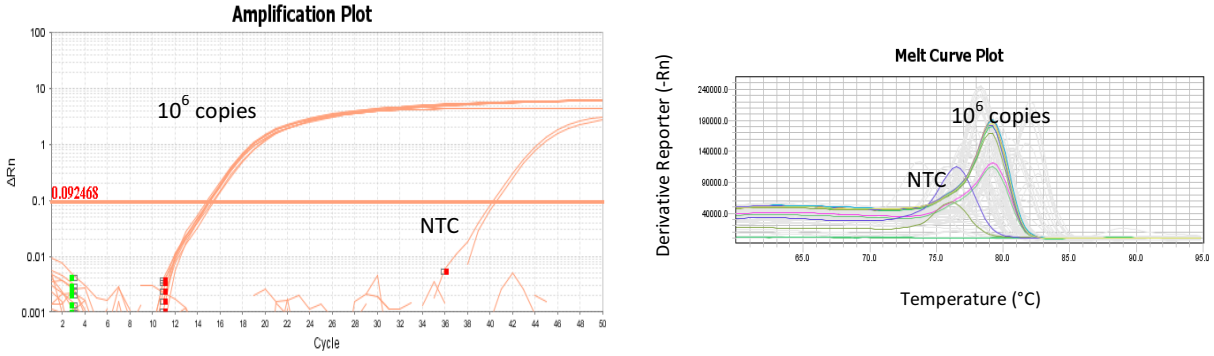


Figure 12 Amplification (left) and dissociation (right) curves corresponding to the DNA samples with 10^6 copies of the target gene for SE303 and the control negative with water (NTC) of each amplification reaction performed by qPCR of the genes: gyrA LI, GAPD LI, Ldh LI, rpoD LI using different concentration of specific primers (100, 300, 600 and 900 nM).

