Inactivation of foodborne pathogens in dairy model systems by radio frequency processing

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Abstract

Food safety was and remains a critical issue, affecting the public, the industry, and governments worldwide. With millions of people being infected by foodborne pathogens globally each year, the food industry is looking for innovative solutions that guarantee the safety of products, while maintaining their high-quality attributes. Traditional thermal processing methods often compromise the quality and nutritional value of food products, and are, in specific cases, such as the treatment of low moisture foods, inefficient. Radio frequency (RF) is a novel processing technology which offers considerable benefits in comparison to conventional thermal treatments (i.e., uniform and rapid heating) and can be applied to a variety of food products. However, the application of a RF-only dynamic heating process has not been explored yet. RF as a dielectric heating process contains an inherent dynamic heating profile stage. However, most studies do not take this stage into account and use it as a heating step prior to conventional thermal heating (i.e., oven holding). Finally, a consensus over the nature of RF inactivation mechanisms has not been reached yet.

The overall objective of this study was to investigate the RF-only dynamic heating process on the inactivation of two foodborne pathogens in dairy model systems. The target pathogens, *Salmonella* Typhimurium and *Listeria monocytogenes,* were chosen due to their frequent occurrence in dairy products and foodborne outbreaks. The dairy model systems were formulated in such way to represent three real food structure categories: powders, liquids, and gels. Additionally, in each structure category, two different fat content levels were used: 0.8% and 35% for the powders, and 0.08% and 5.9% for the liquids and gels. The dairy powders (milk powder) were used as the foundation for the formulation of the liquids and, subsequently, the gels. A lab-scale RF set-up was used for the treatment of liquids and gels while a pilot-scale RF set-up was used for the treatment of powders.

Abstract

Initially, the survival of *S*. Typhimurium and *L. monocytogenes* in the two milk powders was monitored during storage at 20°C for a month. Both microorganisms survived, with *S*. Typhimurium demonstrating higher resistance than *L. monocytogenes*, but also higher levels of sublethal injury (SI), emphasizing the need for an effective, post spray drying treatment.

In order to explore the efficiency of RF-only heating inactivation of the two foodborne pathogens in the two milk powders, four different dynamic heating profiles were tested for the treatment of skimmed milk powder. The heating profiles incorporated two different heating rates (i.e., a fast and a slow heating rate) until two target temperatures (85 and 95°C). None of the four treatments impacted the solubility of the milk powder and only the harshest treatment (i.e., the slow heating profile until 95°C) led to a slight increase in the yellowness of powder's colour. *S*. Typhimurium was used as a case study to identify the most successful treatment, i.e., the slow heating profile until 95°C, which led to 4-log reduction. Subsequently, the established RF-only dynamic heating approach was used to obtain the inactivation kinetics of both microorganisms in the two types of milk powder. *S*. Typhimurium demonstrated higher resistance than *L. monocytogenes*. Nonetheless, a higher SI (%) was also detected in *Salmonella* in comparison to *L. monocytogenes*. Both microorganisms showed a higher inactivation in the whole milk powder, indicating that the high levels of lactose and protein in the skimmed milk powder had a protective effect on the microbial cells. Lastly, *E. faecium* was successfully validated as a surrogate microorganism as it showed higher resistance than either of the two foodborne pathogens.

The same approach of using a RF-only dynamic heating profile was followed for the inactivation of the two foodborne pathogens in the dairy liquid and gel model systems. The effects of fat content and presence of a gel matrix structure on the survival of *S*. Typhimurium and *L. monocytogenes* were investigated. For *S*. Typhimurium, higher fat content (either in liquids or gels) or the presence of a gelled matrix resulted in higher inactivation rates. *L. monocytogenes* demonstrated a more complex behaviour which was temperature-dependent. While in liquid systems, higher fat content resulted in higher inactivation, in gel systems the opposite occurred but only for temperatures above 70°C. *L. monocytogenes* also exhibited the highest SI (%) at 5.9% fat content and high temperatures. Finally, using similar temperature profiles of the dairy liquid and gel treatments, RF and conventional thermal

inactivation treatments were conducted in liquid BHI broth. RF treatments demonstrated higher inactivation rates in comparison to thermal treatments for both microorganisms. Then, aiming at providing knowledge on the RF inactivation mechanisms, the integrity of cell membrane and DNA were evaluated. The level of membrane damage was higher after RF treatments than conventional thermal treatments, which could though be attributed to the lower viable cells densities after RF inactivation. In general, fluorescence analyses indicated a potential DNA damage after both treatments, however further examination with agarose gel electrophoresis showed no considerable differences in the integrity of the DNA.

Overall, this dissertation further elucidated the efficacy and mechanisms of a RF-only dynamic heating for the inactivation of foodborne pathogens in different dairy food model systems. The effects of different factors on the survival of the microorganisms were unravelled, i.e., the microorganism's Gram properties or the intrinsic food properties (composition, fat content, matrix structure). Consequently, this research offered insights to potential future applications of RF dynamic heating treatments.

Beknopte samenvatting

Voedselveiligheid garanderen blijft een belangrijke uitdaging met een invloed op de algemene samenleving, de industrie en regeringen over de hele wereld. Miljoenen mensen over heel de wereld raken jaarlijks besmet door voedselpathogenen. Daarom is de voedingsindustrie constant op zoek naar innovatieve oplossingen die de veiligheid, en tegelijk ook de hoge kwaliteit, van voedingsproducten garanderen. Traditionele warmtebehandelingsmethodes hebben namelijk vaak een negatieve invloed op de kwaliteit en voedingswaarde van voedingsproducten. Bovendien zijn deze warmtebehandelingen in specifieke gevallen ontoereikend om voedselveiligheid te garanderen, met voedsel met een laag vochtgehalte als belangrijk voorbeeld. Radiofrequentie (RF) is een nieuwe behandelingstechnologie die aanzienlijke voordelen biedt in vergelijking met conventionele warmtebehandelingen (bv. uniforme en snelle opwarming), en toegepast kan worden op een verscheidenheid aan voedingsproducten. De toepassing van dynamische *RF-only* verwarmingsprocessen is echter nog niet afdoende verkend. Aangezien RF een diëlectrisch verwarmingsproces is, omvat het steeds een fase met een dynamisch opwarmingsprofiel. De meeste studies nemen deze fase echter niet in rekening, en gebruiken RF als een opwarmingsstap die vooraf gaat aan een conventionele warmtebehandeling (bv. warmhouden in een oven). Ten slotte is er ook nog geen consensus bereikt betreffende de inactivatiemechanismen die optreden bij RF-verwarming.

Het algemene doel van deze studie was het onderzoeken van dynamische *RF-only* verwarmingsprocessing op de inactivatie van twee voedselpathogenen in zuivelmodelsystemen. De doelmicroörganismen, *Salmonella* Typhimurium en *Listeria monocytogenes*, werden gekozen omwille van hun frequente voorkomen in zuivelproducten en hun relevantie in het veroorzaken van voedselvergiftingsuitbraken. De zuivelmodelsystemen behoorden tot drie categorieën van voedselmicrostructuur: poeders, vloeistoffen en gels. Bovendien werden in elke categorie twee verschillende vetgehaltes gebruikt: 0,8% en 35% voor de poeders, en 0,08% en 5,9% voor de vloeistoffen en

gels. De zuivelpoeders (melkpoeder) vormden de basis voor de ontwikkeling van de vloeistoffen en de gels. Een laboschaal-RF-opstelling werd gebruikt voor de behandeling van vloeistoffen en gels, en een pilootschaal-RF-opstelling werd gebruikt voor de behandeling van poeders.

Eerst werd het overleven van *S*. Typhimurium en *L. monocytogenes* in de twee melkpoeders tijdens opslag bij 20°C opgevolgd gedurende een maand. Beide microörganismen overleefden deze opslagperiode, waarbij *S*. Typhimurium een hogere resistentie dan *L. monocytogenes* toonde, maar ook meer *sublethal injury* (SI). Deze elementen tonen de nood aan voor het ontwikkelen van effectieve behandelingen volgend op het *spray-drying*-proces.

Met als doel de efficiëntie van dynamische *RF-only*-verwarmingsprocessen voor de inactivatie van de twee voedselpathogenen in de twee melkpoeders te onderzoeken, werden vier verschillende dynamische verwarmingsprofielen voor mager melkpoeder getest. De verwarmingsprofielen bestonden uit twee verschillende verwarmingssnelheden (snel en traag), naar twee doeltemperaturen (85 en 95°C). Geen van de vier behandelingen had een invloed op de oplosbaarheid van het melkpoeder, en enkel de zwaarste behandeling (het trage opwarmingsprofiel tot 95°C) leidde tot een kleine stijging van de geelheid van het poeder. Een *case study* met *S*. Typhimurium leidde tot de conclusie dat het trage opwarmingsprofiel tot 95°C de meest geschikte behandeling was, aangezien deze tot een 4-log reductie leidde. Het dynamische *RF-only*-verwarmingsproces werd dan gebruikt om de inactivatiekinetieken van beide microörganismen in de twee melkpoeders te verkrijgen. Hierbij toonde *S*. Typhimurium een hogere resistentie dan *L. monocytogenes*. Voor *S*. Typhimurium werd echter wel een hoger percentage SI geobserveerd dan voor *L. monocytogenes*. Beide microörganismen werden meer geïnactiveerd in poeder van volle melk dan in poeder van magere melk. Dit toont aan dat de lactose en proteïnen in poeder van magere melk een beschermend effect uitoefenden op de microbiële cellen. Ten slotte werd *E. faecium* succesvol gevalideerd als een surrogaatmicroörganisme aangezien het meer resistent was dan beide voedselpathogenen.

Dezelfde benadering van het gebruik van een *RF-only* dynamisch verwarmingsprofiel werd toegepast voor de inactivatie van de twee voedselpathogenen in vloeibare en gegelleerde zuivelmodelsystemen. Hierbij werd het effect van vetgehalte en de aanwezigheid van een gegelleerde

matrixstructuur op het overleven van *S*. Typhimurium en *L. monocytogenes* onderzocht. Bij *S*. Typhimurium zorgde een hoger vetgehalte (in vloeistoffen of gels) of de aanwezigheid van een gegelleerde matrix voor hogere inactivatiesnelheden. Voor *L. monocytogenes* gold een meer complex temperatuursafhankelijk gedrag. Hoewel in vloeibare systemen een hoger vetgehalte voor snellere inactivatie zorgde, gold de omgekeerde situatie voor gelsystemen, maar enkel voor temperaturen hoger dan 70°C. *L. monocytogenes* toonde ook de hoogste SI bij 5,9% vetgehalte en bij hoge temperaturen. Ten slotte werden RF en conventionele thermische inactivatiebehandelingen uitgevoerd in vloeibare *BHI broth*, hierbij gebruik makend van temperatuursprofielen gelijkaardig aan die van de vloeibare en gegelleerde zuivelmodelsystemen. Voor beide microörganismen waren de inactivatiesnelheden van RF hoger dan voor de conventionele behandelingen. Vervolgens werd de integriteit van het celmembraan en DNA geëvalueerd, met als doel kennis te voorzien betreffende RF-inactivatiemechanismen. De hoeveeelheid membraanschade was groter na RF-behandelingen dan na conventionele behandelingen, wat deels toegewezen zou kunnen worden aan de lagere *viable cell densities* na RF-behandelingen. Fluorescentie-analyses toonden over het algemeen mogelijke DNA-schade aan volgend op beide behandelingen. Een verdere analyse d.m.v. *agarose gel electrophoresis* toonde echter geen aanzienlijke verschillen in DNA-integriteit aan.

