Challenge test assessing growth potential of *Listeria monocytogenes* in Eldost

Tillväxtpotential för Listeria monocytogenes i Eldost

Louise Helsing

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Swedish University of Agricultural Sciences, SLU
Faculty of Natural Resources and Agricultural Sciences
Department of Molecular Sciences
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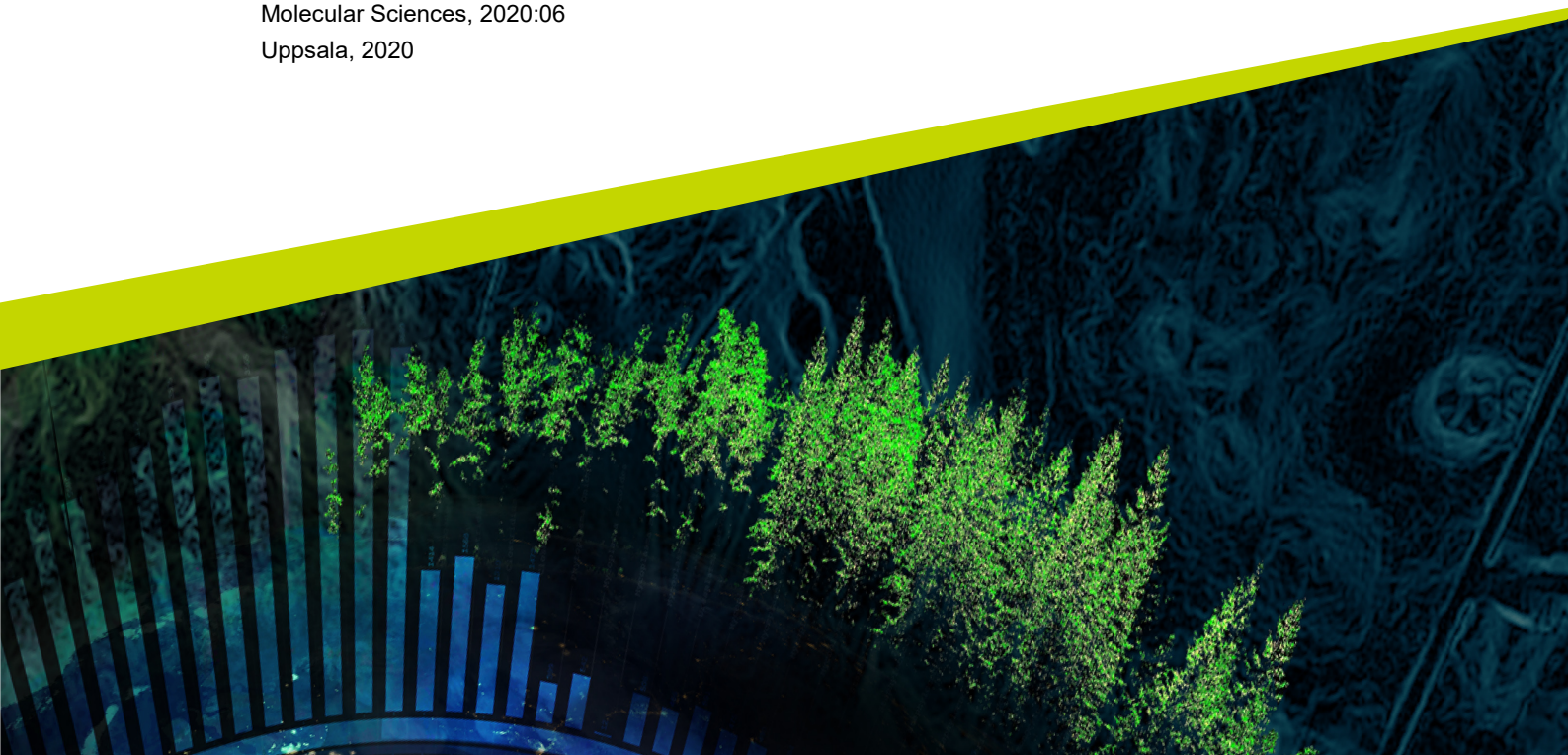

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Louise Helsing

Supervisor:	**Hans Jonsson, Swedish University of Agricultural Sciences, Department of Molecular Sciences**
Assistant supervisor:	Su-lin Hedén, Swedish University of Agricultural Sciences, Department of Molecular Sciences
Examiner:	Stefan Roos, Swedish University of Agricultural Sciences, Department of Molecular Sciences

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Swedish University of Agricultural Sciences
Faculty of Natural Resources and Agricultural Sciences
Department of Molecular Sciences

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Abstract

The demand for Cypriot Halloumi on the Swedish market has increased. However, more consumers request a similar product with origin from Sweden. This is the reason behind the launch of the Swedish Eldost, a cheese similar to Halloumi but produced in Sweden, from Swedish milk and in an artisanal manner.

Listeria monocytogenes is a pathogenic bacterium that poses a risk in Ready to eat (RTE) products. Since cheese is categorized as an RTE product *L. monocytogenes* may be of concern. In order to be compliant with current legislation, knowledge about the potential of the products to support or suppress growth of the pathogen is of importance. This study aims to assess the growth potential of *L. monocytogenes* in Eldost.

The study is conducted using a challenge test assessing the growth potential (δ) of four strains of *L. monocytogenes* which were artificially inoculated in samples of Eldost. The inoculated samples were stored for a total of 60 days and the growth was monitored at start and end of the test. Three batches were tested in total; every batch was tested for pre-existing *L. monocytogenes* at start and end.

No pre-existing *L. monocytogenes* could be found in non-inoculated samples in any of the batches at day 0 and day 60. The results from the inoculated samples indicate that Eldost is able to support growth of *L. monocytogenes.* The growth potential obtained demonstrated that to be compliant with the stated criteria in the law, a zero prevalence of *L. monocytogenes* in 25 grams of Eldost when leaving the manufacturer is required.

Keywords: Eldost, *Listeria monocytogenes,* Halloumi, challenge test, microbiological hazards

Sammanfattning

Efterfrågan på Cypriotisk Halloumi har ökat på den svenska marknaden. Dock har konsumenter utryckt en önskan att kunna köpa en svensk variant av Halloumi. Denna efterfrågan låg till grund för lanseringen av Eldost, vilken är en Halloumi-liknande ost producerad i Sverige, på svenska råvaror och tillverkad på ett hantverksmässigt vis.

Listeria monocytogenes är en patogen bakterie som kan vara en risk i ätfärdiga produkter. Då ost faller inom ramen för ätfärdiga produkter kan *L. monocytogenes* vara en potentiell riskorganism. Rådande lagstiftning gör gällande att insikt i en produkts förmåga att stödja eller inte stödja tillväxt av *L. monocytogenes* är av vikt. Denna studie har för avsikt att avgöra tillväxtpotentialen för *L. monocytogenes* i Eldost.

Studien är utförd efter mallen för ett challenge test som avgör tillväxtpotentialen. Fyra stammar av *L. monocytogenes* inokulerades på konstgjord väg i prover av Eldost. De inokulerade proverna lagrades totalt 60 dagar och tillväxten kartlades vid start och slut av testet. Tre batcher testades totalt och för varje batch testades om *L. monocytogenes* fanns i proverna redan innan inokulering.

Ingen *L. monocytogenes* kunde påvisas i de icke-inokulerade proverna i någon av de testade batcherna dag 0 eller dag 60. Resultatet från de inokulerade proverna indikerar att Eldost kan understödja tillväxt. Den tillväxtpotential som erhölls från försöket, visar att för att följa nuvarande lagstiftning ska ingen *L. monocytogenes* kunna påvisas i 25 gram av Eldost när produkten lämnar tillverkaren.

Keywords: Eldost, *Listeria monocytogenes,* Halloumi, challenge test, mikrobiella faror

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Abbreviations

BPW	Buffered Peptone Water
CCP	Critical control point
GMP	Good manufacturing practices
HACCP	Hazard Analysis and Critical Control Point
L. ivanovii	*Listeria ivanovii*
L. monocytogenes	*Listeria monocytogenes*
mg/PCU	mg per population correction unit
PDO	Protected Designation of Origin
RTE	Ready to eat
TBC	Total Bacterial Count

1. Introduction

In this chapter the background to this study is presented. After the background, a short problem description and aim are given.