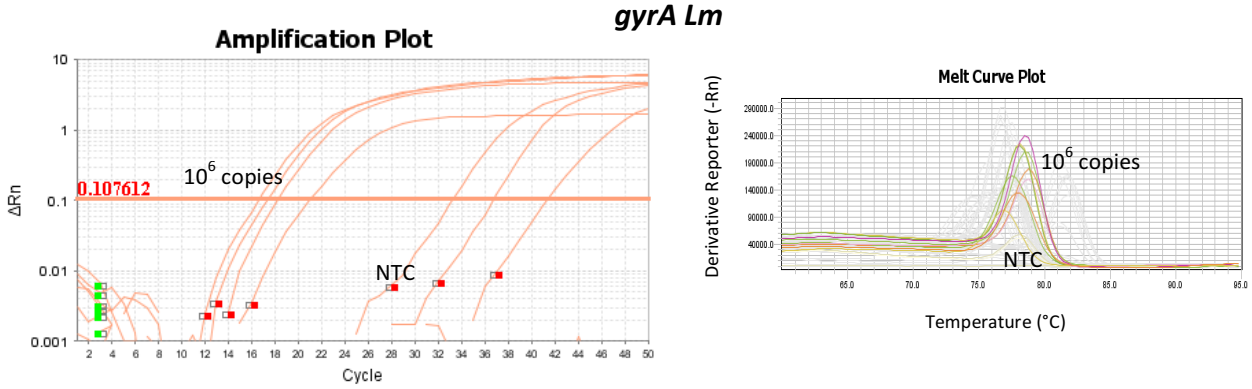
tufA LI



recA LI



gyrA Lm



GAPD Lm

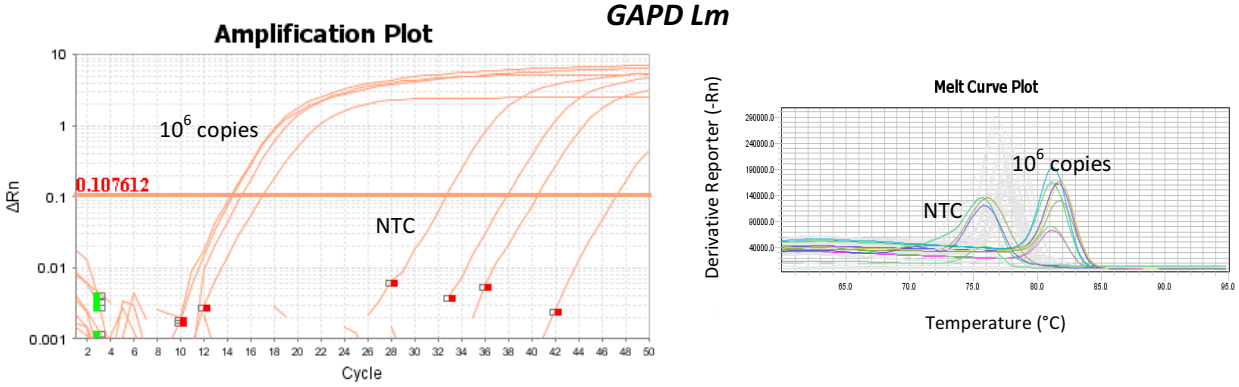


Figure 13 Amplification (left) and dissociation (right) curves corresponding to the DNA samples with 106 copies of the target gene for SE303 or CM160 and the control negative with water (NTC) of each amplification reaction performed by qPCR of the genes: tufA LI, recA LI, gyrA Lm and GAPD Lm using different concentration of specific primers (100, 300, 600 and 900 nM).

