Growth potential for *Listeria monocytogenes* in vegan salami

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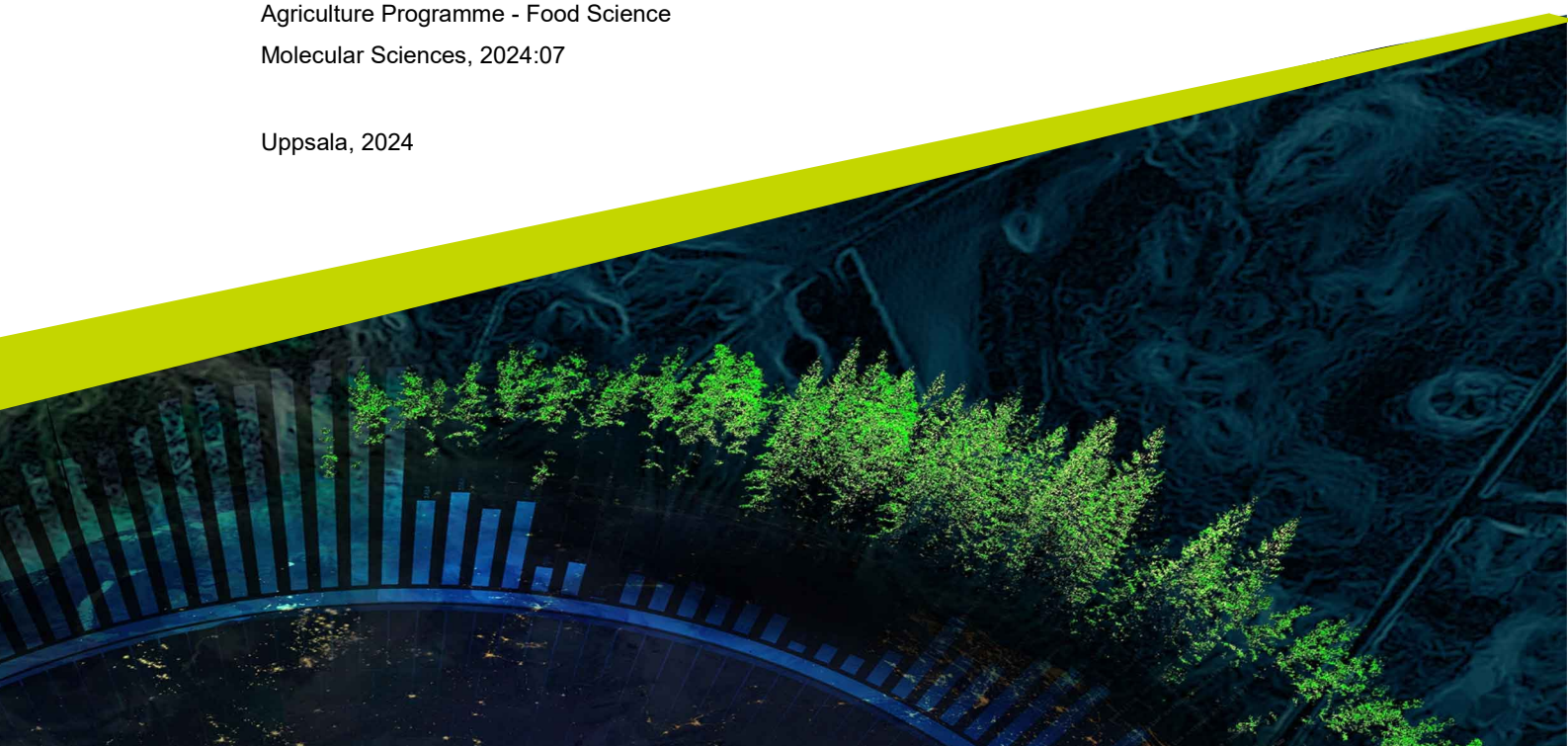

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Abstract

The increasing demand for plant-based products in the Swedish market has raised concerns regarding the potential presence of *Listeria monocytogenes*, a pathogenic bacterium, in ready-to-eat products such as plant-based deli slices. Compliance with current legislation requires assessing a product's ability to support or inhibit the growth of *L. monocytogenes*. Therefore, this study aimed to investigate the growth potential of *L. monocytogenes* in vegan salami.

The "challenge test" method was employed to evaluate the growth potential. Vegan salami samples were inoculated with two strains of *L. monocytogenes* and stored at 8°C for approximately 30 days. The concentration of *L. monocytogenes* was measured at the beginning, three intermediate time points, and at the end of the test. Prior to inoculation, a qualitative method was used to detect the initial presence of *L. monocytogenes*. Physicochemical characteristics were also assessed during the challenge test, and non-inoculated samples were analyzed for the same characteristics and total bacterial count.

The results indicated varying growth among the three different batches (with different production dates), with *L. monocytogenes* levels increasing from an initial inoculation level of 2 $\log_{10}$ to 7 $\log_{10}$ cfu/g during the challenge test. The overall growth potential across all batches was 4.4 $\log_{10}$ cfu/g. Furthermore, the physicochemical characteristics showed a decrease in pH, an increase in CO_2 levels (especially for Batch 2), and varying concentrations of total bacterial count (TBC). In conclusion, these findings demonstrate that vegan salami has the potential to support the growth of *L. monocytogenes*. To comply with existing legislation, it is crucial to ensure the absence of *L. monocytogenes* in 25 grams of the product upon leaving the manufacturer.

Keywords: plant-based deli slices, *Listeria monocytogenes*, vegan salami, growth potential

Sammanfattning

Efterfrågan på växtbaserade produkter har ökat på den svenska marknaden. Dock kan färdiga produkter, inklusive växtbaserade pålägg, utgöra en potentiell risk på grund av närvaron av den patogen bakterien *Listeria monocytogenes*. Enligt gällande lagstiftning är det viktigt att bedöma en produkts förmåga att stödja eller hämma tillväxten av *L. monocytogenes*. Därför var syftet med denna studie att undersöka tillväxtpotentialen för *L. monocytogenes* i en vegansk salami.

Metoden "challenge test" användes för att utvärdera tillväxtpotentialen. Prover av vegansk salami inokulerades med två stammar av *L. monocytogenes* och förvarades vid 8°C i ungefär 30 dagar. Koncentrationen av *L. monocytogenes* mättes i början, vid tre mellantidpunkter och vid slutet av testet. Före inokulering användes en kvalitativ metod för att undersöka om det redan fanns *L. monocytogenes* i produkten. Fysikokemiska egenskaper analyserades också under studien, och icke-inokulerade prover analyserades för samma egenskaper och totala bakterieantalet.

Resultaten visade varierad tillväxt mellan de tre olika batcherna (med olika produktionsdatum), med *L. monocytogenes*-nivåer som ökade från en inokuleringsnivå på 2 $\log_{10}$ till 7 $\log_{10}$ cfu/g under testet. Den totala tillväxtpotentialen över alla batcher var 4,4 $\log_{10}$ cfu/g. Dessutom visade de fysikokemiska egenskaperna en minskning av pH-värdet, en ökning av CO_2-nivåerna (särskilt för batch 2) och varierande koncentrationer av totalt bakterieantal (TBC). Sammanfattningsvis visar dessa resultat att vegansk salami har potential att stödja tillväxten av *L. monocytogenes*. För att följa gällande lagstiftning är det viktigt att säkerställa att *L. monocytogenes* är frånvarande i 25 gram av produkten när den lämnar tillverkaren.

Keywords: växtbaserade pålägg, *Listeria monocytogenes*, vegansk salami, tillväxtpotential

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Abbreviations

CFU	Colony Forming Units
FBO	Food Business Operators
LAB	Lactic acid bacteria
L. monocytogenes	*Listeria monocytogenes*
MAP	Modified atmosphere packaging
PBW	Buffered Peptone Water
RTE	Ready-to-eat
TBC	Total Bacteria Count

1 Introduction

This chapter provides an introduction to the study, presenting the background information regarding plant-based deli slices and the bacterium Listeria monocytogenes. Following this, a description of the problem is outlined with a final aim of this study.

1.1 Plant-based trends – substitutes

In recent years, a noticeable surge in consumer interest has emerged, focusing on reducing meat consumption and embracing plant-based foods and alternative protein sources derived from plants. This shift in consumer preferences has sparked innovations in the food market, tailored to meet this evolving trend (Aschemann-Witzel et al. 2021). These substitutes are designed to replicate the taste, texture, and nutritional profile of foods from animal origin. Manufacturers are actively employing ingredients such as soy, pea protein, wheat gluten, and mycoprotein to create a diverse array of products, including burgers, sausages, nuggets, and other meat analogues that closely resemble their animal-based counterparts (Alcorta et al. 2021).

In today's grocery stores, a wide variety of plant-based substitutes resembling traditional animal products, including deli slices, are available (He et al., 2020). Despite being relatively new to the market, these kind of products are swiftly gaining popularity (Van Paepeghem et al. 2024).

The driving forces behind the development of plant-based meat alternatives arises from concerns regarding the environmental impact, health implications, and animal welfare issues which are typically correlated with traditional meat production and consumption (He et al. 2020). For numerous consumers, the choice to exclude animal products from their diets is also motivated by ethical considerations, while others opt for such dietary changes for health reasons or to diminish their carbon footprint (Livsmedelsverket 2023).