1.1. Halloumi

Halloumi is a cheese produced in Cyprus and has grown in popularity on the Swedish market. The import of the product increased from 21 tonnes in 2010 to 4 000 tonnes in 2018 (Lagerstedt 2019). The cheese is characterized by its feature to withstand heating without melting, making it suitable for consuming fried or grilled (Official Journal of the European Union 2015).

Halloumi is protected by a trademark (EUIPO), which is registered by the "Foundation for the Protection of the Traditional Cheese of Cyprus named Halloumi". An application for a protected designation of origin (PDO) has also been published, but is not granted yet (Forney *et al.* 2018). The products that can obtain the PDO are food, wine and agricultural products (European Commission 2020). The food must have special characteristics that can be linked to the region of production. It is of importance to single out these specific quality parameters and demonstrate how to verify their geographical origin (Martelo-Vidal & Vázquez 2016). The link between the geographical area Cyprus and the specific quality parameters of the Halloumi stated in the application are the climate, and the flora and fauna of the island (Official Journal of the European Union 2015). Another characteristic that links the product to its origin is the presence of *Lactobacillus cypricasei,* which only has been isolated in Cypriot Halloumi. However, the Halloumi has not been granted a PDO yet, and is nowadays protected by the trademark solely.

The cheese can be made from only sheep or goat milk or from a mixture of cow, goat and/or sheep milk (Official Journal of the European Union 2015). Origin of

the milk must be Cyprus. If cow milk is used, the percentage of goat or sheep milk should always be greater than that of cow milk. Rennet, salt and Cypriot mint leaves are other ingredients stated to be used in Halloumi.

1.1.1. Trademark

A trademark is a way of ensuring exclusive rights to use a certain expression or word (Swedish Intellectual Property Office 2019). Applications for trademarks are managed by the Swedish Intellectual Property Office. In order to get a registered trademark, it must fulfil some criteria. A trademark should be unique, distinctive, and not easily confused with other trademarks.

If a trademark is registered, no one else can use the trademark for their products or in marketing (Swedish Intellectual Property Office 2019). If someone intrudes on a trademark, legal action can be pursued with help of The Patent and Market Court.

1.1.2. Antibiotics

As mentioned, Halloumi has been an increasing segment on the Swedish market. However, in 2019, the high amount of prescribed antibiotics for agricultural animals on Cyprus gained attention in Swedish media (Velander 2019).

Statistics over sales of antibiotics show that in 2017, Cyprus had the highest sale when compared amongst 31 European countries (The European Medicines Agency 2019). Cyprus had a sale of 423.1 mg per population correction unit (mg/PCU). This number could be compared to the country with the second largest sale, Italy, with 273.8 mg/PCU. Sweden had a sale of 11.8 mg/PCU whilst Norway had the lowest sale with only 3.1 mg/PCU (The European Medicines Agency 2019). This data shows that Cyprus is by far the country that used the most antibiotics.

The occurrence of bacteria resistant to antibiotics has increased. By over-use of antibiotics, bacteria strains resistant to the compounds are emerging. The extensive use of antibiotics in agriculture is a problem: it is used both as a growth supplement and to prevent infection (Ventola 2015). Bacterial resistance towards antibiotics is both a threat to animal and human health worldwide (McDermott *et al.* 2002).

When the news broke that Cyprus had the highest sale of antibiotics, it resulted in a reaction amongst Swedish customers: many consumers wanted a Halloumi produced in Sweden (Klintö 2019). But since the cheese has a trademark, a Swedish version of the cheese cannot be marketed under the name Halloumi. Therefore, the expanding interest in a similar product gave rise to the urge to find a name for the Swedish alternative.

1.2. Eldost

Sveriges Gårdsmejerister is a trade association for artisan cheese and dairy producers in Sweden (Sveriges Gårdsmejerister n.d.). The association gathers around a hundred members and works to create networks within the industry and support small scale production. They arrange education within their field of expertise and emphasise the importance of bringing the consumer in contact with the local, small-scale producer.

Members of the association have been producing a Swedish version of Halloumi-type cheese suitable for grilling and frying for years. However, the need for a unison name and trademark for the cheese became visible. In 2018 a registration for a trademark of the word Eldost was submitted by Sveriges gårdsmejerister to the Swedish Intellectual Property Office. The registration was granted and from January 2019 Eldost is a registered trademark (PRV 2019).

Eldost is a registered trademark by Sveriges Gårdsmejerister (PRV 2019). It is the word Eldost itself that is the registered trademark and it can solely be used by members of Sveriges Gårdsmejerister. It is registered both as a national (Sweden) trademark as well as an EU-trademark. In the registration form, it is stated that the cheese should be produced in an artisanal manner.

1.2.1. Production process for Eldost

Eldost is always produced from Swedish milk and by members of Sveriges Gårdsmejerister (Sveriges Gårdsmejerister n.d.). The milk can originate from cow, goat, sheep, or buffalo. Mixtures of these types of milk are also accepted.

The production of the cheese starts with the milk being heated to 35-38°C followed by addition of starter culture: mesophilic, thermophilic or a mixture (Elvingson 2020). The addition of rennet for coagulation is conducted, subsequently followed by curd cutting and heating to 40-42°C. Next the curd is placed in cheese molds and left for drainage. The separated whey is then heated to 85°C and the cheese is placed in the heated whey. When the core temperature of the cheese is >76 °C it is placed on a tray for salting and subsequently cooled to below 8 °C under a time span of two hours.
The cheese has a dense texture with some elasticity to it and can have a NaCl concentration ranging from 1-3%; an overview of the characteristics of the product is shown in Table 1.

Table 1 - Characteristics of the finished Eldost

Eldost	
pH	5.8

NaCl (%)	1-3
Shelf-life (days)	Max. 90
Storage temperature (°C)	~ 8

The finished product is vacuum packed and stored in the refrigerator. Even if the product often in consumed fried or grilled, it is not stated by the manufacturer that a heating step is necessary before consumption.

1.3. Listeria monocytogenes

The genus *Listeria* consists of around 17 species, some of which are recently found (Datta & Burall 2018). Two of the species are pathogens: *Listeria monocytogenes* and *Listeria ivanovii.* However, *L. ivanovii* rarely causes human listeriosis but is instead the causal agent of animal listeriosis. *L. monocytogenes* is on the contrary pathogenic to both human and animals and is classified as a zoonotic pathogen (Zunabovic *et al.* 2011).

L. monocytogenes is a Gram-positive, facultative anaerobe, psychrotrophic, non-spore forming bacteria (Adams *et al.* 2016). It is omnipresent in the environment and therefore it can enter the food chain almost anywhere.

L. monocytogenes is stress-tolerant and can survive in a_w as low as 0.92 and in salt concentrations up to 10%. It has an ability to survive in a wide range of temperatures (<1-45 °C) and also in pH between 4.0-9.5 (Melo *et al.* 2015). Optimum temperature for growth is seen between 30 to 35 °C, growth below 5 °C is very slow with generation times between 13 to 130 hours (Adams *et al.* 2016). *L. monocytogenes* is heat susceptible and a pasteurisation step is often enough to kill the bacteria (Jadhav *et al.* 2012). It has a D_{70}[1] of a few seconds (Adams *et al.* 2016). However, due to it being omnipresent in the environment, and its ability to form biofilms and attach to abiotic surfaces, post pasteurisation contamination is at risk (Jadhav *et al.* 2012).

L. monocytogenes is the causative agent for human listeriosis. The disease can occur in two forms: invasive and non-invasive (Jadhav *et al.* 2012). The invasive infection is the more severe form and the consequences of a listeriosis infection can be meningitis, encephalitis, meningoencephalitis, and septicaemia. In pregnant women the invasive form can cause still birth, abortions, and premature deliveries.

[1] Time required at a certain temperature to obtain a 90% reduction of the microbe (Dogruyol *et al.* 2020).