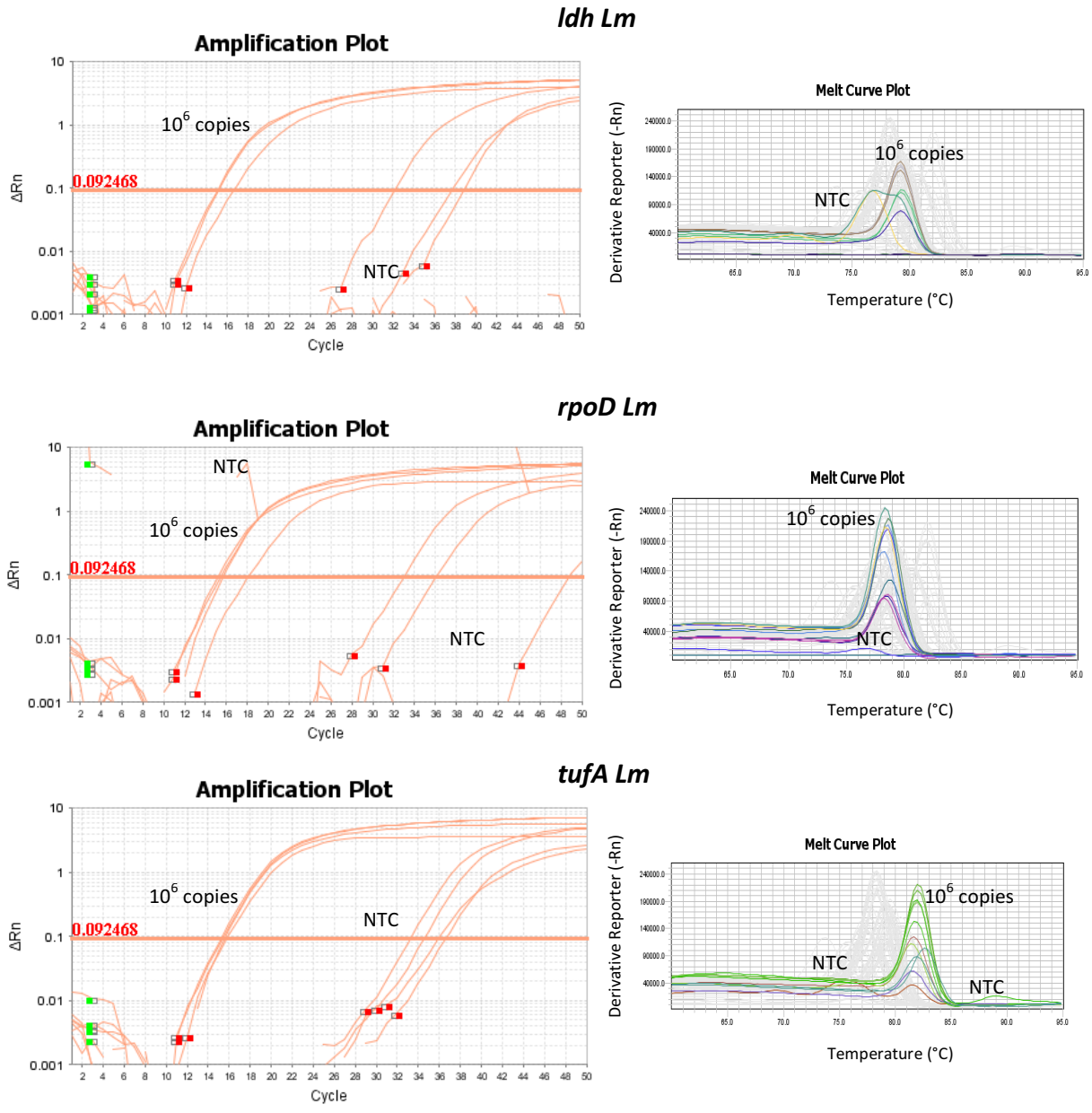


Figure 14 Amplification (left) and dissociation (right) curves corresponding to the DNA samples with 10^6 copies of the target gene for CM160 and the control negative with water (NTC) of each amplification reaction performed by qPCR of the genes: ldh Lm, rpoD Lm and tufA Lm using different concentration of specific primers (100, 300, 600 and 900 nM).

To check if the standard curve was prepared good enough to design the calibration curve, the amplification curves are checked. It is important that the shape of the amplification curves is correct and the space between the following curves is preferred to be as equidistant as possible. In Figure 15 and Figure 16 the amplification plot for each gene is shown.

