Development of a high-throughput screening Queso Fresco model for evaluating antimicrobials

Degree Project in MSc Biotechnology Engineering Programme

Jonna Östberg

Division of Applied Microbiology

Department of Chemistry

Faculty of Engineering

LUND UNIVERSITY

Supervisors
Dr. Michael J. Miller
Associate Professor
Department of Food Science and Human Nutrition, the University of Illinois at Urbana Champaign

Dr. Jenny Schelin
Associate Professor
Division of Applied Microbiology
Department of Chemistry, Faculty of Engineering, Lund University

Examiner
Prof. Peter Rådström
Head of the Division of Applied Microbiology
Division of Applied Microbiology
Department of Chemistry, Faculty of Engineering, Lund University

Abstract

Listeria monocytogenes is a pathogen, sometimes occurring in fresh cheese, such as Queso Fresco. Antimicrobials could be utilized to prevent the presence of *L. monocytogenes* in this type of cheese. In order to be able to screen and assess several novel antimicrobials simultaneously in the future, the validated miniaturized laboratory-scale model for Queso Fresco (MLQF) was further developed into a high-throughput screening model (HTQF). This included studying and comparing the microbial behavior of *L. monocytogenes* 10403S (LM), *L. monocytogenes* 10403S expressing GFP (LM-GFP), and a cocktail of five *L. monocytogenes* (LM-COCKTAIL). Additionally, analysis of luminescent bacteria (LUX) was also part of the study, however, not luminescent *L. monocytogenes*. LM-GFP and LUX was studied due to their light-emitting properties, which enables an efficient screening with a fluorometer instead of performing time consuming plating. In the HTQF, the Queso Fresco was produced in 96-well microplates, with a size of 0.05 ± 0.0004 g/cheese.

The strains and the model were evaluated with three different antimicrobial treatments; Lauric arginate, Ɛ-polylysine and a combination of these two. The aim for the combination of the antimicrobials was to have a variety in order to be able to see if the method is applicable in different scenarios. Preferably one antimicrobial without effect, one bacteriostatic, and a combination.

The result showed that the engineered LM-GFP had similar growth behavior as the parental strain. This was true both with and without treatments. They both grew 4 log CFU/g under no antimicrobial treatment for 28 days. The LM-COCKTAIL was comparable to these two for the control cheese (no treatment), with a growth of 4 log CFU/g and for one out of three treatments (lauric arginate), also for 28 days. The microbial behavior of the LM-COCKTAIL in the Queso Fresco produced in 96-well microplates needs further investigation in order to conclude its correlation to the other *L. monocytogenes* strains in this model. The fluorescence measurements with the LM-GFP gave a result which was difficult to correlate to the viable count (VC) of the same strain. However, the emitted light and the VC from the LUX suggested a better correlation.

In conclusion, based on the comparison of the result from the LM-GFP and the LUX, the luminescent reporter system seems to be preferable to the fluorescent in this HTQF model.

Key words: Queso Fresco, *Listeria monocytogenes*, fluorescence, luminescence, antimicrobial

Preface

This master's thesis was conducted at the Department of Food Science and Human Nutrition at the University of Illinois at Urbana-Champaign (UIUC), Illinois, USA during the spring of 2018. It is registered as the course "KMBM05" and is the last step towards acquiring an MSc in Engineering, Biotechnology, from the Faculty of Engineering (LTH), Lund University.

I would like to thank and acknowledge Luis Alberto Ibarra Sanchez for all the extensive help with the laboratory work and cheese expertise. A great thanks to Shannon Rezac and Maxwell Holle, for the close collaboration, rewarding discussions, support, and proofreading. Thanks to everyone in Dr. Miller's laboratory for all the valuable insights and kindness. Also, a special thank you to my supervisors Dr. Jenny Schelin and Dr. Michael J. Miller for all their assistance and guidance throughout the whole process and continuous encouragement. An extra thanks to Dr. Michael J. Miller for the opportunity of performing this project at UIUC.

I would also like to thank my examiner Prof. Peter Rådström.

You have all been essential during my thesis.

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List of abbreviations

ATP	Adenosine triphosphate
A_w	Water activity
BHI	Brain Heart Infusion
BHICM	Brain Heart Infusion with Chloramphenicol
CFU	Colony Forming Unit
CM	Chloramphenicol
EPL	Ɛ-polylysine
FDA	Food and Drug Administration
FER	Ferulic acid
GFP	Green Fluorescent Protein
HSFC	Hispanic-style fresh cheeses
HTQF	High-throughput Queso Fresco
LAE	Lauric arginate
LB	Luria-Bertani
LM	*L. monocytogenes* 10403S
LM-COCKTAIL	*L. monocytogenes* cocktail of five strains
LM-GFP	*L. monocytogenes* 10403S expressing GFP
LUX	Bacteria that exhibit the property of luminescence
MJM	Michael J. Miller culture collection
MLQF	Miniaturized Laboratory-scale Queso Fresco
NIS	Nisin
NRRL	The Agricultural Research Service Culture Collection (ARS)
OD	Optical Density
PALCAM	Polymyxin Acriflavin Lithium-chloride Ceftazidime Esculin Mannitol
PBS	Phosphate-buffered saline
ppm	Parts Per Million
QF	Queso Fresco
RFU	Relative Fluorescence Unit
RLU	Relative Light Unit
rpm	Revolution Per Minute
SEM	Standard Error of the Mean
UIUC	University of Illinois at Urbana-Champaign
VC	Viable Count

1. Introduction

It is well established that *Listeria monocytogenes* is a common pathogen in nature, and might contaminate foods. Every year it causes several outbreaks worldwide and could have a fatal outcome (CDC, 2017). In the United States, *L. monocytogenes* is the second most common bacteria leading to death caused by outbreaks (Scallan et al., 2011). *L. monocytogenes* is most dangerous for immunocompromised, pregnant or elderly individuals (CDC, 2017).

In the US, there is a zero tolerance policy for *L. monocytogenes* in ready-to-eat foods according to the Food and Drug Administration (FDA) (FDA, 2016). However, in Europe the tolerance level is <100 Colony Forming Unit per g (CFU/g) during the shelf-life of the product (EC No 2073/2005). These legislations prove the necessity not only for antimicrobial treatments against *L. monocytogenes* in the food industry, but also continuous monitoring and carefully controlled hygiene and cleaning routines in the processing areas.

Soft cheeses are prevalent food vehicles associated with *Listeria* outbreaks. From 2009 to 2011 twelve outbreaks were reported in the US, five of which soft cheese, made from pasteurized milk, was the source (CDC, 2013). In 2014, investigations revealed 25% of the outbreaks were caused by Mexican-style cheese (CDC, 2015). Queso Fresco (QF) is one of the most common Hispanic-style fresh cheeses (HSFC) in the United States. The favorable growth conditions for *L. monocytogenes* in QF, combined with the fact that *L. monocytogenes* is a psychrotrophic and can therefore grow despite the refrigerated storage of the cheese contribute to the prevalence of these outbreaks (Ibarra-Sánchez et al., 2017). Additionally, the trend of *L. monocytogenes* outbreaks for QF can be connected to the increasing demographic population in the US (MacDonald et al., 2005).

The research group of Dr. Miller's laboratory at the University of Illinois at Urbana-Champaign (UIUC) have performed and published research on *L. monocytogenes* control in QF. This research confirms the growth conditions for *L. monocytogenes* in this cheese and also suggests promising antimicrobials for this purpose. A modular, miniaturized laboratory-scale QF model (MLQF) was developed for testing the incorporation of novel antimicrobials (Van Tassell et al., 2015). The model and research have been utilized as a foundation for this study – the development of a new, high-throughput screening model for QF (HTQF). The development of the HTQF could enable a more efficient screening of a wide range of potential antimicrobials. This study was carried out to design a model, based on exploiting light-emitting *L. monocytogenes.*

Both bioluminescent and fluorescent bacteria emits light, but differently. An advantage with bioluminescence compared to fluorescence is the more accurate measurement of the emitted light. This is because the bioluminescence is Adenosine triphosphate-dependent (ATP), and therefore, only the intact and viable cells will produce light. Fluorescence however, arises when a fluorophore is excited by light, and emits light while falling back to the ground state. This mechanism suggests that the intensity of the fluorescence signal might be expressed even from dead or dying cells, due to the stability of the Green Fluorescent Protein (GFP) as a reporter system. The wild-type protein has a half-life greater than 24 hours (Corish and Tyler-Smith, 1999; Lesmana and Friedl, 2001). The consequence can be an inaccurate fluorescence detection. Bioluminescence does not require excitation

by light, compared to fluorescence which needs the absorption of photons in order to produce light. Another challenge with measuring fluorescence is autofluorescence, which is background light and contributes to the emitted light, in a non-specific manner. Autofluorescence does not exist for bioluminescence, which is an advantage, since no background light needs to be taken into consideration. However, the bioluminescence might give a low level of emitted light and therefore a weak signal, compared to the signal intensity from fluorescence (Imaging & Microscopy - Research, Development, Production, 2013).

This study included two separate screenings; a preliminary and a final evaluation of the screening method.

1.1 Queso Fresco

Queso Fresco is a type of HSFC. The properties of HSFCs, such as QF, are similar for all cheese within this category. They have a pH close to neutral (6.0-6.5), are perishable with high moisture content (45-55%) and a low salt content. Additionally, they also have a high water activity (A_w) (Soni et al., 2012).

MLQF has been evaluated in comparison to the traditional scale produced QF, in terms of dry matter, fat and protein content (Van Tassell et al., 2015) (Table 1). The composition of the cheese produced in the MLQF is confirmed to be similar to the commercial QF and can therefore be considered to be representative of the original commercial cheese (Van Tassell et al., 2015).

QF found in grocery stores local to the Urbana-Champaign area show that the majority of commercial QF contains no preservatives or antimicrobials. Out of the ones found in this area, only "Fud, Queso Fresco" and "La Chona, Fresco" have potassium sorbate as a preservative. Other brands of QF contain only milk, salt, and enzymes. Locally 5 out of 9 brands contain cultured milk (addition of a culture of bacteria instead of pasteurize it), the rest use pasteurized milk (Holle et al., 2018).

Table 1. Comparison of protein and fat contents and dry matter of miniaturized laboratory Queso Fresco (MLQF) and a traditional-scale Queso Fresco*. The values are means ± SEM displayed (Van Tassell et al., 2015).*

Item	MLQF	Traditional scale
DM (%)	44.43 ± 1.16	46.14 ± 0.56
Protein (%)	18.25 ± 0.31	18.34 ± 0.23
Fat (%)	22.35 ± 1.14	23.54 ± 0.17

1.2 *Listeria monocytogenes* and Food Safety

Listeria monocytogenes is a gram-positive bacterium and a human pathogen, which can cause listeriosis. It is facultatively anaerobic and has the ability to grow over a wide range of temperature, from 0-42°C. *L. monocytogenes* is also salt tolerant and can survive a 16% salt solution at pH 6.0. To develop an infection of listeria, it is not known exactly what the minimum infective dose is. Based on

the cell number in analyzed outbreaks, it is assumed to be relatively high, 10^3 CFU/g or greater. The incubation time varies from 1 to 90 days and this wide range of time makes it very hard to identify food vehicles of outbreaks. Listeriosis is most dangerous for pregnant women, elderly or immunocompromised. The symptoms can vary from a flu-like illness to meningitis. If a pregnant woman is infected, it can transfer to the fetus either through the birth canal or after birth. This could result in abortion, stillbirth or premature labor. Listeriosis in adults has a high mortality rate (Adams and Moss, 2008).

Different microbes, such as bacteria, yeasts, and molds, can contaminate and spoil food. The composition and physico-chemical properties of the food is the main factor enabling the spoilage. Bacteria thrive in foods with high protein content, high a_w, and with a neutral pH. As mentioned, QF has several of the intrinsic factors suitable for *Listeria* and therefore has a high risk of supporting growth of this pathogen. Extrinsic factors, such as temperature are also important to consider when concerning food safety and quality. The storage of QF affects the microbes ability to grow and survive (Adams and Moss, 2008). *L. monocytogenes'* ability to grow under refrigeration conditions contributes to increased *L. monocytogenes*-associated food safety risk (Ibarra-Sánchez et al., 2017). *L. monocytogenes* can even grow in packages with modified atmospheres. It is naturally occurring in nature, and can be found in soil, water, or animals. Its ability for adhesiveness on different surfaces, forming biofilms, increases the risk of its presence in facilities or on equipment where food is produced and handled. It is persistent and hard to eliminate. These are risk factors to why *L. monocytogenes* is a contamination risk for not only QF but also food in general (SVA, 2017).

During the process of cheesemaking, there are different routes for *L. monocytogenes* contamination to occur. One of the highest risk factors during this manufacture is the incoming raw milk before pasteurization. Personnel and equipment are two additional hazards. During the first manufacturing step, when rennet is added to the milk, might involve manual handling and is therefore a risk for *Listeria* contamination. Similarly, following steps (e.g. cutting or pressing) contain additions or preparations of the cheese and are therefore a contamination risk for the same reasons. Another possible route of *L. monocytogenes* contamination, even if rather unlikely, is a mix up of the pasteurized milk with raw milk. This risk could be minimized by an implementation of necessary routines of how to handle incoming raw material. In MLQF (Figure 7) these contamination risks in different steps are considered. The model is designed to have the possibility of evaluating these routes of contaminations if needed. However, for this study, the contamination of the QF occurs at the end of the process, as a post contamination.

In order to avoid contamination of *L. monocytogenes*, Good Manufacturing Practices (GMP), should be implemented. Examples of such are routines for personnel hygiene ensuring efficient washing of hands before entering the production area, and change of production clothes once or several times a week. Also, meticulous cleaning of the facility and the equipment is required. In order to verify that these routines are enough and enforced, randomized sampling can be performed and analyzed.

Outbreaks continue to occur due to *L. monocytogenes* contamination of QF, despite the use of pasteurized milk and following GMPs. Therefore, in order to increase the safety of QF, additional antimicrobial control is required to ensure a QF without *L. monocytogenes*.

1.2.1 *Listeria monocytogenes* 10403S

The *L. monocytogenes* 10403S (LM) was included as one of the strains in this study since it is the parental strain of *L. monocytogenes* 10403S expressing GFP (LM-GFP). Therefore, its main purpose in this research project is to act as a reference to compare the result between the engineered strain and the parental strain. This strain is not isolated from an outbreak, but a widely used laboratory strain (in the Michael J. Miller culture collection; MJM 202).

1.2.2 *Listeria monocytogenes* cocktail

A cocktail of five *L. monocytogenes* (LM-COCKTAIL) strains, isolated from foodborne outbreaks were used in this study (
Table **3**). Commonly, only one strain is isolated from a specific outbreak. However, the reason for the approach of combining five different isolates in this study is to cover several strains at the same time. The research group of Dr. Miller's laboratory has previously shown that the strains might act differently against different antimicrobials etc., and this is why all of the five are combined into one cocktail. In this approach, if one of the strains in the cocktail is not affected by the treatment, then the cell growth of the cocktail will increase. This knowledge is useful in order to be able to draw conclusions of how effective treatments are against a majority of different *L. monocytogenes* strains. However, the different strains in the LM-COCKTAIL are not distinguished from each other in this study.

1.2.3 Luminescent *Listeria monocytogenes*

In this study a luminescent *Listeria monocytogenes* (LUX) was evaluated as an indicator organism. The luminescent *L. monocytogenes* strain was purchased from a company, not mentioned by name because of confidentiality. Its serotype is 1/2a wild-type strain and is genetically engineered by integrating a stable copy of *Photorhabdus luminescence lux* genes, and this operon exists on the bacterial chromosome. LUX is therefore capable to emit light as a result of its luciferase reporter system (Figure 1) (Alves et al., 2011). Later in the project, it was discovered that the luminescent strain utilized was *Klebsiella pneumonia* and not *L. monocytogenes*.

The definition of bioluminescence is the emission of light from a living organism. Originally the bioluminescence gene is naturally found in living organisms, such as the bioluminescent lanternfish (Paxton and Eschmeyer, 1998). Today it can be engineered into a bacterium, such as in the bacteria used in this study. Commonly, these reporter systems are studied in order to determine gene expression or promoter activity of a specific gene. However, in this project it is a tool for evaluating microbial growth behavior as an alternative and more rapid method to the traditional Viable Count (VC).

The detected light emitted from the bioluminescence bacteria is dependent on the metabolic activity of the cells. However, it has been shown that the levels of bioluminescence changes during the bacteria's growth phase (Daghighi et al., 2015).

Bioluminescence is an example of a chemical reaction, produced by a living organism. The most common reaction for bioluminescence is the luciferin oxidation (Chemistry and Light, 2018), an

enzyme-catalyzed reaction including luciferin, ATP, oxygen and luciferase. This reaction leads to a release of a proton and therefore emits light. The oxygen oxidizes the luciferin and the reaction is catalyzed by the enzyme luciferase. Additionally, this reaction needs cofactors in order to work, these can be, for example, ATP and Mg^{2+} (Haddock et al., 2010).

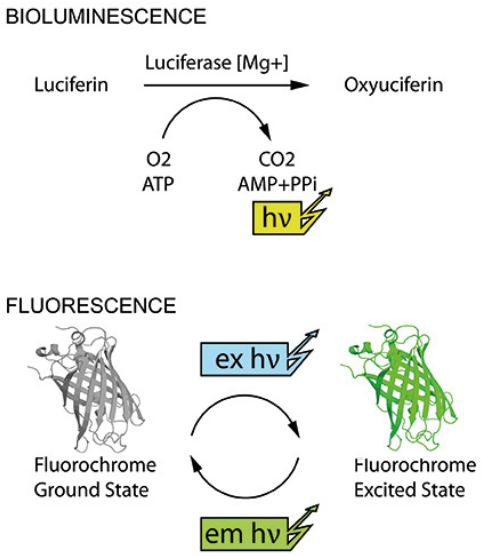


Figure 1. The different reactions for bioluminescence (top) and fluorescence (bottom). *Hv means energy, here energy in forms of light. Ex hv is the excited light and em hv is the emitted light (Imaging & Microscopy - Research, Development, Production, 2013).*

1.2.4 Fluorescent *Listeria monocytogenes*

The fluorescent *L. monocytogenes* in this study was *L. monocytogenes 10403S with pH-hly GFP-PL3* (LM-GFP) (Shen and Higgins, 2005) (MJM 321). This strain contains the green fluorescent protein (GFP) gene and is therefore suitable for measurements in a fluorometer. The plasmid for MJM 321 has a constitutive promoter; Hyper SPO1 (Shen and Higgins, 2005). The plasmid pH-hly GFP-PL3 is also engineered to have chloramphenicol (CM) as a selection marker. CM is an antibiotic which facilitates the stability of the plasmid within the LM-GFP by the antibiotic pressure, and also prevents bacteria without the fluorescent gene to grow.