Neonates can be at risk and an infective listeriosis can cause septicaemia, meningitis and the outcome is often fatal. Risk groups are mainly pregnant women, immunocompromised individuals, and elderly people. Other population groups do not have such a high susceptibility (Jadhav *et al.* 2012; Jamali *et al.* 2013). The non-invasive form mainly gives mild symptoms: gastro-intestinal symptoms such as diarrhoea as well as fever and headache (Berrada *et al.* 2006).

Human listeriosis is mainly linked to foodborne transmission, and cheeses have been identified as one major food vehicle for infection (Loncarevic *et al.* 1995; Berrada *et al.* 2006). The incubation time for listeriosis can range from a few days to several weeks, therefore identification of the possible contaminated food can be problematic (Datta & Burall 2018). Thus, the source of many sporadic cases remains unidentified.

Since the bacteria is ubiquitous in our environment, human exposure to the bacteria is thought to occur repeatedly (Adams *et al.* 2016). The estimated infective dose is suspected to be high, contaminated foods causing listeriosis often have a cfu/g above 10^3. But levels as low as 10^2 cfu/g have been linked to outbreak of listeriosis in immunocompromised groups (Välimaa *et al.* 2015).

When comparing *L. monocytogenes* to other pathogens associated with food, such as campylobacters and salmonellas, the prevalence of *L. monocytogenes* is lower (Alessandria *et al.* 2010). However, listeriosis shows a considerably higher mortality rate, around 30%, which shows the severity of the disease and places *L. monocytogenes* as a significant food borne pathogen (Rocourt *et al.* 2003).

1.3.1. Cases of listeriosis in Sweden

During 2018, 89 cases of listeriosis were reported in Sweden. Most cases affected people over 80 years old. The incidence of listeriosis has increased on average by 14% every year since 1983 in Sweden, and a rise is also seen in the rest of Europe (Public Health Agency of Sweden 2018). A combination of factors such as an aging population, a rise in use of immunosuppressive drugs and a shift in eating habits whereby more Ready-to-eat products are being consumed, are thought to be the reason behind this increase. The source of infection cannot be established in most cases of listeriosis.

A number of outbreaks that could be linked to the same product have occurred over the years (Public Health Agency of Sweden 2016). In 2013-2014 a larger outbreak possibly linked to a charcuterie product was discovered and 47 cases were reported. During the summer of 2001, 30 cases could be linked to a cheese produced on a local farm.

1.3.2. *L. monocytogenes* in Halloumi

Growth of *L. monocytogenes* could be observed in Halloumi in a study assessing growth potential of *L. monocytogenes* in different types of cheeses. Halloumi was placed in a group of semi-hard cheeses such as Gouda and Edam and supported the largest growth of all three (Kapetanakou *et al.* 2017). However, *L. monocytogenes* is rarely found in packed cheese such as Halloumi (Ottoson 2017). The absence of the bacteria could possibly be linked to the production process which includes heating steps.

1.3.3. Detection and quantification methods for *L. monocytogenes*

Traditional culture methods are still the standard for detection and enumeration of *L. monocytogenes.* They are time and labour consuming but nevertheless show a high sensitivity (Gasanov *et al.* 2005). PCR is an alternative that is more time efficient but difficulties could occur for example when selecting primers if multiple virulence genes have to be targeted (Zunabovic *et al.* 2011). However, molecular methods such as PCR are used for identification and/or confirmation of strains obtained from, for example, a culture method targeting *L. monocytogenes*.

Culture methods are approved and used by regulatory agencies in EU and USA and have the requirement to detect one *L. monocytogenes* per 25 grams of food (Gasanov *et al.* 2005; Välimaa *et al.* 2015). In order to meet this criterion for sensitivity, enrichment steps are needed for the bacteria to grow to detectable numbers. Fraser broth is a commonly used enrichment broth for *Listeria* spp. The selective ingredients acriflavine and nalidixic acid and also esculin are used in the broth, the β-D-glucosidase activity performed by *Listeria* is utilized resulting in a blackening of the medium (Gasanov *et al.* 2005).

The addition of antimicrobial agents are utilized in enrichment and plating media due to the slow growth rate of *Listeria* spp.; if any competitive bacteria are present, they can easily out-grow *Listeria* spp. (Law *et al.* 2015).

Commonly used selective plating media are Oxford and PALCAM (Zunabovic *et al.* 2011). However, they do not differentiate pathogenic from non-pathogenic *Listeria* spp., and other organisms such as *Bacillus* spp. can still grow on the media (Gasanov *et al.* 2005; Zunabovic *et al.* 2011). Chromogenic media, such as ALOA, are another type of selective plating media that distinguish between the pathogenic and non-pathogenic *Listeria* spp. The pathogenic *L. ivanovii* and *L. monocytogenes* are separated from non-pathogens by the formation of an opaque halo around the green-blue colonies. The formation of a halo is due to phosphatidylinositol-specific

phospholipase C (PI-PLC) linked to a virulence gene present in *L. ivanovii* and *L. monocytogenes* in combination with β-glucosidase activity.

Further confirmation tests are often needed. *L. monocytogenes* can perform B-haemolysis on blood agar and can be distinguished from *L. innocua* that lacks this ability (Gasanov *et al.* 2005; Adams *et al.* 2016). Carbohydrate fermentation of L-rhamnose and D-xylose can separate *L. monocytogenes* from *L. ivanovii. L. monocytogenes* are L-rhamnose positive and D-xylose negative whilst *L. ivanovii* shows the opposite response.

The high mortality associated with listeriosis, especially for pregnant women, infants and immunocompromised individuals, place it as a significant food borne pathogen (Adams *et al.* 2016). Due to this fact, a reliable method for detection as well as food safety management systems targeting *L. monocytogenes* are of importance for food safety and public health.

1.4. Food safety management systems

According to EC Regulation no. 852/2004 every food producer should have a quality control program that is implemented to prevent potential hazards in their product. This in order to ensure that only safe products are placed on the market. Food safety management systems are built on the principals of HACCP, Hazard Analysis and Critical Control Point.

Before implementation of a HACCP system, good manufacturing practices (GMPs) and good hygienic practices, amongst other, must be established (Drosinos & Siana 2007). These are the foundation on which the HACCP system depends.

1.4.1. HACCP

HACCP analyses the potential risks linked to food production; it could be microbial hazards as well as physical and chemical hazards (Sandrou & Arvanitoyannis 2000).

The HACCP system is based on seven steps and is presented in EC Regulation no. 852/2004:

1) Hazard identification – analysis of all potential risks.
2) Identify critical control points (CCPs). Which steps are essential to control for prevention of identified hazards?
3) Establish the critical limits for every CCP. What is the critical limit that separates accepted levels from unaccepted levels?

4) Implementation of efficient monitoring practices at each CCP.
5) Determination of corrective measurements when a deviation from a CCP is observed
6) Establish procedures that verify that step 1-5 are working efficiently
7) Establish documentation for step 1-6

The HACCP requirements should however offer flexibility in order to be useful in the large span of food businesses. For instance, in some small businesses the need for monitoring a CCP can be substituted with good hygienic practices.

1.4.2. Control of *L. monocytogenes* in cheese production

One way of controlling the prevalence of *L. monocytogenes* is heat treatment. Pasteurization eliminates the hazard caused by the presence of heat-susceptible microorganisms, such as *L. monocytogenes* (Sandrou & Arvanitoyannis 2000). It is considered a robust CCP since some of the pathogens killed during pasteurization may otherwise grow during ripening or storage of the cheese. A way of ensuring correct pasteurization is an alkaline phosphate test or temperature monitoring.

Since cheese is a ready-to-eat product, a thermal step before consumption does not always occur (Melo *et al.* 2015). Cheese is often stored in a refrigerator which could favour the growth of psychrotrophic bacteria such as *L. monocytogenes.* Even if a pasteurization step of the raw milk is conducted, the risk of post pasteurization contamination is real. For example, this could be displayed by the outbreak of listeriosis linked to cheese made from pasteurized milk in Germany 2006.

Environmental sampling in the production facilities is a way of ensuring that the cleaning measurements taken are efficient. The incidence of *L. monocytogenes* in different cheese production facilities was studied by environmental sampling (Melo *et al.* 2015). By following GMP, the occurrence seemed to be lower. At manufacturers where there was a higher prevalence of *L. monocytogenes* in the environmental sample, poor sanitary conditions and inadequate personal hygiene could be observed. This result highlights the importance of good hygiene and GMP in combination with the principle of HACCP to control *L. monocytogenes*. By reducing the occurrence of the bacteria in the production plant, the risk of post-pasteurization contamination can be lowered.