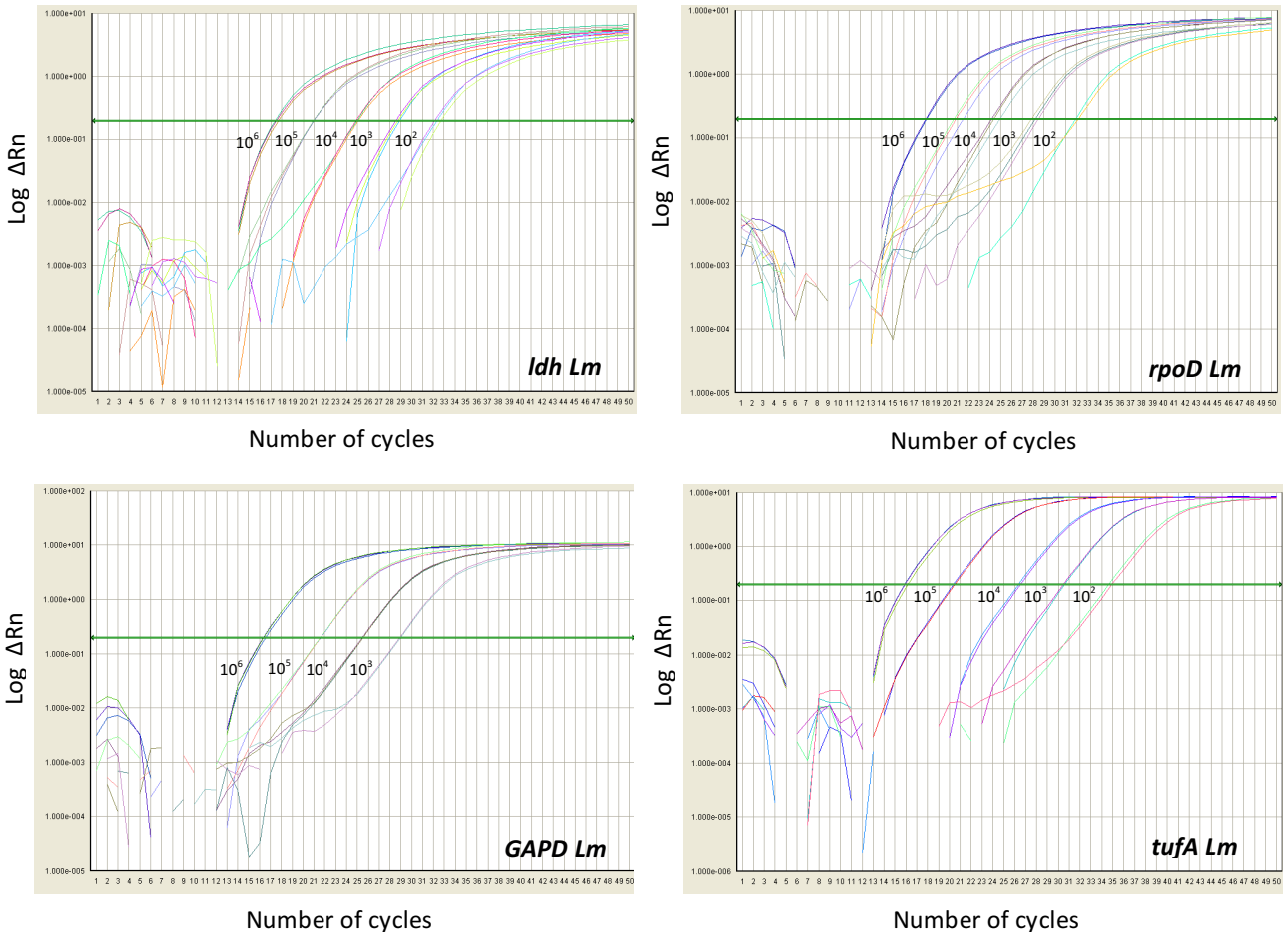


Figure 15 Amplification plots for the reference genes of L. mesenteroides CM160

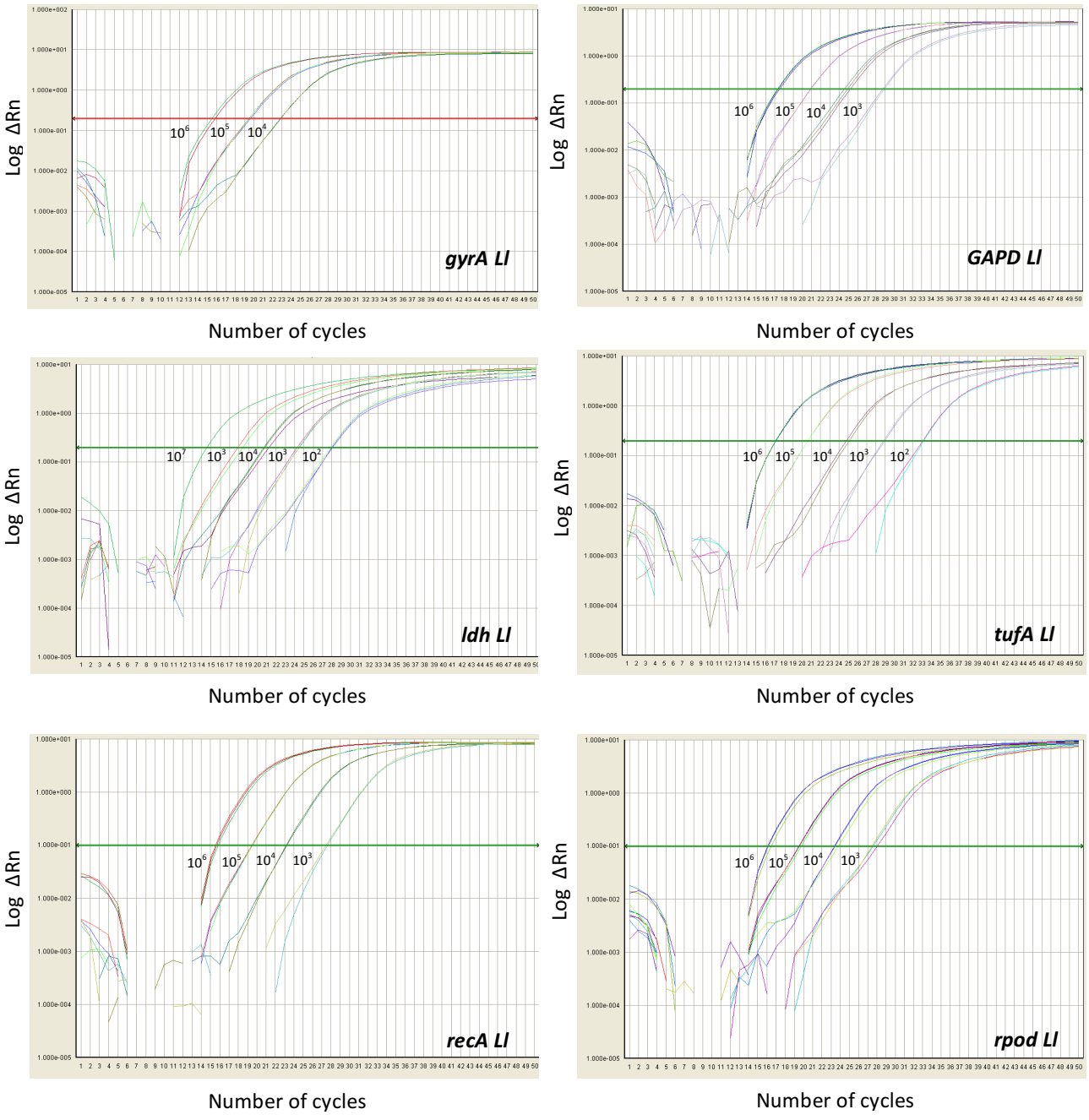


Figure 16 Amplification plots for the reference genes of L. lactis SE303

With the acquired amplification plots, the calibration curves can be designed. The various obtained calibration curves for all the reference genes can be assembled in one graph for each strain (Figure 17). In Table 12 the linear equations for the calibration curves of all the genes are