Based on Axfood’s vegobarometer, an annual survey of Swedes' eating habits, shifts in dietary habits and attitudes are noticeable across all demographic groups,

with notable variations between genders and age groups. In general, women and younger individuals demonstrate a higher consumption of plant-based foods compared to men and older individuals. Additionally, they exhibit a greater inclination to continue transitioning towards plant-based diets in the future (Kantar Sifo 2019).

Plant-based foods play a part in the transition to a more sustainable food consumption. However, dietary recommendations advocating the shift from animal to plant-based products for environmental or health reasons must also address food safety concerns to safeguard consumers, particularly those in vulnerable groups (He et al. 2020).

1.1.1 Vegan salami

Salami, a fermented sausage enjoyed worldwide, boasts a distinctive appearance with its finely chopped fat and meat blend held together by salt-soluble proteins from the meat. This marbling effect, showcasing distinct fat particles, serves not only aesthetic but also technical purposes. The fat in salami intertwines with connective tissue, creating a network that adds both firmness and elasticity. However, replicating this texture in plant-based substitutes proves challenging. Traditional plant-based fats fail to match the structure and behavior of these animal fats. A recent innovation incorporated plant-based oils into a plant protein network through emulsification, followed by transglutaminase-induced covalent bonding, presenting a promising method for mimicking meat texture (Dreher et al. 2021).

Meat analogs, designed to mimic salami, primarily contain plant-based protein sources such as soy and wheat protein isolate, along with legumes such as peas and lupine, and grains such as rice and potatoes. These protein components are critical in creating a meat-like texture while providing important nutritional benefits. Complementing the proteins are lipids and polysaccharides, often sourced from rapeseed oil, selected to replicate the desired texture and mouthfeel of traditional salami. To bring out the taste, aroma and nuance of meat, a mix of spices, herbs and natural dyes are mixed into the formulation (Flores & Piornos 2021).

Central to the success of meat analogues is the replication of the fibrous structure of muscle tissue, crucial to achieving the desired structural properties. One of the key techniques proposed for this purpose is extrusion. Extrusion is a process that transforms dry ingredients into a continuous semi-solid product. This method involves a screw mechanism housed within a barrel, which propels a mixture consisting of dry ingredients, water, and oil through a “die” (a small opening). Throughout this process, the mixture is exposed to heat, mechanical force, pressure,

and moisture. Extrusion is notably favored for its efficiency and scalability in commercial production (Dreher et al. 2021), making it a prominent method in the development of meat analogues.

1.2 Listeriosis

Listeriosis is the infection caused by *Listeria monocytogenes* and poses a significant threat to certain individuals (Kontrollwiki 2023). Those most vulnerable include the elderly (over 65 years), individuals with compromised immune status, and pregnant women and their fetus (Ottoson 2022). In Sweden, reported cases typically range between 70 and 100 annually, with approximately two-thirds of those affected being over 70 years old. Additionally, between three and five reported cases involve pregnant individuals (Livsmedelsverket 2024a).

The incubation period for listeriosis is variable, spanning from a few days to several weeks, and can be even longer, especially in pregnant women (Goulet et al. 2013) Individuals affected by listeriosis can experience severe symptoms such as blood poisoning or meningitis, which can be fatal. Additionally, common gastrointestinal issues such as diarrhea and vomiting may occur. Pregnant women may exhibit flu-like symptoms, and the infection can spread to the fetus, potentially resulting in miscarriage or severe illness in the newborn (Livsmedelsverket 2024a). Listeriosis remains a prominent foodborne illness in the EU, leading to a high rate of hospitalizations and fatalities. Among reported *L. monocytogenes* infections, the majority occur in individuals aged 64 and above. In 2022, the overall fatality rate in the EU was reported to be 18.1% (EFSA 2023).

Unlike more common foodborne illnesses, listeriosis often exhibits a prolonged incubation period, further complicated by the wide range of food items susceptible to *L. monocytogenes* contamination. This variability in sources of infection and the long incubation time often leads to suspicion encompassing products consumed over approximately 30 days. Moreover, many contaminated products have extended shelf-life, allowing for consumption over multiple occasions, making it challenging to pinpoint the exact source of infection (Goulet et al. 2013).

The infective dose of *L. monocytogenes* depends on the immunocompetence of the individual exposed and the virulence of the specific strain of the bacterium. Elevated concentrations containing > 200 cfu/g have been identified in

contaminated food items, correlating with reported cases of the illness (Chen et al. 2003).

1.2.1 Listeria monocytogenes

The bacterial family Listeriaceae comprises two separate genera: Listeria and Brochothrix. Within the genus *Listeria*, there are over 20 species, including *Listeria monocytogenes* (*L. monocytogenes*) and *Listeria ivanovii* (*L. ivanovii*), which are classed as pathogenic (Mansell, 2022). *Listeria monocytogenes* is linked to listeriosis, a bacterial infection affecting both humans and ruminants, while *L. ivanovii* is primarily recognized as a pathogen for ruminants. While the infection can originate from animals, such as through unpasteurized milk, it is more commonly found in the environment, such as in soil (Schlech 1997).

Microscopically, *L. monocytogenes* appears as a gram-positive, rod-shaped bacterium that may be found singly or in short chains (0.5-2 μm × 0.5 μm) and does not produce spores. It exhibits a characteristic tumbling motility at 20-25°C and possesses peritrichous flagella. *Listeria monocytogenes* is classified as an aerobic and facultatively anaerobic bacterium. Furthermore, it demonstrates positive catalase activity and negative oxidase activity (Adams & Moss 2000).

Listeria is known for its ability to ferment glucose without producing gas as a byproduct. Strains of *L. monocytogenes* are commonly characterized by their negative response to D-xylose and their capacity to produce lecithinase. Additionally, they exhibit β-hemolysis and can utilize L-rhamnose as a carbon source (Beaufort et al. 2019)

Listeria monocytogenes displays the ability to grow in various conditions, including low temperatures (from 0 °C), a broad pH range (4.4 – 9.4), and low water activity/high salinity (0.92/12%)(Beaufort et al. 2019), as detailed in Table 1. This resilience means that even if the food is stored correctly, the bacterium can still be present (Miller 1992). As previously mentioned, *L. monocytogenes* is able to grow at a wide range of temperatures, including refrigerator temperatures (Roberts et al., 2020) The growth rate decreases at lower temperatures with growth at 4°C occurring at half the rate when compared to 8°C (Rosengren 2019). Moreover, *L. monocytogenes* can grow in packaging environments without access to oxygen, such as vacuum packaging or packaging with a modified atmosphere (Roberts et al. 2020).

Table 1. *Growth/survival characteristics of L. monocytogenes (Beaufort et al. 2019:5).*

	Min.	Growth opt.	Max.	Survival (no growth)
Temperature °C	-2	30-37	45	-18
pH	4.0-4.3	7.0	9.6	3.3 - 4.3
a_w	0.92	0.99	-	**≥** 0.90
NaCl content	-	-	12	-
Thermal inactivation				
$D_{65°C}$	0.2 to 2 min			

Listeria monocytogenes is frequently encountered in agricultural, aquaculture, and food environments (Roberts et al. 2020). The bacterium can be present in smoked and cured salmon, unpasteurized and pasteurized dessert cheeses, ready-to-eat (RTE) meals, charcutier and packaged cold cuts such as deli slices (Livsmedelsverket 2024a). Most problematic food items are those with a shelf-life of several weeks if kept in the refrigerator. Here *L. monocytogenes* can have time to multiply to levels that are harmful to risk groups before the shelf-life has expired. This particularly applies to foods stored for a few weeks in the refrigerator and that are consumed without heating. Applying heat treatment before consumption is a common method used to mitigate the prevalence of *L. monocytogenes* (Rosengren 2019).

Despite its presence, *L. monocytogenes* does not alter the sensory attributes of food, making contamination difficult to detect (Livsmedelsverket 2024a). While cooking and pasteurization effectively eliminate *L. monocytogenes,* the bacterium can survive freezing (Roberts et al. 2020). However, it cannot multiply in dry foods such as aged hard cheeses and cured meats, nor does it grow in acidic environments, such as those found in pickles and fruit juices (Livsmedelsverket 2024a).

Inadequate sanitation practices increases the risk of *L. monocytogenes* contamination through food processing, with food serving as the primary transmission pathway to humans, accounting for the majority (99%) of listeriosis cases (Beaufort et al. 2019). Consumption of contaminated food can lead to the development of listeriosis, highlighting critical aspects of food safety (Roberts et al. 2020).

1.2.2 Control of *L. monocytogenes* in production

To control the presence of *L. monocytogenes* within the production plant, it is necessary to take samples from areas where contamination could occur. These samples are typically collected from wet areas such as drains and floors, as well as challenging-to-clean equipment sections such as crevices. Slicing machines pose a notable challenge in thorough cleaning (Jordan et al. 2018). Consequently, during slicing, there is an elevated risk of *L. monocytogenes* contamination if cleaning and disinfection protocols are not thorough (Van Paepeghem et al. 2024).

The use of preservatives such as lactate, diacetate, nitrite, and sorbate is common in inhibiting bacteria such as *L. monocytogenes.* However, for food manufacturers of plant-based products who aim to cater to the increasing consumer preference for "clean label" foods, not using preservatives presents a dilemma. "Clean label" foods are highly regarded for their avoidance of "chemical" additives, transparent ingredient lists, and minimal processing. This evolving consumer trend brings forth challenges for food producers in terms of both extending the shelf life of products and effectively managing spoilage bacteria in plant-based products (Aschemann-Witzel et al. 2021).