Dit proefschrift verhelderde over het geheel genomen de efficiëntie en de mechanismen van *RF-only dynamic heating* voor de inactivatie van voedselpathogenen in verschillende zuivelmodelsystemen. De effecten van verschillende factoren op het overleven van de microörganismen werden ook ontrafeld: de Gram-eigenschappen en de intrinsieke voedseleigenschappen (samenstelling, vetgehalte en matrixstructuur). Daarom biedt dit onderzoek inzicht in de mogelijke toekomstige toepassingen van *RF-dynamic-heating*-behandelingen.

List of Abbreviations

ANOVA	Analysis of variance
BHI	Brain Heart Infusion
CFU	Colony forming unit
DL	Detection limit
DNA	Deoxyribonucleic acid
GS1	Gel System 1 (0.08% fat)
GS2	Gel System 2 (5.9% fat)
ISM	Industrial, scientific, and medical
LS1	Liquid System 1 (0.08% fat)
LS2	Liquid System 2 (5.9% fat)
MSE	Mean squared error
PIFM	Powdered infant formula milk
RF	Radio frequency
RMSE	Root Mean Squared Error
RNA	Ribonucleic acid
RTE	Ready-to-eat
SI	Sublethal injury
SICs	Sublethally injured cells
SMP	Skimmed milk powder
TDT	Thermal Death Time
TICC	Time-averaged Cells Coefficient
TSA	Tryptone Soy Agar
TSB	Tryptone Soy Broth
TSAYE	Tryptone Soy Agar with Yeast extract
UHT	Ultra-high temperature
WMP	Whole milk powder
XLD	Xylose Lysine Deoxycholate

List of Symbols

a_w	Water activity [-]
c	The speed of light in free space [m/s]
C_c	Concentration of a critical component for cell survival [-]
$C_c(0)$	The initial concentration of the critical component for cell survival [-]
D-value	Time necessary to reduce the number of bacteria by one log unit under isothermal conditions [min]
d_p	Penetration depth [cm or m]
E	The electric field intensity [V/m]
ε_0	Permittivity of free space, equal to 8.854*10−12 [F/m]
ε'	Dielectric constant [-]
ε"	Dielectric loss factor [-]
F-value	Measure of the lethal effect of a certain heat treatment on the target microorganism [min]
f	Frequency [Hz or MHz]
k	Inactivation rate [1/s, 1/min, or 1/h]
k_{max}	Maximum specific inactivation rate [1/s, 1/min, or 1/h]
N	Cell density [CFU/mL or CFU/g]
N_0	Initial cell density at time zero [CFU/mL or CFU/g]
$N(t)$	Cell density at time t [CFU/mL or CFU/g]
N_{res}	Residual population [CFU/mL or CFU/g]
P	Power [W or kW]
S_l	Shoulder length [s, min, or h]
t	Time [min, h, or d]
tanδ	Loss tangent [-]
z-value	The increase in temperature necessary for a 10-fold decrease of the D-value [°C]

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Chapter 1

Introduction

1.1 Motivation

The ever-present threat of foodborne diseases caused by pathogenic microorganisms keeps governments, industries, and consumers constantly on high alert. From farm to table, food can harbour microbial threats that range from causing mild gastroenteritis to triggering severe systemic infections or even resulting in fatalities (Nyachuba, 2010).

Each year, 600 million cases of foodborne diseases and 420,000 deaths are reported all over the world (Lee & Yoon, 2021). Foodborne diseases in the European Union (EU), contributing to 50,000 cases of illness. Specifically, in 2022, the EU witnessed one of its highest numbers of recorded deaths due to foodborne outbreaks in a decade (EFSA & ECDC, 2023). *Listeria monocytogenes* was the leading cause, accounting for 43.8% of the total deaths while *Salmonella* was the pathogen responsible for the majority of the outbreaks (17.6%) and hospitalisations (50.5%) (EFSA, 2022; Visciano & Schirone, 2023). These numbers highlight the considerable health hazards linked to the two foodborne pathogens.

Foodborne pathogens can lurk in a variety of unsuspected food products, from meat, dairy and seafood to fruits and vegetables and even processed foods if not handled or stored correctly. Some microorganisms can even survive in low-moisture foods like seeds, spices and food powders, which are generally perceived as safe (Acuff et al., 2023). Powdered infant formula milk is an exemplary case, where the perceived safety of low-moisture foods is being challenged. It is especially concerning given that the product is targeted at a highly vulnerable population; infants.

1. Introduction

Powdered infant formula milk has been linked to several outbreaks of foodborne illnesses, particularly due to bacteria such as *Cronobacter sakazakii* and *Salmonella*. Just in 2022, a *C. sakazakii* contamination of powdered infant formula led to 4 hospitalisations and 2 deaths in the United States (U.S. Food and Drug Administration, 2022). In 2018, a multi-country outbreak of *Salmonella* infections was linked to infant formula (EFSA & ECDC, 2018) Overall, recent foodborne outbreaks highlight the significant risks associated with different dairy products. For example, the CDC documented 101 outbreaks of *L. monocytogenes* in the U.S. between 2012 and 2019, primarily linked to cheese, ice cream, and raw milk. Similarly, the EU reported 149 outbreaks in milk and milk products between 2018 and 2022 which subsequently, led to 1,754 cases of foodborne illnesses. *Salmonella* was a major contributor, causing the majority of these outbreaks and hospitalisations. These incidents emphasize the persistent threat these pathogens pose to dairy products, even in low moisture foods, which are generally recognised as safe. Additionally, *L. monocytogenes* remains a critical concern in refrigerated products such as soft cheeses and ice cream due to its ability to thrive at low temperatures. These factors highlight the need for innovative and advanced processing techniques in dairy products targeted at microbial inactivation.

Current food safety technologies, while advanced, do not cover all potential risks, especially when considering the complexities of foodborne pathogens in products like powdered infant formula. Conventional thermal treatments (e.g., pasteurisation, sterilisation) aimed at microbial inactivation also come with drawbacks, such as nutrient loss and high energy consumption (Morales-de la Pena et al., 2019). As consumer demands evolve towards safer, higher-quality food products with minimal processing, the food industry is challenged to innovate and to implement more effective safety measures that address a broad spectrum of food safety concerns while maintaining nutritional and sensory qualities (Rodrigo et al., 2010; Van Impe et al., 2018; Allai et al., 2023).

Innovations in food safety technologies are essential to meet these growing demands and address the limitations of older methods. Novel technologies can offer improved efficiency, safety and sustainability in food processing. One of the novel technologies of interest is radio frequency (RF) (Geveke et al., 2002a; Wang et al., 2022).

Unlike conventional thermal technologies, RF is a dielectric heating technology that provides rapid and uniform heating of the product, reducing processing time while enhancing the efficiency of the microbial inactivation treatments (Altemimi et al., 2019). This technology is especially beneficial for the treatment of low moisture food products, where conventional thermal technologies can result in non-uniform heating, and hence, quality degradation. Additionally, non-uniform heating is particularly problematic when the goal is foodborne pathogen inactivation, as it can lead to cold spots where pathogens may survive, thereby compromising the safety of the product (Ling et al., 2020; Jian et al., 2020).

However, it is essential to point out that the effects of RF inactivation treatments can vary across different types of food products. The interaction between RF energy and the components of food products is complex and can be influenced by different factors such as the product's moisture content, composition, and physical properties (Piyasena et al., 2003; Marra et al., 2009; Jiao et al., 2018). These factors can also either promote (Finn et al., 2013; Enache et al., 2017; Podolak et al., 2017) or inhibit (Chirife et al., 1983; Sprong et al., 2002; Kasra-Kermanshahi & Mobarak-Qamsari, 2015) the survival of foodborne pathogens. Another factor that influences the inactivation of foodborne pathogens is the (micro)structure of food (Hill et al., 2001). The food microstructure can impact the uniformity of RF energy distribution, and hence, the heating uniformity. Therefore, understanding how RF interacts with microbial cells in various food matrices is crucial for optimising this technology for food safety applications.

Additionally, the debate concerning the mechanisms of RF inactivation remains unresolved. While some studies propose the existence of non-thermal effects in RF inactivation (Geveke & Brunkhorst, 2004; Ukuku et al., 2008), more recent research attributes inactivation to (mainly) thermal mechanisms (Kou et al., 2018; Cui et al., 2022). However, these studies either (i) use real food products with complex food microstructure, complicating the isolation of the specific RF effects, or (ii) use RF only at a constant temperature, neglecting the inherent dynamic nature of RF heating. This knowledge gap indicates the need for further research to elucidate the underlying principles of RF-induced microbial inactivation.

1.2 Objectives

The overall objective of this dissertation is to elucidate how Radio Frequency (RF) treatments influence the inactivation of food pathogens in different model systems. This objective can be divided into the following sub-objectives:

- Explore RF-only treatments in the microbial inactivation in dairy powders
- Investigate RF-only treatments on the microbial inactivation in dairy liquids and gels
- Evaluate the RF inactivation mechanism in a minimalistic medium

The innovative aspect of this dissertation lies in the systematic study of the inactivation kinetics of a RF-only dynamic heating treatment by using relevant model systems that correspond to real food products with various microstructures.

Specifically, this dissertation focused on dairy model systems with three structural categories, i.e., solid, liquid and gel, all at two fat content levels. The conceptual framework for developing these systems is illustrated in Figure 1.1, where each category is aligned with a corresponding real food product. Milk powder was used as the basis for the formulation of all model systems. Two different types of milk powder were used, i.e., skimmed milk powder containing 0.8% fat, and whole milk powder containing 35% fat. On one hand, for the development of liquids and gels with 0.08% fat, skimmed milk powder was mixed with the necessary quantities of water and gelling agent. On the other hand, for the emulsions and the gel emulsions with 5.9% fat, whole milk powder was used as the primary ingredient, along with the respective quantities of water and gelling agent. A detailed description of the

formulation of the dairy model systems is provided in Chapter 8, section 8.2.1.2. Following the approach of Verheyen et al. (2018), the liquids, emulsions, gels and gel emulsions exhibited minimal variations in compositional and physicochemical properties. However, the powders presented inherent differences, particularly due to their role as the primary ingredient and the significant variability in fat content, which is challenging to standardise.

Dairy model systems were used because of their relevance in the context of RF treatments targeted at microbial inactivation. The dairy sector currently faces significant challenges, particularly in ensuring the microbial safety of low moisture products (i.e., milk powder and powdered infant formula), and RF technology is a promising solution to these challenges (Dag et al., 2022).