Fluorescence arises when light is emitted because of absorption of energy. The fluorescence process is governed by three important events; excitation, vibrational relaxation and emission. The energy excites an atom, or so called fluorophore, which undergo vibrational relaxation during the excited state and then emits light while returning to its ground state (Figure 2) (Jameson, 2014).

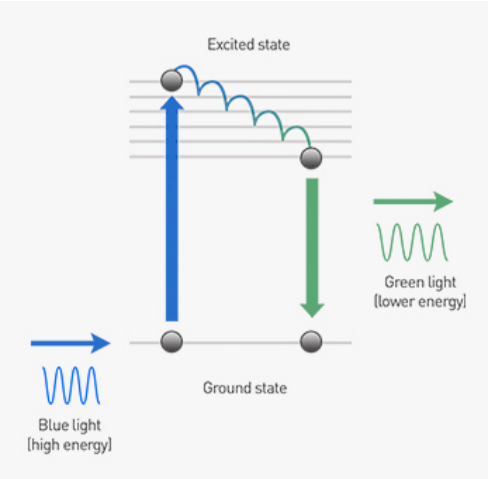


Figure 2. Facilitated figure of the mechanism of fluorescence. *The excitation, vibrational relaxation and emission of the fluorescence process are displayed (ThermoFisher, 2018).*

1.3 Antimicrobials

Today there are no commercial antimicrobials against bacteria for QF in the US. During production of cheese different physical and chemical methods exist in order to preserve quality. Two examples are high pressure and sterilization (Henriques et al., 2013). However, high-pressure processing, has been shown to be ineffective for *Listeria* control, because it unacceptably alters textural properties of the cheese (Hnosko et al., 2012).

At the end of the cheesemaking process, salt is added which acts as a chemical preservative since it regulates the A_w in the food (Van Tassell et al., 2015). Despite these preservation methods the addition of antimicrobial compounds is necessary for *Listeria* control as highlighted by the ever occurring outbreaks of listeriosis. Currently few additives have been shown to be effectively listericidal in fresh cheeses and often do not inhibit eventual regrowth in cold storage (Soni et al., 2010).

Four different antimicrobials were utilized in this study in order to have an antilisterial effect on the *L. monocytogenes*. The aim for the combination of the antimicrobials was to have a variety in order to be able to see if the method is applicable in different scenarios. Preferably one antimicrobial without effect, one bacteriostatic, and a combination. The antimicrobials were selected based on previous research in the Miller laboratory to target these different scenarios (Martinez Ramos, 2017; Van Tassell et al., 2015).

1.3.1 Lauric arginate

Lauric arginate (LAE) was added to the cheese at a concentration of 200 Parts Per Million (ppm), which is the highest amount accepted by the Food and Drugs Administration in the US (JHeimbach, LCC, 2005, p. 6). The LAE used in this study is food grade. Lauric arginate is the commercial name of ethyl lauroyl arginate and is derived from the natural components lauric acid, L-arginine and ethanol. LAE is a cationic surfactant which reduces bacterial levels by altering the cytoplasmic membrane of the microbe. This causes a disruption of the membrane lipid bilayer which prevents the cells' survival and growth. Overall, LAE has a broad spectrum of antimicrobial efficacy and acts against gram-

positive and negative bacteria, yeast and molds ("CytoGuard LA," 2014, "The Lowdown on Lauric Arginate," 2005; Hawkins et al., 2009).

This antimicrobial has been shown to easily hydrolyze in the human body during digestion. It becomes lauric acid and arginine, compounds which is naturally found in diets (Hawkins et al., 2009). All of these factors indicate LAE to be an appropriate antimicrobial for use in foods. LAE is commercially used primarily in meat products, such as ham and hot dogs for example (Luchansky et al., 2005; Martin et al., 2009). The LAE was expected to not have any effect on the *L. monocytogenes* in QF (Martinez Ramos, 2017).

1.3.2 Ɛ-polylysine

The concentration of the Ɛ-polylysine (EPL) in the study was 250 ppm, the same as the maximum permissible concentration in the US (Biochem, 2010, p. 42). Based on previous research in the Dr. Miller laboratory (Martinez Ramos, 2017), EPL was expected to have an effect between none and bacteriostatic on *L. monocytogenes*. The same research indicates that combined with LAE, these two antimicrobials have a bacteriostatic effect. LAE and EPL have a synergistic interaction for *L. innocua* and *L. monocytogenes* 10403S. The research indicated the best combination of the LAE and EPL to be 1:1 and with the concentrations set to the highest acceptable (Martinez Ramos, 2017).

EPL antimicrobial is GRAS and nontoxic towards humans (Drugge, 2015). The antimicrobial spectrum against microorganisms is wide for EPL, including yeast, molds, gram-positive and negative bacteria. The antimicrobial effect of EPL has shown to cause leakage of ions in the cell's cytoplasmic membranes. Additionally EPL also damage the cellular proteins (Li et al., 2014). Commercial use of EPL as an antimicrobial includes staple foods of the Oriental diet and fish (Hiraki et al., 2003).

1.3.3 Nisin

Nisin (NIS) was applied only in the preliminary screening. It has a mild antilisterial effect on *L. innocua* and *L. monocytogenes* in QF (Van Tassell et al., 2015). The concentration of NIS for this study was 250 ppm, which is the highest acceptable level in the US (FDA, 2017).

The cell membrane lipid composition of *Listeria* is altered by NIS. This entails decreased membrane fluidity and further pore formation is prevented (Mazzotta and Montville, 1997; Ming and Daeschel, 1995).

Nisin is a commercially utilized preservative in the food industry. It is a bacteriocin, produced by *Lactococcus lactis*, with a broad spectrum and inhibits gram positive bacteria, such as *L. monocytogenes* (Gálvez et al., 2007). The antimicrobial effect of NIS has been shown to be higher at low pH (Benkerroum and Sandine, 1988; Davies et al., 1997). Van Tassel et. al (2015) confirms nisin as an ineffective antilisterial in QF with its near-neutral pH.

1.3.4 Ferulic acid

For the preliminary screening of this study, a concentration of 2.5 mg/g of Ferulic acid (FER) was used. Previous studies in the Miller laboratory showed that this is the MIC (Minimum Inhibitory

Concentration) of FER for *L. innocua* and *L. monocytogenes* in BHI (Brain Heart Infusion) (Van Tassell et al., 2015). FER is a phenolic phytochemical which has been shown to have an antilisterial effect (Takahashi et al., 2013). Phenolic acids has a general mechanism of inhibiting bacterial growth by increasing the permeability of cell membranes. This often leads to leakage of cytoplasmic components (Borges et al., 2013; Campos et al., 2009). FER is currently not available as food-graded and is therefore still a novel antilisterial without any commercial use. This antimicrobial was expected to have a bacteriostatic effect on the *L. monocytogenes* (Van Tassell et al., 2015).

1.4 Analytical tool box

The MLQF and HTQF are two different models, both designed to analyze the growth behaviour of *L. monocytogenes* in different scenarios. The MLQF was originally developed to produce QF in microcentrifuge tubes. In this study, the MLQF was adapted to produce QF in 96-well microplates instead of tubes, but still treated the same and using plating (VC) for enumeration. In the development of the HTQF, the plating and measurements in the fluorometer were performed in parallel, due to the desire of correlate the two (Figure 3).

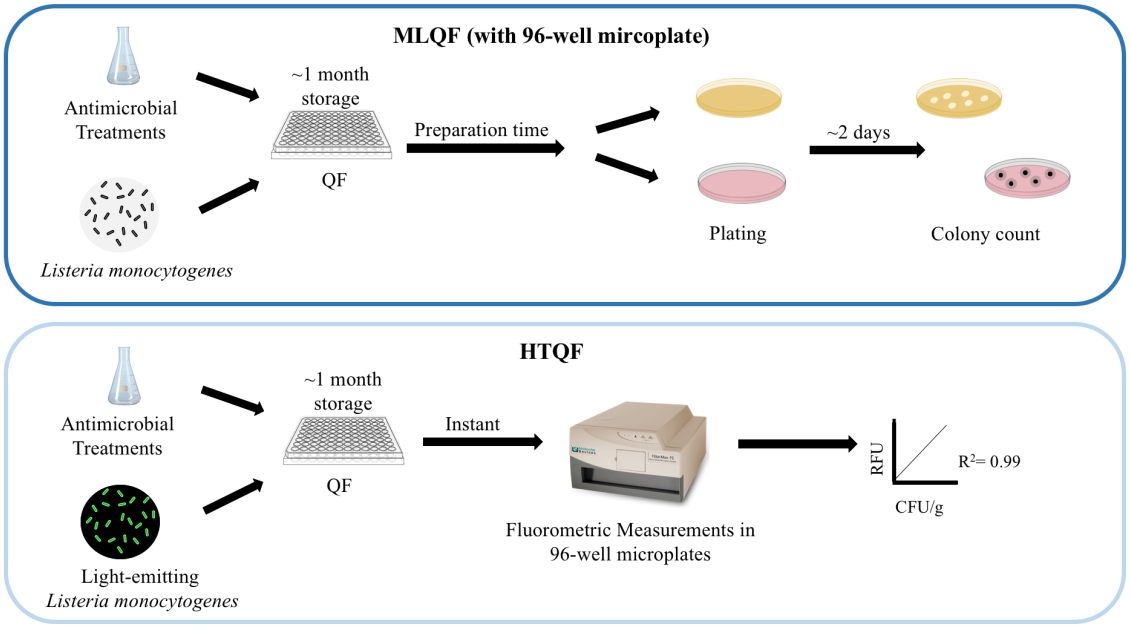


Figure 3. An overview of the MLQF model versus the HTQF model. *MLQF) QF was produced in 96-well microplates, treated with and without antimicrobials and spiked with L. monocytogenes. Then plated for VC. HTQF) QF was produced in 96-well microplates, treated with and without antimicrobials and spiked with light-emitting L. monocytogenes. Then luminescence and fluorescence was measured with a fluorometer.*

1.4.1 MLQF

The MLQF is a validated method developed by the Miller Laboratory. It includes small-scale production of QF, plating, and enumeration. Using this methodology, different antimicrobial treatments can be evaluated quicker than traditional QF processing or pilot plant scale processing. The MLQF accurately replicates the composition and cheesemaking process of commercial QF (Van Tassell et al., 2015).

1.4.1.1 Plating

Microbial growth measurements are traditionally performed using plating on agar to obtain Viable Count (VC). This methodology is time consuming and costly. When plating, the aim is to select a dilution that results in a proper amount of colonies on the plate. Between 30 and 300 colonies is ideal. If there are less than 30 colonies, the statistical error increases. The colony count of the plate can then be calculated into a microbial concentration of the original sample, taking the dilution factor into account. Colony Forming Units per gram (CFU/g) is the commonly used concentration (Harrigan, 1998).

A further developed method of plating is spiral plating (Figure 4). The spiral plating machine distributes a small amount of the sample across the agar plate, in a spiral pattern, diluting the sample more and more along the spiral. The dish is rotated in the machine, while simultaneously dispensing the liquid. The movement is at constant speed and the spiral creates a lover concentration on the outside of the plate, enabling a dilution of the liquid which facilitates counts of single colonies. This machine is automatic, compared to the traditional plating method which is performed manually. It has been shown there is no significant difference between the two methods (Harrigan, 1998). In addition to this less labor intensive technique, the result from the spiral plater can easily be recorded by a colony counter. The colony counter calculates the correct microbial concentration based on the colony's position in the spiral (IUL Instruments, 2017).

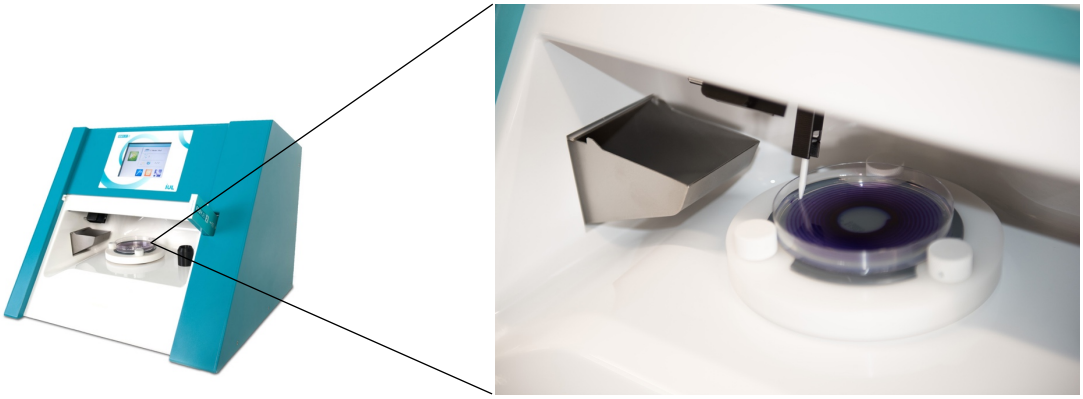


Figure 4. Spiral plater. *To the left is the instrument, and to the right is the spiral plater zoomed in while plating (IUL Instruments, 2017).*

1.4.2 HTQF

The HTQF is a proposed method to facilitate a more efficient analysis of microbial growth. It consists of measurements with a fluorometer, of light-emitting *L. monocytogenes* in 96-well microplates. This method can rapidly evaluate antimicrobial effectiveness, compared to the MLQF. Based on the result from the HTQF screening, the antilisterials exhibiting potential effect against *L. monocytogenes* will be evaluated with the MLQF model. Developing the HTQF is the focus area of this study. During the development of this method, a comparison between viable cell counts and light generated in the measurements with the fluorometer is required to investigate the correlation between the different methods to monitor growth. Results from both luminescent and fluorescent bacteria were evaluated.

1.4.2.1 Fluorometer

Measurements of fluorescence and luminescence in a fluorometer could be an alternative to the traditional method for measurements of microbial growth. The GFP has an excitation at 485 nm and the emission wavelength is 535 nm. When measuring fluorescence, it is ideal to have a dark background, in order to avoid possible background fluorescence and dims this light, which is why the black 96-well microplates are chosen in this study. For the same reason, a white plate is optimal for luminescence measurements. In this study, the light source is an LED. Both the excited light from the light source, and the emitted light, is filtered with specific filters in the fluorometer which isolate selected wavelengths (Jameson, 2014). Finally, the emitted light is measured by a detector (Figure 5).

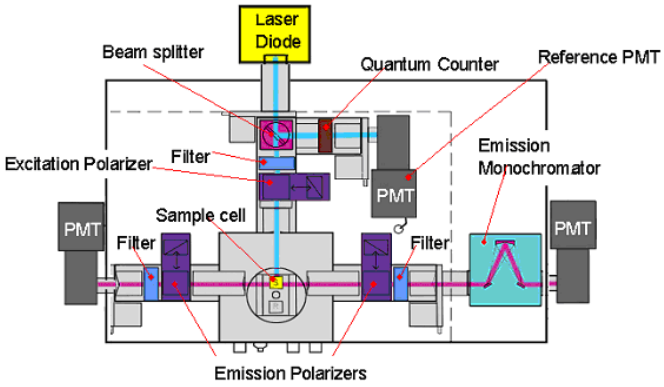


Figure 5. Schematic of a fluorometer depicting the different pieces. *Filters and light source are some instrumental details displayed (ISS, 2015).*

2. Scope

The scope of this master thesis project is to develop a high-throughput screening model with the purpose of using it to evaluate antimicrobials for Queso Fresco. This was studied by using bioluminescent (LUX) and fluorescent (GFP) bacterial strains, measuring emitted light with a fluorometer, and correlated with plate count.

The long-term goal of this project is to improve the safety of Hispanic-style fresh cheeses. The main questions are: can high-throughput screening be implemented to simplify the existing analysis model for antimicrobials and make it more efficient? Are the different *L. monocytogenes* strains comparable? Additionally, which reporter system is most representative for analyzing the bacterial growth in the HTQF model, GFP or LUX?

This master thesis is part of the research performed at Dr. Millers' laboratory, in the department of Food Science and Human Nutrition, at the University of Illinois at Urbana-Champaign.

3. Materials and Method

Prior to this project, the research group at Dr. Miller's laboratory has developed a validated method to produce Queso Fresco in a laboratory scale. They have also assessed different antimicrobials for *L. innocua, L. monocytogenes* 10403S, and a cocktail of five *L. monocytogenes* strains. This method and its results were used as a foundation for this study.

3.1 Bacterial strains and growth condition

The included strains were *L. monocytogenes* 10403S (LM), *L. monocytogenes* 10403S expressing GFP (LM-GFP), five strains cocktail of *L. monocytogenes* (LM-COCKTAIL) (Table 3), and luminescent bacteria (LUX). One day prior to each experiment, all strains (Table 2) were inoculated in broth from a glycerol stock stored at -80°C (1:1, 25% glycerol and bacteria). The recovered bacteria was cultured overnight in 10 ml BHI/BHICM broth (Brain Heart Infusion/Brain Heart Infusion with Chloramphenicol) (Difco, Becton Dickinson and Co., Sparks, MD) (Chloramphenicol, Fisher Scientific, NJ) for 20 hours at 37°C with an agitation of 250 Revolution Per Minute (rpm) (INCU-SHAKER Mini, Benchmark Scientific Inc., Edison, NJ). The LUX and the LM-COCKTAIL were cultured in BHI, while BHI with 7.5 mg/ml CM was used for the LM-GFP (for the evaluation of the screening method) (kindly received from the Department of Food Science, Cornell University) (Milillo et al., 2008) (Shen and Higgins, 2005). The overnight culture obtained a cell concentration of approximately 9 log CFU/g.