1.4.3. Rules and legislation

The legislation applied for food products aims to protect the consumers by ensuring that food products on the market are safe (Swedish Food Agency 2020). The

legislation applied in Sweden is mainly based on the EU-legislation. EU regulation (EC) No 178/2002 states that only safe products should be introduced to the market and it is the manufacturer's responsibility to fulfil this criterion.

A product that does not need to be cooked or undergo other processing before consumption is defined as a "ready-to-eat" or RTE food. In the commission regulation (EC) No 2073/2005, the criteria regarding *L. monocytogenes* prevalence in ready-to-eat products:

- If a food stuff is classified as able to support growth of *L. monocytogenes* the bacteria must be absent in 25 g of the product. This is applied before the food has left the immediate control of the manufacturer.
    - After the product has been placed on the market, the cfu/g are not allowed to exceed 100 cfu/g during the stated shelf-life.

- If a product is classified as unable to support growth of *L. monocytogenes*, the product does not have the demand of ensuring zero prevalence in 25 g of food stuff at the manufacturer. The limitation is then that the product placed on the market never can exceed a concentration above 100 cfu/g during the stated shelf-life.

1.5. Challenge test

It is stated in Regulation (EC) No. 2073/2005 in Annex II that food business operators shall, if necessary, evaluate the growth of *L. monocytogenes* in the product during the shelf-life. However, how this study should be performed is not stated. Therefore, the European Reference Laboratory for *Listeria monocytogenes* and ANSES have developed a technical guidance document on how to conduct shelf-life studies (Beaufort *et al.* 2019).

A challenge test is one form of shelf-life study described in the EURL *Lm* Technical Guidance Document (Beaufort *et al.* 2019). It is designed to give information about the behaviour of *L. monocytogenes* in artificially inoculated samples of RTE-foods. A challenge test can be used assessing either the growth potential or the maximum growth rate, depending on the information needed.

The challenge test assessing growth potential can be used to determine whether a product can support growth of *L. monocytogenes* or not (Beaufort *et al.* 2019). The test period starts at the day of contamination and stops at the end of shelf-life. The growth potential, δ, is the difference between $\log_{10}$ cfu/g at the end of the test and the $\log_{10}$ cfu/g at the start of the test. If the standard deviation for δ between samples in a batch is >0.5 $\log_{10}$ cfu/g the challenge test for that batch is not accepted.

δ can vary depending on several factors. The most influential are:

- the strains used for inoculation,
- the physical state of the inoculated cells,
- intrinsic properties such as pH, NaCl, associated microflora, other antimicrobial constituents,
- extrinsic properties

If the food is thought to support growth of *L. monocytogenes*, three batches should be tested. If the probability of growth is < 10% only one batch is needed. Also, inter-batch variability affects if more than one batch should be tested. When choosing batches to test, the batch most likely to support growth should be used.

The strains used for inoculation should preferably be in early stationary phase and at least one of the strains have known characteristics, such as the strains from the collection of EURL Lm strains. A minimum of two strains should be used.

1.5.1. Adaptation of strains

If *L. monocytogenes* is exposed to non-lethal environmental stresses it is known to demonstrate an adaptive response which results in an ability to survive even more severe challenges (Melo *et al.* 2015). This ability could for example be observed with regard to acid conditions as well as the stress of high NaCl content.

By exposing the cells to stress such as sub-optimal pH, salt concentration, temperature, a more resistant strain could be obtained compared to a non-adapted strain (Wusimanjiang *et al.* 2019). Cells exposed and adapted to acid and salt stress could survive doses of salt and acid that were lethal to non-adapted cells.

This can be used to adapt strains to foreseeable conditions in order for better survival and growth (NACMCF 2010).

2. Aim and purposes

2.1. Problem description

The characteristics, the package, and the storage conditions for Eldost imply that *L. monocytogenes* could be a potential risk organism. In theory the bacteria seem to be competitive under these conditions.

Depending on whether a product can or cannot support growth of *L. monocytogenes*, the rules that apply are different. If a product can support growth, a zero prevalence in 25 g of product is the legal requirement when the product leaves the manufacturer. If the product is unable to support growth, the prevalence of the bacteria can never exceed 100 cfu/g during the stated shelf-life. Thus, to know which legislation that applies for a particular food stuff, its ability to support growth or not, is crucial.

No studies addressing the potential of Eldost to support or not support growth have been found. This implies that there is a lack of knowledge about *L. monocytogenes* behaviour in Eldost and a study addressing this could be of importance for the producers as well as public health.

2.2. Aim

The aim of this project is to assess the potential of Eldost to suppress, or support growth or survival of *L. monocytogenes* during storage. To evaluate growth, a challenge test was chosen as the most appropriate method.

3. Materials and methods

This study was conducted using a challenge test to assess the growth potential (δ) of *L. monocytogenes* in Eldost.

3.1. Test points

Three different batches of Eldost cheese were tested, the NaCl content in all batches was 2.25%. They were produced by the same manufacturer but on different occasions. All batches used were opened and re-packed in the laboratory 5 days after the production date, both inoculated samples and non-inoculated samples. The date for re-packing is referred to as day 0.
As displayed in Table 2, inoculated samples were tested at day 0 and day 60 with the enumeration method. The enumeration method is described in section 3.5.

Table 2 - Overview of number of inoculated samples tested with the enumeration method.

No. of samples tested per batch	Day 0	Day 60
Enumeration method, inoculated test units	3	3

Non-inoculated samples were tested with the detection method, as described in section 3.6. Total bacterial count and physico-chemical measurements were also conducted on non-inoculated samples. An overview can be seen in Table 3.

Table 3 - Overview of analyses preformed on non-inoculated samples.

No. of samples tested per batch	Day 0	Day 60
Detection method	3	3
Measurement of pH and a_w	1	1
Total bacterial count	1	1

A total of 16 samples from each batch were tested during the challenge test.

3.2. Selection of bacterial strains

In order to find relevant strains of *L. monocytogenes* that could endure the growth conditions chosen for the challenge test, a selection based on the following parameters was conducted:

1) Origin: from which food stuff the strain was isolated
    a) preferably dairy or cheese products
2) Growth capacity with regards to
    a) pH
        i) Strains capable of growing at pH 5.8
    b) Temperature
        i) Strains capable of growing at low temperature, i.e. 9 °C

One of the chosen strains must originate from the set of strains kept by the European reference laboratory for *Listeria monocytogenes.* This in order to meet the criteria for the challenge test.

3.3. Bacterial growth curve

The strains used for inoculation should preferably be in early-stationary phase when inoculated on the sample. To determine when the four strains enter the early stationary phase, a growth curve was generated for each strain.

Each strain was first inoculated in BHI-broth with a NaCl content adjusted to 2.25 %. The inoculated broth was incubated at 30 °C for 17±1 h. Next 0.1 ml of the over-night culture was transferred to a fresh BHI-broth (NaCl 2.25 %). This culture was next incubated at 9 °C. Three replicates of each strain were performed.

Once a day, for 10 days, the culture for each strain was transferred to a cuvette and the optical density was measured at a wavelength of 600 nm. Any sample obtaining a measured value above 1, were diluted and re-measured.

3.4. Inoculum preparations

3.4.1. Adaption of strains

The strains were adapted to a salt content of 2.25% and cold storage.

One colony of each strain (2068, 4079, LM96 and LM98) was aseptically taken from a fresh BHI-plate and inoculated in 18 ml BHI-broth with a NaCl content of 2.25 %, Hereafter called subculture 1. Subculture 1 was incubated for 17±1 hour at 30 °C. Next 0.1 ml of subculture 1 was transferred to 18 ml of BHI-broth (NaCl 2.25 %) and incubated at around 9 °C for 5 days for batch 1 and 6 days for batches 2 and 3. Hereafter referred to as subculture 2.

At day 5 respectively 6, OD600-values were measured for each strain and compared to the reference curve, see Figure 1. The aim was to inoculate the strains when in early stationary phase.