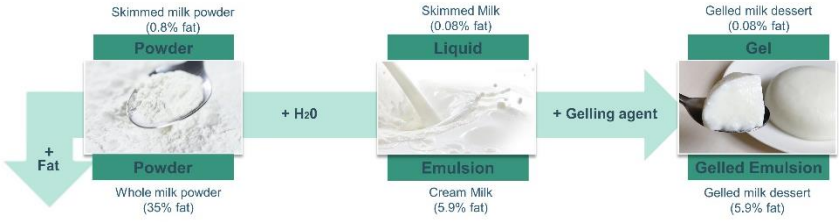


Figure 1.1: Dairy model systems development

Two microorganisms were selected as the targeted foodborne pathogens, i.e., (i) *L. monocytogenes*, a Gram-positive bacterium, and (ii) *Salmonella* Typhimurium, a Gram-negative bacterium. These bacteria were selected because of their association with dairy products, with *L. monocytogenes* commonly associated with outbreaks in cheese and other ready-to-eat dairy products, and *Salmonella* often linked to contamination in milk powder (El-Gazzar & Marth, 1992; Angulo et al., 2008; Melo et al., 2015; Ulsusoy & Chirkena, 2019; Paswan et al., 2020). Another reason for their selection is that *Salmonella* is known for its significant role in foodborne illnesses and hospitalisations, while *L. monocytogenes* is known for its high fatality rates (Visciano & Schirone, 2023). Specifically, the strains used throughout this thesis are *S.* Typhimurium ATCC 14028, isolated from bovine, septicaemic liver and *L. monocytogenes* LMG 23356, isolated from Jalisco Cheese. The specific *Salmonella* strain was selected due to its ability to survive and exhibit

thermotolerance in various low moisture food products (Nascimento et al., 2018; Ruiz-Hernandez et al., 2021; Tong et al., 2022; Wang & Yang, 2022) while the specific *L. monocytogenes* strain was used because it was isolated by a dairy product, highlighting its relevance to dairy-related microbial safety studies.

Additionally, the non-pathogenic microorganism *E. faecium* ATCC 8459 was used as a surrogate microorganism. *E. faecium* is commonly used as a surrogate for studying the microbial inactivation of *S.* Typhimurium in low moisture foods due to its high thermotolerance and its ability to survive in low moisture conditions (Villa-Rojas et al., 2017, Wei et al., 2018, Ozturk et al., 2019, Dag et al., 2022). In this thesis, the surrogate was used (i) to prevent food processing hall contaminations during specific temperature measurements of the RF process and (ii) to verify if it is suitable to be used in future validation experiments following a specific RF treatment process.

The RF treatments were conducted using two different RF set-ups. The treatments of the powders were conducted in a pilot-scale 4 kW, 27.12 MHz, 50 Ω RF set-up (SAIREM, Lyon, France), whereas the treatments of the liquids and gels were conducted in a lab-scale low-power free-running radio frequency (RF) oscillator operating at a frequency of 27.12 ± 0.50 MHz (Kozak et al., 2021). The reason for the use of different RF set-ups was to align with the specific requirements and characteristics of each material type. The overall design of each system accommodates unique properties of specific materials. The limitation of this setting was that it further complicated the comparison of inactivation effectiveness between powders and liquids/gels.

The first sub-objective of this dissertation was to delve into the RF inactivation of two specific microorganisms in milk powders. To achieve this, three steps were taken: Initially, it was essential to establish the survival of these microorganisms in both skimmed and whole milk powder. Following this, the focus shifted to developing a RF-only dynamic heating protocol, aiming to achieve effective inactivation of foodborne pathogens without compromising the overall quality of the product. Lastly, the aim was to document the inactivation kinetics of the two pathogens during the RF-only treatment process and unravel the influence of the fat content and the microorganism's Gram properties on the RF inactivation.

The second sub-objective of this dissertation was to investigate the RF inactivation of liquids and gels. First, the four different systems, i.e., liquid, gel, emulsion and gel emulsion, were developed. Subsequently, RF inactivation kinetics experiments were conducted for the two microorganisms in all respective systems. Then, in order to accurately compare the effects of fat content and structure, a unique modelling approach was used.

The third sub-objective of this dissertation was to elucidate the RF inactivation mechanisms. To simplify the analysis and avoid the complexities of more intricate systems as encountered in previous similar studies, a simple liquid laboratory medium was used for both microorganisms. Thermal treatments, mirroring the temperature profiles of the RF-only treatments, were also conducted. Subsequently, microbial kinetics were examined for a first view on the inactivation at the macroscopic level, whereas analyses on the sublethal injury, membrane damage, and DNA integrity were carried out to offer an insight on the inactivation at the microscopic level.

1.3 Chapter-by-chapter overview

Part I – State of the art

This section provides an overview of the scientific background for this dissertation, organised into three chapters.

Chapter 2: Microbiological food safety
This chapter introduces key concepts in microbiological food safety. It covers the foodborne pathogens of interest, *Salmonella* spp. and *L. monocytogenes*, describing their characteristics and prevalence. Then, thermal inactivation of foodborne pathogens is discussed, including basic principles, microbial inactivation models, sublethal injury, and thermal inactivation mechanisms. The last section discusses the microbial safety of dairy products and the current thermal processes that the food industry uses.

Chapter 3: Food microstructure
In this chapter, the food model systems and their relevance in understanding microbial dynamics are discussed. Initially, information on the food microstructure, including classifications and the influence of microstructure on microbial behaviour is provided. Then, the different types of food model systems are analysed. Finally, the conducted research on dairy model systems is described.

Chapter 4: Radio frequency
This chapter provides a detailed background on RF heating. In the first part, the fundamentals of radio frequency (RF) heating, starting with its definition, principles, and heating mechanism, are thoroughly discussed. The concept of the dielectric properties of food and the different types of RF heating systems are also included. Additionally, the applications of RF in food processing along with its advantages and disadvantages are discussed. In the second part, the focus shifts to RF inactivation, providing general information on targeted products and microorganisms. Finally, the RF pasteurisation of powders, liquids, and complex foods, as well as treatment of dairy products, are highlighted.

Part II – Research and publications

This section presents the results of the dissertation. An overview of the structure of this section is illustrated in Figure 1.2. The three objectives are marked by the distinctive dotted lines, with the relevant chapters numbered accordingly.

Chapter 5: Survival of *S.* Typhimurium and *L. monocytogenes* in dairy model systems: powder
The aim in Chapter 5 was to monitor the behaviour of the two foodborne pathogens in skimmed and whole milk powder during storage at 20°C for the duration of approximately one month. The evolution of sublethal injury was calculated for both microorganisms and the impact on the DNA integrity of *L. monocytogenes* was evaluated.

Chapter 6: Development of a RF-only dynamic heating treatment for dairy model systems: powder
This Chapter focused on implementing a RF-only heating process for the treatment of skimmed milk powder. Two heating rates, a fast and a slow were used to heat milk powder using a dynamic temperature profile. *Salmonella* Typhimurium was used as a case study to validate the inactivation efficacy of each treatment. Colour and solubility attributes of the milk powder were also evaluated after each type of RF treatment.

Chapter 7: RF inactivation of *S.* Typhimurium and *L. monocytogenes* in dairy model systems: powder
In Chapter 7, the most efficient RF heating process obtained from the results of Chapter 6 was used to conduct inactivation treatments for *S.* Typhimurium and *L. monocytogenes* in two types of milk powders, skimmed and whole. The influence of the milk powder's composition and the microorganisms' Gram properties were assessed. The microorganism *E. faecium* was evaluated as a surrogate for the specific treatments and target microorganisms.

Chapter 8: RF inactivation of *S.* Typhimurium and *L. monocytogenes* in dairy model systems: liquids and gels
In this Chapter, the two foodborne pathogens were introduced to dairy liquids and gels. Specifically, four different dairy model systems were developed, using the same skimmed and whole milk powder from the

previous Chapters as the basis. Then, the RF inactivation kinetics of the two microorganisms in the four different systems were acquired. In order to compare the effect of two properties of the dairy systems i.e., fat and structure, a different modelling approach was used.

Chapter 9: Exploring the RF inactivation mechanisms of *S.* Typhimurium and *L. monocytogenes*: liquid broth

In Chapter 9, the focus turns to the identification of the RF inactivation mechanisms. Using the average temperature profiles from Chapter 8, the two foodborne pathogens are subjected to RF and conventional thermal treatments with the same dynamic heating profile, in liquid broth. The inactivation was first compared on a macroscopic level (inactivation kinetics, sublethal injury), and then microscopic analyses, i.e., membrane and DNA damage evaluations, were conducted after specific treatment times.

Part III – Conclusion

Chapter 10: Conclusions & future perspectives

An overview of the main conclusions of the research conducted in this dissertation is presented and discussed, along with perspectives for future research.

Part IV – Exploitation of research

Chapter 11: Valorisation plan

The last part of this dissertation presents a valorisation plan on the possibilities of the exploitation of the results of this dissertation.

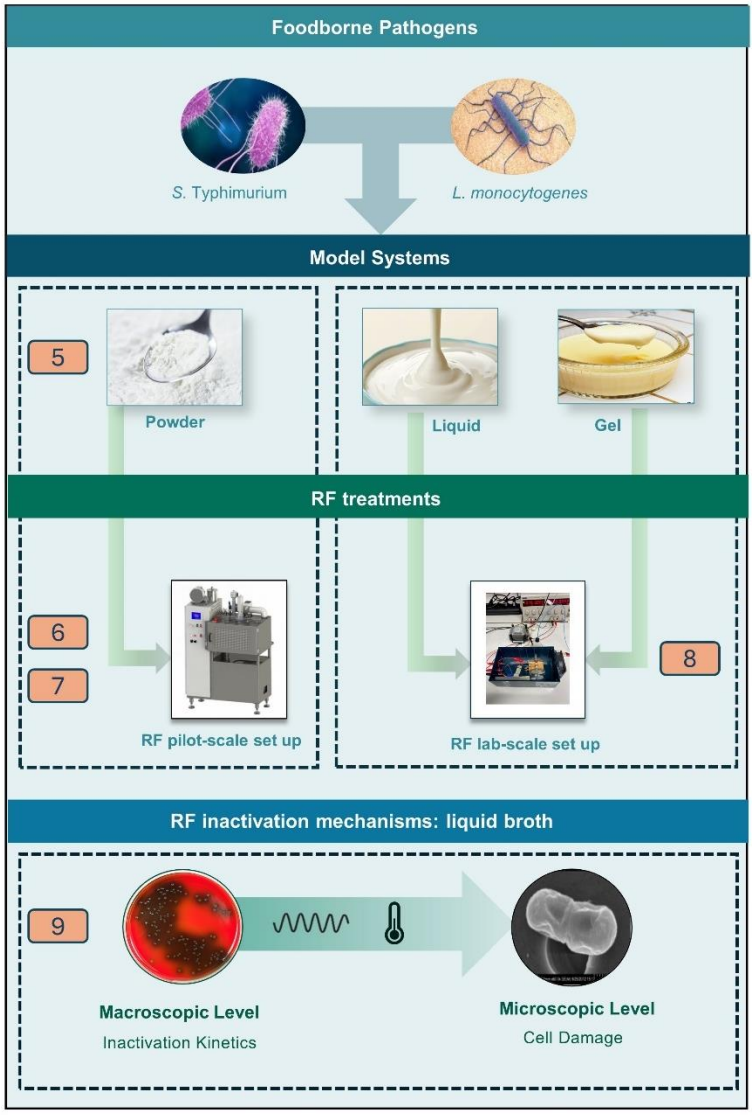


Figure 1.2: Schematic overview of objectives and chapters.