Before the cheese was inoculated with the different strains of *L. monocytogenes*, each strain was washed twice with Phosphate-buffered saline (PBS) (KCl 200 mg/L; KH_2PO_4, 200 mg/L; NaCl, 8 g/L; Na_2HPO_4, 1.15 g/L, pH 7.2, MP Biomedicals, LLC, OH) and diluted to approximately 4 log CFU/g. LM-COCKTAIL was prepared with equal parts of each strain to obtain the same final total cell concentration in all the solutions.

Table 2. Bacterial strains employed in this study. *The MJM number for each strains; LM, LM-GFP and the five strains of the LM-COCKTAIL. * The previous names of the LM-GFP was FSL B2-0107 and DH1039.*

Strains	MJM nr.
Listeria monocytogenes 10403S	202
Listeria monocytogenes 10403S expressing GFP *	321
Listeria monocytogenes NRRL B-33419	433
Listeria monocytogenes NRRL B-33424	436
Listeria monocytogenes NRRL B-33420	440
Listeria monocytogenes NRRL B-33513	441
Listeria monocytogenes NRRL B-33104	442
Luminescent bacteria	-

Table 3. The Foodborne outbreak-associated bacterial strains of L. monocytogenes included in the cocktail in this study. *The serogroup and source of isolation for each of the five strains in the LM-COCKTAIL (Ibarra-Sánchez, Van Tassell and Miller, 2018).*

Strains	Serogroup	Source of isolation
Listeria monocytogenes NRRL B-33419	1/2a	Human, epidemic, sliced turkey
Listeria monocytogenes NRRL B-33424	1/2b	Human, epidemic, chocolate milk
Listeria monocytogenes NRRL B-33420	4b	Food, epidemic, Ready-to-eat meat products
Listeria monocytogenes NRRL B-33513	4b	Food, epidemic, pate
Listeria monocytogenes NRRL B-33104	4b	Food, epidemic, Jalisco cheese

3.1.1 Preliminary screening

Standard BHI was used as the growth media for the overnight cultures, for all of the different strains, including the LM-GFP. For this experiment, LUX was not yet available and therefore not included in this part of the study.

3.1.2 Evaluation of screening method

For the LM-GFP, BHI with CM was used, as explained in the protocol above, as an added optimization step concerning the growth conditions. Since this was not included in the preliminary screening, it was unclear if the construction of the LM-GFP was stable without this antibiotic pressure. Therefore, it was included as a precautionary measure in the evaluation of the screening method.

3.2 Antimicrobial compounds

Table 4 shows an overview of the four different antimicrobials utilized in this study, including the concentration of each antimicrobial; NIS [250 ppm], LAE [200 ppm], FER [2.5 mg/g], EPL [250 ppm], LAE/EPL [200 ppm/250 ppm].

Table 4. An overview of the antimicrobial treatments in this study. *The concentration of each antimicrobial.*

Antimicrobial treatment	Concentration
Nisin	250 ppm
Lauric arginate	200 ppm
Ferulic acid	2.5 mg/g
ε-polylysine	250 ppm
Lauric arginate/ε-polylysine	1:1 (200 ppm/250 ppm)

3.2.1 Preliminary screening

For the first experiments NIS (Nisaplin® 2.5%, Danisco, New Century, KS), FER (Ferulic Acid, MP Biomedicals, LLC, OH), LAE (CytoGuard LA20, A&B Ingredients, Inc, NJ) and EPL (epsilon-Poly-L-lysine >98%, Wilshire Technologies©, NJ) was selected as the treatments for *L. monocytogenes*.

3.2.2 Evaluation of the screening method

In the evaluation of the screening method, the FER and NIS were excluded from the treatments. LAE and EPL were still applied, with the additional combination of these two. These selections were based on the goal of having a range of a different effectiveness of treatments in the experiment, with three different scenarios. This was why the NIS and FER were excluded. Previous research (Martinez Ramos, 2017; Van Tassell et al., 2015) was used to set the concentrations for this study (Table 4).

3.3 Production of Queso Fresco manufactured in a 96-well microplate

The cheese production in this study was performed to imitate commercial manufacturing. This process was performed the same for both the preliminary screening and the evaluation of the screening method.

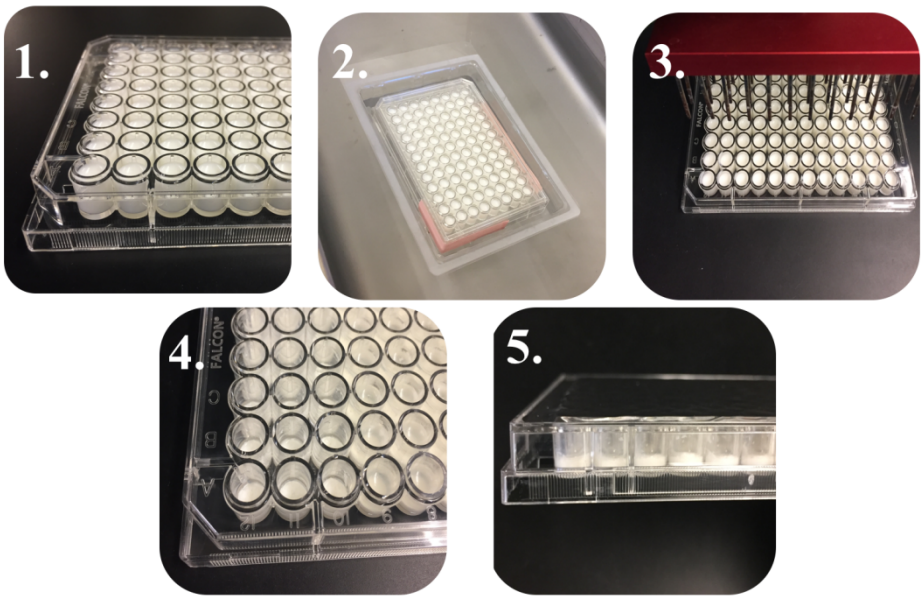


Figure 6. Production of QF in 96-well microplate. *1) wells filled with 250µl milk 2) cooking in water bath 3) cutting the curd 4) whey removal from the three left columns in the plate, whey still remaining in the two right columns 5) finished QF, covered with aluminum foil (Aluminum Foils for 96-well plates, VWR, NY) for storage.*

Queso Fresco was produced by using the method previously described by Van Tassel el al. (2015) (Figure 7). However, it was performed with some modifications to suit this particular study with an even smaller scale production in 96-well microplates (Figure 6). The protocol was as follows.

Each batch contained 100 ml of pasteurized whole milk (Vitamin D Milk, Schnuck Market Inc., MO). The milk was heated to 35°C in a water bath (Isotemp® waterbath, Thermo Fisher Scientific, NJ). 200 µl of diluted $CaCl_2$ solution (40 µl of 50% w/v stock in 160 µl ultrapure water) and 200 µl of diluted rennet (30 µl in 170 µl ultrapure water) was added to the milk. 250 µl of milk was added into the 96-well microplates. For measurements in the fluorometer white plates (Microplate, 96 well, white,

Greiner bio-one, NC) was used for LUX and black plates (Microplate, 96 well, black, Greiner bio-one, NC) for LM-GFP. Clear plates (Polystyrene Microplates 96-well, Falcon ®, NY) were used for the other samples. The plates were incubated in a water bath for 45 minutes at 35°C. The curds were cut by a sterile 96 pin replicator (Boekel Scientific, PA) and plates were replaced in the water bath for additionally 30 minutes, with an increase of 1°C every 6 minutes, starting at 36°C and ending at 40°C. In order to remove whey, the plates were centrifuged at 4 000 rpm for 1 minute (Centrifuge 5810 R, Eppendorf, NY) with rotor (A-4-62, Eppendorf, NY). 200 µl of whey was removed from each well and curd was gently mixed with an addition of 12.5 µl of NaCl (Fisher Scientific, NJ) solution (0.16 g/ml) per well. The NaCl solution was filter sterilized with 33 mm syringe filters (Fisherbrand, Fisher Scientific, NJ). An additional cooking of the cheese occurred for 20 minutes at 40°C. Plates were further centrifuged at 4 000 rpm for 15 minutes. Excessive whey was removed and the antimicrobials were added to each cheese, and stirred gently. An additional centrifugation at 4 000 rpm for 10 minutes was performed. The whey was removed completely. Each cheese was inoculated with 12.5 µl of *L. monocytogenes* on the surface of the cheese, to represent a post processing contamination. It rested for 30 minutes on bench-top to allow for bacterial attachment to the cheese and to dry. Residual liquid was completely removed from the cheese's surface. The cheese was stored in 4°C.

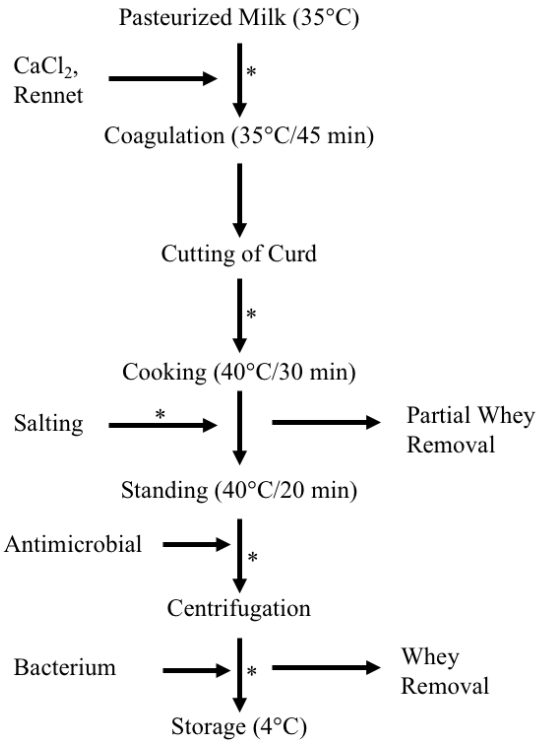


***Figure 7. Flow diagram of Queso Fresco production in small scale**. * means potential route for microbial contamination (Van Tassell et al., 2015).*

The cheese produced with MLQF described by Van Tassel et al. (2015) gave a cheese of 0.148 ± 0.001 g. The QF produced in 96-well microplates weighed 0.05 ± 0.0004 g (Figure 8). Both values are means ± Standard Error of the Mean (SEM). The value of the 96-well microplate cheese was based on the weighing of 20 different cheeses from one batch.

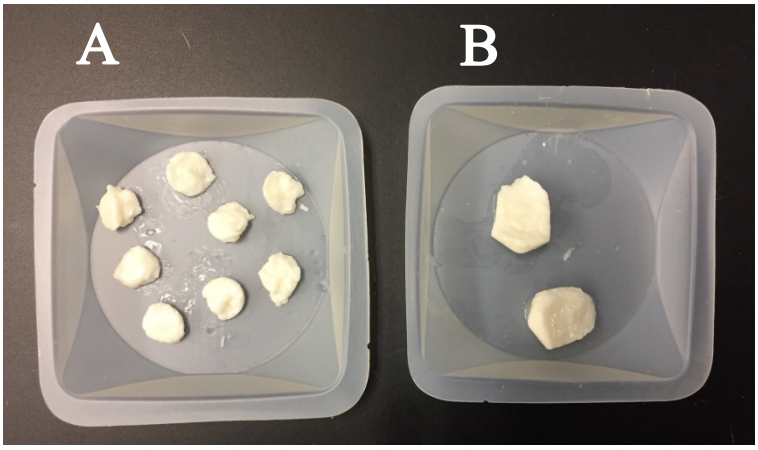


Figure 8. Two different size of QF. *A) QF produced in 96-well microplate, B) QF produced with MLQF in tubes.*

3.4 Experimental set up

3.4.1 Preliminary screening

Before all the final material, strains and equipment were available, a preliminary screening was performed. This experiment gave useful knowledge to determine an appropriate procedure and experimental set up.

In this first experiment, all the strains as seen in Table 5, were included (LM, LM-GFP, LM-COCKTAIL). The experiment had four different treatments for the *L. monocytogenes* strains. The black 96-well microplate used for the LM-GFP to measure in the fluorometer, did not contain any standard or any blank cheeses without any treatment or inoculation. However, a positive control, an contaminated cheese without treatment, was included. For this screening experiment, three independent batches of cheese were made and with duplicates of each sample within the batch.

Table 5. Overview of the preliminary screening. *Different experiments, trials, strains and treatments of the preliminary screening in this study.*

Trial within the experiment	Strains	Treatments for the strains
1	LM	a) Lauric arginate
	LM- GFP	b) ε-polylysine
	LM- COCKTAIL	c) Ferulic acid
		d) Nisin
2	LM	a) Lauric arginate
	LM- GFP	b) ε-polylysine
	LM- COCKTAIL	c) Ferulic acid
		d) Nisin
3	LM	a) Lauric arginate
	LM- GFP	b) ε-polylysine
	LM- COCKTAIL	c) Ferulic acid
		d) Nisin

Note: LUX was not yet available.

3.4.2 Evaluation of the screening method

Identical experiments were performed on two separate days, with three replicates of each sample for respectively experiment. The positive control was an contaminated cheese without treatment. A blank was added for the fluorometer measurements, which consisted of an uninoculated cheese.

The experimental set up of this experiment consisted of LM, LM-GFP, LM-COCKTAIL and the LUX. All of these were treated with the same antimicrobials (LAE, EPL, LAE/EPL) and all had a positive control (untreated cheese with inoculum) (Table 6).

Table 6. Overview of the evaluation of the screening method. *Different experiments, trials, strains and treatments in the evaluation of the screening method of this study.*

Trial within the experiment	Strains	Treatments for the strains
1	LM LM- GFP LM- COCKTAIL LUX	a) Lauric arginate b) ε-polylysine c) Lauric arginate/ε-polylysine
2	LM LM- GFP LM- COCKTAIL LUX	a) Lauric arginate b) ε-polylysine c) Lauric arginate/ε-polylysine

3. 5 Growth measurements

3.5.1 Viable Count and Optical Density

The LM, LM-GFP AND LM-COCKTAIL, enumeration was carried out on Polymyxin Acriflavin Lithium-chloride Ceftazidime Esculin Mannitol (PALCAM) Listeria-Selective agar (EMD-Millipore) including 20 µg/ml of ceftazidime (Tokyo Chemical Industry Co. Ltd., Tokyo), incubated at 37°C for 48 hours (Symphony, VWR, NY). Same conditions were applied for the LUX, with the exception of plating on Luria-Bertani agar (LB Broth, Miller, Fisher Scientific, NJ; Agar, Fisher Scientific, NJ) supplemented with 200 µL/mL of kanamycin (kanamycin sulfate from *Streptomyces kanamyceticus*, Sigma -Aldrich, MO) and incubation for 24 hours. Both ceftazidime and kanamycin act as selective markers in the agar. At each time point, each sample of cheese was moved from the well into a tube and diluted 1:10 (wt/vol) with PBS, using a spatula. Before serially dilutions, 10 fold, each sample was vortexed (Vortex-Genie 2, VWR, NY) to further disrupt the curd structure. Two dilutions of each sample were spiral plated (Eddy Jet - Spiral plater, Neutec group inc., NY).

3.5.1.1 Preliminary screening

Instead of using a spiral plater, the conventional plating technique was performed during the sampling of the first experiments.

3.5.1.2 Evaluation of the screening method

To see if the overnight culture of BHICM and its antibiotic pressure helped the construction of the LM-GFP to be stable or not, a validation step of the presence of the plasmid was performed after plating. One colony from each plate in the evaluation of the screening method was transferred. The colonies were recovered from PALCAM plates at each measurement point and cultured on replicaplates of BHI and BHICM respectively in order to verify the presence of the plasmid.

3.5.2 Fluorometer

3.5.2.1 Standard curve – evaluation of the screening method

For the evaluation of the screening method, a standard curve was introduced in the 96-well microplates for the fluorometer measurements. Based on the measurements from each standard curve, a linear regression and its equation (y=kx+m) were utilized to standardize the CFU/g of the samples. This was performed in order to enable an equivalent determination of the CFU/g in the samples compared to the known relation between Relative Fluorescence Unit (RFU) and CFU/g, and Relative Light Unit (RLU) and CFU/g respectively. For each individual measurement, a specific equation was formulated and applied.

Prior to the day of each measurement, LM-GFP and LUX were inoculated respectively from a glycerol stock (-80°C), according to the method described above. The same day as the experiments 2 ml of each inoculum culture was transferred to two centrifuge tubes which was centrifuged for 2 minutes at 14 000 rpm and washed with PBS. Two washing steps were performed. Each overnight culture was diluted 10 fold and the Optical Density at 600 nm (OD_{600}) was measured (YSI EcoSense 9 500 Photometer, airmet, VIC). Then, the overnight cultures were diluted to reach an OD_{600} of 2.

Two different standard curves were designed and implemented in the fluorometer measurements. One with the standard in broth, and the other with the standard inoculated on cheese. Both of these were included in order to compare which one was the better alternative for this model.

During the development of the protocol for the standard, each dilution was plated in duplicate on LB with kanamycin and PALCAM with ceftazidime for LUX and LM-GFP respectively, in order to enumerate and determine the CFU/g. After the CFU/g was confirmed for three independent overnight cultures, a protocol was set for the methodology of the standard curve for the experiment. This in order to ensure a consistency of the standard curve for each measurement, even if performed on different days.

For the broth, a serial dilution was made from the solution (OD_{600} = 2), with 10 fold dilutions. In total, one blank and seven different known dilutions were added to the plate in triplicate. The highest was 9 log CFU/g and the lowest 1 log CFU/g. 70 µl of the standard was added to the wells of triplicates.

The blank cheese was inoculated with 12.5 µl of the standard dilutions. This series of standards were diluted 1:5, with values ranging from 6 log CFU/g to 1.8 CFU/g. The measurements with a cheese standard was first implemented on day 7 of the experiment (and then with the same dilutions as in the broth). The protocol described was first applied at day 14.