Plant-based deli slices are intended as a RTE food, which is defined as a product that does not require cooking or any further processing before consumption. These innovative RTE products, often pre-packed and refrigerated with an extended shelf-life (>5 days), are challenging to assess for listeriosis risk due to insufficient data (Van Paepeghem et al. 2024). Currently, the lack of data on the presence and proliferation of *L. monocytogenes* in plant-based RTE foods poses a challenge for manufacturers and food safety authorities. Therefore, more research is needed to determine the presence of *L. monocytogenes*, other pathogenic bacteria, and molds in these types of products.

1.2.3 Occurrence and guidelines of *L. monocytogenes* in charcutier and plant-based substitutes on the Swedish market

The occurrence of *L. monocytogenes* in both cured meats and plant-based substitutes has been examined on the Swedish market. Cured meats such as pâté, sliced ham, cooked sausages, and turkey are known to potentially harbor *L. monocytogenes,* which can proliferate during the products' shelf-life, typically spanning several weeks. It is recommended that individuals in risk groups consume these products early in their shelf-life, ideally within a week from packaging date. Exceptions include air-dried hams and smoked sausages, which, due to their dry, salty nature and lower pH values, respectively, tend to inhibit *L. monocytogenes*

growth throughout their shelf-life (Livsmedelsverket 2024a). In a small-scale study performed in 2022 by the Swedish Food Agency, *L. monocytogenes* was not present in any of the 20 products tested of pre-packaged plant-based deli slices available on the Swedish market (Flink 2023).

The Swedish Food Agency conducted a risk assessment on 15 plant-based deli slices, using predictive modeling tools such as Combase, Pathogen Modeling Program (PMP), and Food and Spoilage and Safety Predictor (FSSP). Their simulations emphasized the importance of pH in limiting *L. monocytogenes* growth in these products (Ottoson, 2022). According to the simulations, *L. monocytogenes* could grow in three products with a pH above 6 at 4°C and in four products at 8°C (Flink 2023). It is important to note that these tests were solely simulations of Listeria growth based on factors such as the product's pH, water activity (a_w), and other relevant parameters.

The Swedish Food Agency has recently issued new guidelines for risk groups concerning *L. monocytogenes* in plant-based deli slices. These guidelines affirm that such products are safe for consumption by risk groups throughout their entire shelf-life, up to the specified best-before date. This recommendation is based on the previous risk assessment, which indicated slow *L. monocytogenes* growth in plant-based deli slices, attributed to their low pH value and frequent use of preservatives (Flink 2023). Additionally, the Swedish Food Agency suggests that beyond the best-before date, bacterial levels could potentially increase to levels exceeding 100 cfu/g. As a general precaution, as for all refrigerated food which has passed its best-before date, consumers are advised to inspect the food's appearance, smell, and taste to determine its suitability for consumption after the best-before date. However, it is important to note that this advice applies exclusively to individuals not belonging to the risk groups for *L. monocytogenes* (Livsmedelsverket 2024b).

Until now, there have been no reported cases of listeriosis linked to plant-based deli slices on the Swedish market (Flink 2023). However, findings of *L. monocytogenes* in plant-bases deli slices have been reported in the Rapid Alert System for Food and Feed (RASFF) due to mandatory analyses performed by the producers in accordance to EG 2073/2005. Examples of RTE products in which *L. monocytogenes* has been detected include apple pâté (Belgium), vegan RTE falafel (France), sliced cooked parsley sausage (Belgium), vegetarian smoked sausage (Germany), and vegan RTE falafel (The Netherlands) (Van Paepeghem et al. 2024).

1.3 Food safety management systems

According to Commission Regulation (EC) No. 852/2004, food producers are obligated to establish a Food Safety Control Program aimed at preventing potential hazards associated with their products. This regulation applies universally to all food businesses, irrespective of their size. It mandates the development, implementation, and maintenance of an internal system ensuring safe and hygienic production practices. Central to this food safety management system is the adoption of the Hazard Analysis and Critical Control Points (HACCP) principle, internationally recognized as a practical tool for food business operators (FBOs) to effectively manage hazards in food production (EFSA et al. 2017)

Food business operators are tasked with identifying significant hazards inherent in their manufacturing processes, such as the presence of *L. monocytogenes*, and taking appropriate measures to prevent, eliminate, or mitigate these hazards to an acceptable level. Particularly for food companies manufacturing products that may pose a risk of listeria contamination, it is crucial that they implement measures within their control to prevent the occurrence and spread of *L. monocytogenes* (Flink 2023).

1.3.1 Rules and legislation

Commission Regulation (EC) No 2073/2005 outlines the guidelines that FBO's must follow regarding microbiological standards for food products. It defines specific rules for compliance and establishes microbiological criteria for certain microorganisms. In Annex I of this regulation, microbiological food safety criteria are detailed for *L. monocytogenes* in RTE foods. According to Commission Regulation (EC) No 2073/2005, the criteria for *L. monocytogenes* prevalence in RTE products are as follows:

- For food category 1.1 - "RTE-foods intended for infants and special medical purposes", *L. monocytogenes* must be absent in 25 g of the product before leaving the manufacturer's immediate control.
- For food category 1.2 - "RTE-foods capable of supporting the growth of *L. monocytogenes*", *L. monocytogenes* must be absent in 25 g of the product before leaving the manufacturer's immediate control. Alternatively, when

placed on the market, the concentration of *L. monocytogenes* must not exceed 100 cfu/g during the specified shelf-life period.
- For food category 1.3 - "RTE-foods unable to support the growth of *L. monocytogenes*", the concentration of *L. monocytogenes* must not exceed 100 cfu/g during the specified shelf-life period.

In Article 3, paragraph 2, it is stated that, when necessary, the FBO responsible for manufacturing the product must conduct studies as outlined in Annex II to assess compliance with the criteria throughout the product's shelf-life. This requirement particularly applies to RTE foods capable of supporting the growth of *L. monocytogenes* and thus posing a risk to public health.

Annex II outlines that studies mentioned in Article 3 should encompass the following aspects:
- Examination of the physicochemical characteristics of the affected product, including pH, water activity (a_w), salt content, concentration of preservatives, and the type of packaging system.
- Consideration of storage and processing conditions, potential sources of contamination, and the expected shelf-life of the product.
- Consultation of available scientific literature and research data concerning the growth and survival characteristics of the relevant microorganisms.

If necessary, further studies may be warranted, which could encompass predictive mathematical modeling, examinations of microorganism growth, and assessments of their survival over the course of the shelf-life period, such as conducting a challenge test.

1.4 Challenge test

As outlined in Commission Regulation (EC) No. 2073/2005, FBO's must assess the growth of *L. monocytogenes* during a product's shelf-life when deemed necessary. However, the specific methodology is not explicitly provided. To address this, the European Reference Laboratory for *L. monocytogenes* and The French Agency for Food, Environment and Occupational Health and Safety (ANSES) collaborated on a technical guidance document, detailing procedures for conducting shelf-life studies.

The challenge test examines the behavior of *L. monocytogenes* in artificially inoculated RTE food samples. It gives insights into either the potential for growth or the maximum growth rate achievable for *L. monocytogenes*. When assessing the

growth potential, it determines whether a product can support *L. monocytogenes* growth or not.

The testing period starts on the day of inoculation and extends until the expiration date of the product. Growth potential (δ) is calculated as the difference between the $\log_{10}$ cfu/g counts logged at the expiration date and the inoculation date. The magnitude of δ is influenced by various factors, including the strains employed, inoculation level, physiological condition of cells, competing microflora, intrinsic properties of the food product, and external factors such as temperature and time (Beaufort et al. 2019).

In the context of Commission Regulation (EC) No. 2073/2005, the δ value classifies food as follows:

- If $\delta > 0.5$ $\log_{10}$ cfu/g, the food is categorized as "*RTE foods able to support the growth of L. monocytogenes."*
- If $\delta \leq 0.5$ $\log_{10}$ cfu/g, the food falls into the category of "*RTE foods unable to support the growth of L. monocytogenes*

For foods presumed to support *L. monocytogenes* growth, it is recommended to test three batches. When selecting batches for testing, preference is given to the one most likely to support growth. Inoculation procedures should involve strains in the early stationary phase, preferably including at least one strain with known characteristics from the EURL Lm strain collection. It is recommended to use at least two strains of *L. monocytogenes* in the challenge test (Beaufort et al. 2019)

The findings derived from challenge tests play an essential role in establishing the shelf-life of a food product and identifying any alterations in its composition that might impact safety and stability with greater reliability. Integrating challenge tests with predictive models underscores their efficacy and convenience in evaluating the immediate viability of current product formulations and predicting the microbiological safety and stability of future formulations in real-time. This emphasizes the superior reliability of challenge tests compared to predictive models alone (Komitopoulou 2011).

2 Aim and purpose

2.1 Problem description

The analysis of key characteristics such as pH and water activity in vegan salami indicates a potential risk associated with *L. monocytogenes* proliferation. Given the extended shelf-life of the product, this potential for bacterial growth raises concerns regarding food safety.

As previously mentioned, it is essential to ascertain whether a food product can or cannot support the growth of *L. monocytogenes*. If the product supports growth, regulatory standards require either no presence of *L. monocytogenes* per 25 g at the producer or a concentration below 100 cfu/g throughout the shelf-life. Conversely, if the product cannot support growth, *L. monocytogenes* concentration must remain below 100 cfu/g during the specified period. Therefore, understanding whether a particular food product can or cannot support growth is essential for determining applicable legislation.