Part I

State of the art

Chapter 2

Microbial food safety

Microbiological food safety is centred on ensuring that food products are free of harmful microorganisms, such as bacteria, viruses, and parasites, whose presence can lead to foodborne illnesses upon consumption. These microorganisms can contaminate food at various points in the food chain, from production to consumption, thus making the implementation of safety practices essential (Meng & Doyle, 2002). Microbiological food safety is typically the principal concern in the food industry, as it impacts consumer health and also determines the overall quality of food products (Ripolles-Avila et al., 2019). Additionally, consumers' demands for healthy, nutritious and convenient food products present new challenges in ensuring their microbial safety (Sofos et al., 1993). Mitigating foodborne illnesses is a global concern, as pathogenic microorganisms can spread through the increasingly globalised food supply chain, affecting individuals in multiple countries (Havelaar et al., 2015).

2.1 Foodborne pathogens

Foodborne illnesses are caused by consuming food or beverages contaminated with pathogenic microorganisms. These pathogens include bacteria, bacterial toxins, viruses and parasites, and can contaminate the food products at any point during production, processing, or preparation. In the European Union (EU) approximately 50,000 human cases of foodborne illnesses are reported each year, with the severity of the cases ranging from mild symptoms to life-threatening conditions. Moreover, in 2022 there was a 44% increase in foodborne outbreaks in the EU in comparison to 2021 (EFSA & ECDC, 2023).

Three foodborne pathogens were established as the most significant bacterial food safety hazards to public health, due to their persistence in the food and feed processing environments. Specifically, *Salmonella enterica* is significant in

feed, meat, and eggs; *Listeria monocytogenes* in meat, fish, seafood, dairy, fruits, vegetables, and low moisture foods; and *Cronobacter sakazakii* in low moisture foods (EFSA Panel on BIOHAZ, 2024).

2.1.1 *Salmonella* spp.

Salmonella is a genus under the family Enterobacteriaceae and it is classified primarily into two species: *Salmonella bongori* and *Salmonella enterica*. *S. bongori* affects mostly cold-blooded animals and is occasionally found in the environment. This species is more likely to be isolated from reptiles, such as lizards and turtles, and is relatively rare in warm-blooded hosts such as humans (Giammanco et al., 2002). When human infections do occur, they are typically associated with exposure to reptile pets and the pathogenicity in humans is generally considered to be lower than that of *S. enterica* (Lamas et al., 2008). *S. enterica*, plays a significant role in public health due to its association with numerous foodborne illnesses, and has received a lot of scientific attention. This attention is primarily focused on the subspecies *S. enterica subsp. enterica*. This subspecies is divided into numerous serovars, each exhibiting distinct pathogenic traits and host specificities. The most notable serovars are *S.* Typhimurium and *S.* Enteritidis, which often cause gastroenteritis in humans, and *S.* Typhi and *S.* Paratyphi, responsible for severe illnesses like the typhoid fever (Andino et al., 2015, Brown et al., 2021).

Structurally, *Salmonella* is a Gram-negative, non-spore-forming bacterium, and it is characterised by a rod shape and flagellar motility. This motility plays a significant role in the bacterium's ability to infect and cause diseases in hosts. The flagella enable *Salmonella* to move toward favourable environments and away from hostile ones, a behaviour known as chemotaxis. This motility is essential for the bacterium to penetrate the intestinal mucus layer, allowing it to attach to and invade intestinal epithelial cells (Fàbrega & Vila, 2013; Chaban et al., 2015). Moreover, *Salmonella* has the ability to survive macrophages and trigger strong immune responses in the host (Pham & McSorley, 2015).

Salmonella enterica is present in a variety of food products and most commonly in poultry, eggs, meat, and dairy (Ehuwa et al., 2021). It has the ability to survive under harsh conditions, including varying ranges of temperature and

pH levels. Additionally *Salmonella* can survive in products with low water activity (a_w), i.e., the availability of water in a substance required for chemical reactions and microbial growth (Doyle & Mazzotta, 2000; Spector & Kenyon, 2012).

Salmonella can cause a highly infectious illness, salmonellosis, which typically includes symptoms such as diarrhea, fever, and abdominal cramps. Severe cases can result in severe dehydration, spread of the infection to the bloodstream and other life-threatening complications, particularly in vulnerable populations such as infants, the elderly, and individuals with weakened immune systems (Cherubin et al., 1969). Reports indicate that there are approximately 93.8 million cases of non-typhoidal salmonellosis globally every year, leading to around 155,000 fatalities, of which 86% can be attributed to the consumption of food products contaminated with *Salmonella* (Sher et al., 2021). In EU, salmonellosis is the second most reported food related illness with a rate which corresponds to 20 per 100,000 population (Schirone & Visciano 2021).

From 2018 to 2022, *S*. enteritidis was linked to 3,061 outbreaks, affecting 21,808 individuals, with 17 deaths and 4,824 hospitalisations, while *S*. Typhimurium accounted for 354 outbreaks, 4,330 cases, 2 deaths, and 519 hospitalisations in different country members of the EU (EFSA & ECDC, 2019, 2021a, 2021b, 2022).

Consequently, European legislation has enforced necessary measures to monitor and control *Salmonella* presence in food. Specifically, the regulation (EC) No. 2073/2005 requires food business operators to ensure the absence of *Salmonella* in their products, specifying "absence in 25 or 10 grams" during marketing and shelf life, with tests of 5 or 30 samples per batch depending on the food category. Regulation (EC) No. 1086 mandates "absence in 25 grams" for specific *Salmonella* serovars in poultry meat batches within the EU (Cesare et al., 2018).

2.1.2 *L. monocytogenes*

Listeria is a genus of bacteria that belong to the family Listeriaceae. The genus consists of several species, among which the most well studied are the non-

pathogenic species *L. innocua* and the pathogenic species *L. monocytogenes*. *Listeria* is a Gram-positive, rod-shaped, non-spore forming and facultatively anaerobic bacterium.

L. monocytogenes is able to grow under various environmental stress conditions, including low pH (<5.0), low a_w levels (<0.90) and even refrigerator temperatures (0°C) (George et al., 1988; Dooley & Roberts, 2000). Additionally, the microorganism has demonstrated high thermotolerance to heat treatments (Pagán et al., 1997). The pathogen is commonly found in various food products but most often it is reported in dairy products, such as raw milk and soft cheeses, and ready-to-eat (RTE) products (Rodriguez-Lazaro et al., 2014). Due to its ability to thrive in cold environments, foods that are not cooked prior to consumption can be particularly susceptible to contamination. This adaptability contributes to the bacterium's role in foodborne illnesses, as it can establish infections even when ingested in small numbers, especially in susceptible populations (Buchanan et al., 2017).

The virulence of *L. monocytogenes* is attributed to its ability to invade and replicate within the cytoplasm of host cells. This way, it manages to hide from the host's immune response (Carbalho et al., 2014). *L. monocytogenes* is especially harmful for pregnant women, newborns, the elderly, and immunocompromised individuals due to their weak immune system. The bacteria can soon spread to the bloodstream, causing septicemia, or to the nervous system, causing meningitis; both serious, life-threatening conditions if they are not treated promptly. Pregnant women are also at risk because the bacteria can cross the placental barrier leading to miscarriage, stillbirth, or severe infection in newborns. The infection caused by *L. monocytogenes* is called listeriosis (Schlech, 2019; Quereda et al., 2021). Between 2007 and 2011, it was estimated that approximately 500 and 1,500 cases of listeriosis were reported annually in the United States (US) and in the EU, respectively (Lomonaco et al., 2015). More recently, from 2018 to 2020, *L. monocytogenes* caused 90 outbreaks, resulting in 621 infections, 94 fatalities, and 405 hospital admissions in the EU member states. Additionally, *Listeria monocytogenes* is the leading cause of deaths among foodborne pathogens, accounting for 43.8% of all fatalities linked to foodborne illnesses (EFSA & ECDC, 2019, 2021a, 2021b, 2022).

The regulation (EC) No. 2073/2005 provides the relevant information to control the risk of *L. monocytogenes* in different products. For foods intended for infants and for special medical purposes, the absence of the microorganism is required in 25 g of the product. For RTE food products, the acceptable limit is below 100 CFU/g at the end of the shelf-life (Zhang et al., 2021a).

2.2 Thermal inactivation of pathogens

2.2.1 Basic Principles and applications of thermal inactivation

The thermal inactivation of pathogens is a cornerstone of safety and shelf life extension of food products in the food industry. The process involves using heat to reduce or eliminate pathogenic microorganisms in food. The effectiveness of this method depends on several factors, including the temperature, duration of heat application, and the nature of the microorganisms targeted (Jujena et al., 2011; Smelt & Brul, 2014).

Pasteurisation and sterilisation are the two primary thermal processes employed in the food industry. Pasteurisation is a mild heat treatment that reduces the number of pathogens and spoilage organisms in food, aiming to extend shelf life while maintaining the food's nutritional and sensory qualities. It's commonly applied to liquid foods like milk and juices. On the other hand, sterilisation is a more intense heat treatment designed to destroy all viable microorganisms, including spores, in a product. This process is essential for producing shelf-stable canned foods and long-life products, often involving heating at 121°C for a specific time depending on the product (Welch & Mitchell, 2000; Matser et al., 2004; Wilson et al.. 2008; Micali et al., 2016).

In the conventional thermal processing of foods, there are two main methods of heat transfer, conduction and convection (Ziaiifar et al., 2021). In conduction, thermal energy is transferred from one particle to another, through direct contact, gradually heating the entire product. It is usually the

dominant heat transfer mechanism in solid and high-viscosity foods such as canned goods. Convection involves the movement of heat through a fluid medium, which can be either liquid or gas. It is usually the dominant heat transfer mechanism in liquids and semi-liquids, such as soups and milk, as the circulating medium facilitates uniform heat distribution (Barigou et al., 1998; Fryer, & Robbins, 2005; Kaviany, 2011).

However, conventional thermal treatments often lead to nutrient degradation, energy inefficiency, and potential loss of sensory qualities, prompting a shift towards novel thermal technologies (Barba et al., 2017; Zhang et al., 2019; Chacha et al., 2021). The current emerging thermal technologies include microwave heating, radio frequency heating, and ohmic heating (Van Impe et al., 2018).

2.2.2 Microbial inactivation models

Predictive microbiology plays a crucial role in understanding and controlling microbial behaviour in food safety management. It involves the use of mathematical and statistical models to predict the growth, survival, or inactivation of microorganisms under various environmental conditions (Van Impe et al., 1995).

Thermal inactivation treatments were initially described by the Thermal Death Time (TDT), a concept first introduced by Bigelow (1921). TDT quantifies the time it takes to sterilise a product at a specific temperature. Specifically, two parameters are defined, i.e., (i) the D-value, the time required at a specific temperature to reduce the microbial population by 90% or one log, and (ii) the z-value, the temperature increase required to decrease the D-value by one log unit. The F value is also a term used in thermal processing. It represents the lethal effect of a heat treatment on microorganisms, specifically the time required to kill a specified number of microorganisms at a certain temperature (Li et al., 2018; Peleg, 2021). D-values, z-values, and F-values are key in designing thermal processes for food safety, but they have limitations. The assumption of log-linear microbial inactivation may not always hold true, leading to inaccuracies in predicting the effectiveness of heat treatments. Microbial populations are heterogeneous, which can result in variability in resistance to thermal processes. Environmental factors like food

composition can influence the effectiveness of thermal inactivation, making it challenging to generalise findings across different food products (Whiting, 1995; van Boekel, 2002).