3.5.2.2 Evaluation of luminescence and fluorescence Listeria monocytogenes

3.5.2.2.1 Preliminary screening

The fluorometer FLx800 (Microplate Fluorescence reader, BioTek Instruments Inc.,VT) was used for the preliminary screening. The excitation and emission wavelengths for the measurements of LM-GFP in this equipment were set to 485 and 530 nm. In this fluorometer, the endpoint fluorescence was measured from the top of the wells. Since pathogens were not approved to be measured in this equipment, a film was used to seal the 96-well microplate as protection during the measurement.

3.5.2.2.2 Evaluation of the screening method

Another fluorometer (FilterMax F5 Multi-Mode Microplate Reader, Molecular devices, CA) was used to measure fluorescence of the LM-GFP and luminescence of the LUX in the evaluation of the screening method. For these measurements, a blank was used with plain cheese in order to recognize the background noise. For this experiment, the excitation and emission wavelengths for the LM-GFP was set to 485 and 535 nm (since a different instrument as in the preliminary screening). Regarding LUX, all wavelengths were chosen in order for all light to be detected. The FilterMax had a more advanced software than FLx800, including a well scan operation, with nine different measurement points, that was utilized for the fluorescence measurements. This operation was unavailable for the luminescence assays. No lid or film was required in this instrument and a optimization step was performed before new measurements in order to define the most suitable read height of the wells. The integration time was set to 1 000 ms.

3.5.3. Enumeration and statistical analysis

Statistical analysis was performed in JMP 14.0.1 (SAS Institute Inc., Cary, NC). The significance was determined by the Multivariate Analysis of Variance (MANOVA) for the analysis of repeated measured data. The significance level was set to a p value of 0.05. This statistical analysis was only applied to the result of the viable cell counting of the evaluation of the screening method.

3.5.3.1 Preliminary screening

The enumeration was executed by traditional VC, in order to determine the microbial growth. Three different dilutions of each sample were plated at each measurement point.

3.5.3.2 Evaluation of the screening method

For every measurement, each sample was plated at two different dilutions. The dilutions depended on how many days the experiment had lasted based on an estimation of the cell growth in the cheese.

The enumeration was performed with a colony counter (The IUL Flash and Go, Neutec group inc., NY) (Figure 9). An average value was calculated based on the triplicates from the same sample in both experiments, which gave an average of approximately 12 different results for each sample, for each measurement.

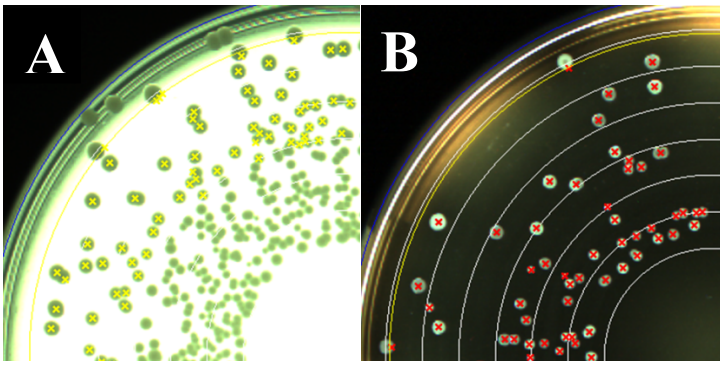


Figure 9. Pictures from VC with the colony counter. *A) Screenshot from colony counter for L. monocytogenes on LB with kanamycin, B) Screenshot from colony counter for L. monocytogenes on PALCAM with ceftazidime.*

3.6 DNA sequencing

In order to verify which bacteria grew on the LUX plates, colony PCR and 16S sequencing was performed.

3.6.1 DNA extraction

A single colony was picked from the plate, and suspended in 100 µl PCR water (molecular grade) (Mo Bio Laboratories, Inc., IL). The suspension was heated for 10 minutes in heating block (Gene Mate Dry Bath, Bioexpress, NY), at 95°C.

3.6.2 PCR

Using the small PCR tubes, a total reaction mix (EconoTaq PLUS 2X Master Mix, Lucigen, WI) of 50 µl was prepared, including 25 µl Master Mix 2X, 0.5 µl forward primer (V4 FW v1) 100 µM, 0.5 µl reverse primer (V4 RV v1) 100 µM, 23 µl nuclease free PCR water, 1 µl DNA from the colony extraction. Start PCR using the PCR machine (Applied Biosystems 2720 Thermal Cycler, Thermo Fisher Scientific, NJ) with following settings from EconoTaq PLUS 2X Master Mix Manual; 94°C for 2 minutes and with 1 cycle for initial denaturation, 30 cycles with denaturation (94°C for 30 seconds), annealing (65°C for 30 seconds) and extension (72°C, 1 min/kb of DNA, usually approximately 1 minute). The final extension was 72°C for 10 minutes with 1 cycle and finally the instrument had an indefinitely hold at 4°C.

3.6.3 Quantification with agarose gel electrophoresis

Before 16S sequencing, gel electrophoresis was performed to ensure the success of the PCR, with the ability to quantitatively analyze amount of DNA present. 0.6 g of agarose (Fisher Bioreagents, Fisher Scientific, NJ) was added to 40 ml of TAE Buffer (Thermo Fisher scientific, NJ) in a large flask. A napkin was placed on top of flask and microwaved for 20 seconds of intervals until all of the agarose was melted, approximately 2 minutes. It was cooled down for 5 minutes on bench top. 4 µl of EtBr solution, 10% of total volume, was added and swirled to mix. The solution was poured into cast, wedged horizontally into the apparatus (Owl Separation Systems, Thermo Fisher Scientific, NJ) with comb in place. It was cooled on bench top for 20 minutes. Thereafter, the comb was removed, the cast was flipped to vertical and the apparatus was filled with TAE buffer to cover the cast. A 100 bp ladder was used and at least 3 µl was added in the well. The same amount of the DNA sample was used and

mixed with equal parts dye, before added to the wells. The instrument was set to 80 V for 50 minutes, the lower the voltage, the more distinct bands.

3.6.4 DNA clean up

The DNA clean-up was performed using the ExoSAP-IT kit (Applied Biosystems, Thermo Fisher Scientific, NJ). 2 µl of ExoSAP-IT was mixed with 5 µl of PCR product. It was held for 15 minutes in a heating block at 37°C. Thereafter, moved to a heating block at 80°C for an additional 15 minutes.

3.6.5 16S rRNA sequencing

7 µl of the ExoSAP-IT product was added to a 1.5 ml Eppendorf tube. Another tube was filled with 5 µl of the utilized primers [10 pmol/µl] for each DNA sample to be sequenced. The tubes were marked and submitted for sequencing. A BLAST (Basic Local Alignment Search Tool) was utilized and performed of the traces from the sequencing in order to analyze the result.

4. Results

4.1 Preliminary screening

The results from assessing the HTQF in the preliminary screening including viable cell count and fluorescence measurements are presented in the following section.

4.1.1 Growth measurements with VC

The main purpose of this experiment was to determine if the validated MLQF and newly developed HTQF models were comparable. The plate counts from the preliminary screening consisted of triplicate trials for days 0, 7 and 14, and of a single trial for days 21 and 28. Since this was first planned to be the main experiment it started out with three trials within. After deciding for it to be a screening experiment, only one out of three trials was analyzed for the last two measurements.

The preliminary screening was executed with *L. monocytogenes* 10403S (LM), *L. monocytogenes* 10403S expressing GFP (LM-GFP) and a five strain cocktail of *L. monocytogenes* (LM-COCKTAIL). Both the two strains and the cocktail were treated with NIS and FER separately. Only LM-GFP had two additional treatments of LAE and EPL (Figure 11). The results from the different treatments of the two strains and the cocktail in this screening experiment were quite different (Figure 10, Figure 11, Figure 12). After 28 days of storage, the growth of the *L. monocytogenes* in the control cheese (no treatment) was 1.5 log CFU/g for the LM (Figure 10), after decreasing 1 log after day 21. The growth was 2 log CFU/g for LM-GFP (Figure 11), and LM-COCKTAIL (Figure 12) grew 3 log CFU/g over 28 days.

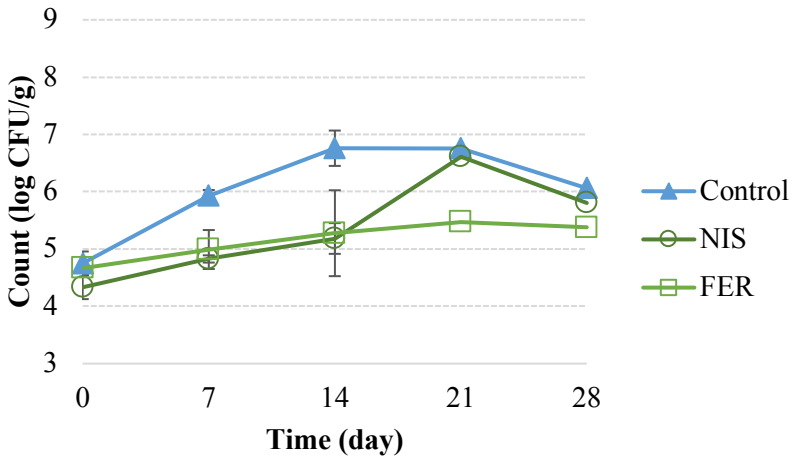


Figure 10. L. monocytogenes 10403S (preliminary screening). *Growth curves of LM in QF treated with different antimicrobials (NIS and FER), including a positive control with no treatment. Measurements obtained by VC. The mean ± SEM of three independent batches for measurements days 0, 7, 14 and the mean for one batch days 21 and 28, is displayed.*

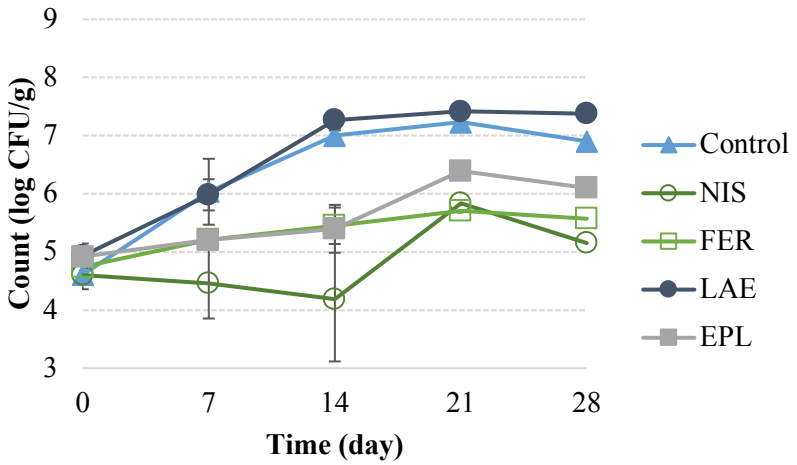


Figure 11. L. monocytogenes 10403S expressing GFP (preliminary screening). *Growth curves of LM-GFP in QF treated with different antimicrobials (NIS, FER, LAE and EPL), including a positive control with no treatment. Measurements obtained by VC. The mean ± SEM of three independent batches for measurements days 0, 7, 14 and the mean for one batch days 21 and 28, is displayed.*

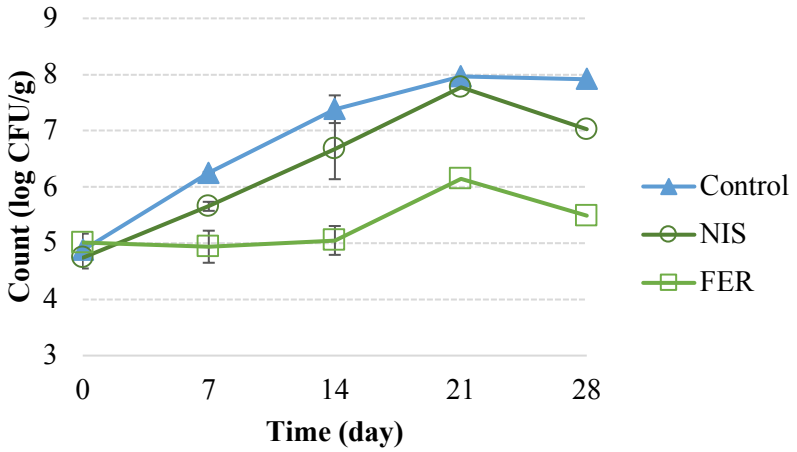


Figure 12. L. monocytogenes five strain cocktail (preliminary screening). *Growth curves of LM-COCKTAIL in QF treated with different antimicrobials (NIS and FER), including a positive control with no treatment. Measurements obtained by VC. The mean ± SEM of three independent batches for measurements days 0, 7, 14 and the mean for one batch days 21 and 28, is displayed.*

The NIS treatment also resulted in different values for the different strains in the HTQF. After 28 days, LM with NIS (Figure 10) showed a growth of 1.5 log CFU/g. The growth was the same for NIS and the control, as expected from the previous MLQF result (Martinez Ramos, 2017). For LM-GFP (Figure 11), NIS seemed to be the most promising treatment with only an increase of 0.5 log CFU/g, which was the opposite to previous MLQF research (Martinez Ramos, 2017). The LM-COCKTAIL (Figure 12) grew 2 log CFU/g with the NIS treatment, it was 1 log less than the positive control of the same cocktail.

The treatment with FER was the one treatment with the most similar result between the two strains and the cocktail in the preliminary screening. For the LM (Figure 10) and LM-GFP (Figure 11), both grew 1 log CFU/g over 28 days. For the LM-COCKTAIL (Figure 12) there was no significant increase or decrease in cell count.

For the preliminary screening, LM-GFP had four different treatments instead of only two as for the LM and LM-COCKTAIL. The LAE indicated to be least promising as a treatment while EPL was between bacteriostatic and no effect.

The result above, from the preliminary screening, was not comparable to the previous result from the validated MLQF. The control for the different graphs above grew 1, 2 and 3 log CFU/g which shows inconsistency within the experiment. For the MLQF the control usually grows 4 log CFU/g. The different treatments did not behave similarly either, in this experiment compared to MLQF. Additionally, the overall result for the two *L. monocytogenes* strains and the cocktail were different. This was also deviate from what previous MLQF studies have shown (Martinez Ramos, 2017; Van Tassell et al., 2015).

4.1.2 Growth measurements with fluorometer

In order to evaluate the correlation between fluorescence and cell growth, measurements of the LM-GFP were made in the fluorometer. The measurements from the fluorometer for the preliminary screening did not contain any standard curve, such as contaminated cheese or broth with a series of defined concentrations of cells. The signal gain was set to one of the positive controls, contaminated cheese without treatment, as the highest sample. This gave a relative result based on this signal gain. Therefore, the value for the different samples did not increase particularly much, and they were all based on the ratio compared to this highest sample. As mentioned for the VC of the preliminary screening, also these measurements from day 21 and 28 were only from 1 trial, and the other three measurements points were from three independent trials.

The results are presented as a percentage of the positive control (Figure 13). The result indicated no difference between controls and treatments for the LM-GFP. Additionally, there was no particular increase or decrease in the fluorescence during the 28 days, it was almost just straight lines. NIS and EPL were all consistent at around 80% growth of the control and FER stayed at 60% during all of the measurements. LAE decreased from about 80% to 70% over 28 days.

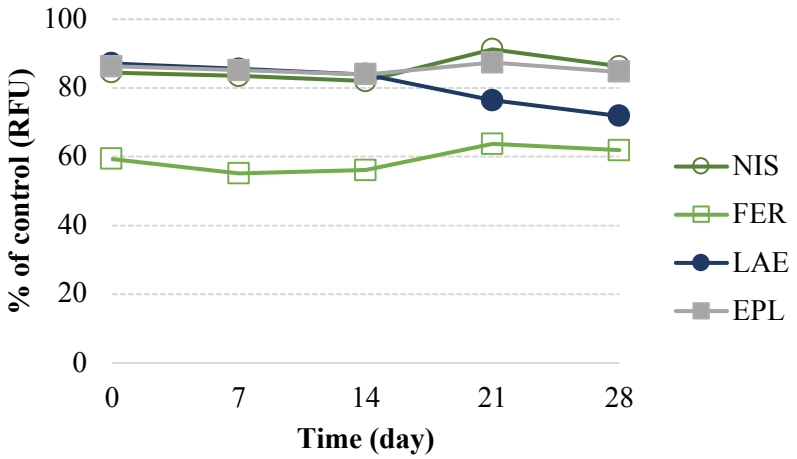


Figure 13. Fluorescence measurements of L. monocytogenes 10403S expressing GFP (preliminary screening). *Growth curves of LM-GFP in QF treated with different antimicrobials (NIS, FER, LAE and EPL), including a positive control with no treatment. Measurements obtained with fluorometer. The mean of three independent batches for measurements days 0, 7, 14 and for one batch days 21 and 28, is displayed.*

The result of the preliminary screening was not reliable because of difficulty with drying cheese due to the storage conditions during the experiment. No correlation could be determined between the viable cell count and the fluorescence measurements of the LM-GFP. The lack of correlation from the result above, entailed in the implementation of an improved screening – the evaluating of the screening method, which is presented in the following section.

4.2 Evaluation of the screening method

The main purpose of this experiment was, similar to the preliminary screening, to determine if the validated MLQF and newly developed HTQF models were comparable. Additionally, in order to assess which reporter system, GFP or LUX, was most representative for analyzing the growth of *L. monocytogenes* in the HTQF model, an evaluation of the screening method was executed.

4.2.1 Verification of the luminescent bacteria

The luminescent bacteria (LUX) was purchased from a company as *L. monocytogenes*. The experiments were performed based on this knowledge. However, after the experiment were completed, the results did not show a growth behavior of *L. monocytogenes.* At that point, the LUX was evaluated and verified to be *Klebsiella pneumoniae.* The strain was received from the company via an agar plate, from which colonies were isolated. Two different agar plates with LUX colonies were sent by the company, in order to verify if any contamination might have occurred after receiving them. As presented below, the two different plates gave similar result.

This deviation was discovered after all experiments were completed but described first in this section in order to better understand the presentation of the results below.

4.2.1.1 First received agar plate with LUX

As seen in the growth behavior (Figure 18) of the luminescent bacteria (LUX), assumed to be luminescent *L. monocytogenes*, it does not appear the same as the parental strain (Figure 15). Therefore investigation of this strain was necessary and performed after all the experiments were completed. Gram staining and 16S sequencing were performed in order to determine what strain the LUX was. Additionally, plating was also performed, on PALCAM with ceftazidime and on LB with kanamycin. These experiments were executed both on the LUX, and with a *L. monocytogenes* 10403S as a reference.