3.4.2. Inoculum preparation

1 ml derived from subculture 2 was aseptically taken for each strain (2068, 4079, LM96 and LM98) and subsequently mixed. OD600-value and bacterial count using a Helber Chamber in a microscope were conducted to acquire the concentration (cfu/ml) of the inoculum.

Thereafter dilutions in peptone water was conducted in order to obtain the wanted inoculum size. Targeted inoculum size was 100 cfu/g for the first batch and 500 cfu/g for batches 2 and 3. The reason for adjusting the targeted inoculum size was to ensure that the samples were inoculated well above the detection limit of the method.

3.4.3. Inoculation of the cheese

Each cheese sample, with a weight of around 45-65 grams, was placed on a sterile petri dish. 0.4 ml of the inoculum was aseptically taken with a pipette and distributed evenly on the upper surface of each cheese. The inoculated samples were left to dry for a minimum of 30 minutes before placed in a vacuum bag and subsequently vacuum packed using an Audionvac Digital (VMS 43).

3.4.4. Storage

The samples were stored in a refrigerator at a temperature around 9°C to mimic the conditions at which the cheese would be stored by the consumer. The temperature and relative humidity were monitored using a temperature logger (Winlog, EBI 20-TH).

Total storage time for the challenge test was set to 60 days.

3.5. Enumeration method, quantification

In order to obtain the number of colony forming units (cfu) of *L. monocytogenes* in each sample, an enumeration method was used (see Table 2 for sampling points). The protocol is based on the NMKL Method No 86 and ISO 11290-2.

3.5.1. Sample preparation

The vacuum pack was opened by cutting the side and pulling the cheese out. 10 g of inoculated sample was aseptically taken from the upper surface of the cheese and placed in a stomacher bag with 90 ml of Buffered Peptone Water (BPW). Sampling was mainly done on the surface. The sample was homogenized together with BPW in a stomacher (Seward) for 60 seconds at normal speed.

3.5.2. Plating on ALOA

Since *L. monocytogenes* often needs to be detected in low amounts, 1 ml can be spread instead of 0.1 ml. 1 ml homogenate was spread using a sterile T-Shaped Cell Spreader (VWR®) divided on two ALOA plates á 0.5 ml each. Prior to performing the spreading, the agar-plates were placed in an incubator at 37°C for 30 minutes in order to dry the medium.

After spread-plating, the plates were left to dry, with lid closed, for 30 minutes at room temperature, followed by incubation in an inverted position at 37 °C for 24 hours.

After 24 hours, if any visible colonies were obtained, they were counted. Then the plates were incubated for an additional 24 hours before a final count was conducted. All colonies, typical and atypical were counted.

3.5.3. Plating on BHI

To perform the confirmation steps, five colonies were aseptically taken from the incubated ALOA-plate and each colony was streaked onto a separate BHI-agar plate. The BHI-plates were incubated at 37 °C for 24 hours. Each colony was marked with a number to ensure traceability.

3.5.4. Confirmation steps

According to ISO-standards, the tests necessary for confirmation of *L. monocytogenes* are β-haemolysis and carbohydrate fermentation of L-rhamnose and D-xylose (ISO 2017).

β-haemolysis

One isolated colony from BHI-agar was aseptically taken, using a sterile loop. The colony were inoculated on a line on the surface of the horse-blood agar plate and the loop was last pressed down in the agar. The plate was next incubated in an inverted position for 24 hours in an incubator at 37 °C. After incubation, the plates were examined for β-haemolysis as indicated by a clear zone in the agar due to complete lysis of red blood cells.

L-rhamnose and D-xylose

One isolated colony from BHI-agar was aseptically taken using a sterile loop. The colony was then inoculated in a carbohydrate fermentation tube containing rhamnose. The tube was incubated for 48 hours at 37 °C. After incubation turbidity and any change in colour was observed. A colour change to yellow indicates a positive result.

The same procedure was conducted for the xylose fermentation tube.

All confirmation steps were repeated for each of the five colonies originally picked from the ALOA plate.

In case any of the presumptive *L. monocytogenes* colonies tested negative, the numbers of quantified *L. monocytogenes* from the ALOA-plates were corrected in accordance with the ratio obtained.

3.6. Detection method, qualitative

In order to determine if the samples tested were free from pre-existing *L. monocytogenes*, a detection method were used. The protocol is based on the standard protocols from NMKL Method No 86 and ISO 11290-1.

The method contains enrichment steps selective for *Listeria* spp.

3.6.1. Pre-enrichment

225 ml of Half-Fraser broth was prepared according to manufacturer's instructions. After autoclaving, the selective supplement reconstituted in 3 ml ethanol:sterile water was added. The Half-Fraser broth was heated to 45 °C. The vacuum pack was opened by cutting the side and pulling the cheese out. 25 g of non-inoculated sample was aseptically taken using a sterile scalpel and placed in a stomacher bag. Sampling was done mainly on the surface of the cheese as described in 3.5.1. 225 ml Half-Fraser broth was added to the stomacher bag and then homogenized for 30 seconds.

The sample was then incubated at 30 °C for 24±3 hours.

Cultures of incubated Half-Fraser broth were streaked on to selective media ALOA and PALCAM and subsequently incubated at 37 °C for 24±4 hours followed by 24±4 hours.

Presumptive colonies of *L. monocytogenes* were streaked on to BHI-agar before confirmations tests, see section 4.5.4, were conducted.

3.6.2. Enrichment

Fraser broth was prepared according to manufacturer's instructions. After autoclaving, the Fraser selective supplement, reconstituted with 5 ml ethanol:sterile water was added to the broth.

0,.1 ml of the pre-enriched culture (3.6.1) was aseptically taken and inoculated into 10 ml of Fraser broth. A negative control was used as reference.

The culture was incubated at 37 °C for 48±4 hours

3.6.3. Isolation

By using a sterile loop, the enriched culture (3.6.2) was aseptically taken and streaked on to selective media ALOA and PALCAM and subsequently incubated at 37 °C for 24±4 hours followed by 24±4 hours.

3.6.4. Confirmation steps

Any colonies of presumptive *Listeria monocytogenes* were tested according to the ones performed in the enumeration method, see section 3.5.3.- 3.5.4.

3.7. Total bacterial count

3.7.1. Sample preparation

The vacuum pack was opened by cutting the side and pulling the cheese out. 25 grams of the non-inoculated sample was aseptically taken at the surface of the cheese and added to 225 ml of peptone water in a stomacher bag. The sample was homogenised for 30 seconds at normal speed using a stomacher. A serial-dilution from the homogenate in BPW was conducted.

3.7.2. Inoculation

1 ml of the homogenate was transferred to an empty, sterile petri dish. Within 20 minutes, 15-20 ml of TGE-agar holding a temperature of 45 °C was poured into the petri-dish. Instantly the petri dish was moved in an eight-shaped movement in order to mix the contents fully. The plates were left to solidify in room temperature.

The same procedure was conducted for each dilution tested.

3.7.3. Incubation

The plates were placed in an inverted position in an incubator at 30 °C for 72±6 hours.

3.7.4. Reading

Plates containing, if possible, 25-250 colonies were counted using a stereo microscope to ensure that even small colonies were counted.

3.8. Physico-chemical properties

3.8.1. Water activity, a_w

Water activity was measured using the AquaLab CX-2
(Decagon devices Inc., Pullman, WA, USA). The sample was placed in the sample cup and subsequently placed in the AquaLab for measurement.

3.8.2. pH

10 g of sample was aseptically taken and placed in a Falcon tube and 15 ml of de-ionized water was added. The sample and water were mixed using a glass wand. Next the pH was measured using a pH-meter (Mettler Toledo SevenCompact™ S220).

3.9. Calculations and statistics

3.9.1. Calculation of growth potential (δ)

The growth potential of each batch was calculated by converting the concentration of *L. monocytogenes* into $\log_{10}$ cfu/g. Next the median amongst the three replicate samples at day 60, minus the median amongst the three replicate samples at day 0, was calculated which resulted in the growth potential.

If the standard deviation between the replicate samples in one batch is higher than 0.5 $\log_{10}$ cfu/g, that batch are invalid to be used as a result of the challenge test.