Various models have been proposed to address the limitations of the TDT concept. This dissertation focuses on the Geeraerd et al. (2000) model widely used in thermal inactivation studies.

$$\frac{dN(t)}{dt} = -\left(\frac{1}{1+C_C)(t)}\right) \cdot k_{max} \cdot \left(1 - \frac{N_{res}}{N(t)}\right) \cdot N(t) \quad (2.1)$$

$$\frac{dC_c(t)}{dt} = -\cdot k_{max} \cdot C_c(t) \quad (2.2)$$

$$S_L = \frac{lm\,(1+C_c(0)}{k_{max}} \quad (2.3)$$

with $N(t)$ (CFU/mL), the cell density at time t; N_{res} (CFU/mL), the residual cell density; k_{max} (1/min), the specific inactivation rate; C_c, the concentration of critical component for cell survival; $C_c(0)$, the initial concentration of the critical component for cell survival; and S_L, the shoulder length i.e., the time period necessary for the cells to adapt to the stress. The second and fourth parts of Equation 2.1 correspond to the classical log-linear inactivation kinetics, whereas the first and third parts represent a shoulder and tailing effect, respectively. In the experimental section of the dissertation, the shoulder phase was not incorporated into the RF inactivation kinetics. This is attributed to the dynamic nature of the heating process, which makes it challenging to distinguish between microbial adaptation and the effects of low temperatures.

2.2.3 Sublethal injury

Sublethal injury (SI) in microorganisms refers to the state where cells are subjected to stress conditions such as thermal, chemical, or radiative exposure, which result in impairment of their vital functions without causing immediate death (Wesche et al., 2009). This injury makes the microorganisms

more sensitive to further stress or selective environments. For instance, heat-treated bacteria might not grow on standard media but can recover on media that provide optimal conditions for injured cells (Smet et al., 2017; Smet et al., 2019; Govaert et al., 2019; Govaert et al., 2020a).

Sublethally injured microorganisms are significant in food safety because they have the ability to repair and regrow in favourable conditions later in the food processing chain, potentially leading to foodborne illnesses (Akkermans et al., 2021). Additionally, these cells can undergo physiological or genetic changes that results in increased resilience to subsequent stressors. This process, known as stress adaptation or stress response, can lead to the development of cross-protection, where exposure to one type of stress enhances resistance to another (Wesche & Ryser, 2013; Khan et al., 2022). For instance, bacterial cells that have previously been exposed to environments with a low a_w environment demonstrate increased resistance to thermal stress (Tiganitas et al., 2009; Lang & Sant'Ana, 2021).

2.2.4 Thermal Inactivation mechanisms

The thermal inactivation of bacteria involves several key mechanisms that disrupt critical cellular components and processes, leading to cell injury and death (Wesche et al., 2009; Van Impe et al., 2018).

At high temperatures (e.g., temperatures above 65°C), one of the primary mechanisms of bacterial inactivation is the denaturation of proteins. Proteins, essential for numerous cellular functions, lose their three-dimensional structure and functionality. This denaturation process affects enzymes and structural proteins, leading to metabolic dysfunction.

Similarly, the bacterial cell membrane, crucial for maintaining cellular integrity and homeostasis, becomes compromised under thermal stress. Heat alters membrane fluidity and permeability, causing leakage of vital cellular contents and eventually leads to cell lysis (Hurst, 1977; Lund et al., 2000).

Another significant thermal inactivation mechanism is the degradation of nucleic acids. Elevated temperatures (e.g., temperatures above 65-75°C) can cause the denaturation of deoxyribonucleic acid (DNA) and ribonucleic acid

(RNA), disrupting replication, transcription, and subsequent protein synthesis (Ray, 1986; Tolker-Nielsen & Molin, 1996).

Finally, enzymatic activity and metabolism are also affected by heat. Enzymes that catalyse essential biochemical reactions, are sensitive to temperature changes. Thermal exposure (e.g., temperatures above 65°C) can lead to the inactivation of these critical proteins, preventing vital metabolic pathways and energy production, which is integral to bacterial survival and replication (Jaenicke, 1981).

2.3 Microbial safety of dairy products

Dairy products, derived from the milk of mammals such as cows, goats, and sheep, play a pivotal role in the diets of people globally. They cover a vast array of food items, including milk, cheese, yogurt, butter, puddings, powders and ice cream, each with distinctive nutritional benefits, flavours and textures (Lagrange et al., 2015). While some products such as ice cream may not offer significant nutritional benefits, they are still a popular choice for their unique taste and texture. The diversity in dairy products is an outcome of various processing methods that modify milk's physical and chemical properties (Butler et al., 2011).

2.3.1 Impact on public health

Globally, the dairy industry is a crucial food sector, yet it faces challenges, with dairy items at risk of contamination by various microbial, physical and chemical hazards from farm to consumption (Motarjemi et al., 2014). Microbiological hazards are more common in dairy products than chemical and physical hazards, with *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella*, and pathogenic *E. coli* being the most significant. The Centre for Disease Control and Prevention (CDC) reported 101 outbreaks of *L. monocytogenes* between 2012 and 2019 in the U.S. linked to dairy products like cheese, ice cream, and raw milk. In the EU, between 2018 and 2022, there have been 149 outbreaks in milk and milk products resulting in 1,754 cases, 298 hospitalisations and 22 deaths. The microorganisms responsible for these outbreaks are depicted in Figure 2.1. *Salmonella* is the leading microorganism

in outbreaks in milk and milk products, causing 54 hospitalisations and 1 death. It is also crucial to note, that even though *L. monocytogenes* was responsible for only 6 outbreaks, it caused 53 hospitalisations and 14 deaths.

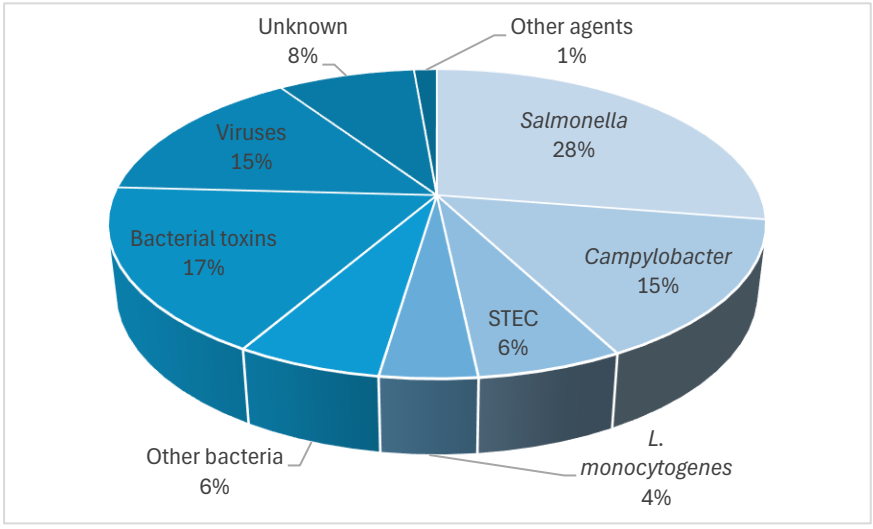


Figure 2.1: Causative agents for foodborne outbreaks in EU between 2018-2022, created using EFSA's interactive dashboard, https://www.efsa.europa.eu/en/microstrategy/FBO-dashboard

2.3.2 Significance of *Salmonella* and *L. monocytogenes* in dairy products

The significance of *L. monocytogenes* and *Salmonella* in dairy products cannot be overstated, as these pathogens pose serious health risks to consumers. Dairy products, given their nutrient-rich composition, can provide an ideal environment for the survival and growth of these bacteria.

Salmonella is a critical pathogen in the dairy sector. Although pasteurisation effectively kills *Salmonella* in liquid dairy products, it remains a concern in raw milk and products made from raw milk (Verraes et al., 2015). Furthermore, the risk of cross-contamination exists for powdered and structured dairy foods post-processing (LeLièvre et al., 2019). Additionally, *Salmonella* can survive the spray drying process during the manufacturing of dairy powders

and remain in the powder for prolonged time period (LiCari, J. A., & Potter, 1970). The growth of *Salmonella* in dairy products is influenced by factors such as temperature, pH, moisture content and a_w.

L. monocytogenes is particularly concerning for the dairy industry due to its ability to survive and grow at refrigeration temperatures, making it a challenging pathogen to control in dairy products. It can be detected in a variety of dairy products, from soft cheeses to ice cream. Its survival and growth dynamics in dairy products depend on several factors, including the specific dairy matrix, temperature, pH, and a_w.

2.3.3 Thermal treatments of dairy products

In order to ensure the safety of (certain) dairy products, thermal treatments, such as pasteurisation and ultra-high temperature (UHT) processing, are widely used in the dairy industry. Pasteurisation, which typically involves heating milk to at least 72°C for 15 seconds, has been proven to significantly reduce the population of both these pathogens. UHT processing subjects milk to even higher temperatures (around 135°C) for a few seconds, offering an even higher inactivation level, extending the shelf life of dairy products without the need for refrigeration (Lewis & Deeth, 2008).

Conventional thermal treatments of dairy products, while effective for ensuring safety and extending shelf life, come with several drawbacks. These methods can lead to nutrient loss, particularly of heat-sensitive vitamins and minerals, and often result in alterations to the flavour, colour, and texture of dairy products. Thermal treatments are also energy-intensive, contributing to higher production costs and environmental impacts. Although thermal treatments can inactivate many microorganisms, they may not effectively counter all spoilage agents, like spores, and there's always the risk of post-processing contamination. These issues are prompting the dairy industry to explore alternative thermal or non-thermal technologies to preserve the quality and safety of dairy products more efficiently (Nunes & Tavares, 2008; Villamiel et al., 2008; Dash et al., 2022).

Chapter 3

Food Microstructure

Foods are inherently heterogeneous mixtures, composed of various elements, that contribute to their complex structures and diverse characteristics (Wimpenny et al., 1995). Food microstructure refers to the spatial arrangement and organisation of structural components within food at the microscopic level, including cells, proteins, fats, and carbohydrates. This microstructure plays a crucial role in determining the physical properties, texture, flavour, and shelf life of food products. Understanding food microstructure is essential for food processing, as it impacts the processing behaviour, sensory attributes, and nutritional quality of food. (Aguilera et al., 2000; Muthukumarappan & Swamy, 2018).

Aiming to model the microbial growth in structured foods, Wilson et al. (2002) classified the food microstructures into six categories: liquids (e.g. juices), aqueous gels (e.g. jellies), oil-in-water emulsions (e.g. milk), water-in-oil emulsions (e.g. butter), gelled emulsions (e.g. liver pate) and surfaces (e.g., vegetable or meat tissues). However, this classification can be expanded or further detailed by considering additional categories or subcategories that account for the wide variety of food structures encountered in the food industry. For instance, the categories do not take into account food powders, which consist of dry, finely divided solid particles. Powders interact differently in comparison to other structures due to their low a_w and high surface area, affecting how microorganisms can act within these systems. As microorganisms are able to adhere to the surface of the powder particles (Beveridge & Todd, 1973), one could include powders in the surface category. It is, however, important to note that microorganisms are not able to grow on powders, contrary to other surface products such as meats.