The result from the 16S sequencing (Appendix 1) showed that the bacterium was *Klebsiella pneumoniae* (with a 97% identity). However, the gram staining (Figure 14) indicated gram positive bacteria with a spherical shape. The *K. pneumoniae* is gram negative and rod-shaped (Boone and Castenholz, 2001).

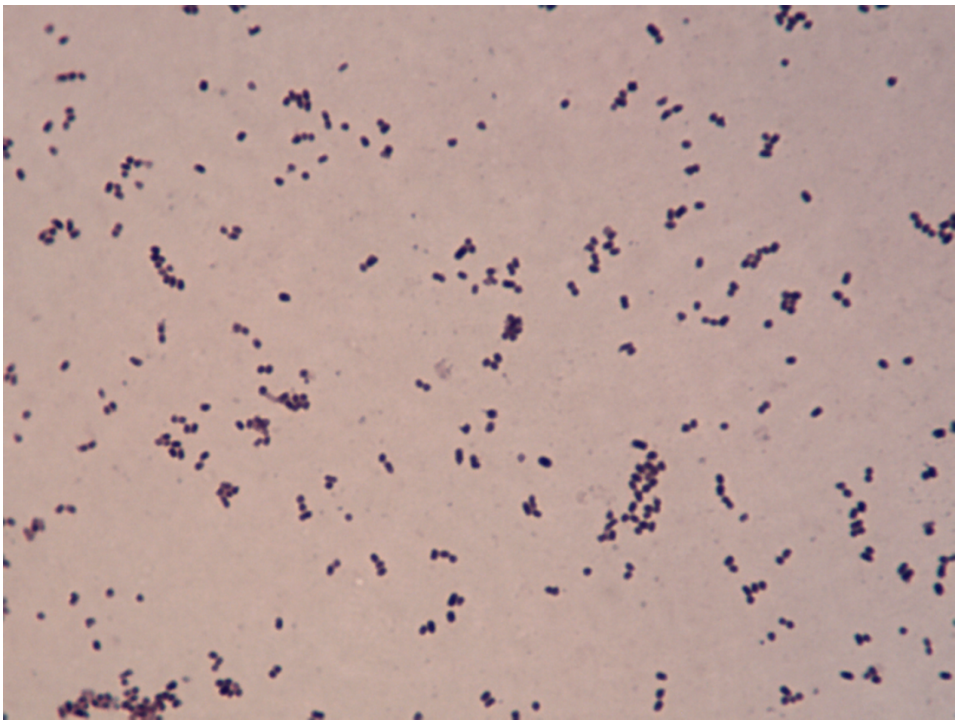


Figure 14. Gram staining of the luminescent bacteria. *Picture taken with microscope.*

4.2.1.2 Second received agar plate with LUX

The second plate with colonies, received from the company, was also analyzed with 16S sequencing. Different colonies was isolated and sequenced, and the result (Appendix 1) indicates a mix of *L. monocytogenes* and *K. pneumoniae.*

4.2.2 Growth measurements with VC

This experiment took precautionary measures, addressing the difficulty with drying cheese, found in the preliminary screening.

The LM, LM-GFP and LM-COCKTAIL contaminated cheeses were treated with LAE, EPL and LAE/EPL (Figure 15, Figure 16, Figure 17) in the evaluation of the screening method. The additional LUX had the same treatments (Figure 18). The plate counts from this experiment consisted of duplicate trials, with triplicate samples within each trial.

The control and treatment with LAE gave similar results for the two *L. monocytogenes* strains and the LM-COCKTAIL. The control cheese (no treatment) grew 4 log CFU/g with an initial value of 4 log CFU/g and a final at 8 log CFU/g after 28 days. Similarly, the LAE treatment also gave identical growth for the two strains and the cocktail over 28 days, with an increase of 4 log CFU/g, same as for the control. The VC results for the growth of the control and treatment with LAE in the HTQF, was the same as previously shown in the MLQF model (Martinez Ramos, 2017).

The LM (Figure 15) and LM-GFP (Figure 16) were comparable for the two additional treatments, EPL and LAE/EPL. After 28 days, they grew 1 log CFU/g with EPL and no change for the bacteriostatic LAE/EPL treatment. However, the result for LM-COCKTAIL (Figure 17) was different. For the EPL it grew 2.5 log CFU/g over 28 days compared to the 1 log for the other two. With a LAE/EPL treatment it also grew 2.5 log CFU/g, exactly the same as with only EPL.

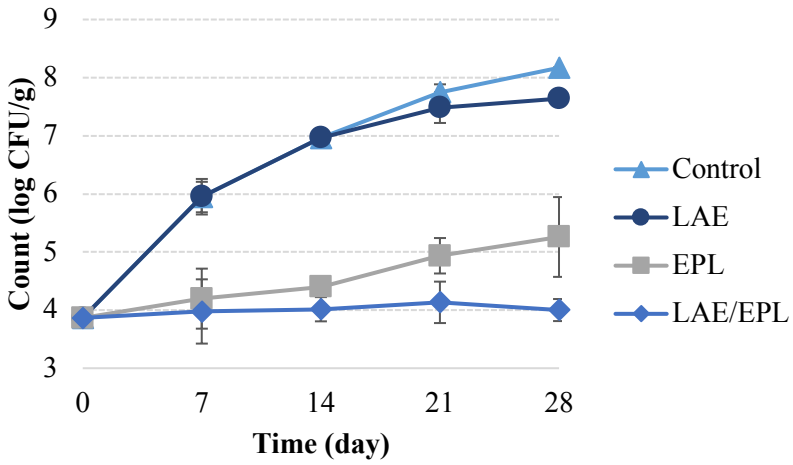


***Figure 15. L. monocytogenes 10403S (evaluation of screening method)**. Growth curves of LM in QF treated with different antimicrobials (LAE, EPL and LAE/EPL), including a positive control with no treatment. Measurements obtained by VC. The mean ± SEM of two independent batches is displayed.*

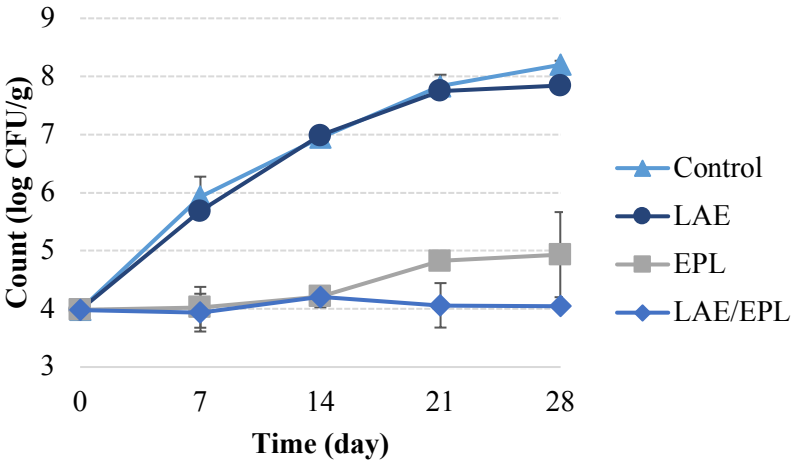


***Figure 16. L. monocytogenes 10403S expressing GFP (evaluation of screening method)**. Growth curves of LM-GFP in QF treated with different antimicrobials (LAE, EPL and LAE/EPL), including a positive control with no treatment. Measurements obtained by VC. The mean ± SEM of two independent batches is displayed.*

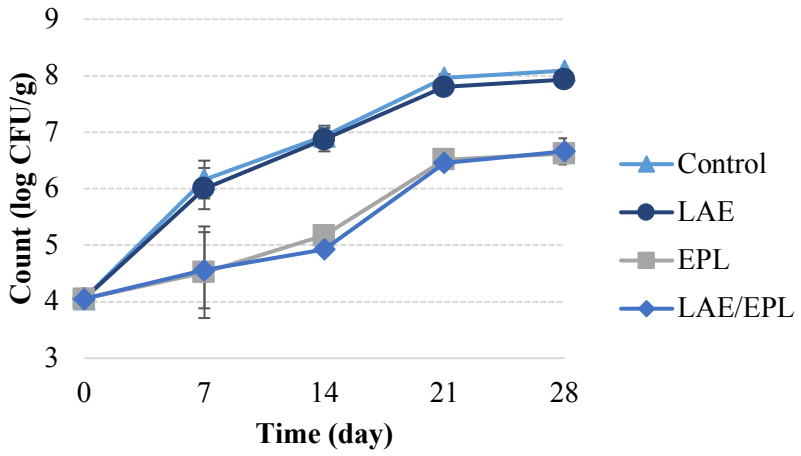


***Figure 17. L. monocytogenes cocktail (evaluation of screening method)**. Growth curves of LM-COCKTAIL in QF treated with different antimicrobials (LAE, EPL and LAE/EPL), including a positive control with no treatment. Measurements obtained by VC. The mean ± SEM of two independent batches is displayed.*

The LUX (Figure 18) grew the same for all the treatments and the control. This was deviate compared to the graphs of the different *L. monocytogenes* presented above. Over 28 days, the growth was 0.5 log CFU/g, compared to an increase of 4 log CFU/g for the control of the *L. monocytogenes*. The growth behavior of the LUX showed no difference between treatments or control, and therefore none of the antimicrobial seemed to work on *K. pneumoniae*.

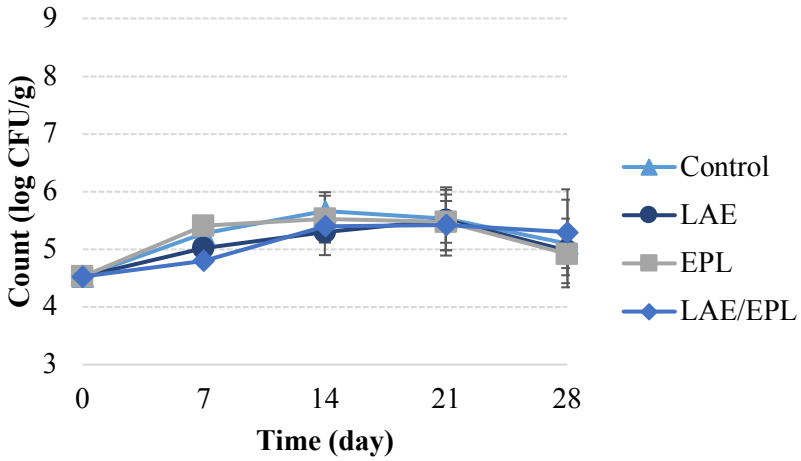


Figure 18. ***K. pneumoniae (evaluation of screening method)**. Growth curves of LUX in QF treated with different antimicrobials (LAE, EPL and LAE/EPL), including a positive control with no treatment. Measurements obtained by VC. The mean ± SEM of two independent batches is displayed.*

The result above for the LM and the LM-GFP was similar to the previous result from the validated MLQF. This implies that the HTQF and MLQF were comparable. The growth behavior with the different treatments were the same, and the control grew the same in this experiment as in previous MLQF studies (Martinez Ramos, 2017; Van Tassell et al., 2015).

As a comparison between the preliminary screening and the evaluating of the screening method, the results from these two experiments were very different. The difference was mainly because of dried cheese and lack of knowledge in the preliminary screening, as result show.

The growth behavior of the control for the LM were 1 log CFU/g (Figure 10) and 4 log CFU/g (Figure 15) respectively for the different screenings. The included treatments were different for the LM in the two screenings and were therefore not comparable. For the LM-GFP however, two of the treatments were the same; LAE and EPL. The control of the LM-GFP grew 2 log CFU/g (Figure 11) and 4 log CFU/g (Figure 16) respectively. The LAE did not have any effect in either of the screenings (grew as the control), but the total increase over the 28 was different. The LM-COCKTAIL had different treatments in the two screenings, hence could not be compared. The controls however, grew with 1 log of difference; 3 log CFU/g (Figure 12) and 4 log CFU/g (Figure 17) respectively. The combined result, based on the two *L. monocytogenes* strains and the cocktail, shows a significant difference between the two screenings.

4.2.2.1 Statistics

A comparison between the *L. monocytogenes* strains and the cocktail, for each treatment of the result from the evaluation of the screening method is presented in Table 7. This result confirms what was already apparent from Figure 15, Figure 16 and Figure 17. The LM and LM-GFP had no significant difference for any treatments. However, the LM-COCKTAIL was significantly different from the other two strains for the treatment with EPL and LAE/EPL. The significant difference is indicated with an asterisk and red font color in the table.

***Table 7. Statistical analysis with MANOVA**, Analysis of comparison between strains (LM, LM-GFP and LM-COCKTAIL) for each treatment. * and red color indicates significant difference between strains.*

Treatment	Strains	Prob > F
LAE	LM, LM-GFP, LM-COCKTAIL	0.7471
	LM-COCKTAIL excluded	0.7115
EPL	LM, LM-GFP, LM-COCKTAIL	0.0263*
	LM-COCKTAIL excluded	0.3046
LAE/EPL	LM, LM-GFP, LM-COCKTAIL	0.0373*
	LM-COCKTAIL excluded	0.9024
Control	LM, LM-GFP, LM-COCKTAIL	0.8357
	LM-COCKTAIL excluded	0.8288

The canonical centroid plots, produced in JMP, are an additional tool to analyze the significance of the strains with different treatments. They present the significance visually instead of with values, for the LM, LM-GFP and LM-COCKTAIL. Based on Figure 19 and Figure 20, it was seen that the LM-COCKTAIL was significantly different from LM and LM-GFP for the treatments with EPL and LAE/EPL.

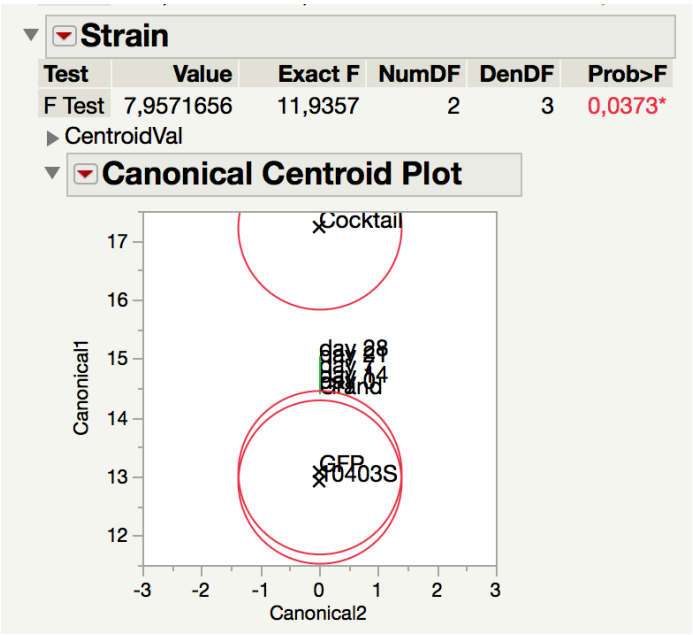


Figure 19. Result of the statistical analysis of L. monocytogenes 10403S expressing GFP, L. monocytogenes 10403S and L. monocytogenes five strain cocktail. *The analyzed treatment is LAE/EPL.*

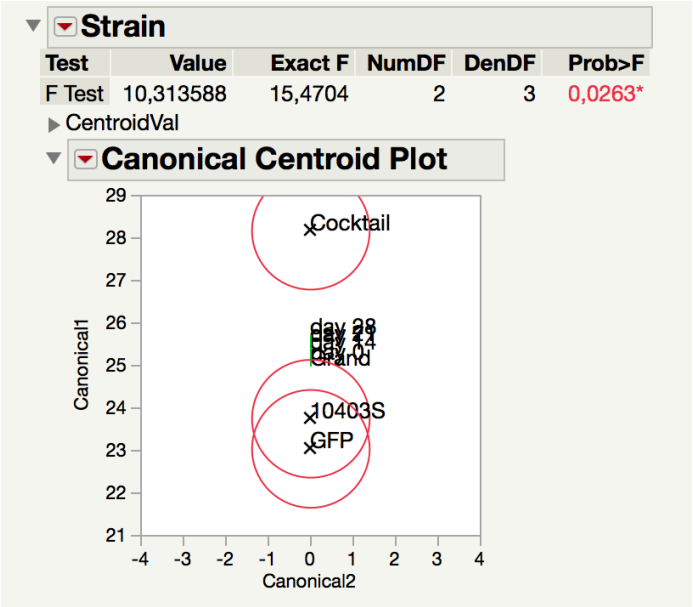


Figure 20. Result of the statistical analysis of L. monocytogenes 10403S expressing GFP, L. monocytogenes 10403S and L. monocytogenes five strain cocktail. *The analyzed treatment is EPL.*

4.2.3 Growth measurements using the fluorometer

Measurements with LM-GFP and LUX was performed to conclude if the GFP or LUX was the better alternative as a reporter system for this model.

4.2.3.1 Standards

In order to enable an equivalent determination of the CFU/g in the samples, based on the light intensity from each type of reporter system, a standard curve was incorporated in the measurements with the fluorometer for the evaluation of the screening method. Two different kinds of standards were applied; in broth and on cheese. The standard curve was used to correlate the result from the fluorescence and the equivalent CFU/g with the known CFU/g of the standard. This equivalent result

was based on the equation from the relation between Relative Fluorescence Unit (RFU) and CFU/g, and Relative Light Unit (RLU) and CFU/g respectively.

The following results present different measurements of fluorescence and luminescence in the fluorometer. There were two different highest values for the measurements. Some had the highest value of 9 log CFU/g and some had 6 log CFU/g, which can be seen in the graphs (Figure 21, Figure 22, Figure 23, Figure 24). The reason for the different scales was that the standard curves were evaluated and had small changes along the experiment, and therefore also change in concentrations.

The standard with LM-GFP in cheese (Figure 21) had a highest value of 9 log CFU/g for days 7 and 14. For days 21 and 28 the highest value was 6 log CFU/g.