Importantly, there is a noticeable lack of studies in existing literature that specifically examine whether plant-bases deli slices support or hinder the growth of *L. monocytogenes*. This research gap underscores the necessity for studies focusing on the behavior of *L. monocytogenes* within plant-based RTE products. Such studies can offer valuable insights beneficial to producers and public health authorities alike. Therefore, conducting research to address this gap is essential for authorities to make informed decisions and implement effective risk mitigation strategies within the food industry.

2.2 Aim

The purpose of this study was to test the growth potential of *L. monocytogenes* in vegan salami during its shelf-life period. To evaluate the growth, a challenge test has been chosen as the most suitable method.

3 Materials and methods

A challenge test was conducted in accordance with the EURL Lm Technical Guidance Document on shelf-life studies for *L. monocytogenes* in RTE foods (Beaufort et al. 2019), to assess the growth potential (δ) of *L. monocytogenes* in a product of plant-based deli slices.

3.1 Selection of product

A pre-study was conducted to identify the product with the highest potential for *L. monocytogenes* growth. The selection criteria focused on a combination of high pH and water activity (a_w) levels, as well as the suitability of the packaging for inoculation purposes. Eight different food items underwent physiochemical testing, see Table 2.

Table 2. *Values represents the measured pH and water activity (a_w) of each food item.*

Product	**pH**	**a_w**
Vegan salami	6.01	0.96
Vegan slices (smoked)	5.84	0.96
Vegan slices (paprika)	5.63	0.97
Vegoslices (tomato & basil)	5.14	0.96
Vegoslices - original	5.04	0.97
Vegoslices (paprika)	5.02	0.95
Vegan smoked flavor slices	4.85	0.97
Vegoslices salami	4.83	0.97

The food item “vegan salami” exhibited the highest pH value among all the food items tested, measuring at 6.01. Moreover, its water activity level was notably high, reaching 0.96. Additionally, the product exhibited a homogenized structure, and its packaging was suitable for inoculation. These characteristics indicated that the selected vegan salami could offer a favorable environment for microbial growth, including *L. monocytogenes*.

Figure 1 illustrates the selected product when opened and with top lid removed.

Figure 1. *A picture of the selected product, vegan salami.*

The following tables provide details on the ingredients, storage instructions, shelf-life, packaging conditions, and nutritional values of the selected product (refer to Tables 3 and 4).

Table 3. *Ingredients, storage instructions, shelf-life, manufacturing country and packaging conditions of the selected product.*

Ingredients: Water, rapeseed oil, potato protein (8% EU), pea protein (7% EU), thickeners (E407, E425, E461, E412), modified starch, crushed linseed, salt, natural food coloring (paprika extract, fenugreek extract, beetroot extract), flavorings, spices (paprika, parsnip), spice extract (paprika, black pepper), sugar, color (E172), barley malt extract.
Storage Instructions: Refrigerate at or below +8°C. Once opened, store in the refrigerator at the same temperature for up to 2 days.
Shelf-life: sealed product - 42 days, open product - 2 days.
Manufacturing Country: Germany.
Packaging Conditions: Modified atmosphere - 80% N_2 and 20% CO_2.

Table 4. *Nutritional values of the selected product.*

Nutritional values	Per 100 grams
Energy	894 kJ / 216 kcal
Protein	5 g
Carbohydrates	5.5 g
of which sugars	3.5 g
Fat	19 g
of which saturated fat	1.1 g
Fiber	1.5 g
Salt	1.8 g

3.2 Test points

The challenge test was repeated three times in independent trials, with each trial involving a new batch of vegan salami sourced from the same manufacturer but produced on separate occasions. Test units were inoculated with *L. monocytogenes* and subsequently analyzed for *L. monocytogenes* (quantitative method), physicochemical characteristics, and modified atmosphere. Food control samples were tested for the presence of *L. monocytogenes* (qualitative method), physicochemical characteristics, modified atmosphere, and enumeration of total bacteria count (TBC). Control units were inoculated with the same volume as the test units but only with sterile physiological water (NaCl 8.6 g/L). The control units were tested for physicochemical characteristics, modified atmosphere, and enumeration of TBC. For more details, see Table 5 below.

Table 5. *Total number of units required for the challenge test based on Beaufort et al. (2019).*

Type of units	**Type of analysis**	**Number of units and date of analyses per batch**	
Test unit	Enumeration of *L. monocytogenes*	**7**	3 test units at t_0 3 test units total for three intermediate points (t_1, t_2, t_3) 1 test unit at t_{end}
	Measurement of physico-chemical characteristics and modified atmosphere		1 at t_0 and 3 intermediates and 1 at t_{end}

Food control sample	Detection of *L. monocytogenes*	**5**	1 at t_0
	Measurement of physico-chemical characteristics and modified atmosphere		1 at t_0 and 1 at t_{end}
	Enumeration of total bacteria count		1 test unit at t_0 and 3 intermediates and 1 at t_{end}
Control units	Measurement of physico-chemical characteristics	**5**	1 test unit at t_0 and 3 intermediates and 1 at t_{end}
	Modified atmosphere		1 at t_0 and 1 at t_{end}
	Enumeration of total bacteria count		1 at t_0 and 1 at t_{end}
Total number required per batch		**17**	
Total number required for 3 batches		**51**	

3.2.1 Selection of bacterial strains

According to EURL Lm Technical Guidance Document on shelf-life studies for *L. monocytogenes* in RTE foods, a mixture of at least two strains should be used in the challenge test to accommodate variations in growth and survival of different strains. The selected strains should be obtained from the collection maintained by the EURL Lm Technical Guidance Document on shelf-life studies for *L. monocytogenes* in RTE foods (Beaufort et al. 2019). The selection of suitable strains of *L. monocytogenes* was made according to the following preference criteria:

1. **Origin:** strains isolated from meat or plant-based products.
2. **Conditions:**
   a. **pH:** Strains which can grow in an environment at pH 5-6.
   b. **Temperature:** Strains which can grow at low temperatures, 8 °C.
   c. **Water activity:** Strains capable of growing at water activity (a_w) 0.95.

The strain 12MOB045LM originates from meat and has been reported to have a high growth rate at low temperature (8°C), low pH (pH 5) and low a_w (0.95) (Guillier et al. (2013)). The decision to choose a strain from a meat origin was based

on the assumption that plant-based deli slices might be produced in the same facility as meat deli slices, likely utilizing the same equipment for both products. This situation presents a risk of potential cross-contamination between the two products. In contrast, 12MOB048LM was chosen under the assumption that it would be derived from a plant-based source (listed as “other products”), as it did not originate from dairy, fish, or meat (which were the alternative sources in the collection). This decision aligns with the tested product, predominantly composed of rapeseed oil, potato, and pea protein, all of which are plant-based.

The strains were selected based on their demonstrated advantages in various tests, including low temperature, low pH, and low water activity, across their respective origins. The strains were obtained from the Swedish Food Agency (Livsmedelsverket).

3.3 Preparation of the inoculum

3.3.1 Adaption of strains

The two different strains underwent acclimatization to cold storage conditions. Colony material from each strain (12MOB045LM and 12MOB048LM) was collected from prepared Brilliance Listeria agar plates (Thermo Scientific™) and mixed with 9 mL of BHI broth. The sample (referred to as subculture 1) was then incubated at 37°C for 24 hours. After 24 hours, 0.1 mL from subculture 1 was transferred to a new tube along containing 9 mL of BHI broth. This sample (referred to as subculture 2) was then incubated at 10°C for 3 days.

3.3.2 Inoculum preparations

After 3 days, 0.5 mL from subculture 2 was obtained from each strain (12MOB045LM and 12MOB048LM) and mixed with 9mL sterile physiological water. Dilutions in physiological water were performed as a pre-experiment to achieve the desired inoculum suspension as determined by plating each dilution on Brilliance Listeria agar plates, incubate 48 hours in 37°C and then counting colonies to determine concentration from the beginning. The inoculated volume per sample was 0.8mL and the initial target was set at 100 cfu/g of product, within the range of 50-200 cfu/g for each test unit. This target was aimed at verifying that the test samples were inoculated in compliance with the method's detection limit. The overall volume of the prepared inoculum suspension had to be sufficient to inoculate all the designated test units.

3.3.3 Inoculation of the test units

The inoculation of *L. monocytogenes* was carried out on the product while it remained within its packaging, performing inoculation with a sterile syringe and needle through a septum (Septum white Ø15 mm, Dansensor®), which was promptly covered by a second septum to preserve the modified atmosphere within the package. Before inoculation, this method was tested by inoculating with a diluted dye to visualize the distribution of the inoculated volume. Additional confirmation of the targeted concentration of cfu/g was achieved by diluting the inoculum and plating it on Brilliance Listeria agar plates. The plates were then incubated at 37°C for 2 days, and the colonies were counted which confirmed the final inoculated concentration of cfu/g.

Brilliance™ Listeria Agar, formerly referred to as OCLA, is a chromogenic medium employed for the detection and differentiation of various Listeria strains. It effectively differentiates pathogenic strains such as *L. monocytogenes* and *L. ivanovii* from non-pathogenic strains (Willis et al. 2006). It contains 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside, which reacts with β-glucosidase, resulting in blue-green colonies. This medium selectively inhibits other bacterial and fungal growth (Park et al. 2014). Both *L. monocytogenes* and pathogenic *L. ivanovii* produce lecithinase enzymes, forming an opaque halo around the blue colonies, which is diagnostic for pathogenic Listeria species (Willis et al. 2006).