3.1 Influence of food microstructure on microbial dynamics

Microstructure exerts a crucial influence on the growth of microorganisms in foods. As a general rule, microbial growth occurs in the aqueous phase of the food products. Therefore, the structural aspects of this phase, such as the distribution of water, nutrients and other components, affect the physical mobility of microorganisms (Aspridou et al., 2014). Similarly, the microstructure also significantly impacts the survival and inactivation of microorganisms within food products. The arrangement and characteristics of the food matrix can either provide protective sectors that enhance the microbial resistance to different inactivation treatments, or enhance the efficacy of these treatments (Noriega et al., 2013; Velliou et al., 2013).

In the following sections, the microstructural influence on growth and (thermal) inactivation will be described in detail. Since this thesis is mainly considering thermal inactivation, this aspect is also the primary focus of this explanation.

3.1.1 Influence on growth

In homogenous food products such as liquids, microorganisms exist in a planktonic form, able to move freely and interact with nutrients and metabolites within the matrix. In these systems, growth or inactivation is mostly influenced by compositional and physicochemical factors (Robins & Wilson, 1994).

In heterogenous food products, bacterial growth occurs in two types, i.e., colonial and planktonic, depending on the type of product (Skandamis & Jeanson, 2015; Verheyen et al., 2021). For instance, in aqueous gels, bacteria can grow as immobilised submerged colonies within the gel, or they may also form surface colonies on the gel's surface (Verheyen et al., 2018a, 2018b). In emulsions, bacteria typically grow as planktonic cells within the aqueous phase. Specifically, in oil-in-water emulsions with a fat content less than 80% v/v, bacteria grow as planktonic cells, but at higher fat contents, where

mobility is restricted, they form colonies (Brocklehurst et al., 1995; Parker et al., 1995). In water-in-oil emulsions, bacteria can grow within the water droplets dispersed in the oil phase as microcolonies or individual cells. However, the type of growth depends on factors such as the water droplet size and distribution, as well as the presence of nutrients, and growth is typically quite limited (Hammer & Long, 1941; Voysey et al., 2009; El-Hajjaji et al., 2020). Gelled emulsions can be considered as a combination of oil-in-water emulsions and aqueous gels, hence the microorganisms typically grow as colonies within the aqueous phase between the fat droplets (Verheyen et al., 2018a, 2018b, 2019). Finally, on food powders, bacterial cells cannot grow and they are typically in a desiccated or dry state due to the low a_w of these environments. Desiccation is a stress condition that causes bacteria to enter a state of metabolic dormancy to survive the lack of water. This dormant state significantly slows down their metabolism, growth, and cell division (Breeuwer, 2014).

3.1.2 Influence on (thermal) inactivation

A first microstructural factor that affects microbial inactivation is the general degree of complexity of the food product. In homogenous food products like liquids, where the microorganisms are moving freely and interacting within the matrix, the inactivation is largely influenced by the food's composition and physicochemical properties (pH, temperature). Inactivation methods such as heat treatments or chemical sanitisers can uniformly target the planktonic cells and inactivate them (Wilson et al., 2002). In contrast, heterogeneous food products (i.e., products with variability in texture and structure) present a more complex situation for inactivation. In gels, colonies that are immobilised within the matrix or located on its surface exhibit increased thermotolerance. In addition, the structure of the gel limits heat transfer, which additionally hinders the inactivation (Velliou et al., 2013; Wang et al., 2017).

Moreover, the influence of fat content on the thermal inactivation of microorganisms within food matrices is complex (Juneja & Eblen, 2000; Verheyen et al., 2020). On the one hand, some studies suggest that fat can protect bacteria from heat, potentially by altering thermal conductivity or providing microenvironments that shield microbial cells (Chhabra et al.,

1999; Van Asselt& Zwietering, 2006; Schultze et al., 2007). On the other hand, some studies have concluded that fat does not increase microbial resistance to thermal processes (Szlachta et al., 2010) and may even facilitate inactivation under certain conditions (Li et al., 2014). However, it's important to recognise that fat content is not the sole factor influencing the inactivation of microorganisms in fat-containing food products. For instance, altering the fat level in a food product necessitates adjustments in other components to maintain the product's overall composition and characteristics (e.g., proteins or carbohydrates), and these components can also affect the thermal inactivation (Kim et al., 2017; Tonti et al., 2024). The effect of other physicochemical factors such as the treatment temperature may also change the effect of the fat content on microbial resistance (Gurman et al., 2016; Brar et al., 2018). This variability underscores the necessity of evaluating thermal inactivation on a case-by-case basis, taking into account the specific food matrix and its fat composition to accurately predict and control microbial survival during thermal processing.

The desiccated state of the microorganisms present on food powders significantly enhances their resistance to thermal inactivation. When bacteria are in a desiccated state, the lack of water in their environment leads to alterations in their cellular structures and metabolic processes, resulting in an increase their thermal tolerance (Chen et al., 2013; Shaker et al., 2008; Lang et al., 2017). The low a_w of food powders slows down the chemical reactions that contribute to thermal death and stabilises the proteins and membranes of microorganisms, making them less susceptible to heat damage. Additionally, the reduced water content limits the efficacy of (conventional) heat transfer (i.e., convection and conduction) within the microbial cells, thereby requiring higher temperatures or longer exposure times to achieve effective inactivation (Rifna et al., 2019). This increased resistance poses a challenge in ensuring the microbiological safety of powdered food products, and highlights the need for novel thermal processing strategies that consider the resilience of microorganisms present in low moisture foods.

3.2 Food model systems

Food model systems are used in scientific studies to simulate the microstructural and compositional properties of real food products, enabling the investigation on how various microstructural factors influence microbial behaviour. These systems offer several advantages over using actual food products, such as simpler microbiological practices, better repeatability, and the ability to modify factors independently (Noriega et al., 2008; Tack et al., 2015; Baka et al., 2017a).

Initially, liquid media were predominantly used, but recent trends have expanded to include model systems that more accurately mimic the food's microstructural properties, such as liquids, emulsions, gels, and surfaces (Verheyen et al., 2018a). Three types of model systems approaches can be distinguished, each with a specific goal: (i) relatively complex model systems, (ii) relatively simple model systems, and (iii) model systems varying in microstructural complexity.

3.2.1 Complex food model systems

Complex food model systems are designed to mimic the microstructural attributes of specific food products or categories, enabling a study of the microbial behaviour without the difficulties of using real food products. These systems can have different complexities, aligning with the microstructural category of the target food (Wilson et al., 2002; Noriega et al., 2008). Complex food model systems typically incorporate various components like gelling agents, fats, and preservatives, all contributing to the desired structural complexity, and supporting either microbial growth or inhibition (Castro et al., 2009; Costello et al., 2018).

3.2.2 Simple food model systems

The simple food model systems approach is relatively minimalistic, concentrating on isolating the effect of particular specifics on microbial behaviour, typically using fewer components than complex systems. These models are instrumental in studying the effects of food composition and

structural properties on bacterial microorganism. For instance, the focus can be on the effect of different fat contents in emulsions or varying gelling agent concentrations in gelled systems to observe microbial responses (Prachaiyo & McLandsborough, 2003; Aspridou et al., 2014; Costello et al., 2018). By simplifying the model, it is possible to identify how individual factors influence microbial activity, providing valuable insights that can inform broader food safety and preservation strategies (MacKey et al., 2006).

3.2.3 Model systems varying in microstructural complexity

Model systems that vary in microstructural complexity are used to assess how different structural aspects of food influence microbial behaviour. Such studies typically compare microbial growth or inactivation in different model matrices, like liquids and gels, to understand the role of microstructure in microbial dynamics (Noriega et al., 2008; Baka et al., 2015; Baka et al., 2016; Boons et al., 2015). Researchers might use a range of systems, from simple liquids to complex gelled emulsions, to explore how factors like gel concentration affect microbial activity (Verheyen et al., 2018b, 2019, 2020). While these models offer a nuanced view of microbial behaviour across different structures, interpreting the results can be challenging due to the interplay of compositional and physicochemical factors inherent to the varied microstructures (Verheyen et al., 2020).

3.3 Dairy model systems

Model systems simulating dairy products are simplified versions of these products. The aim is to replicate certain compositional and microstructural properties in order to investigate fundamental aspects, such as the effect components and their interactions on the system's behaviour, without the complexity of full-scale food matrices. Most studies thus far have focused on the isolating the effect of component interactions on microstructure, rheology, and texture (Verbeken et al., 2004; Sabadini et al., 2006; Vélez-Ruiz et al., 2006; Verbeken et al., 2006; Lubbers et al., 2007; Allen et al., 2008; Considine et al., 2011; Gentès et al., 2013; Zahn et al., 2013; Silva et al., 2015). The components and their interactions can influence gelation properties, viscosity, and stability of dairy products, providing knowledge about how ingredients and processing conditions can be tailored to achieve desired product attributes. Thus far, there have been very few studies on the effect of different dairy model systems on microbial behaviour, growth or inactivation (Choi et al., 2008). Therefore, more research on the impact of dairy model systems on microbial behaviour is necessary to improve food safety and product quality in the dairy industry.

Chapter 4

Radio frequency processing

4.1 Principles of radio frequency (RF)

RF processing is an innovative technology which uses electric energy to generate electromagnetic radiation. When this radiation is applied to a material, its molecules absorb it, causing them to oscillate. This movement generates friction between molecules and ions, ultimately converting the electromagnetic energy into thermal energy (Piyasena et al., 2003).

RF technology's initial application in food processing dates back to 1947 when it was first used for blanching. In the following years, it gradually extended to cooking, dehydration, thawing, and post-bake drying. It was not until the 1990s that RF technology began to be considered for pasteurisation processes (Zhang H. et al., 2020a). Jiao et al. (2018) and Altemimi et al. (2019) provide a comprehensive overview of relevant studies regarding the specific RF applications.

The RF portion of the electromagnetic spectrum covers a wide range of frequencies used for various purposes, from communication and broadcasting to navigation and sensing. It can be categorised into these bands: Low Frequency (30 kHz-300 kHz), Medium Frequency (300 kHz-3 MHz), High Frequency (3 MHz-30 MHz) and Very High Frequency (30 MHz-300 MHz) (Raab et al., 2002). RF typically refers to frequencies between 3 and 300 MHz, corresponding to wavelengths ranging from 1 m to 100 m (Lagunas-Solar, 2012).

Three different frequencies are permitted for industrial, scientific and medical (ISM) applications by the Federal Communication Commission to prevent

disturbance in telecommunications (cell phone, broadcasting, satellites): 13.56 MHz, 27.12 MHz and 40.68 MHz (Jones and Rowley, 1996).

Among industrial RF setups, 27.12 MHz is the most widely used and internationally accepted frequency. The higher ISM frequency 40.68 MHz is not allowed in every country. Additionally, it has the drawbacks of generating the lowest electric field strength (V/cm) and the lowest penetration depth. The ISM frequency of 13.56 MHz offers the highest electric field strength, highest penetration depth and the most uniform voltage distribution. However, in some cases, this high electric field might approach the limit of 10 kV/cm. When this limit is surpassed, air can become conductive, leading to current flow, sparking, and potentially arcing (Mehdizadeh, 2015). Additionally, at the frequency of 27.12 MHz, the bandwidth is the largest, with a tolerance of ± 0.6%. In contrast, frequencies of 13.56 MHz and 40.68 MHz have narrower bandwidths, with tolerances of ± 0.05% (Tang et al., 2004).