summed up together with the linearity and efficiency. This efficiency is calculated with the slope of the linear equation as mentioned in the material and methods. For a reference gene to be useful, it is recommended that the value for the efficiency lies between 80% and 100%.

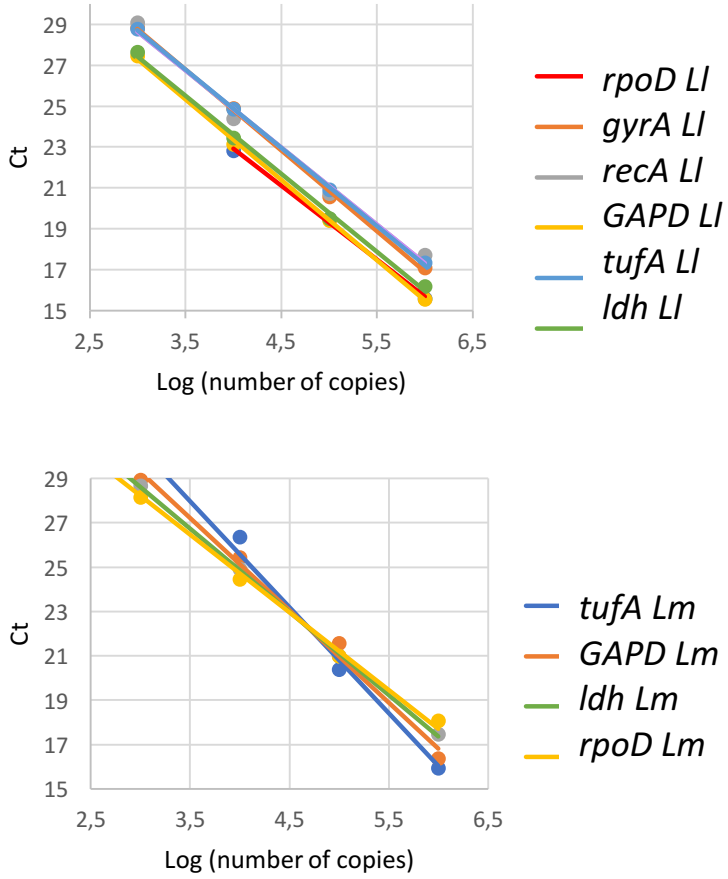


Figure 17 Calibration curves of the reference genes for L. lactis SE303 (above) and L. mesenteroides CM160 (bottom)

Table 12 Overview of the linear equation, linearity and efficiency for each reference gene