After inoculation, it was crucial to ensure that *L. monocytogenes* was homogenously distributed throughout the food sample. This was evaluated based on the standard deviation of *L. monocytogenes* counts obtained from the three replicates on t_0 (the day of inoculation). If the standard deviation exceeded 0.3 $\log_{10}$ cfu/g, the batch would be deemed unacceptable, and a new inoculation must be performed. This calculation was thus performed once for every batch, so in total three times.

3.3.4 Storage temperature and duration

Inoculated samples were stored in an incubator at 8°C to replicate conditions recommended for consumer storage. Ideally, it would have been preferable to simulate various temperatures to mimic the product's journey from manufacturing to retail, covering retail storage and ultimately consumer storage temperatures. However, this was not practically feasible.

Originally, the challenge test duration was intended to be 42 days, aligning with the complete shelf-life of the product. However, adjustments were necessary due to the logistics, since the production was in Germany and then stored in a warehouse in Sweden before being distributed to the retailers. The products collected for Batch 1

for example, were produced on February 26th but not available at retail level until March 8th, 12 days after production. To ensure that the challenge test accurately reflected the remaining shelf-life of the product, the duration of the challenge test was adjusted accordingly.

3.3.5 Enumeration method, quantification

To quantify the cfu/g of *L. monocytogenes* in inoculated samples, an enumeration methodology was employed. The procedural approach adhered to the guidelines outlined in ISO 11290-2.

3.3.6 Sample preparation

The inoculated product's upper lid was cut and removed to open it. The product was sliced using a sterile scalpel using the same technique for each sample, see Figure 2.

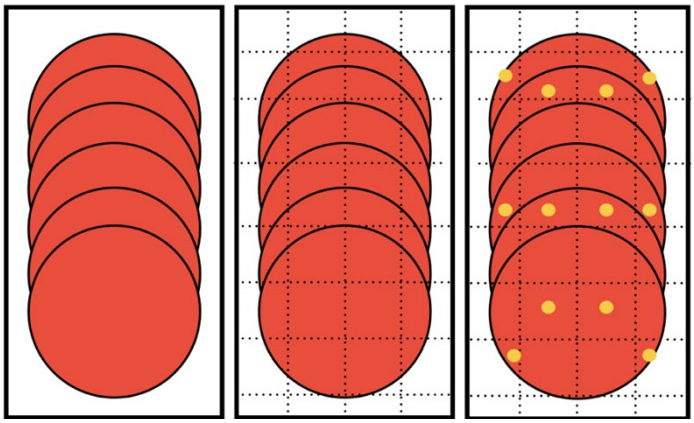

Figure 2 *An illustrative image that presents the product (left), the method of slicing the product (middle), and the parts collected indicated by yellow dots (right).*

Subsequently, 10g of the inoculated sample was aseptically collected according to the yellow dots in Figure 2 and placed in a stomacher bag. Thereafter, 90 ml of sterile Buffered Peptone Water (BPW) was added. The sample with BPW was homogenized in a stomacher (easyMIX®, AES Laboratoire) for 60 seconds.

3.3.7 Plating on selective medium

Brilliance Listeria plates were placed in a 37°C incubator for 10 minutes to allow the medium to dry, enabling the spreading of the sample volume on the medium. To enhance the detection of *L. monocytogenes* in low concentrations, 1 mL of homogenate was evenly spread across four dried Brilliance Listeria plates, approximately 250 µL per plate. Additionally, serial dilutions of the homogenate were prepared and plated on Brilliance Listeria agar plates to detect higher concentrations. Following spread plating, the plates were allowed to dry at room temperature for 30 minutes. Subsequently, the plates were placed in a bag and

incubated in an inverted position at 37°C for 24 hours. After 24 hours, presumptive colonies of *L. monocytogenes* were enumerated. A final count was conducted after 48 hours.

3.4 Detection method, qualitative

To confirm the absence of pre-existing *L. monocytogenes* in the tested samples, a detection method was applied to the food control sample at t_0. The protocol used derived from standard procedures outlined in ISO 11290-1.

3.4.1 Primary enrichment

The top lid was removed from the food control sample. 25g of the food control sample was added into a stomacher bag containing 225 mL of Half-Fraser broth (Thermo Scientific™ Oxoid™) and homogenized for 60 seconds. Next, the sample was incubated at 30 °C ± 1 °C for 24 ± 2 hours.

3.4.2 Secondary enrichment

After incubation, 0.1 ml of the primary-enriched sample (3.4.1) was transferred into 10 ml of secondary enrichment medium (Fraser broth, Thermo Scientific™ Oxoid™). The culture was then incubated at 37 °C ± 1 °C for 24 ± 2 hours.

3.4.3 Plating out and identification

Sample from the primary enrichment (3.4.1), incubated at 30 °C for 25 ± 1hours was streaked onto the selective media Brilliance Listeria agar and subsequently incubated at 37 °C ± 1 °C for 48 hours. Sample from secondary enrichment (3.4.2), incubated at 37 °C ± 1 °C for 24 ± 2hours, underwent the same procedure on the selective plating medium. After incubation for 48 hours, the plates were observed for any presumptive colonies of *L. monocytogenes.*

3.5 Assessment of total bacteria count (TBC)

3.5.1 Sample preparation

The food control (non-inoculated) samples were tested at each timepoint for assessment of TBC. In addition, the control unit (inoculated with physiological water) was tested at t_0 and t_{end}. The samples were opened by removing the top lid. 10g of the sample was collected according to the yellow dots in Figure 2 and placed in a stomacher bag with 90 mL of BPW. The sample was homogenized for 60 seconds. Subsequently, the homogenized sample was serially diluted.

3.5.2 Inoculation

1 ml of diluted samples was dispensed onto the center of the bottom film of the 3M^{TM} Petrifilm^{TM} Aerobic Count Plate. Subsequently, top film was carefully placed onto the sample. The 3M Petrifilm Spreader was then positioned with the ridge side down on the top film, covering the sample. Pressure was applied to the Petrifilm spreader to evenly distribute the sample over the entire area. After the spreader was removed, the gel solidified within 1 minute.

3.5.3 Incubation and reading

The 3M Petrifilm plates were incubated with clear side up for 72 h ± 3 hours at 30 °C ± 1 °C. Following the incubation period, the plates were examined, and all red colonies were enumerated, regardless of their size and intensity, in accordance with the Interpretation Guide.

3.6 Physico-chemical parameters

3.6.1 Water activity

Water activity was measured using an Aqualab CX-2 (Decagon devices Inc., Pullman) water activity meter. All samples, including both test and control units, were assessed for water activity at time points t_0, 3 intermediate time points t_1, t_2 and t_3, and finally at t_{end}. The food control sample was tested at time points t_0 and t_{end}. Each sample was collected and placed in a sample cup and inserted into the AquaLab for measurement. Two measurements were conducted to ensure consistency in the obtained values. Similar values needed to be observed for confirmation.

3.6.2 pH

The pH was measured using a pH/temperature measurement instrument (Testo 205, Testo, North America). Both test and control units were tested at time points t_0, t_1, t_2 and t_3, and finally at t_{end}. Additionally, the food control sample was tested at time points t_0 and t_{end}. Samples were measured by inserting the probe into the top layers of the product.

3.6.3 Modified atmosphere packaging (MAP)

To ascertain the absence of any packaging leakage for all test units during the storage period, atmospheric conditions were assessed using a portable headspace analyzer (PBI Dansensor, Mocon). Testing was conducted on samples throughout the entire challenge test, see Table 5.

3.6.4 Temperature (°C)

Temperature measurements were conducted using an infrared thermometer (Standard ST-8810, Waeco) on one control unit repeatedly from time point t_0 to t_{end}, confirming that the incubator maintained a consistent temperature of 8 °C ± 1 °C.

3.6.5 Ocular Assessment

A comparison between inoculated product (test unit), product inoculated with only physiological water (control unit), and untouched product (food control) was conducted at each time point by the same person to observe if any changes in color, odor, or surface layer had occurred. This was performed as a form of quality control to assess if any visual alterations in the product took place during the test period.

3.7 Calculation

3.7.1 Calculation of growth potential

The growth potential of each batch was assessed by converting the *L. monocytogenes* concentration into $\log_{10}$ cfu/g. Then, for each batch, the growth potential Δ was calculated using the formula: $\Delta = \log_{max} - \log_i$

In this equation:

- Log_i represents the average *L. monocytogenes* counts of the 3 test units assessed at t_0.
- Log_{max} represents the highest counts of *L. monocytogenes* obtained from the four sampling points (excluding the t_0 sample), with one test unit analysed per sampling point.

The growth potential (δ) for the tested batches corresponds to the highest Δ value obtained.

4 Results

4.1 Enumeration method

The counts of *L. monocytogenes* at t_0(10 days from manufacture), t_1(15 days), t_2(22 days), t_3(33 days), and t_{end}(42 days, end of shelf-life according to best-before date) in inoculated test units are illustrated in Figure 3 below. All samples underwent analysis using the enumeration method according to ISO 11290-2. It is important to note that the growth period for Batch 3 commences at t_1, as this batch was already 16 days old when inoculated, whereas Batch 1 and 2 were 15 days old at timepoint t_1. Consequently, the test period for Batch 3 was shortened due to this discrepancy. Batch 1 exhibited rapid growth of *L. monocytogenes*, reaching levels close to 7 $\log_{10}$ cfu/g at the end of the shelf-life. In contrast, Batch 3, inoculated on day 16 from the production date, initiated its growth curve at t_1. Despite the delayed inoculation, Batch 3 exhibited a growth curve similar to that of Batch 1, reaching a level of 5.4 $\log_{10}$ cfu/g at the end of the shelf-life.