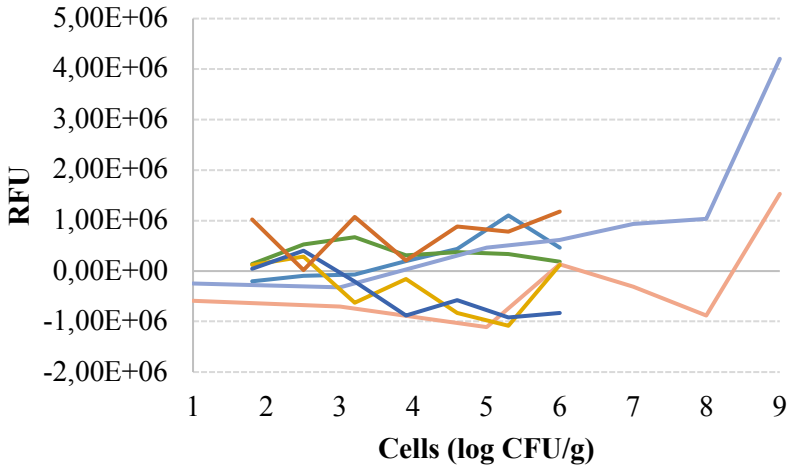


Figure 21. Fluorescence measurements of L. monocytogenes 10403S expressing GFP standard in cheese. *Standard curves of LM-GFP in QF, for concentrations from 1 to 9 log CFU/g. Measurements obtained with fluorometer. Each colored line represents one measurement point, during days 7 to 28. Graphs from both independent trials, for the each day are displayed.*

The standard with LM-GFP in broth (Figure 22) had a highest value of 9 log CFU/g for all days of the measurements.

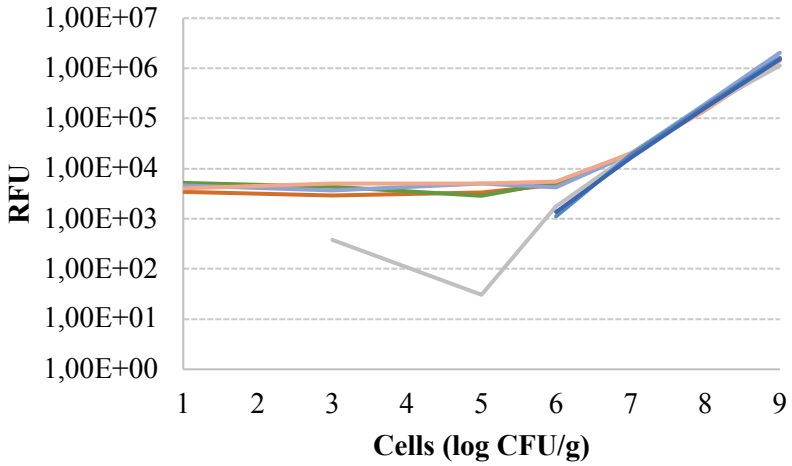


Figure 22. Fluorescence measurements of L. monocytogenes 10403S expressing GFP standard in broth*. Standard curves of LM-GFP in QF, for concentrations from 1 to 9 log CFU/g. Measurements obtained with fluorometer. Each colored line represents one measurement point, during days 0 to 28. Graphs from both independent trials, for the each day are displayed..*

The standard with luminescent *K. pneumoniae* in cheese (Figure 23) had a highest value of 9 log CFU/g for days 7 and 14. For days 21 and 28 the highest value was 6 log CFU/g.

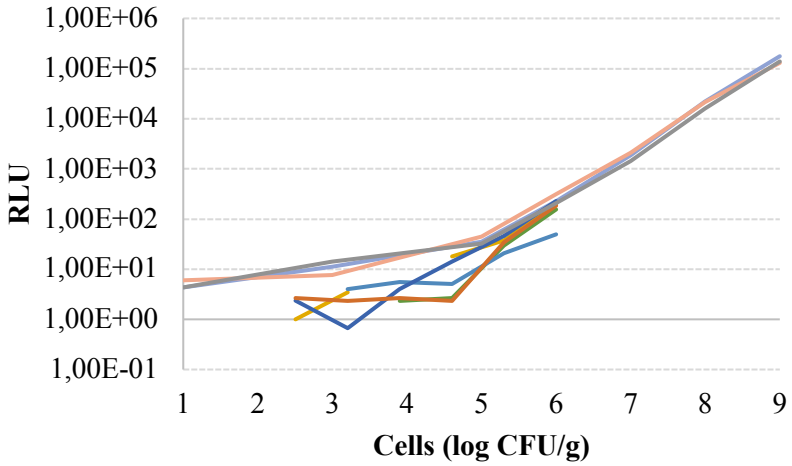


Figure 23. Luminescence measurements of K. pneumoniae standard in cheese. *Standard curves of LUX in QF, for concentrations from 1 to 9 log CFU/g. Measurements obtained with fluorometer. . Each colored line represents one measurement point, during days 7 to 28. Graphs from both independent trials, for the each day are displayed.*

The standard with luminescent *K. pneumoniae* in broth (Figure 24) had the highest value of 9 log CFU/g for all days of the measurements.

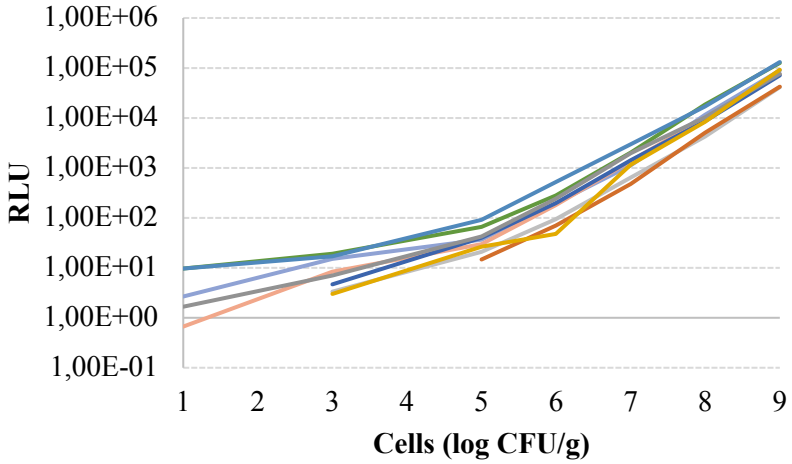


Figure 24. Luminescence measurements of K. pneumoniae standard in broth. *Standard curves of LUX in QF, for concentrations from 1 to 9 log CFU/g. Measurements obtained with fluorometer. Each colored line represents one measurement point, during days 0 to 28. Graphs from both independent trials, for the each day are displayed.*

4.2.3.2 Standardized CFU

Depending on whether the standard was in broth (Figure 26) or on cheese (Figure 25), the fluorescence results for the LM-GFP were different. Also, neither of the standards gave a good correlation to the VC. The broth standard gave a result where the control was highest at all measuring points, while the treatments were all lower than the control but quite the same as each other for all days. Between days 14 and 28 there was no increase in fluorescence, for either of the samples.

The cheese standard had the same trend as the broth standard for the first two measurements, with a difference between control and treatments. For days 21 and 28, both treatments and control were all the same and showed no particular increase in fluorescence.

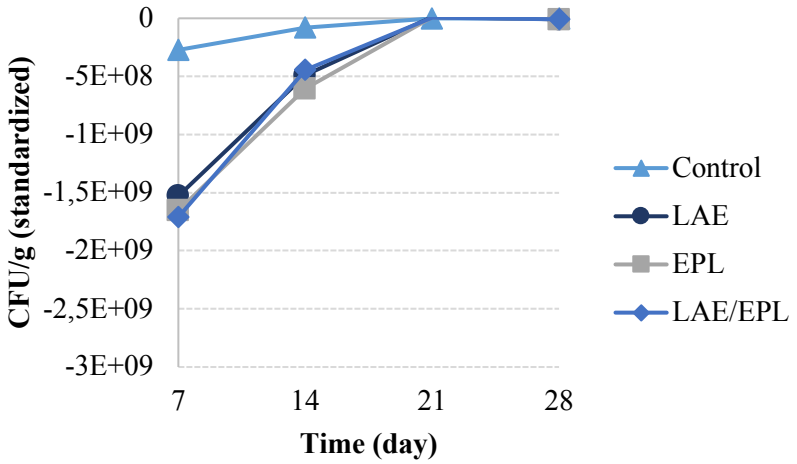


Figure 25. L. monocytogenes 10403S expressing GFP, result from the fluorescence measurements standardized by the cheese standard. *Growth curves of LM-GFP in QF treated with different antimicrobials (LAE, EPL and LAE/EPL), including a positive control with no treatment. Measurements obtained with fluorometer. The mean of two independent batches is displayed.*

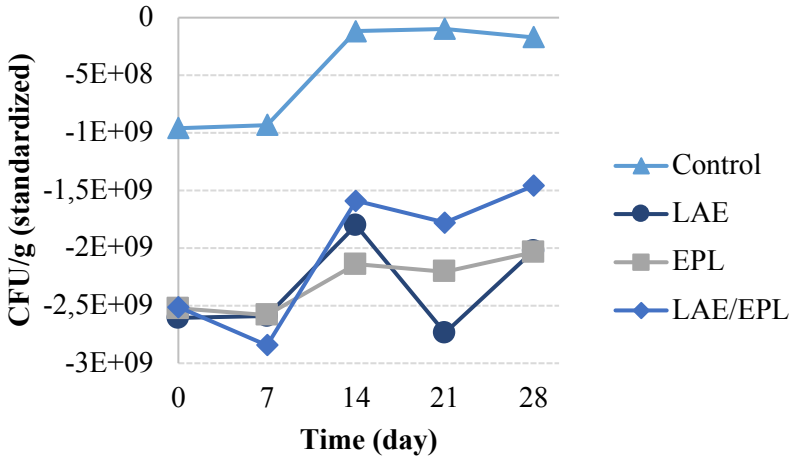


Figure 26. L. monocytogenes 10403S expressing GFP, result from the fluorescence measurements standardized by the broth standard. *Growth curves of LM-GFP in QF treated with different antimicrobials (LAE, EPL and LAE/EPL), including a positive control with no treatment. Measurements obtained with fluorometer. The mean of two independent batches is displayed.*

The luminescence was the same for control and treatments from day 0-28, for both standard variants (Figure 27, Figure 28), they had the same trend but with different values. The increase in luminescence was apparent during all of the measurements. However, the equivalent CFU/g of the samples differed between results based on the standard in broth and cheese. These standards were placed in the same plate, measuring the same samples for each standard respectively. This same procedure was applied for the fluorescence measurements.

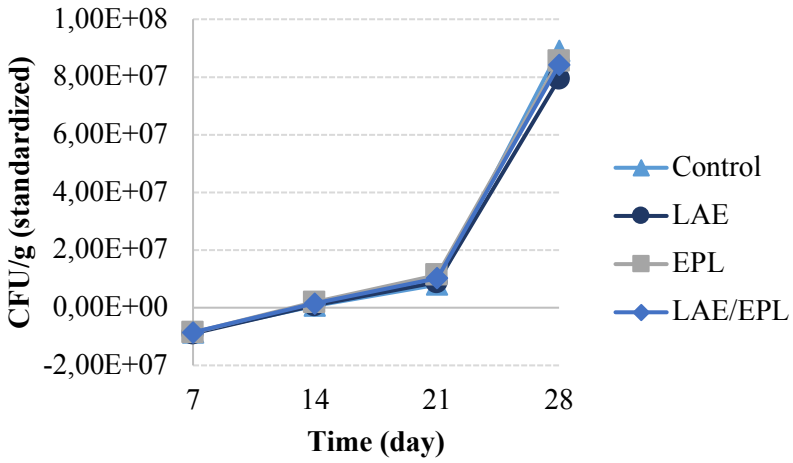


Figure 27. K. pneumoniae with LUX, result from measurements with fluorometer standardized from the cheese standard. *Growth curves of LUX in QF treated with different antimicrobials (LAE, EPL and LAE/EPL), including a positive control with no treatment. Measurements obtained with fluorometer. The mean of two independent batches is displayed.*

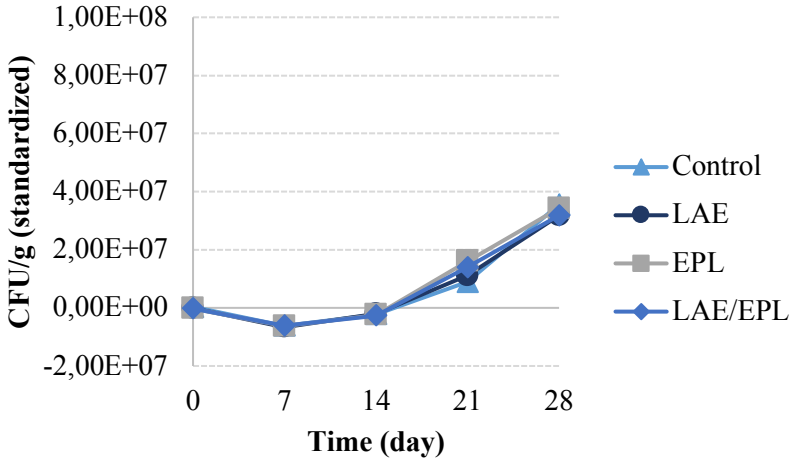


Figure 28. K. pneumoniae with LUX, result from measurements with fluorometer standardized from the broth standard. *Growth curves of LUX in QF treated with different antimicrobials (LAE, EPL and LAE/EPL), including a positive control with no treatment. Measurements obtained with fluorometer. The mean of two independent batches is displayed.*

The result above were based on an assessment of the HTQF model, in order to determine which reporter system was the most representative one. The result show potential in correlating the VC with luminescence (Figure 18, Figure 27, Figure 28). However, the correlation between the VC and fluorescence (Figure 16, Figure 25, Figure 26) was not as promising.

4.2.4 Verification of the presence of the plasmid

In order to verify that the plasmid was still inside the cell of LM-GFP, during the entire experiment, colonies from the VC of this strain was evaluated. Every LM-GFP colony moved from each plate, for all measurements, onto BHI and BHICM gave a positive result, verifying that the plasmid was present in the cell throughout the experiment. However, colonies from day 21 and 28 were not analyzed for either trial of the evaluation of the screening method because of the destruction of plates. The plates were stored in a 4°C refrigerator, awaiting the colonies to be recultured on BHI and BHICM. Before this was executed, the refrigerator froze and the plates with the colonies were destroyed and could no longer be evaluated.

4.2.5 Investigation of autofluorescence

To determine the prevalence of autofluorescence of the QF, blank cheeses were measured with a monochromatic fluorometer. The emission spectrum was measured, with a set excitation at 485 nm, since this is the same excitation wavelength as used for GFP. The peak at 485 nm in every figure was because of the excitation at that wavelength. The measurements of the blank QF (Figure 29) showed no emission at 535 nm, the normal wavelength of emission for GFP.

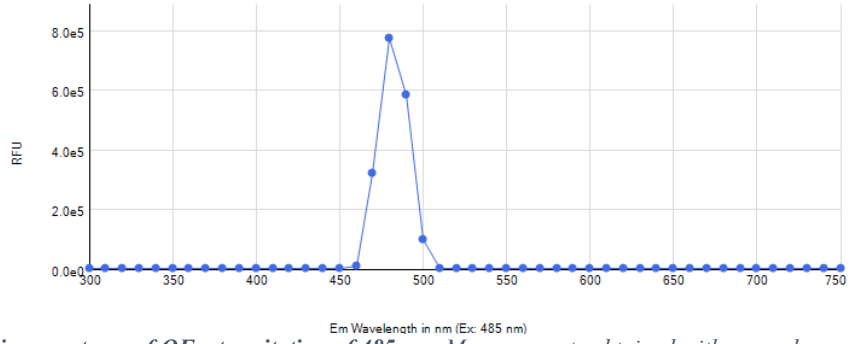


Figure 29. Emission spectrum of QF, at excitation of 485 nm. *Measurements obtained with monochromatic fluorometer.*

The measurements of the PBS, as a reference for the background of the diluted inoculum in the experiments (Figure 30) showed no emission at 535 nm.

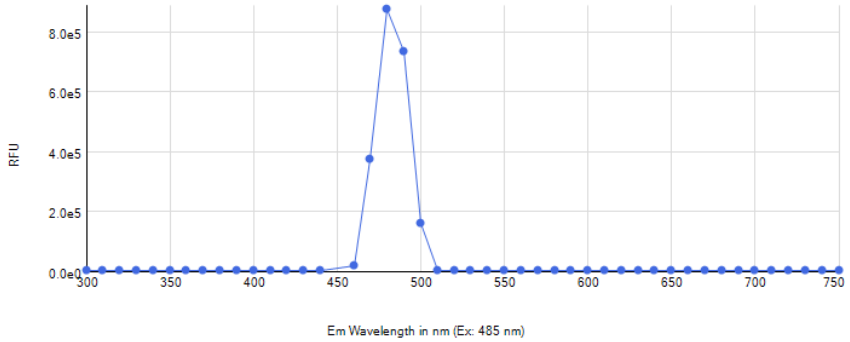


Figure 30. Emission spectrum of PBS, at excitation of 485 nm. *Measurements obtained with monochromatic fluorometer.*

The measurements of the E. coli expressing GFP (Figure 31) indicated a tail of the first peak, showing emission at 535 nm, which is normal emission for GFP.

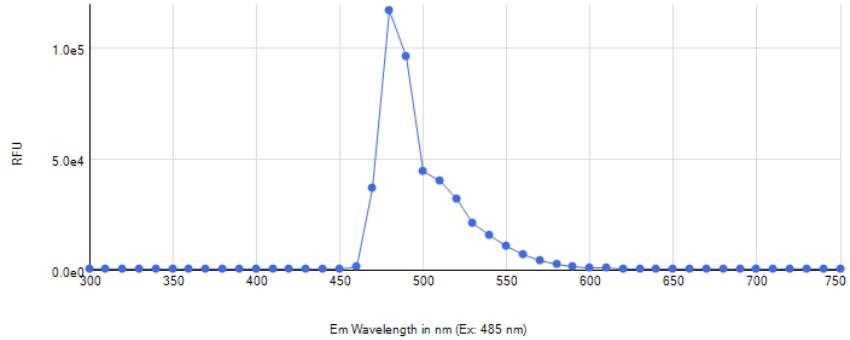


Figure 31. Emission spectrum of E.coli expressing GFP (as a reference for GFP expression), at excitation of 485 nm. *Measurements obtained with monochromatic fluorometer.*

5. Discussion

For this study, the focus was initially to compare the validated MLQF (Van Tassell et al., 2015) with the proposed HTQF model. Also, this study aimed to investigate the applicability of *L. monocytogenes* with two different reporter genes; LUX and GFP for the HTQF. This comparison was interesting since these reporter systems have very different behavior, such as their different mechanism in emitting light (Fan and Wood, 2007). They were therefore evaluated to see their difference in food matrix and correlation to VC. To my knowledge, QF has never been analyzed with fluorescence or luminescence before. Thus, the result in this study is new information within this area of research.