Gene	Linear equation	Linearity R^2	Efficiency (%)
rpoD Ll	y = -3,830644x + 38,935860	0,995255	82,41%
gyrA Ll	y = -3,620934x + 39,785244	0,961119	88,87%
recA Ll	y = -3,938108x + 39,126232	0,998712	79,44%
GAPD Ll	y = -3,816553x + 40,150581	0,997988	82,82%
tufA Ll	y = -3,229147x + 37,824730	0,940510	95,98%
ldh Ll	y = -3,343099x + 37,842720	0,995852	99,12%
tufA Lm	y = -3,723932x + 40,065113	0,959466	85,58%
GAPD Lm	y = -3,944520x + 39,952770	0,996396	79,27%
ldh Lm	y = -3,745746x + 39,845360	0,999041	84,91%
rpoD Lm	y = -3,508253x + 38,706787	0,993770	92,77%

5.2 SELECTION OF REFERENCE GENES

Different growth conditions were used to culture target bacteria in order to study the influence of the culture conditions in the expression level of the reference genes.

Different media were used to culture the strains CM160 and SE303 in order to select one media allowing an optimal growth and another with a low growth. In Figure 18 (above) the growth curves for SE303 are shown and in Figure 18 (bottom) the ones for CM160. The best media for SE303 was LBP in which the strain had a maximal OD of 1. MRS was the optimal media for CM160, in this case also the maximal OD was around 1. The media with the lowest growth were MRS 1/10 for SE303 and MRS0.2 for CM160.