The generation of heat following RF applications is attributed to two dominant mechanisms: dipolar polarisation and ionic conduction.

Dipolar molecules exhibit an uneven distribution of electrical charge, resulting in a partial positive charge on one end and a partial negative charge on the other. A typical example of a dipolar molecule is water, where the oxygen atom attracts the electrons more strongly than the hydrogen atoms, leading to the formation of a dipole moment within the molecule. Under the influence of an electromagnetic field, the dipolar molecules tend to orient themselves in way that their dipole moments align with the field direction, a phenomenon known as dipolar polarisation. As the polarity of the electromagnetic field changes continuously, the molecules reorient, and this alignment results in the transfer of energy (Jiang et al., 2019).

Ions are charged particles formed from atoms or molecules by gaining or losing electrons, resulting in either a positive or negative charge. Under the influence of an electromagnetic field, ions migrate towards oppositely charged electrodes, a phenomenon called ionic conduction. As the ions migrate towards oppositely charged electrodes, they collide with other ions and the surrounding atoms or molecules in the material. These collisions lead

to friction and resistance, causing the kinetic energy of the ions to be converted into thermal energy, which manifests as heat (Di Rosa et al., 2019).

4.2 Dielectric Properties of Food

The interaction of RF electromagnetic energy with materials is significantly influenced by their dielectric properties. These properties include the dielectric constant (ε') and the dielectric loss factor (ε"). The dielectric constant represents a material's ability to store electromagnetic energy and affects the electric field distribution. The higher the dielectric constant, the more electrical charge a material can store. When a dielectric material stores electrical energy, some of it dissipates in the form of heat and this energy dissipation is quantified by the dielectric loss factor. The higher the dielectric loss factor, the more thermal energy is generated (Sosa-Morales et al., 2010). The ratio of ε" over ε' is commonly known as tanδ (Equation 4.1) and describes the ability of the material to dissipate energy as heat when subjected to an electric field (Grant & Halstead, 1998). Materials with higher tanδ values tend to exhibit more efficient RF heating behaviour, as they dissipate energy more fast.

$$\tan\delta = \frac{\varepsilon''}{\varepsilon}, \qquad (4.1)$$

Understanding the dielectric properties of a material is essential for predicting both the penetration depth (d_p) of electromagnetic waves into the material and the amount of power absorbed by it. Penetration depth describes how far electromagnetic waves can travel into a material before their intensity diminishes to a specified fraction of the original value (Equation 4.2). Power absorption quantifies the amount of electromagnetic energy that is absorbed by the material as the waves penetrate it and it represents the conversion of electromagnetic energy into thermal energy, within the material (Equation 4.3) (Clark et al., 2000; Jiao et al., 2017).

Both penetration depth and power absorption are important parameters in understanding how materials interact with electromagnetic fields.

$$d_p = \frac{c}{2\sqrt{2}\pi f[\varepsilon'(\sqrt{1+(\frac{\varepsilon''}{\varepsilon'})^2}-1]^{\frac{1}{2}}}, \quad (4.2)$$

$$P = 2\pi f \varepsilon_0 \varepsilon'' |E|^2, \quad (4.3)$$

Where c is the speed of light in free space ($3*10^8$ m/s), P is the power absorption (W), ε_0 is the permittivity of free space, equal to $8.854*10^{-12}$ (F/m), and E is the electric field intensity (V/m).

Knowledge on the dielectric properties of materials will provide insight on the RF heating mechanism and aid to the optimisation of each treatment. For example, surface heating is more likely to occur at high frequencies and increased values of the dielectric properties, while at lower frequencies and smaller values of the dielectric properties, more volumetric heating will be achieved (Clark et al., 2000). Thus far, there is a lack of a scientific database on the dielectric properties of different materials with respect to temperature and frequency.

Overall, the values of dielectric properties exhibit variability among different food matrices as they can be influenced by several factors. For food products, moisture content is the principal factor which can impact the dielectric properties, as water molecules have high polarisability and therefore, food products with higher moisture content have higher dielectric properties (Nelson, 2015). In low-moisture foods, where dipole dispersion of free water molecules is minimal, the dielectric properties are primarily influenced by the presence of bound water. Bound water refers to water molecules with low mobility, ranging between that of ice and free water, leading to low values of dielectric properties for these products (Jiang et al., 2019). Another factor which impacts the dielectric properties, is the chemical composition of the material. Different types of atoms and molecules (dipoles, ions) may exhibit varying degrees of polarisation and conductivity. Moreover, dielectric properties may change with temperature and frequency, as higher temperatures and frequencies can lead to increased molecular movement (Altemimi et al., 2019).

Recently, there has been an effort in acquiring information regarding the dielectric properties of dairy products. Specifically, the dielectric properties of various dairy products, including liquid milk (Nunes et al., 2006; Guo et al., 2010; Zhu et al., 2014; Zhu et al., 2015a; Zhu et al., 2015b; Liu et al., 2018; Muñoz et al., 2018; Zhu et al., 2018), milk powder (Silalai & Roos et al., 2010; Lin et al., 2016; Dag et al., 2019; Lin et al., 2022) and more structure dairy food systems such as cheese (Green, 1997; Herve et al., 1998; Everard et al., 2006; Velázquez-Varela et al., 2018), yogurt (Guo et al., 2018) and butter (Ahmed et al., 2007a), have been recorded. These studies have examined their dielectric properties under varying conditions, including temperature, frequency, and their interactions with specific food components. Overall, the diverse nature of dairy products leads to considerable complexity in their dielectric properties.

4.3 Types of RF Heating Systems

A simplistic configuration of a typical RF heating system consists of a power source, an oscillator generator, a capacitor(s), a transmission line (usually a coaxial cable), and an applicator or antenna. More sophisticated systems often integrate monitoring and control systems to improve efficiency, ensuring precise regulation of parameters such as power levels, frequency and temperature.

The power source supplies electrical energy to the system, which is then used by the oscillator generator to produce RF waves. The capacitor plays a crucial role in this process by storing electrical energy from the power source and assisting in fine-tuning the frequency of the RF waves generated by the oscillator generator. The transmission line facilitates the transfer of RF energy generated by the oscillator to the applicator or antenna. In most RF setups, two parallel electrodes are used, one being grounded and the other being a movable applicator and the food product is being positioned between them. These electrodes are responsible for delivering the RF energy to the food product (Dag et al., 2020). Different types of configurations and RF set-ups are available, each tailored to address specific applications and demands (Soto-Reyes et al., 2022; Gao et al., 2023) .

RF systems are often categorised into two types, the Free-Running Oscillator (FRO) system and the 50-Ohm system. On the one hand, FRO systems contain an oscillator generator that operates independently without requiring external signals to determine its oscillation frequency. These systems are characterised by their simplicity, making them suitable for applications where precise frequency control is not necessary and are most commonly used in continuous operations such as bulk heating of food products or thawing frozen foods. On the other hand, 50-ohm RF systems offer a fixed frequency output and precise regulation of power and feedback. This is achieved by maintaining a consistent impedance of 50 ohms throughout the system, which results in accuracy over power distribution and heating uniformity. These systems are more expensive than FRO and they are used in more sophisticated heating processes such as cooking or pasteurisation of packaged foods.

4.4 RF advantages and challenges

RF technology presents several advantages in food processing. Firstly, it enables rapid and relatively uniform heating of food products, reducing processing time and enhancing efficiency. Specifically, RF heating has reduced the process times by 1/3 compared to conventional thermal treatments (e.g., in the thermal treatment of food powders or spices). This rapid heating also helps in preserving the nutritional content and sensory qualities of food items, better than traditional heating methods. Additionally, RF heating systems are often more energy-efficient than conventional methods, leading to cost savings and reduced environmental impact (Ramaswamy & Tang, 2008; Jiao et al., 2018). Also, RF systems can be automated for continuous processing in tunnels, reducing the need for manual intervention and enhancing productivity (Geveke et al., 2018). RF presents advantages particularly for processing low moisture food products. Conventional heating of such products typically requires extended processing time and high temperatures. The mechanisms of conventional thermal heating, conduction and convection, are less effective in these cases because of the small water content and reduced thermal conductivity, leading to prolonged heating times. Moreover the heat is applied externally and must penetrate inwards, further complicating the process for bulk foods products (Deng et al., 2021).

However, RF technology also comes with certain limitations. The primary challenge thus far has been the non-uniform heating. RF is a dielectric heating method, and the temperature distribution and heating uniformity of the sample depend on many factors. These include the food composition, the size, shape and the characteristics of each sample such as moisture content, density and the dielectric properties. For example, in non-uniform samples, the areas with higher ε" values will absorb more energy and heat up more quickly than the other areas. This non-uniform absorption of RF energy can lead to localised overheating and the creation of hot spots. Then, as the temperature increases, the ε" of these hot spots will increase even further, as with higher temperatures, the values of the dielectric properties also increase. This phenomenon is called runaway heating and it commonly results in surface over-heating (Cai et al., 2019; Llave & Erdogdu, 2022). RF heating systems require special operations to prevent overheating and ensure operator safety, particularly in high-power applications (Guo et al., 2019).

4.5 RF inactivation

The advantage of using RF energy targeted at pasteurisation or sterilisation lies in its potential for selective inactivation of microorganisms, due to the rapid heating and large penetration depth (Jojo and Mahendran, 2013).

Most available studies have focused on achieving a designated log reduction of different foodborne pathogens, relevant to a specific food product. Some studies also investigate the reduction of the total bacteria counts (Zhao et al., 2017; Rosa et al., 2018; Lu et al., 2018; Ozturk et al., 2018). Research on RF also includes the treatment of fungi (Jiao et al., 2016; Zheng et al., 2017, Ling et al.; 2020) or bacterial spores (Jiao et al., 2019; Xu et al., 2023).

RF has been used for the treatment of products with different types of microstructure., i.e., powder, liquids, gels, emulsions and products with more complex structure (e.g. tissues, meat). Furthermore, RF has been applied to grains (corn, wheat) (Jiao et al., 2016; Zheng et al., 2017), nuts (Salazar et al., 2018) and seeds (Zheng et al., 2017; Chen et al., 2019). Zhang et al. (2020) provide a comprehensive summary of RF pasteurisation in different types of agricultural products. Moreover, Dag et al. (2022) and Soto-Rayes et al. (2022)

present a compilation of studies on RF pasteurisation of powder and liquid foods, respectively.

While microwave technology has been approved by the FDA for industrial use as a sterilisation process in 2009, the application of RF pasteurisation in the industry is still under research and development (Barbosa-Cánovas et al., 2022). Currently, there are no regulations which establish specific directives for RF pasteurisation. RF pasteurisation in food processing is thus subject to the general regulatory framework that governs food safety and processing technologies. Hence, research studies focus on specific food products achieving the required microbial reduction on those products. For example, Wei et al. (2021) state that a thermal process which achieves more than a 5-log reduction of *Salmonella* in milk powders, can be considered a pasteurisation process. In some cases, industry groups or organisations might establish best practices or standards for using RF pasteurisation with specific food products and might need to seek approval on a case-by-case basis. Therefore, further research is essential to provide legislation bodies with the necessary data and information necessary to create and shape the legal frameworks of RF pasteurisation or sterilisation techniques.