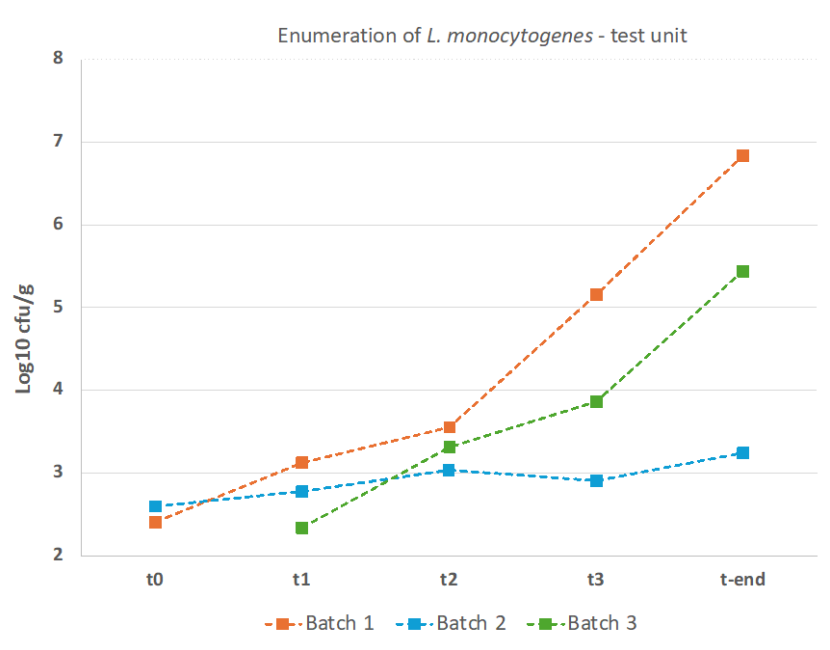

Figure 3.*Growth of L. monocytogenes for test unit batches 1, 2 and 3 for all timepoints.*

Batch 2 demonstrated a slight increase in the population of *L. monocytogenes* during the storage period. However, this growth was significantly less pronounced compared to Batch 1 and Batch 3. There was a difference of more than 3 $\log_{10}$ cfu/g between Batch 1 and Batch 2 at t_{end}.

4.1.1 Growth potential (δ)

To determine whether the tested batches of vegan salami can be classified as able or unable to support the growth of *L. monocytogenes,* in accordance with Commission Regulation (EC) No 2073/2005, it is essential to calculate the growth potential (δ). The results of these calculations are detailed in Table 6 below.

Table 6. *Growth potential calculation for 3 batches - 5 sampling points - 1 test unit per sampling point, except for timepoint t_0 which is presented as the mean of 3 test units.*

Time-points	**Time 0 (t0)**		**Time 1 (t_1)**	**Time 2 (t_2)**	**Time 3 (t_3)**	**Time 4 (t_{end})**	**Δ batch**	**(δ)**
	[Lm] $\log_{10}$ cfu/g	Mean ± sd*	Lm] $\log_{10}$ cfu/g	Lm] $\log_{10}$ cfu/g	Lm] $\log_{10}$ cfu/g	Lm] $\log_{10}$ cfu/g		
Batch 1	2.49 2.54 2.17	2.40 ± 0.16	3.12	3.55	5.15	6.83	6.83-2.40= 4.43	**4.43**
Batch 2	2.58 2.61 2.59	2.59 ± 0.01	2.77	3.03	2.9	3.24	3.24-2.59 =0.65	
Batch 3	2.63 2.38 2.00	2.34 ± 0.26	2.34	3.31	3.86	5.44	2.34-5.44 = 3.10	

*sd: standard deviation

The highest "Δ" value among the three batches was 4.43 $\log_{10}$ cfu/g. The growth potential (δ) was therefore 4.43 $\log_{10}$ cfu/g which clearly exceeds the limit of 0.5 $\log_{10}$, which defines whether the RTE food is able or unable to support the growth of *L. monocytogenes*. Therefore, this RTE food is classified as able to support the growth of *L. monocytogenes* and falls under category 1.2 of Regulation (EC) 2073/2005.

4.2 Detection method, qualitative

The results of the analysis indicated negative findings for *L. monocytogenes* on day t_0 across all three batches. This serves as confirmation that all batches were free from contamination of *L. monocytogenes* at the start of the challenge test.

4.3 Enumeration of total bacteria count (TBC)

The results of TBC for food control samples are presented in Figure 4.

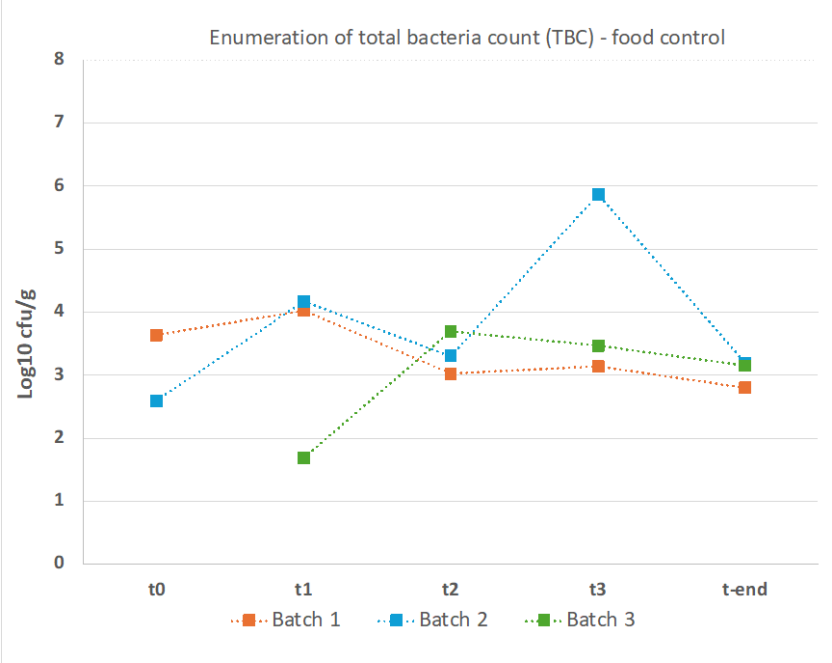

Figure 4. *Total bacteria count for food control samples from Batches 1, 2 and 3 collected at each time point.*

Batch 1 displayed a decreasing growth trend in TBC. In contrast, Batch 2 demonstrated a significant increase between t_2 and t_3, followed by a decline towards the end of the test period. Batch 3 showed rapid initial growth, which gradually decreased towards the test's end. Since Batch 3 was already 16 days old, the test period commenced at t_1. In summary, the concentrations of TBC in all food control samples ranged between 2-6 $\log_{10}$ cfu/g.

The TBC concentrations for the control units, which were inoculated with only physiological water, varied between 2 and 4 $\log_{10}$ cfu/g.

4.4 Physio-chemical properties

4.4.1 Water activity (a_w)

The a_w for test units remained consistently around the values of 0.95 to 0.97 throughout the entire duration of the challenge test for all inoculated batches.

The a_w for both food control and control units remained consistently between the values of 0.96 to 0.98 throughout the entire duration of the challenge test.

4.4.2 pH

The pH measurements are shown in Figure 5. All batches initially had a pH around 6.0. During the challenge test, the pH decreased for batches 1 and 2 to a value of 4.5. Meanwhile, for Batch 3, the pH decreased during the challenge test but increased towards the end, remaining stable at the value of 6.0.

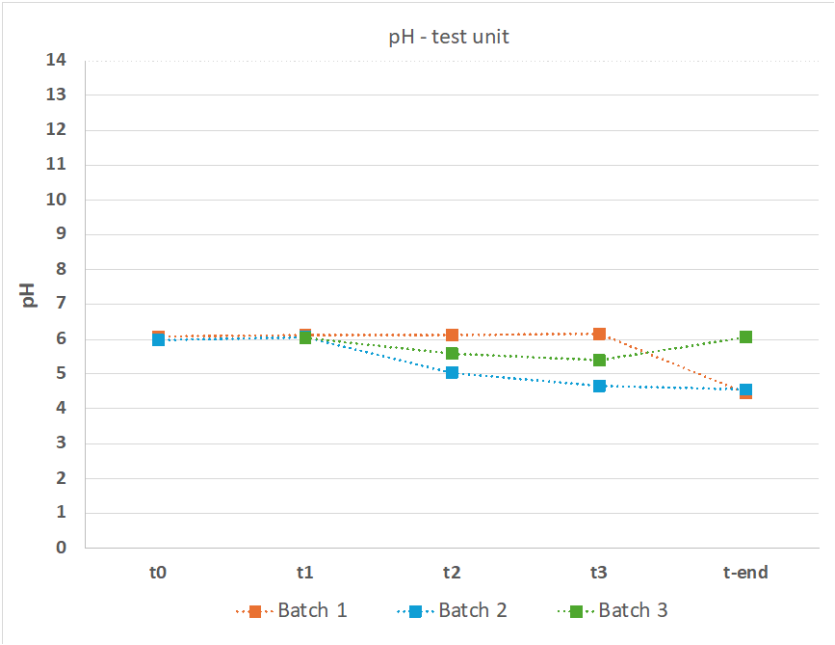

Figure 5. *pH measurements for inoculated test units - Batch 1, 2 and 3 at each time point.*