If δ, for the batch with the highest growth potential, is lower or equal to the limit of 0.5 $\log_{10}$ cfu/g, the food is then assumed not to be able to support growth of *L. monocytogenes* for the shelf-life of the product / duration of the test. On the contrary, if δ is higher than 0.5 $\log_{10}$ cfu/g, the food is assumed to support growth of *L. monocytogenes*.

3.9.2. Statistics

Minitab was used for descriptive statistic of the result from the temperature logger regarding temperature and relative humidity. The standard deviation amongst the batches for calculation of growth potential was also derived from Minitab.

4. Results

The result will be presented in the order it was presented in section 3, materials and methods.

4.1. Selection of strains

Four strains of *L. monocytogenes* were chosen. Two strains originated from the EURL Lm, LM96 and LM98, see Table 4.

Table 4 - Known characteristics for strains LM96 and LM98.

Strain:	**LM96** (12MOB096LM)	**LM98** (12MOB098LM)
Genoserotype	IV	II
Origin	Dairy products	Dairy products
Geographical origin	France	France
Max. growth rate, (h^{-1}) at 8°C, pH 7, a_w 0.98	0.096±0.002	0.094±0.002
Grows at 9°C	Yes	Yes
Grows at NaCl 2.25%	Yes	Yes

Both strains LM96 and LM98 are well characterised and fulfil the criteria set for the strains to be selected; they are also part of the strain set of the EURL. They can both grow at low temperatures and originate from dairy products.

Strain 2068 and 4079 originated from cheese, see Table 5. They were received from the strain collection of Wilhelm Tham, now held at the Dept of Biomedical Sciences and Veterinary Public Health, SLU. However, they are not as well characterized as LM96 and LM98.

Table 5 - Known characteristics for strains 2068 and 4079.

Strain:	**2068**	**4079**
Genoserotype	unknown	unknown
Origin	Gorgonzola	Goat cheese
Geographical origin	unknown	unknown

Max. growth rate, (h^{-1}) at 8°C, pH 7, a_w 0.98	unknown	unknown
Grows at 9°C	Yes	Yes
Grows at NaCl 2.25%	Yes	Yes

4.2. Bacterial growth curve

The mean OD600-value of each strain s plotted against time can be seen in Figure 1. Early stationary phase was observed around day 5 for every strain and they reached stationary phase around the same time. LM96 and 4079 seemed to have a longer lag-phase than 2068 and LM98.

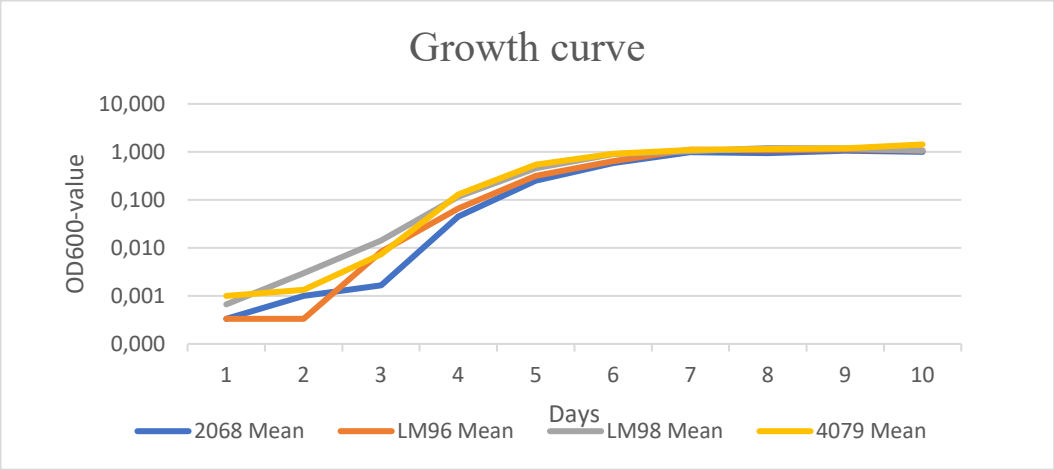

Figure 1- Growth curve based on means of OD600-values for strain 2068, LM96, LM98 and 4079. OD600-values are plotted against time on a logarithmic scale. Three replicates of each strain were measured once a day for 10 days.

The decision of when to inoculate the strains on the samples was based on the result from the growth curve.

4.3. Enumeration method

The numbers of *L. monocytogenes* at day 0 (day of inoculation) and day 60 (end of storage) in inoculated samples are presented for each batch in Figures 2, 3 and 4. The samples were analysed with the enumeration method.

The growth in batch 1 can be observed in figure 2, all samples showed an increase in cfu/g during the storage period of 60 days. Thus, growth of *L. monocytogenes* in all inoculated samples of Eldost in batch 1 could be observed.

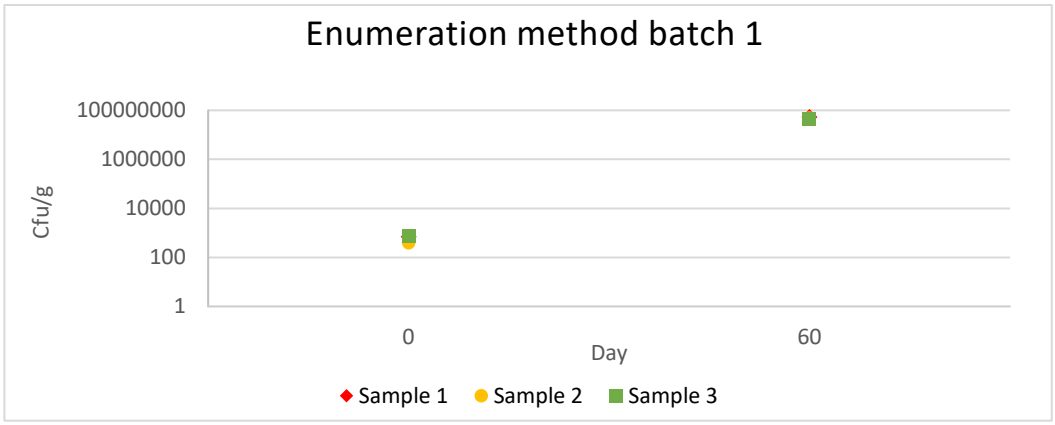

Figure 2 - Growth of L. monocytogenes in inoculated samples in batch 1.

For batch 2 all inoculated samples tested also showed an increase in cfu/g on day 60 compared to day 0, see figure 3. The inoculation levels in batch 2 was higher

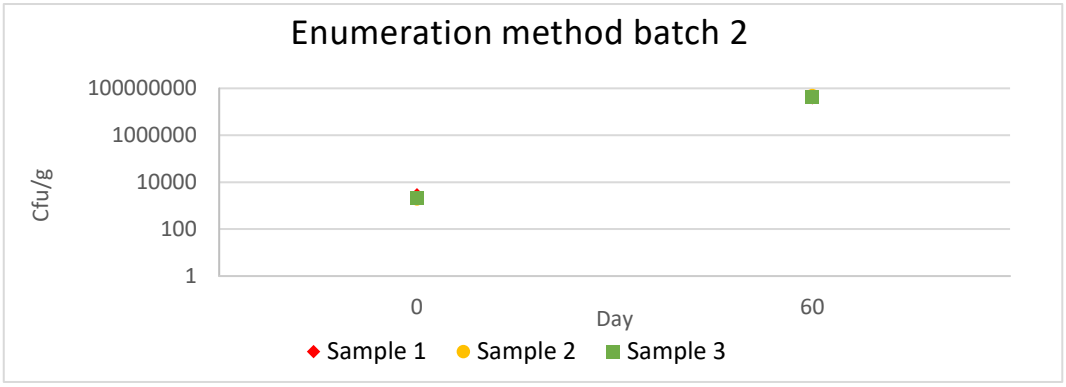

Figure 3 - Growth of L. monocytogenes in inoculated samples in batch 2.

than batch 1, but the concentration of cfu/g on day 60 is similar to the ones observed in batch 1.