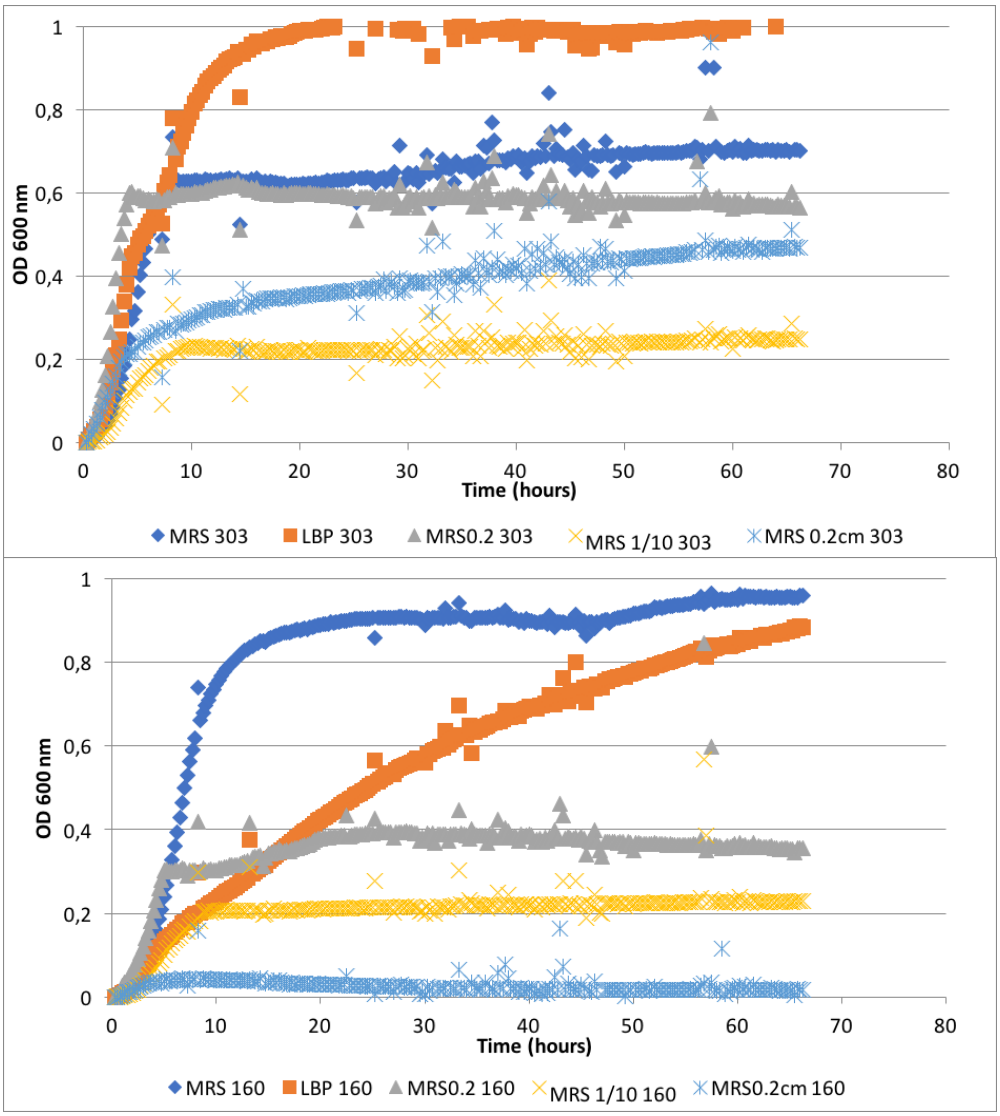


Figure 18 Growth curves in different media for) SE303 (above) and CM160 (bottom)

CM160 strain was cultured in two media and two temperatures in order to verify that the expression level of reference genes was not influenced by different conditions. The media selected were MRS and MRS0.2 and the temperatures were 20°C and 37°C (Figure 19). The different conditions used in this experiment allowed the strain CM160 to grow at different rates. CM160 reached the highest value of optical density and cfu/ml in MRS at 37°C after 7,5 h of growth whereas in the same media at 20 °C attained the lowest OD value and cfu/ml (Table 13).

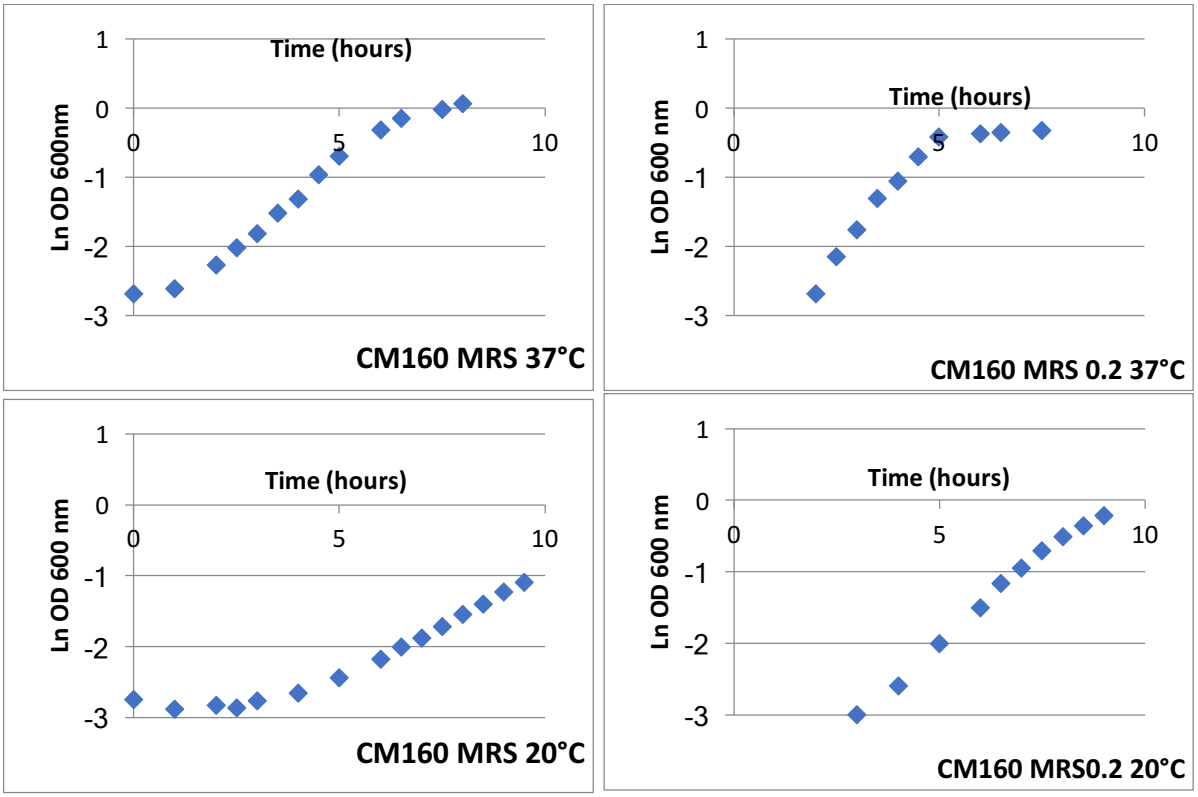


Figure 19 Growth curves of CM160 in different conditions

Table 13 Overview of the different values for OD_{600nm} at 7,5 hours, maximum cfu/ml, linear equation and linearity