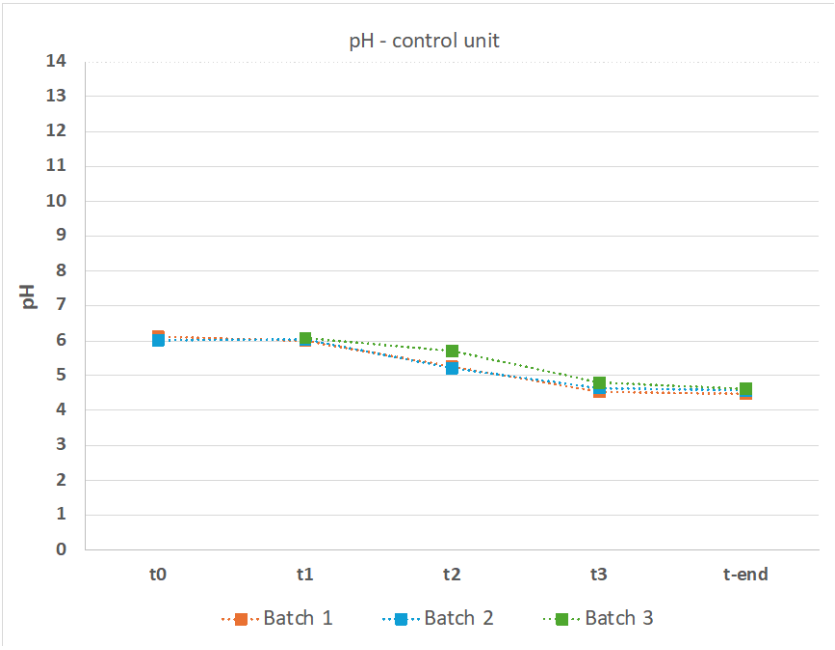

Figure 6. *pH measurements for control units - Batch 1, 2 and 3 at each timepoint.*

Initially, all control units began with a pH of approximately 6.0, as depicted in Figure 6 above. Throughout the challenge test, they exhibited a consistent decline in pH towards the conclusion of the shelf-life period, ultimately reaching a final value of 4.6.

4.4.3 Modified atmosphere packaging (MAP)

The modified atmosphere measurements of the inoculated test units are provided in Table 7. The O_2 values ranged between 0.0% and 1.9%, indicating no signs of leakage. The CO_2 values varied from 11.1% to 51.8%.

Table 7. *Modified atmosphere results for inoculated test units – Batch 1, 2, 3 at each time point before opening the package.*

MAP O_2:$C0_2$ %	Time 0 (t_0)	Time 1 (t_1)	Time 2 (t_2)	Time 3 (t_3)	Time 4 (t_{end})
Batch 1	0.6 : 13.2	1.0 : 12.7	1.2 : 12.4	1.5 : 12.2	1.9 : 11.1
Batch 2	0.4 : 13.5	0.9 : 14.3	0.0 : 40.2	0.0 : 51.8	0.0 : 45.4
Batch 3	0.9 : 16.1	0.9 : 16.1	0.4 : 22.5	0.0 : 17.2	0.0 : 18.4

For Batch 2, the CO_2 percentage notably increased for timepoints t_2 and t_3. Batch 1 and 3 displayed consistent values ranging from 11.1% up to 22.5% CO_2.

The modified atmosphere measurements of the food control samples are detailed in Table 8 below. Throughout the challenge test, the O_2 values consistently remained

below 1%, indicating no signs of leakage. However, the CO_2 values exhibited a significant increase over time, ranging from 32.7% to 66.8% at the end of the challenge test.

Table 8. *Modified atmosphere measured for food control sample at timepoint t_0 and t_{end}.*

MAP O2:C02 %	Time 0 (t_0)	Time 4 (t_{end})
Batch 1	0.8 : 13.0	0.0 : 66.8
Batch 2	0.1 : 29.3	0.0 : 54.3
Batch 3	0.8 : 16.5	0.0 : 32.7

Table 9. *Modified atmosphere measured for control unit at timepoint t_0 and t_{end}.*

MAP O2:C02 %	Time 0 (t_{end})	Time 4 (t_{end})
Batch 1	0.6 : 13.4	0.0 : 60.5
Batch 2	0.0 : 26.1	0.0 : 55.7
Batch 3	0.5 : 23.1	0.0 : 47.5

The modified atmosphere measurements of the control units are presented in Table 9 above. Across the challenge test duration, the O_2 values consistently remained below 0.6%, suggesting no indications of leakage. The CO_2 values displayed a notable increase over time, ranging from 47.5% to 60.5% at the end of the challenge test.

4.4.4 Temperature (°C)

The temperature measurements consistently showed a temperature around 8°C for all control units at all time points during the challenge test. This confirmed that the incubator maintained its temperature at 8°C.

4.4.5 Ocular assessment

From a visual quality control perspective, no difference could be detected between the test unit, control unit, and food control samples. No alterations in color or odor were evident in any of the respective products. However, variations were observed within the packaging, where certain samples, notably Batch 2, food samples and control unit, exhibited high pressure similar to an inflated balloon, while other samples such as Batch 1 and 3, maintained consistent pressure throughout the entire test period, consistent with the initial pressure.

5 Discussion

The results of the vegan salami challenge test revealed that all tested batches supported the growth of *L. monocytogenes*. Notably, two batches exhibited significant growth, with growth potential values of 4.4 and 3.1 $\log_{10}$ cfu/g over the shelf-life period. It is important to highlight that due to the production logistics, all batches were inoculated relatively late, about 10 days after the production date. This delay potentially limited the growth observed in the batches. Had the inoculation occurred within 2-3 days after production, even higher concentrations of *L. monocytogenes* might have been reached. This delay was due to the product's manufacturing process in Germany, followed by storage in a Swedish warehouse before distribution to stores. Thus, Batch 2, despite not reaching high concentrations, could have shown increased growth if inoculated earlier. However, all batches exceeded the 0.5 $\log_{10}$ cfu/g growth potential threshold, indicating their capacity to support growth of *L. monocytogenes*.

The notable variability observed among the batches concerning growth potential, modified atmosphere, and pH reduction is noteworthy. In Batch 1, *L. monocytogenes* exhibited a rapid growth curve, while maintaining a stable TBC at 3 $\log_{10}$ cfu/g without significant increase. Important to note that the TBC is derived from a different sample than the inoculated test unit; however, they serve as an indication of the background microflora for each batch tested. The low TBC observed in the control units could potentially suggest that *L. monocytogenes* was not inhibited by a competitive background microflora. According to the Swedish Food Agency, *L. monocytogenes* may have an advantage in products with low background flora, as it does not need to compete with other microorganisms for resources (Ottoson 2022). Batch 1 exhibited relatively low CO_2 levels, ranging from 11.1% to 13.2%, compared to Batch 2 and 3. Batch 3 exhibited a growth curve similar to Batch 1 but was inoculated on day 16 instead of day 11, providing Batch 1 with approximately 5 additional days of bacterial growth. Aside from this timing difference, the growth curves did not notably differ otherwise. Batch 2 exhibited a growth pattern different from the other two batches, marked by a lower growth curve of *L. monocytogenes* and a notable rise in CO_2 levels from 13.5% to 51.8%. During the testing period, an increase in TBC was observed in the control units of Batch 2. Unlike Batch 1, which had a TBC count of 3 $\log_{10}$ cfu/g at timepoint t_3,

Batch 2 reached a count of 6 $\log_{10}$ cfu/g. This difference suggests that a highly competitive microflora, potentially including lactic acid bacteria (LAB), may have suppressed the growth of *L. monocytogenes* in Batch 2. In a study conducted by Francis and O'Berine (1997), the impact of atmospheric conditions on the growth of *L. monocytogenes* and other background microorganisms was explored. Their findings indicated that under modified atmosphere conditions with 3% O_2 and 5–20% CO_2, *L. monocytogenes* growth might be stimulated. However, they also observed that elevated CO_2 levels could promote the growth of lactic acid bacteria while inhibiting *L. monocytogenes*. This observation suggests a potential mechanism by which lactic acid bacteria appear to antagonize *L. monocytogenes* (Francis & O'Beirne 1997).

When comparing the inoculated samples (test units) with the control units (inoculated with physiological water) and the food control samples, no variations were found in the physiological parameters, including water activity and pH, among the different sample groups. Interestingly, all samples demonstrated a decrease in pH from initial levels of 6 to 4.5. Brown et al. (2018), conducted a challenge test on cheese and observed that the pH of non-inoculated (control units/food control) cheeses decreased after 7 days in modified atmosphere conditions containing CO_2. They suggested that the observed increase in acidity was likely a result of carbonic acid formation. When carbon dioxide reacts with water, it generates carbonic acid, which then dissociates into hydrogen ions (Brown et al., 2018). Therefore, this could potentially explain the decrease in pH observed in this study. Additionally, the production of lactic acid by the microbiota could also contribute to the observed pH decrease.