In batch 3, the initial inoculum levels were similar to the ones in batch 2. However, sample 3 in batch 3 showed a minor increase in cfu/g compared to the two other samples, see figure 4. Sample 1 and 2 showed a similar growth of *L. monocytogenes,* like the samples in batch 2.

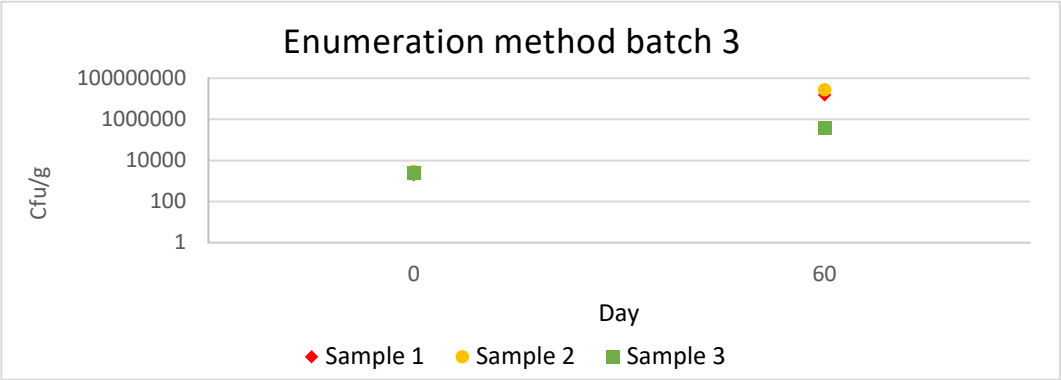

Figure 4 – The growth of L. monocytogenes in inoculated sample 1, 2 and 3 in batch 3.

All three batches tested demonstrates an increase in cfu/g over the storage period of 60 days. Thus, growth of *L. monocytogenes* could be observed in all tested samples.

4.3.1. Growth potential (δ)

A growth could be observed in all batches. However, in order to establish if the tested batches of Eldost should be classified as able or unable to support growth of *L. monocytogenes,* in alignment with the Regulation (EC) No 2073/2005, growth potential, δ, must be calculated. The result of this calculation can be seen in Table 6.

Table 6 - Calculation of the growth potential. The median (in bold) of the result for each sample in log_{10} cfu/g at day 60 minus the result in log_{10} cfu/g at day 0 gives the growth potential.

Batch	Day	Concentration cfu/g	log_{10} cfu/g	Growth potential, δ	Highest δ among the 3 batches (log_{10} cfu/g)
1	0	7.00E+02 4.00E+02 7.60E+02	**2.85** 2.60 2.88	7.69–2.85= 4.84	**4.84**
	60	5.44E+07 4.96E+07 4.56E+07	7.74 **7.69** 7.66		
2	0	2.87E+03 1.93E+03 2.04E+03	3.46 3.29 **3.31**	7.63–3.31= 4.32	
	60	4.00E+07 4.90E+07 4.34E+07	7.60 7.69 **7.63**		
3	0	2.05E+03 2.93E+03 2.39E+03	3.31 3.47 **3.38**	7.18–3.38= 3.80	
	60	1.53E+07 2.72E+07 3.50E+05	**7.18** 7.43 5.54		

The growth potential was 4.84 log_{10} cfu/g, see Table 6. Since the product has a δ higher than 0.5 log_{10} cfu/g, for these tested batches the food is classified as able to support growth of *L. monocytogenes.*

Batch 3 however, did not meet the criterion of having a standard deviation of 0.5 $\log_{10}$ cfu/g or less amongst the sample on day 60. Therefore batch 3 is invalid for assessing the growth potential according to the challenge test.

4.4. Detection method

The detection method was performed on day 0 and day end, i.e. day 60 on non-inoculated samples. This in order to ensure that the batches used for the challenge test were free from pre-existing *L. monocytogenes*.

Table 7 - Results from the detection method for batch 1, 2 and 3

Detection method	Day 0 **Negative (-)/positive (+)**	Day 60 **Negative (-)/positive (+)**
Batch 1	(-)	(-)
Batch 2	(-)	(-)
Batch 3	(-)	(-)

The samples from batch 1, 2 and 3 all tested negative for *L. monocytogenes* both day 0 and day 60, see Table 7. Thus, the batches are assumed to be free from *L. monocytogenes*.

4.5. Total bacterial count

The results of total bacterial count (TBC) from day 0 are presented in Table 8. Two out of three batches had a cfu/g around 100 whilst batch 3 only had 30 cfu/g on day 0.

Table 8 - Results from total bacterial count for batch 1, 2, and 3 for day 0.

Batch	Day 0 (cfu/g)	Day 60 (cfu/g)
1	1.0E+02	6.1E+03
2	1.3E+02	3.6E+07
3	3.0E+01	3.1E+03

At day 60 the number of cfu/g had increased in all batches. Batch 2 however, stood out since it had the highest amount of cfu/g at day 60, see Table 8. Batch 1 and 3 had more similar numbers of cfu/g compared to batch 2.

4.6. Physico-chemical properties

4.6.1. Water activity, a_w

The measurement of water activity using the AquaLab are presented in Table 9. The a_w was identical at both timepoints for batch 2.

Table 9 - Result of water activity measurement for each batch at day 0 and day 60.

a_w	Day 0	Day 60
Batch 1	0.986	0.958
Batch 2	0.992	0.992
Batch 3	0.962	0.974

The measurement conducted on batch 1 showed a slight decrease in a_w whilst batch 3, on the contrary, displayed a small increase in a_w.

4.6.2. pH

Batch 1 displayed the highest pH-value at day 0 as well as day 60, see Table 10. Batch 2 and 3 both had a pH around 5.7-5.8 at day 0.

Table 10 - Results of pH measurement for each batch at day 0 and day 60.

pH	Day 0	Day 60
Batch 1	6.09	6.42
Batch 2	5.75	6.38
Batch 3	5.73	5.98

All batches showed an increase in pH at the end of the storage period. Batch 1 and 2 had a pH around 6.4 at day end. Batch 3, however, showed a smaller increase in pH than the other batches at day 60.

4.7. Storage conditions

4.7.1. Temperature and relative humidity

The measurement of temperature and relative humidity in the refrigerator showed that the mean temperature during the storage time was 9.5±0.05 °C whilst the average relative humidity (%) was 76.6±0.7

5. Discussion

The result from the challenge test assessing growth potential showed that the batches tested supported growth of *L. monocytogenes.* That growth could be observed is consistent with the result of a study conducted on Halloumi, which is a similar product to Eldost (Kapetanakou *et al.* 2017). Batch 1 displayed the highest growth potential amongst the batches tested. One possible factor that could have affected the growth is the inoculated cells in batch 1 was in early stationary phase whilst the cells inoculated in batch 2 and 3 could have entered the stationary phase. However, batch 3 were not valid since the standard deviation between the replicates were larger than 0.5 $\log_{10}$ cfu/g at day 60 (see table 6 in the results). So, technically one more batch should have been tested to get 3 valid batches in alignment with criteria for the challenge test (Beaufort *et al.* 2019). If an additional batch were tested, the outcome of Eldost supporting growth of *L. monocytogenes* would most likely not change. If the growth potential for the new batch is lower than batch 1, it is still the batch with the highest growth potential that sets the result. If the new batch, on the contrary, would express a higher growth potential than batch 1, the result from the new batch would set the growth potential. Hence, giving Eldost an even higher growth potential. Thus, testing an additional batch would most likely not change the fact that Eldost supports growth of *L. monocytogenes.*

The high growth potential of *L. monocytogenes* in the tested samples of Eldost implies that risk management of the pathogen is of relevance. A proper HACCP-plan targeting *L. monocytogenes* and strict use of GMP and basic hygiene routines is of importance since it is an effective way to minimize the occurrence of the bacteria at the production plant (Melo *et al.* 2015).