Media	Temperature	OD_{600nm} t = 7,5 h	μ (h^{-1})	Cfu/ml (max)	Equation	R^2
MRS	20°C	0,18	0,29	$2,3 * 10^8$	y = 0,2919x – 3,89	0,996
	37°C	0,98	0,48	$6,8 * 10^8$	y = 0,4752x – 3,17	0,994
MRS0.2	20°C	0,50	0,50	$5,5 * 10^8$	y = 0,4983x – 4,49	0,998
	37°C	0,72	0,78	$6,9 * 10^8$	y = 0,7849x – 4,17	0,993

The expression of the reference genes (*rpoD, GAPD, ldh* and *tufA*) was quantified by qPCR in cells of CM160 cultured in different conditions. In Table 14 the different Ct values are summed up for each gene in each condition. To quantify the level of expression of reference genes, samples of CM160 cells were collected around the same concentration. The most suitable reference gene was selected taking into account that the growth of cells under different conditions will not influence the expression of genes. In general, *GAPD Lm* showed the best results and can be considered as a suitable internal control in the expression of *L. mesenteroides* CM160. The Ct values are very similar for each condition except for MRS0.2(37°C). However, this bouncer is found for the 4 genes. A reason could be the RNA concentration which is significantly lower than the other ones. Therefore, these values could be eliminated. The other genes exhibit fluctuating Ct values and are not suitable for the expression of *L. mesenteroides* CM160.

Table 14 Overview of the mean Ct values for each condition together with OD_{600nm}, cfu/ml and RNA concentration (ng/µl) for rpoD Lm, ldh Lm, GAPD Lm and tufA Lm

Media	T (°C)	OD_{600nm}	cfu/ml	[RNA] (ng/µl)	Mean Ct *rpoD Lm*	Mean Ct *GAPD Lm*	Mean Ct *ldh Lm*	Mean Ct *tufA* Lm
MRS	20°C	0,213	$1,3 * 10^8$	10,8	18,56	15,18	21,87	15,04
		0,245	$1,5 * 10^8$	9,2	20,53	16,62	23,72	16,82
	37°C	0,219	$9,0 * 10^7$	9,4	18,20	15,01	20,02	15,55
		0,269	$1,5 * 10^8$	15,0	17,04	14,04	18,76	-
MRS0.2	20°C	0,224	$1,3 * 10^8$	14,5	16,48	14,30	18,71	13,57
		0,314	$2,2 * 10^8$	23,4	16,14	14,06	18,31	13,40
	37°C	0,270	$1,2 * 10^8$	**2,3**	**32,14**	**26,27**	**35,18**	**29,31**
		0,347	$2,3 * 10^8$	12,7	17,20	13,97	19,38	13,74

6 CONCLUSION

The primer concentration for the amplification of reference genes for *L. lactis* SE303 was optimal at 100 nM for gyrA, *GAPD*, *ldh*, *tufA*, and *recA* Ll and at 300 nM for *rpoD*. In the case of *L. mesenteroides* CM160, *tufA* had an optimal primer concentration of 100 nM, *GAPD*, *ldh* and *rpoD* of 300 nM and *gyrA* of 500 nM. The efficiency of amplification lies between 80% and 100% for all the studied genes.

rpoD, GAPD, ldh and *tufA* were validated as reference genes for expression studies in *L. mesenteroides* CM160. The expression of *GAPD* was not affected by growth conditions therefore, it could be a suitable reference gene for the normalization of the quantification of the expression of *mesY*, produced by CM160.

Future perspectives on this project are the validation of the reference genes for Lactococcus lactis SE303 and the quantification of the expression for *mesY* and *nisA*.

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