Alterations in gas composition were observed within each sample during storage. Control units and food control samples exhibited a notably higher concentration of CO_2 at the end of the challenge test compared to inoculated test units. Emser et al. (2011) conducted a study on various gas compositions in modified atmosphere packaging for minced meat. The author explained that the gaseous environment within modified atmosphere packs is dynamic and influenced by factors such as microbial activity, packaging material permeability, product respiration, and gas absorption by the food. In their study, the O_2 concentration decreased, and CO_2 concentration increased in all samples from the first day of storage. This trend was particularly pronounced in samples with lower initial O_2 concentrations. According to their suggestion, the increase in CO_2 levels during storage could be attributed to the consumption of available oxygen by microorganisms in the food item, along with the production of CO_2 as a metabolic byproduct by certain microflora, such as *Brochothrix thermosphacta* and LAB (Esmer et al. 2011). This could potentially explain the observed changes in gas composition in control units and food control

samples where *L. monocytogenes* was not present. It is possible that other microorganisms, particularly LAB, may have proliferated more prominently in these samples.

The TBC values varied among the samples. It is important to note that there were difficulties in interpreting the results obtained from the Petrifilm AC method. The colonies observed were unclear and notably small, despite a 3-day incubation period. This raises concerns regarding the reliability and accuracy of these findings. In this particular instance, it may have been more suitable to employ standard agar for TBC analysis, as it tends to yield more precise and dependable results. Important to note that LAB prefer anaerobic conditions, and so grow slowly in aerobic conditions such as on Petrifilm AC. Morin et al. (2021) validated Petrifilms against standard bacteriology test methods and concluded that they do not consistently provide identical results. While Petrifilms are recommended for detecting high levels of bacterial contamination, they are less specific for low levels of bacteria. However, they serve as good indicators when bacterial contamination/proliferation is high (Morin et al. 2021).

In light of the challenge test, the vegan salami falls under the RTE foods category capable of supporting *L. monocytogenes* growth. As per Regulation (EC) No. 2073/2005, *L. monocytogenes* must be absent in 25 g of the product before leaving the manufacturer's immediate control. Alternatively, the concentration of *L. monocytogenes* shall not exceed 100 cfu/g during the specified shelf-life period.
The absence of *L. monocytogenes* detected in all three batches using the qualitative method ISO11290-1 suggests that the manufacturer has effectively implemented Good Manufacturing Practices (GMP) and maintained satisfactory hygiene standards throughout the production process for these three tested batches.

Few similar studies are available to compare the results from this study with the results from other challenge tests for plant-based deli slices. However, Van Paepeghem et al. (2024) conducted a challenge test on two plant-based deli slices available on the Belgian market: one vegan deli slice and one vegetarian deli slice with garden herbs. Both had a water activity above 0.97 and a pH above 5.4 when inoculated. The vegan deli slice had a maximal growth potential of 0.03 $\log_{10}$ cfu/g, while the vegetarian deli slice with garden herbs had a growth potential of 0.42 $\log_{10}$ cfu/g. These two products did not exceed the 0.5 $\log_{10}$ cfu/g limit, indicating that growth of *L. monocytogenes* was not supported during the challenge tests. The authors suggested that the limited growth could have been affected by the presence of additives possessing antimicrobial properties, such as potassium and sodium acetate, alongside competition for growth from other bacteria such as LAB and the utilization of modified atmosphere with elevated CO_2 levels from 30-50%. In

contrast, the tested product in this present study did not contain any preservatives possessing antimicrobial properties, which may have affected the growth of *L. monocytogenes*. However, it should be noted that Batch 2 exhibited high levels of TBC, potentially including LAB, aligning with the findings of the Belgian study, which reported a concentration of 8.0 $\log_{10}$ cfu/g of LAB in one of their tested products. Furthermore, the elevated levels of CO_2 (51.8%) observed for Batch 2 are consistent with the Belgian study's explanation that high CO_2 levels can contribute to a reduction of *L. monocytogenes*.

The Swedish Food Agency has conducted predictive models indicating that plant-based deli slices could potentially support *L. monocytogenes* growth in 4 out of the 15 examined products at 8 °C (Ottoson 2022). Interestingly, three of the products that supported growth based on predictive modeling did not contain any preservatives, similar to the tested product in this study. Furthermore, the pH levels of these three products were also above 6, mirroring the initial pH values of the tested product during the challenge test. Additionally, their water activity levels were similar to the tested product, which exceeded 0.96. As a result, the predictive models for these plant-based deli slices align with the tested product, indicating that all of them have the potential to support the growth of *L. monocytogenes*. The findings here confirm that predictive models can serve as useful indicators of potential growth and may be employed prior to conducting a challenge test on a specific product.

The Swedish Food Agency's general advice for individuals who are not part of the risk group is to inspect the appearance, smell, and taste when consuming plant-based deli slices past their expiration date (Flink 2023). However, these recommendations may not be as effective for detecting the presence of the bacterium *L. monocytogenes*. During the ocular assessment, no differences were observed between inoculated samples with concentrations of up to 7 $\log_{10}$ cfu/g of *L. monocytogenes* and uninoculated food control samples. All samples appeared and smelled the same, with no visible differences. This poses a challenge with *L. monocytogenes*, as it does not cause any noticeable changes in odor or color. The concentrations of *L. monocytogenes* detected during the challenge test (7 $\log_{10}$ cfu/g) can lead to severe illness, especially for individuals in risk groups. For individuals not belonging to a risk group, these concentrations can cause common food poisoning symptoms such as diarrhea and vomiting (Bell & Kyriakides 1998). However, it is important to acknowledge that the initial inoculation concentration employed in the challenge test was higher than what would typically be encountered in a production environment. Consequently, the resulting final concentrations observed in this study may not accurately reflect realistic levels of natural contamination in a commercial production process.

The vegan salami selected for the challenge test was chosen due to its pH level and water activity which were considered the most favorable for microbial growth compared to the other plant-based deli slices. Following the guidelines from the Swedish Food Agency, a pH within the range of 6-7 is categorized as high risk for microbial growth (Rosengren 2019). By selecting the product with the highest pH value, the test was aimed at assessing the product with the greatest potential risk of supporting microbial growth. However, it would have been valuable to test multiple plant-based products to determine which intrinsic or extrinsic factors have the most significant impact on the proliferation of *L. monocytogenes*. In terms of extrinsic factors, the focus would be on packaging, modified atmosphere composition, storage time, and temperature. By taking this approach, it would have been possible to ascertain whether the elevated pH and water activity values were the primary contributors to microbial growth or if other factors, such as specific ingredients or temperature played a more substantial role. This broader examination would have provided a more comprehensive understanding of the factors influencing the growth of *L. monocytogenes* in plant-based deli slices.

When conducting a challenge test, it is crucial to note that the results are only applicable to the specific batches tested under the defined temperature and time conditions for this food item. Any alterations in the product's recipe, production process, or storage conditions would render the test results invalid for the tested product, necessitating the performance of a new challenge test. However, the test results can still serve as an indication, as they demonstrated significant growth well above the growth potential threshold of *L. monocytogenes*.

In summary, the findings of the study indicate that the tested batches of vegan salami have the ability to support the growth of *L. monocytogenes*, placing them in food category 1.2 - "RTE-foods capable of supporting the growth of *L. monocytogenes*". To comply with current regulations, it is essential for food producers to ensure the absence of *L. monocytogenes* in 25g of the product before it is released from their control. However, to generalize these conclusions beyond the specific tested batches, further studies investigating the behavior of *L. monocytogenes* in plant-based deli slices are necessary. These additional studies would provide a broader understanding of the growth potential and behavior of *L. monocytogenes* in a range of plant-based deli slice products.

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Popular science summary

Investigating the Safety of Plant-Bases Deli Slices: The Case of *Listeria monocytogenes*

Have you ever tried vegan salami? It is a plant-based alternative to traditional meat-based salami that has been gaining popularity in recent years. However, ensuring the safety of these products is highly important. One particular concern is the potential presence of *Listeria monocytogenes*, a harmful bacterium that can pose a risk in ready-to-eat products such as plant-based deli slices.

Listeria monocytogenes is a bacterium with the ability to grow in refrigerated temperatures. When consumed, it can lead to the development of a severe and potentially life-threatening illness called listeriosis. This disease poses a significant threat, particularly to individuals belonging to the risk- group such as the elderly (over 65), those with weakened immune systems, and pregnant women and their unborn babies.

To address this concern, a comprehensive study was conducted to investigate the growth potential of *L. monocytogenes* in vegan salami. The study involved deliberately introducing *L. monocytogenes* into samples of vegan salami and subjecting them to controlled storage conditions at refrigeration temperatures. During the storage period, samples were collected, and the concentration of *L. monocytogenes* was monitored.

The findings revealed that *L. monocytogenes* could grow in vegan salami and potentially reach levels that may cause disease in individuals which are part of the risk-group. These findings underscore the importance of food safety and hygiene measures during the manufacturing process. In order to meet safety regulations, it is mandatory for food producers to ensure the absence of *L. monocytogenes* before foods are released for sale.

This study highlights the need for continuous research to ensure the safety of plant-based products. By understanding the potential risks associated with these foods, manufacturers can implement necessary protocols to safeguard consumers and meet safety standards. As a consumer, it is essential to be aware of these potential risks

and make informed choices, prioritizing the consumption of safe and high-quality products.

In conclusion, while plant-based deli slices offer an appealing alternative, the growth potential of *L. monocytogenes* underscores the importance of strict safety procedures. Through continued research and collaboration among scientists, manufacturers, and health authorities, we can enjoy plant-based products while ensuring our safety.

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