The results of the challenge test only apply to the product tested. It is stated by the EURL that if any changes in production methods, recipes and other factors affecting the product is done, the result of the test would be invalid (Beaufort *et al.* 2019). Due to time limitations within this project, the storage time for the challenge test was set to 60 days. In practice the shelf-life is set to a maximum of 90 days for the product. It would be of relevance to perform the challenge test on the full 90 days in order to get results that apply for the whole shelf-life period. However, it is not likely to get a different outcome at day 90 compared to day 60 in terms of the product being able to support growth. This, since the bacteria count had increased

so much already at day 60. Originally a sampling at day 30 was planned to be carried out, but due to covid-19 that had to be cancelled. Data from day 30 would have been of interest to obtain. Maybe the concentration of bacteria was at high levels of cfu/g already at day 30 or perhaps the largest increase in cfu/g was from day 30 to day 60. If the first 30 days would not show a significant (<0.5 $\log_{10}$ cfu/g) increase in cfu/g, this could in theory affect if the product would be classified as able or unable to support growth for a 30 days period. If so, the manufacturers could hypothetically discuss if the shelf-life should be shortened to 30 days. However, this must be addressed in further studies.

Another aspect that could be of importance is the adaption of strains. As described in material and methods, the NaCl content in the BHI broth was set to simulate the salt concentration in the cheese of around 2.25 %. The cold storage, during the growth of the strains was also set to mimic the storage conditions of the cheese in the consumer's fridge. To improve the adaption of strains, additional factors could be taken into consideration. For example, the pH values of the broth could have been adjusted to the 5.8 simulating the pH of the cheese. However, since the outcome of the challenge test showed a growth of *L. monocytogenes*, an even more adapted strain would probably only enhance the growth further. Another aspect of the strain adaption is that it was done to optimize the strains to grow well in specific conditions, which might not be the case in a real-life scenario. If comparing the adapted strains to a non-adapted strain that possibly would contaminate the cheese in a production plant, the growth of the non-adapted strains could perchance be less than that of the adapted strains inoculated in the laboratory environment. So, in theory, the growth in the challenge test, with the adapted strains, could possibly be larger than if the cheese would be inoculated with non-adapted strains. Nevertheless, since there is a risk that the strains that are present in the production facilities may have been exposed to cold-temperature and salt, resulting in an adapted strain that can contaminate the product. Usage of adapted strain for inoculation of the samples, even the worst-case scenario where an adapted strain contaminates the product, is covered.

The pH in batches 2 and 3 both was around 5.7-5.8 at day 0. This value is consistent with the pH obtained during the production process. Batch 1 on the contrary showed a higher pH at day 0. This could possibly be linked to problems with calibration of the pH-meter, so the reliability of this result could be questioned.

In the performed challenge test, non-inoculated samples only were tested for TBC, a_w, and pH. It would be of relevance to include a measurement of physico-chemical properties such as a_w, pH and TBC also on the inoculated samples. This in order to see if any changes in these parameters could be linked to the growth or suppression of growth for *L. monocytogenes*. An improvement could also be to test more

samples, not only 1 per batch, for these parameters. Because Eldost is an artisanal product and there could be a little more variability within a batch than with foods produced by a completely mechanised process.

The total bacterial count, TBC, showed a 1- and 2-log increase in batches 1 and 3. Batch 2 showed a 5-log increase, going from 1.3E+02 cfu/g on day 0 to 3.6E+07 cfu/g on day 60. The microbiota in the cheese could possibly compete with *L. monocytogenes*. If assuming that the inoculated samples had similar levels of bacteria as the non-inoculated samples tested for TBC, the sample that showed a 5-log increase could hypothetically compete with *L. monocytogenes* and perhaps suppress its growth. However, this was not observed in the tested batches, with batch 2 still supporting growth. This could be due to *L. monocytogenes* being competitive in cold-temperature environments such as refrigerators and in vacuum-packed products (Law *et al.* 2015; Melo *et al.* 2015; Adams *et al.* 2016). Testing more samples for TBC and conducting analyses of the inoculated samples as well, to see if any connection between suppressed growth of *L. monocytogenes* and high prevalence of associated microflora could be of interest.

Based on the results from the detection method, no prevalence of *L. monocytogenes* could be confirmed. These results indicate that GMP, as well as sufficient hygiene standards, have been implemented by the manufacturer for these three batches.

The measures taken at the manufacturer to reduce the occurrence of the bacteria seemed effective in the tested batches. However, if *L. monocytogenes* would contaminate the product somehow, heat treatment is an effective way to kill the pathogen. If the consumer was to fry or grill the cheese, the bacteria would be reduced significantly since the D_{70} for *L. monocytogenes* is only a few seconds (Adams *et al.* 2016). Another measurement that could be an alternative is that the producer conducts an in-package heat treatment. That would minimize the risk that post-pasteurization contamination poses, and the product would fulfil the criteria of zero prevalence in 25 g of cheese when leaving the producer.

When choosing batches to test, the batch most likely to support growth should be singled out and tested. This, since it is the batch with the highest growth potential that sets the growth potential for all batches. Thus, it would be of interest to test batches with parameters that possibly could inhibit growth, such as a higher NaCl-content. If a NaCl-concentration that both could inhibit growth and is accepted by consumers with regard to sensory characteristics could be singled out, perhaps producers could consider allowing higher salt concentrations. Nevertheless, the sensory characteristics are important to ensure consumers acceptability towards the product. But testing variation in the formula and its effect on growth of *L. monocytogenes* could be implemented in future challenge tests.

In summary, the result of the study implies that the tested batches of Eldost support growth of *L. monocytogenes.* To be compliant with current legislation, a zero prevalence of *L. monocytogenes* is accepted in 25 g of product when leaving the manufacturer. However, to be able to generalise the result beyond the three batches tested, further studies assessing the behaviour of *L. monocytogenes* in Eldost are needed.

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Appendix 1 - Popular Scientific Summary

Listeria hysteria – do we need to worry?

Maybe you have tried the Swedish version of Halloumi, Eldost? Many of us have discovered Halloumi and hopefully also Eldost. It has a fantastic taste and texture when grilled during summer barbeques and it is perfect to use as a vegetarian option in many dishes. But could hazardous bacteria *Listeria monocytogenes* be a potential risk in these kinds of products?

The sales of Halloumi have increased in Sweden during the latest years. However, it is an imported product that originates from Cyprus. Cyprus has by far the highest use of antibiotics when compared to other European countries. This is one reason behind the increased demand from consumers for a Swedish alternative to Halloumi, such as Eldost.
Even if the cheese often is consumed fried or grilled, it falls under the category of Ready to eat products. According to the labelling, you do not have to heat it prior to consumption. Generally, in Ready-to eat products, *Listeria monocytogenes* is a bacterium that could potentially grow and cause illness amongst consumers. The bacteria are competitive in cold-temperature environments such as refrigerators and in vacuum-packed products. *L. monocytogenes* can be lethal or cause severe illness, especially in immunocompromised groups, elderly people, and pregnant women.

The characteristics of Eldost, such as being stored in refrigerator, having a suitable pH and being vacuum packed implies that this pathogenic bacterium could grow well if the product is contaminated in some way. The current Swedish food legislation states that if a product can support growth, no *L. monocytogenes* should be present in 25 g of product when leaving the manufacturer, and the total concentration cannot exceed 100 colony forming units/g during the shelf-life. If the product cannot support growth, the legislation just states that the cfu/g cannot exceed 100 cfu/g during the shelf-life.
The conducted study tested if *L. monocytogenes* could grow or not in Eldost. The method for monitoring growth was a challenge test. It is based on an artificial contamination of the cheese with four different strains of *L. monocytogenes.* The cheese was then stored in a refrigerator, and in this way the potential growth could be tested. By testing the concentration of *L. monocytogenes* in the samples at the

start and end of the storage, any growth or reduction in concentration could be monitored.
By knowing if the product can support growth or not, the producer knows which legislation to follow and we as consumers can be sure that the products we eat are safe.

The result showed that the three tested batches of Eldost could support growth of *L. monocytogenes.* So, the producer must make sure that no *L. monocytogenes* is present in the product when leaving the production plant. The prevalence of pre-existing *L. monocytogenes* was tested in non-contaminated samples and no occurrence of the bacterium could be found. This result seems reassuring, no need for *Listeria* hysteria! Another calming information is that, even if the product can support growth, Swedish Food Agency states that it is not often *L. monocytogenes* is found in this type of product. However, the small scale of this study makes it hard to generalize beyond the three batches tested and further studies could be relevant.