The strains utilized in the comparisons were *L. monocytogenes* 10403S (LM), a five strain cocktail of *L. monocytogenes* (LM-COCKTAIL), *L. monocytogenes* 10403S expressing GFP (LM-GFP), and a luminescent bacteria (LUX). During the final stages of the evaluation of the screening method, the LUX bacteria proved to be *Klebsiella pneumoniae* (with a 97% identity from the 16S sequencing) instead of *L. monocytogene*s. Therefore, comparing these two with equal conditions became technically impossible. The company, from who the LUX strain was purchased, genetically altered the product in order to create a luminescent *L. monocytogenes*. Based on the result from the investigation of LUX colonies, it was believed that the company sent a contaminated plate, with a mix of *L. monocytogenes* and *K. pneumoniae*, both luminescent. Consequently, the focus of investigation has primarily been on comparing different strains and their microbial behavior based on different microbial treatments, in order to see if the *L. monocytogenes* strains were comparable. In this matter, the HTQF was compared to MLQF in order to decide whether or not they were comparable. The results of the evaluation of the screening method were more reliable than from the preliminary one. The trials within the evaluation of the screening method were performed with more knowledge, practice and also with more consistency. These are the reasons why they are the results focused on in this report. One of the learning outcomes from the primary screening was the dried cheese. In the evaluation of the screening method, this was an necessary improvement in order to gain better, more reliable result.

The different *L. monocytogenes* 10403S strains evaluated in this study had some limitations due to their origin. They were originally laboratory strains and not natural isolates. However, this limitation must be considered when evaluating the result comparing with the result from the LM-COCKTAIL. Also, it would be even more interesting if one of the isolates was from an outbreak with QF. Nevertheless, one of the five food-borne isolated *L. monocytogenes* in this study were at least from a cheese outbreak. There was also the limitation of only evaluating one particular strain in this study, *L. monocytogenes*. Therefore, the results were mainly regarding the behavior of this strain. If other pathogenic strains could be engineered and evaluated with the same method, it would have very broad analysis possibilities.

Based on previous research, the strains did not behave as predicted in the preliminary screening. The antimicrobials did not show the same effect as in earlier studies with MLQF (Martinez Ramos, 2017; Van Tassell et al., 2015). Also, there was difference between the strains for the same treatment. LAE and NIS was expected to not have any antimicrobial effect. The treatment with EPL was predicted to have an effect between none and bacteriostatic on *L. monocytogenes*. FER should have a bacteriostatic

effect. The reason for the different result compared to previous research was believed to be due to the dried out cheese, which probably affected the result significantly. The cheese dried because of the lack of protection. The cheese in this experiment was only sealed with a lid, and no additional film. However, the LM-GFP in the black 96-well microplate had an extra cover with a plastic film, since it was required for the measurements in the fluorometer. This plate had a slightly less dried cheese than the cheeses in the clear plate in this round of experiments. Due to the drying cheese issue, the above results for the preliminary screening should be viewed with suspicion.

Concerning the growth behavior from the evaluation of the screening method, the results for the control and treated cheese of LM and LM-GFP showed that they grew similar as in previous research with MLQF (Martinez Ramos, 2017; Van Tassell et al., 2015). However, compared to the same research, the result for the LM-COCKTAIL with the EPL and LAE/EPL treatment, had a different growth behavior in this study. Therefore, the LM-COCKTAIL needs to be further investigated. Since the result for the LM in this study was similar to previous evaluated growth behavior of this strain in MLQF, it suggest there was no shortages for HTQF. Instead, the LM-COCKTAIL results seems deviate and additional trials with the cocktail need to be performed and evaluated in order to see if it was an error only in this set of experiments. However, since the cocktail was a combination of five different strains, it was not possible to know if it was a specific strain that affects the results, since they were not distinguished from each other in this experiment. What also needs to be considered when comparing these two different results of the LM-COCKTAIL though, was the fact that the cocktails in the two scenarios were different. In the published result (Van Tassell et al., 2015) the cocktail consisted of clinical *L. monocytogenes* and not natural, as in this study. Due to time limitations, the evaluation of the screening method was only performed twice with triplicates instead of three times with duplicates. This decreases the certainty of the data.

For all the plates in the evaluation of the screening method, a temperature abuse might have occurred during the storage. The refrigerator, where the plates were stored, broke a few weeks after the experiment ended. Therefore it must be considered that the refrigerator might not have been fully functional during the weeks prior, and this could be an explanation of the result of the LM-COCKTAIL. Another explanation for the deviate LM-COCKTAIL results in the evaluation of the screening method could be how the EPL behaves in the cheese, which has not been evaluated in MLQF before. Based on previous research, with the MLQF, it can be assumed that the lower the concentration, the lower antimicrobial effect on the *L. monocytogenes* in QF (Martinez Ramos, 2017). EPL is water soluble, but there is lack of knowledge about how well it is retained in the cheese or if it is removed with the whey in the final step of the cheesemaking. Therefore, it would be of interest to evaluate its retention coefficient. The maximum permissible concentration in the US for the EPL was used in this study. If the EPL was decreased during the whey removal, it might give an decreased antimicrobial effect which could explain the result of the LM-COCKTAIL for EPL and LAE/EPL in this study.

The result from the luminescence and fluorescence measurements is what will be discussed in the forthcoming parts. Despite the same bacteria not being employed in the fluorescence and luminescence measurements, there can be some conclusions drawn from comparing the results from

these two. However, these comparisons were not completely reliable because of this drawback. The results indicated that measuring the luminescence could be a better method than measuring fluorescence, since the trend of the graphs from the fluorometer and the VC were similar for the LUX. It was more difficult to find a similarity for the LM-GFP. A limitation of the fluorescence measurement was that all the results were negative (Figure 25, Figure 26). This due to the subtraction of the blank for each sample. The blank (cheese without any LM-GFP or treatment), had a higher fluorescence value than the samples which resulted in a negative value after subtraction. This could be due to autofluorescence or another fluorescence disturbance resulting in the difficulty for measuring the fluorescence on the food matrix (Fan and Wood, 2007). However, measurements with a monochromatic fluorometer (Appendix 3) suggested there was no autofluorescence from the QF. Further investigation into this phenomena will be necessary for future implementation of LM-GFP in the HTQF.

The detected luminescence measurements indicated that the amount of luminescence increased with the amount of cells. This applies both to the measurements for the 28 days in the cheese, but also for the different concentrations of the samples in standard curves. A possible advantage with the luminescence, compared to the fluorescence, is the independently evolved light. No light source was involved for the detection of the luminescence, which means there is no risk of photobleaching, unlike with GFP. Photobleaching means when the emitted fluorescence decrease with time (Diaspro et al., 2010). In order to address the most accurate measurement possible, the integration time was set to 1 000 ms. The higher integration time results in a longer amount of time for the measurement per plate.

It is also worth noting that the result from the LM-GFP might be less accurate because of the long half-life of GFP (Lesmana and Friedl, 2001). The numbers of bacteria was not for certain correlated to the detection level of fluorescence. The bacteria could still be fluorescent even if not necessarily alive. In a study by Lowder et al., (2000) *pseudomonas* was studied in order to determine if fluorescence from GFP was effected in starved, viable but nonculturable and dead or dying cells. Their result showed that the viable but nonculturable cells were 80% fluorescent compared to the viable, non-stressed cells and they remained fluorescent for 11 months. Most of the dead cells were not fluorescent but some still had a lower GFP concentration compared to the concentration in the live cells. Lowder et al., (2000) suggests that viable but nonculturable cells remain fluorescent but it is lost when the cells die. They implies the reason is because of membrane integrity is lost. This result could be true for *L. monocytogenes* also, however, it could be quite challenging to introduce a step to detect live/dead bacteria in this study with the HTQF. Overall, the result of the LM-GFP, both from VC and fluorometer, do not make it possible to draw any conclusions concerning a possible correlation between the two.

Another kind of temperature abuse than mentioned earlier concerns the black and white plates in this study. They were exposed to a greater fluctuation in temperature than the clear plates. Approximately 30 minutes were required to prepare the standards prior to an additional 30 minutes during the measurements in the non-temperature controlled fluorometer. This resulted in some of these cheeses becoming a bit drier than the cheese in the clear plates. It also seemed like the cheeses located in the

outer wells of the plate were drier than the other positions. An additional consequence of the temperature abuse might be stressed *Listeria* cells, because of the drastically difference in temperature. The physiological state of the cell has an significant impact on the intensity of the cellular fluorescence (Dupont and Augustin, 2011). Perhaps stressed cells were not a consequence of temperature abuse for *L. monocytogenes* since it is psychrotrophic. Regardless, it should be considered for the result from the plates measurements. Also, in comparison to the black and white plates. The cheese on these entire 96-well microplates was exposed to air and light every week since the protective aluminum foil was removed for the fluorometer measurements. The clear plates however, had the aluminum foil protection all the time since only one part was removed in order to take the HTQF cheeses out for the VC measurements.

The HTQF model had a higher throughput than the MLQF model which makes it a more efficient screening tool. If this model can be further developed and validated, in the future it would save time and money by eliminating numerous VC. The HTQF has come a long way in its development and shows a great potential as a time efficient screening tool.

One point of possible error from the fluorometer measurements would be if diffusion was a problem. The cheese model is made of a soft and complex matrix with a lot of fat globules that might interact with the *L. monocytogenes* over time, making it harder for the fluorometer to detect and measure its activity. Perhaps this could be evaluated visually with fluorescence microscopy, by following the development of the *L. monocytogenes* and the cheese during the 28 days. Additionally, this might explain the comparison between the standards in broth versus cheese. These results indicated the food matrix's impact on the measurements in the fluorometer, compared to measuring *L. monocytogenes* in broth. The standard on the food matrix did not give as good result as in broth. In the methodology employed in this study, the *L. monocytogenes* was inoculated on the surface of the QF. This was performed primarily to get more accurate measurements in the fluorometer when measured from top. Surface inoculation is more representative of post processing contamination, however, mixing it into the cheeses is more representative of processing contamination. Since this model only was evaluated with a surface inoculation, it could be thought of as a limitation.

One of the biggest setbacks in this study was the evaluated luminescent bacteria, since it turned out not to be *L. monocytogenes*, and therefore did not contribute to as much knowledge as desired. The gram staining of the LUX strain, received from the company, indicated it to be gram positive and coccus (Figure 14). The characteristics of *K. pneumoniae* is rod-shaped and gram negative. However, since the 16S sequencing gave a result of 97% identity of *K. pneumoniae* (Appendix 1). The latter is a more rigorous testing, and therefore it was assumed that the LUX strain evaluated in this study was *K. pneumoniae*. *K. pneumoniae* is not psychrotrophic, unlike the *L. monocytogenes*, which explains the lack of cell growth in the cheese at 4°C for this particular strain. However, it still grew 0.5 log CFU/g, even if there should not be any growth at all, although still alive (Tsuji et al., 1982). This could be because of the variation in temperature, mentioned earlier, occurring while measurements in the fluorometer was performed, hindering the plate to be held at 4°C at all time. Also occurring when standard was removed and added to the plate, which therefore might enable the growth of *K. pneumoniae* in QF.

6. Conclusion

In summary, this project has brought new insight on how downscaling the production of QF in tubes versus in 96-well microplates may affect the results. It has been shown that both models give similar results, and therefore the HTQF could be used to asses novel antimicrobials in future research. It may be suggested that the luminescence reporter system seems to be the better option for the specific purpose of measuring microbial growth in a food matrix such as QF. However, this needs to be confirmed with experiments using a luminescent *L. monocytogenes*. Finally, this study has taught me the importance of controlling purchased material and to not always rely on that professional companies not make mistakes.

7. Future Perspectives

The work performed in this study has contributed to more knowledge of the microbial behavior of engineered *L. monocytogenes*. There is still more work ahead in order to have a reliable and well-functioning model. Future work could focus on optimizing the standard for the measurements in the fluorometer, or evaluating other alternatives to optimize the measurements with this instrument. Additionally, the model could be further developed to evaluate how the novel antimicrobial treatments are affected by a modified atmosphere since many QF are distributed in this way. It would be of interest to evaluate how the HTQF model distribute the bacteria in the food matrix, and if the outcome is different if *L. monocytogenes* is mixed into the QF compared to inoculated on the surface. It would be valuable to further examine a luminescent *L. monocytogenes* for use in this model.

References

Adams, M.R., Moss, M.O., 2008. Food microbiology, 3rd ed. ed. RSC Publishing, Cambridge, UK.

Alves, E., Costa, L., Cunha, Â., Faustino, M.A.F., Neves, M.G.P.M.S., Almeida, A., 2011. Bioluminescence and its application in the monitoring of antimicrobial photodynamic therapy. Appl. Microbiol. Biotechnol. 92, 1115–1128. https://doi.org/10.1007/s00253-011-3639-y

Benkerroum, N., Sandine, W.E., 1988. Inhibitory Action of Nisin Against Listeria monocytogenes. J. Dairy Sci. 71, 3237–3245. https://doi.org/10.3168/jds.S0022-0302(88)79929-4

Biochem, P., 2010. GRAS Notice 000336: epsilon-Polylysine.

Boone, D.R., Castenholz, R.W., 2001. Bergey's Manual® of Systematic Bacteriology: Volume One The Archaea and the Deeply Branching and Phototrophic Bacteria. Springer New York : Imprint : Springer, New York, NY.

Borges, A., Ferreira, C., Saavedra, M.J., Simões, M., 2013. Antibacterial Activity and Mode of Action of Ferulic and Gallic Acids Against Pathogenic Bacteria. Microb. Drug Resist. 19, 256–265. https://doi.org/10.1089/mdr.2012.0244

Campos, F.M., Couto, J.A., Figueiredo, A.R., Tóth, I.V., Rangel, A.O.S.S., Hogg, T.A., 2009. Cell membrane damage induced by phenolic acids on wine lactic acid bacteria. Int. J. Food Microbiol. 135, 144–151. https://doi.org/10.1016/j.ijfoodmicro.2009.07.031

CDC, 2017. Listeria (Listeriosis) [WWW Document]. URL https://www.cdc.gov/listeria/index.html (accessed 4.12.18).

CDC, 2015. National Enteric Disease Surveillance: Listeria Annual Summary, 2014 8.

CDC, 2013. Vital Signs: Listeria Illnesses, Deaths, and Outbreaks — United States, 2009–2011 [WWW Document]. URL https://www.cdc.gov/mmwr/preview/mmwrhtml/mm6222a4.htm (accessed 4.15.18).

Chemistry and Light, 2018. Bioluminescence | Chemistry and Light.

Corish, P., Tyler-Smith, C., 1999. Attenuation of green fluorescent protein half-life in mammalian cells. Protein Eng. Des. Sel. 12, 1035–1040. https://doi.org/10.1093/protein/12.12.1035

CytoGuard LA [WWW Document], 2014. . AB Ingred. URL http://abingredients.com/ingredients/lauric_arginate/ (accessed 3.5.18).

Daghighi, S., Sjollema, J., Harapanahalli, A., Dijkstra, R.J.B., van der Mei, H.C., Busscher, H.J., 2015. Influence of antibiotic pressure on bacterial bioluminescence, with emphasis on Staphylococcus aureus. Int. J. Antimicrob. Agents 46, 713–717. https://doi.org/10.1016/j.ijantimicag.2015.09.007

Davies, E.A., Bevis, H.E., Delves-Broughton, J., 1997. The use of the bacteriocin, nisin, as a preservative in ricotta-type cheeses to control the food-borne pathogen Listeria monocytogenes. Lett. Appl. Microbiol. 24, 343–346.

Diaspro, A., Chirico, G., Usai, C., Ramoino, P., Dobrucki, J., 2010. Photobleaching, in: Handbook of Biological Confocal Microscopy: Third Edition. pp. 690–702. https://doi.org/10.1007/978-0-387-45524-2_39

Drugge, H., 2015. Support for ε-poly-lysine as Natural Antibacterial Preservative [WWW Document]. ThermoFisher Sci. URL https://www.thermofisher.com/blog/food/support-for-%c9%9b-poly-lysine-as-natural-antibacterial-preservative/ (accessed 3.5.18).

Dupont, C., Augustin, J.-C., 2011. Relationship between the culturability of stressed Listeria monocytogenes cells in non-selective and selective culture media and the cellular esterase activity measured by solid phase cytometry. J. Microbiol. Methods 87, 295–301. https://doi.org/10.1016/j.mimet.2011.08.018

EC No 2073/2005, 2005. Commission regulation (EC) No 2073/2005.

Fan, F., Wood, K.V., 2007. Bioluminescent Assays for High-Throughput Screening. ASSAY Drug Dev. Technol. 5, 127–136. https://doi.org/10.1089/adt.2006.053

FDA, 2017. CFR - Code of Federal Regulations Title 21 [WWW Document]. URL https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=184.1538 (accessed 4.8.18).

FDA, 2016. Compliance Policy Guides - CPG Sec. 555.320 Listeria monocytogenes [WWW Document]. URL https://www.fda.gov/ICECI/ComplianceManuals/CompliancePolicyGuidanceManual/ucm136694.htm#III_A (accessed 4.12.18).

Gálvez, A., Abriouel, H., López, R.L., Omar, N.B., 2007. Bacteriocin-based strategies for food biopreservation. Int. J. Food Microbiol. 120, 51–70. https://doi.org/10.1016/j.ijfoodmicro.2007.06.001

Haddock, S.H.D., Moline, M.A., Case, J.F., 2010. Bioluminescence in the Sea. Annu. Rev. Mar. Sci. 2, 443–493. https://doi.org/10.1146/annurev-marine-120308-081028

Harrigan, W.F., 1998. Laboratory methods in food microbiology, 3rd. ed. ed. Academic Press, San Diego.

Hawkins, D.R., Rocabayera, X., Ruckman, S., Segret, R., Shaw, D., 2009. Metabolism and pharmacokinetics of ethyl Nα-lauroyl-L-arginate hydrochloride in human volunteers. Food Chem. Toxicol. 47, 2711–2715. https://doi.org/10.1016/j.fct.2009.07.028

Henriques, M., Santos, G., Rodrigues, A., Gomes, D., Pereira, C., Gil, H., 2013. Replacement of conventional cheese coatings by natural whey protein edible coatings with antimicrobial activity. J Hyg. Eng. Des. 34–47.

Hiraki, J., Ichikawa, T., Ninomiya, S., Seki, Hideaki, Uohama, K., Seki, Hiroshi, Kimura, S., Yanagimoto, Y., Barnett, J.W., 2003. Use of ADME studies to confirm the safety of ε-polylysine as a preservative in food. Regul. Toxicol. Pharmacol. 37, 328–340. https://doi.org/10.1016/S0273-2300(03)00029-1

Hnosko, J., San-Martin Gonzalez, M.F., Clark, S., 2012. High-pressure processing inactivates Listeria innocua yet compromises Queso Fresco crumbling properties. J. Dairy Sci. 95, 4851–4862. https://doi.org/10.3168/jds.2011-5028

Holle, M.J., Ibarra-Sánchez, L.A., Liu, X., Staswiecz, M., Miller, M.J., 2018. Microbial analysis of commercially available US QF (In Review). J. Dairy Sci.

Ibarra-Sánchez, L.A., Van Tassell, M.L., Miller, M.J., 2017. Invited review: Hispanic-style cheeses and their association with Listeria monocytogenes. J. Dairy Sci. 100, 2421–2432. https://doi.org/10.3168/jds.2016-12116

Imaging & Microscopy - Research, Development, Production, 2013. Bioluminescence Microscopy | Imaging & Microscopy - Research, Development, Production [WWW Document]. URL https://www.imaging-git.com/science/light-microscopy/bioluminescence-microscopy (accessed 4.16.18).

ISS, 2015. ISS | Application Notes | Fluorescence Lifetime Measurements of BODIPY and Alexa Dyes on ChronosFD and K2 [WWW Document]. URL http://www.iss.com/resources/research/application_notes/K2CH_LifeMeasBODIPYAlexa.html (accessed 4.18.18).

IUL Instruments, 2017. Flash and Go - Automatic Colony Counter [WWW Document]. IUL Instrum. URL http://www.iul-inst.com/en/air-sampling/9-iul/86-flash-and-go-en (accessed 4.18.18).

Jameson, D.M., 2014. Introduction to fluorescence. CRC Press, Taylor & Francis Group, Boca Raton.

JHeimbach, LCC, 2005. GRAS Notice 000164: Lauramide arginine ethyl ester.

Lesmana, J., Friedl, P., 2001. Destabilization of Green Fluorescent Protein by Substitution of Its Amino-Terminal Residue, in: Animal Cell Technology: From Target to Market, ESACT Proceedings. Springer, Dordrecht, pp. 6–9. https://doi.org/10.1007/978-94-010-0369-8_2

Li, Y.-Q., Han, Q., Feng, J.-L., Tian, W.-L., Mo, H.-Z., 2014. Antibacterial characteristics and mechanisms of ε-poly-lysine against Escherichia coli and Staphylococcus aureus. Food Control 43, 22–27. https://doi.org/10.1016/j.foodcont.2014.02.023

Lowder, M., Unge, A., Maraha, N., Jansson, J.K., Swiggett, J., Oliver, J.D., 2000. Effect of starvation and the viable-but-nonculturable state on green fluorescent protein (GFP) fluorescence in GFP-tagged Pseudomonas fluorescens A506. Appl. Environ. Microbiol. 66, 3160–3165.

Luchansky, J.B., Call, J.E., Hristova, B., Rumery, L., Yoder, L., Oser, A., 2005. Viability of Listeria monocytogenes on commercially-prepared hams surface treated with acidic calcium sulfate and lauric arginate and stored at 4°C. Meat Sci. 71, 92–99. https://doi.org/10.1016/j.meatsci.2005.04.006

MacDonald, P.D.M., Whitwam, R.E., Boggs, J.D., MacCormack, J.N., Anderson, K.L., Reardon, J.W., Saah, J.R., Graves, L.M., Hunter, S.B., Sobel, J., 2005. Outbreak of Listeriosis among Mexican Immigrants as a Result of Consumption of Illicitly Produced Mexican-Style Cheese. Clin. Infect. Dis. 40, 677–682. https://doi.org/10.1086/427803

Martin, E.M., Griffis, C.L., Vaughn, K.L.S., O'Bryan, C.A., Friedly, E.C., Marcy, J.A., Ricke, S.C., Crandall, P.G., Lary, Jr., R.Y., 2009. Control of Listeria Monocytogenes by Lauric Arginate on Frankfurters Formulated with or without Lactate/Diacetate. J. Food Sci. 74, M237–M241. https://doi.org/10.1111/j.1750-3841.2009.01196.x

Martinez Ramos, A., 2017. Evaluación de mezclas a partir de la endolisina PlyP100, nisina, arginato laúrico y Ɛ-polilisina con efecto antimicrobiano sobre L. monocytogenes en queso fresco.

Mazzotta, A.S., Montville, T.J., 1997. Nisin induces changes in membrane fatty acid composition of Listeria monocytogenes nisin-resistant strains at 10°C and 30°C. J. Appl. Microbiol. 82, 32–38. https://doi.org/10.1111/j.1365-2672.1997.tb03294.x

Milillo, S.R., Badamo, J.M., Boor, K.J., Wiedmann, M., 2008. Growth and persistence of Listeria monocytogenes isolates on the plant model Arabidopsis thaliana. Food Microbiol. 25, 698–704. https://doi.org/10.1016/j.fm.2008.03.003

Ming, X., Daeschel, M.A., 1995. Correlation of Cellular Phospholipid Content with Nisin Resistance of Listeria monocytogenes Scott A. J. Food Prot. 58, 416–420. https://doi.org/10.4315/0362-028X-58.4.416

Paxton, J.R., Eschmeyer, W.N. (Eds.), 1998. Encyclopedia of fishes, 2nd ed. ed. Academic Press, San Diego, CA.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.-A., Roy, S.L., Jones, J.L., Griffin, P.M., 2011. Foodborne Illness Acquired in the United States—Major Pathogens. Emerg. Infect. Dis. 17, 7–15. https://doi.org/10.3201/eid1701.P11101

Shen, A., Higgins, D.E., 2005. The 5′ untranslated region-mediated enhancement of intracellular listeriolysin O production is required for *Listeria monocytogenes* pathogenicity: *hly* 5′ UTR-mediated regulation of LLO production. Mol. Microbiol. 57, 1460–1473. https://doi.org/10.1111/j.1365-2958.2005.04780.x

Soni, K.A., Desai, M., Oladunjoye, A., Skrobot, F., Nannapaneni, R., 2012. Reduction of Listeria monocytogenes in queso fresco cheese by a combination of listericidal and listeriostatic GRAS antimicrobials. Int. J. Food Microbiol. 155, 82–88. https://doi.org/10.1016/j.ijfoodmicro.2012.01.010

Soni, K.A., Nannapaneni, R., Schilling, M.W., Jackson, V., 2010. Bactericidal activity of lauric arginate in milk and Queso Fresco cheese against Listeria monocytogenes cold growth1. J. Dairy Sci. 93, 4518–4525. https://doi.org/10.3168/jds.2010-3270

SVA, 2017. Listerios - SVA [WWW Document]. URL http://www.sva.se/djurhalsa/zoonoser/listerios (accessed 5.2.18).

Takahashi, H., Kashimura, M., Koiso, H., Kuda, T., Kimura, B., 2013. Use of ferulic acid as a novel candidate of growth inhibiting agent against Listeria monocytogenes in ready-to-eat food. Food Control 33, 244–248. https://doi.org/10.1016/j.foodcont.2013.03.013

The Lowdown on Lauric Arginate, 2005. . Food Qual. Saf.

ThermoFisher, 2018. Physical Properties That Define Fluorescence [WWW Document]. URL https://www.thermofisher.com/us/en/home/life-science/cell-analysis/cell-analysis-learning-center/molecular-probes-school-of-fluorescence/imaging-basics/fundamentals-of-fluorescence-microscopy/physical-properties-that-define-fluorescence.html (accessed 4.19.18).

Tsuji, A., Kaneko, Y., Takahashi, K., Ogawa, M., Goto, S., 1982. The effects of temperature and pH on the growth of eight enteric and nine glucose non-fermenting species of gram-negative rods. Microbiol. Immunol. 26, 15–24.

Van Tassell, M.L., Ibarra-Sánchez, L.A., Takhar, S.R., Amaya-Llano, S.L., Miller, M.J., 2015. Use of a miniature laboratory fresh cheese model for investigating antimicrobial activities. J. Dairy Sci. 98, 8515–8524. https://doi.org/10.3168/jds.2015-9967

Appendix 1. Traces and sequences from the 16S sequencing of LUX

Sequences producing significant alignments:

Select: All None Selected:0

Alignments | Download | GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Klebsiella pneumoniae subsp. pneumoniae strain NF90 16S ribosomal RNA gene, partial sequence	1415	1415	63%	0.0	97%	KP772070.1

Figure 32. Sequencing of a colony from the first plate from the company, the analyzed colony did not grow on PALCAM. *97% certainty of K. pneumoniae.*

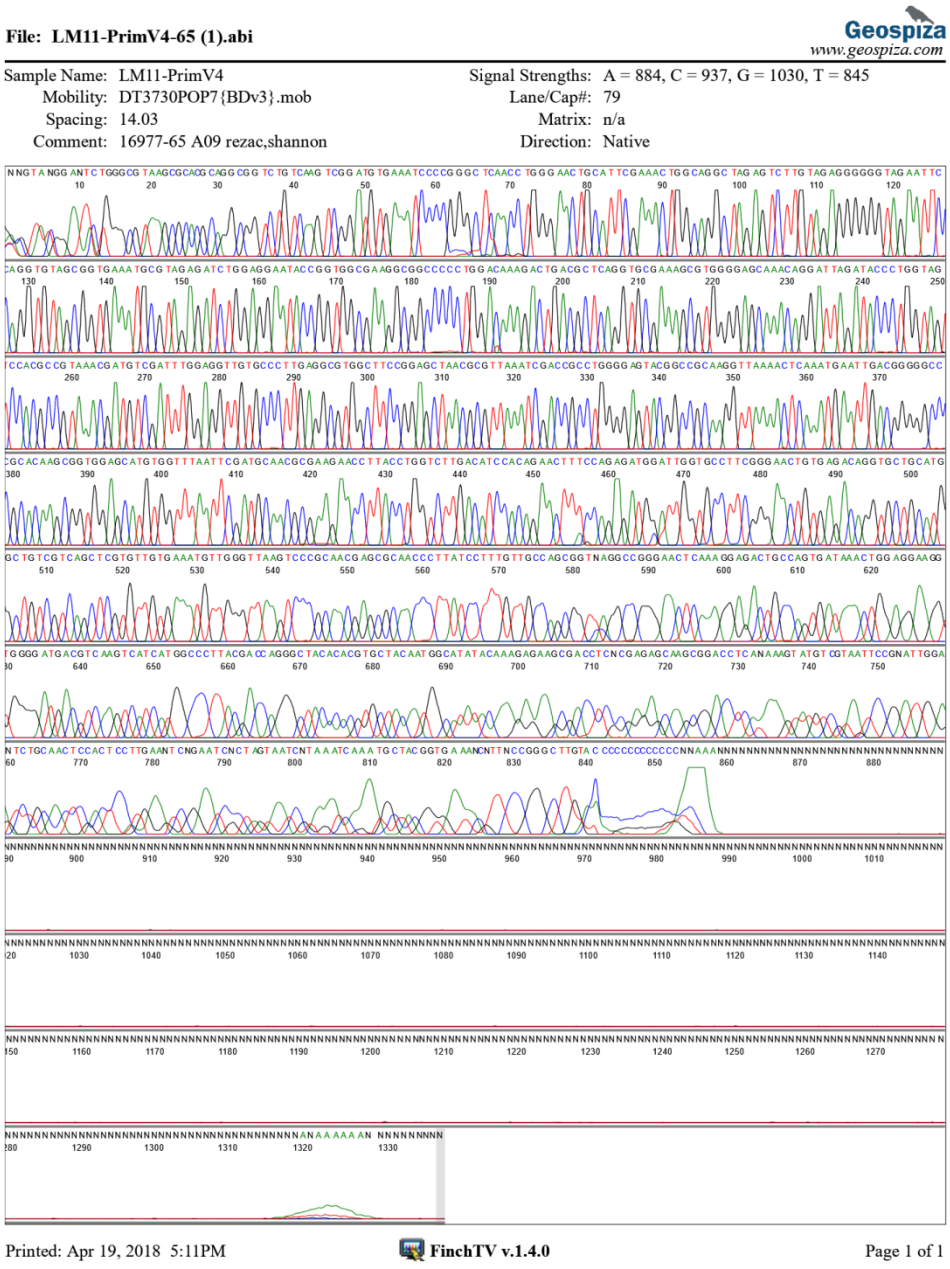


Figure 33. Traces from the 16S sequencing of a colony (LUX strain) from the first plate from the company. *The sequenced colony did not grow on PALCAM.*

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Listeria monocytogenes strain LM_3296_2 16S ribosomal RNA gene, partial sequence	423	423	25%	7e-114	94%	KF588557.1

Figure 34. Sequencing of a colony from the second plate from the company, the analyzed colony grew on PALCAM. *94% certainty of L. monocytogenes.*

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Uncultured Enterobacter sp. isolate DGGE gel band db1251 16S ribosomal RNA gene, partial sequence	405	405	20%	2e-108	96%	KF881846.1
Enterobacter sp. PSB-2 16S ribosomal RNA gene, partial sequence	401	401	20%	3e-107	96%	KP115595.1
Klebsiella pneumoniae strain 16 16S ribosomal RNA gene, partial sequence	401	401	20%	3e-107	96%	KM377645.1

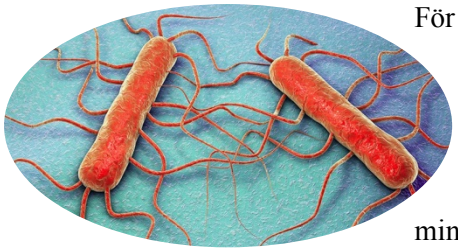
Figure 35. Sequencing of a colony from the second plate from the company, the analyzed colony did not grow on PALCAM *96% certainty of Klebsiella pneumoniae.*

Appendix 2. Popular science summary

Utveckling av en modell för att öka livsmedelssäkerheten för mexikansk färskost

Då säkerheten av livsmedel är väldigt viktig för alla individer, försöker industrin och forskare alltid hitta nya lösningar för att öka livsmedelssäkerheten. Hur kan man enkelt kolla flera konserveringsmedel samtidigt? Hur kontrollerar man att de fungerar? De här frågorna kan besvaras genom att studera hur bakterien *Listeria monocytogenes* kan överleva och växa i den mexikanska osten Queso Fresco. I den här studien har detta undersökts mer specifikt genom att använda en variant av bakterien *Listeria* som kan avge ljus. Det gör att det blir både snabbare och lättare att mäta om den finns och växer i osten.

L. monocytogenes är en patogen, vilket innebär att den är sjukdomsframkallande. I synnerhet är den här bakterien extra farlig för gravida, äldre och andra personer med nedsatt immunförsvar. Sjukdomen kallas listerios och för riskgruppen innebär det en dödlighet mellan 20-30%. Då *Listeria* kan växa även under kylda förhållanden likt de vi har i vårt kylskåp är den ett extra stort hot för livsmedel och dess konsumenter. Den mexikanska osten Queso Frescos egenskaper är dessutom passande för att *Listeria* ska trivas och frodas. Därför är det viktigt att försöka hitta konserveringsmedel som kan tillsättas för att hindra den här tillväxten och förebygga att människor utsätts för risken att bli drabbade.



För att kunna iscensätta ett verkligt scenario i osten, har en modell tagits fram för att utvärdera påverkan av *L. monocytogenes* av olika konserveringsmedel. Olika stammar av bakterien har utvärderats för att kunna jämföra om de beter sig likvärdigt i modellen. I modellen används Queso Fresco som tillverkats i miniformat, i storlek av 0,05 g per ost. De olika *Listeria* som jämförs är en ursprunglig moderstam, en modifierad version av moderstammen med en gen som avger ljus, samt en blandning av andra *Listeria,* isolerade från livsmedelsutbrott.

Studien visar att moderstammen och den modifierade stammen beter sig liknande, vilket är användbar information. Det betyder att den modifierade *Listerian* fördelaktigt kan användas i modellen, och ge ett resultat som är direkt jämförbart med liknande experiment med moderstammen. Däremot finns där fortfarande utmaningar med att genomföra analyser med den modifierade bakterien. Det handlar om att den mäts i en så kallad fluorometer, där det inte är optimalt att använda livsmedel vid mätningar. Vanligtvis utförs mätningarna i en homogen lösning, och inte med komplexa livsmedel. Ostens färg,

sammansättning och oregelbundna form stör mätningarna och gör det svårt att utläsa ett resultat. Två olika sätt att avge ljus analyserades, så kallade luminescens och fluorescens.
Det som skiljer de båda åt är främst att fluorescens uppstår när bakterien utsätt för ljus. Luminescens är automatiskt, och ingen ljuskälla är inblandad för att ge upphov till ljus. De här två har olika sätten visade sig ge olika resultat. Den mest lovande verkar vara luminescens.

Kunskapen från den här studien har lett till en ökad förståelse av hur väl den här modellen fungerar, hur de olika stammarna är jämförbara, samt vilka begränsningar som kan utvärderas för att förbättra framtida forskning om hur man kan kontroller *Listeria* i mexikansk ost.

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