4.5.1 RF pasteurisation of powders

RF treatments aiming at the inactivation of foodborne bacteria are conducted on a variety of food powders, such as milk powder (skimmed and whole), powdered infant formula milk, cocoa powder, wheat flour, corn flour, grass powder, broccoli powder, and various spices such as cumin, red pepper, white pepper and black pepper (Michael et al., 2014; Wang et al., 2016; Zhang H. et al., 2020b; Lin et al., 2020; Wei et al., 2021; Dag et al., 2022; Tonti et al., 2024). The most common targeted foodborne pathogens are *Salmonella* spp., *Cronobacter sakazaaki*, *Escherichia coli* and *L. monocytogenes* (Dag et al., 2022; Jeong et al., 2020; Zhang Y. et al., 2020a; Ballom et al., 2021; Tonti et al., 2024).

Salmonella is most frequently identified as the leading cause of foodborne outbreaks, especially in low moisture foods (Zhang H. et al., 2020a). The microorganism can survive under hostile environmental conditions for a prolonged period of time (Sekhon et al., 2021). Moreover, the microorganism

has the ability to enter a state of metabolic dormancy, reducing its a_w requirements and enhancing its survival in low a_w environments (Gruzdev et al., 2011). These stresses can influence the thermal resistance of the bacterium. Specifically, *Salmonella* activates various stress response pathways when exposed to low a_w environments, which increases its resistance not only to desiccation but also to other stresses, such as heat (Alvarez-Ordóñez et al., 2015). Most *Salmonella* outbreaks are associated with poor sanitation measures, inadequately designed facilities and equipment, and the ineffective implementation of Good Manufacturing Practices (Podolak & Black, 2017). These factors are responsible for the selection of *Salmonella* as the main target foodborne pathogen in most RF inactivation studies. Dag et al. (2022) review different RF inactivation studies of *Salmonella* in various food powders and the potential to achieve effective pasteurisation against the foodborne pathogen. In the case of powdered infant formula milk, the emphasis is often on targeting *Cronobacter sakazakii*, a pathogen known for its association with severe infections in infants, such as meningitis and septicemia (Lin et al., 2016; Wang et al., 2016; Lin et al., 2020; Zhang Y. et al., 2020a).

Thus far, there have been no known outbreaks of *L. monocytogenes* in powder foods. However, if present in these products, *L. monocytogenes* can survive for over a year during storage (Ballom et al., 2020). Additionally, in low a_w, the thermal resistance of *L. monocytogenes* is increasing (Tsai et al., 2019). Consequently, recent studies explored the potential of different thermal treatments on the inactivation of *L. monocytogenes* in powders (Ballom et al., 2021; Quinn et al., 2021; Sekhon et al., 2021; Yang et al., 2022; Tonti et al., 2024). Lastly, *E. coli* is a Gram negative microorganism commonly found in the intestines of mammals, though certain strains known as Shiga toxin-producing *E. coli* (STEC) can cause severe illness and even lead to death (Daryaei et al., 2018). While *E. coli* cannot grow in food powders, it has the ability to survive in milk powder for up to a year (Paswan & Park, 2020). Furthermore, in 2016, a US multi-state outbreak of Shiga toxin-producing *E. coli* on all-purpose flour was reported by CDC, which led to 17 hospitalisations (Marshall et al., 2020). Kim et al. (2012) and Jeon & Kang (2014) focused on the RF inactivation of *E. coli* in spices.

Many inactivation studies use surrogate microorganisms to assess the efficacy of RF pasteurisation. Surrogates are chosen based on their resistance to the pasteurisation process, which often exceeds that of the actual target

pathogens. The use of surrogates provides a conservative estimate of microbial inactivation, ensuring that the pasteurisation process is effective against the more resistant microorganisms and, by extension, against less resistant pathogenic microorganisms. Additionally, this approach diminishes the risk of working with highly pathogenic organisms in processing halls and minimises the risk of cross-contamination (Hu & Gurtler, 2017). The most common surrogate used in microbial inactivation studies, either thermal or RF validation processes, is *Enterococcus faecium*. *E. faecium* is a Gram-positive, facultative anaerobic bacterium, primarily used as a surrogate for *Salmonella* due to its similar thermal resistance and behaviour under processing conditions (Kopit et al., 2014). Another microorganism commonly used as a surrogate specifically for *L. monocytogenes* due to their similar heat resistance and growth characteristics is *L. innocua* (Milillo et al., 2012).

4.5.2 RF pasteurisation of liquids

The interest in the RF pasteurisation of liquid foods is driven by the need to maintain the nutritional and sensory properties of the products while ensuring microbial safety. Liquid foods can be particularly challenging to sterilise because they can easily lose their quality attributes when subjected to conventional processing thermal treatments (Geveke et al, 2002). The following products have been subjected to RF treatments aiming at inactivation of the non-pathogenic microorganism *E. coli* K12: milk, apple cider, apple juice, orange juice, beer, deionised water, liquid whole egg, and tomato juice (Awuah et al., 2005; Geveke et al., 2002b; Geveke & Brunkhorst, 2004; Geveke et al., 2007; Ukuku et al., 2008; Geveke & Brunkhorst, 2008; Geveke et al., 2012). Regarding the pathogenic microorganisms, Zhu et al. (2021) identified the best treatment conditions for the 5-log reduction of *Salmonella enterica* in liquid whole egg, liquid egg white and liquid egg yolk, while Kou et al. (2018) investigated the inactivation mechanism of *Staphylococcus aureus* in apple juice. The RF systems which are used for RF pasteurisation studies are most commonly FRO systems, either on continuous mode or on batch mode (Di Rosa et al., 2018; Kou et al., 2018; Zhu et al., 2021).

4.5.3 RF pasteurisation of complex foods

As discussed in section 4.4, a significant challenge in the RF processing of non-homogeneous food products is the uneven heating distribution. This inconsistency is due to the different dielectric properties of the different food components, resulting in certain areas heating more quickly or slowly than others. This uneven heating can impact the effectiveness and uniformity of the pasteurisation process.

Nonetheless, some studies explore the application of RF heating on more complex, heterogenous food matrices. Wang et al. (2003) achieved relative uniform RF sterilisation temperatures while heating macaroni and cheese for 30 min and Wang et al. (2012) also using sterilisation temperatures, identified the smallest temperature differences in the position of the components of pre-packaged meat lasagna. However, neither of these studies involved actual microorganisms. Ha et al. (2013) achieved a >4-log reduction of *S.* Typhimurium and *E. coli* in peanut butter cracker sandwiches after 30 s of RF treatment and without detecting temperature differences in the product. Even so, the study noted that the uniform RF heating was a result of the uniformity of the peanut butter spread in between the two crackers. Recently, RF heating was applied to pre-packed non-intact beefsteaks inoculated with Shiga toxin-producing or non-pathogenic *Escherichia coli* strains (Rincon & Singh, 2016). The steaks were subjected to heating temperatures which correspond to rare (60°C for ~14 min) or medium-rare (65°C ~16.5 min) cooking type and a 5-log reduction of the STEC strain was achieve only by RF treatment at 65°C with a holding time at room temperature of 5 min.

4.6 RF treatment of dairy products

RF pasteurisation is considered advantageous over conventional thermal pasteurisation methods due to its ability to rapidly and uniformly heat products, reducing the overall process time and potentially lowering energy consumption. Conventional methods often cause issues like fouling, where residues build up on the heating surfaces due to the heat transfer mechanisms of conduction and convection, leading to operational inefficiencies and the need for frequent cleaning. Furthermore, traditional methods can cause

overheating, nutrient degradation and changes in the sensory qualities of dairy products due to the prolonged exposure to high temperatures (Cappato et al., 2017). RF could provide a more uniform heat distribution and hence, minimise the negative impacts of conventional thermal treatments and align with the dairy industry's growing emphasis on more sustainable and efficient processing practices.

In view of this, several RF inactivation studies centre on processing different dairy products. Table 4.1 provides an overview of the documented studies focused on the RF inactivation of dairy products.

The comparison between RF and conventional thermal treatments has been established mostly on the microbiological or the quality aspects of dairy powders. So far, there have been no reported statistical differences between RF and conventional thermal treatments targeted at inactivation. When comparing hot air-assisted RF to hot water treatment, Zhang et al. (2020) observed no statistically significant differences in D values at 60, 65 and 70°C of *C. sakazakii* at 0.2 a_w present in powdered infant formula milk. Similarly, Michael et al. (2014) documented similar log reductions for both *C. sakazakii* and *Salmonella* spp. using thermal-death-time disks and radio frequency dielectric heating in skimmed milk powder (i.e., non-fat dry milk) . Regarding the quality aspects, RF treatments frequently yield improvements in the overall quality attributes of dairy products. Zhang et al. (2023) reported lower Maillard reaction levels and browning in comparison to conventional (hot water) thermal treatments in powdered infant formula milk. However, both treatments promoted the aggregation of powder particles, a negative phenomenon which can cause implications later in the dairy processing chain. Lin et al. (2020) also concluded that RF assisted traditional thermal processing is more suitable for the treatment of powdered infant formula milk than traditional thermal processing, as it led to lower lipid oxidation and considerably shorter processing time.

Table 4.1: Overview of the RF inactivation studies on dairy products.

Microstructural category	Product	Treatment	Target	Major Results	Reference
Powder	Low-heat SMP High-heat SMP	RF used until target temperature, convection oven holding for specific time periods. RF set-up: 27.12 MHz, 3 kW and 120 mm electrode distance. Convection oven preheated at target temperature.	*C. sakazakii* *Salmonella* spp.	-- D-values of *Salmonella* spp. were 23.02 and 24.94 min at 75°C, 10.45 and 12.54 min at 80°C, 8.63 and 8.68 min at 85°C, and 5.82 and 4.55 min at 90°C for HH- and LH-NFDM, respectively. -- D-values of *C. sakazakii* were 24.86 and 23.00 min at 75°C, 13.75 and 7.52 min at 80°C, 8.00 and 6.03 min at 85°C, and 5.57 and 5.37 min at 90°C for HH- and LH-NFDM, respectively.	Michael et al., 2014
Powder	PIFM	Combined RF and hot air treatment. RF set-up: 6 kW, 27.12 MHz. Auxiliary 5.6 kW hot air system preheated at target temperature.	*C. sakazakii*	-- D-values for sample with 0.3 a_w at 60, 65, 70 and 75°C were 77.9, 50.3, 29.9 and 15.6 min, respectively.	Wang et al., 2016

Microstructural category	Product	Treatment	Target	Major Results	Reference
				-- 1-log reduction of *C. sakazakii* was obtained during come up time of 70°C. -- Increase of sample's a_w led to decrease of D and z values.	
Powder	PIFM	Combined RF and hot air pasteurisation (RF-HA). RF set-up: 6 kW, 27.12 MHz. Auxiliary 6.3 kW hot air system preheated at target temperature.	*C. sakazakii*	-- D-values for sample with 0.3 a_w at 55, 60, 65 and 70°C were 209.1, 48.7, 24.0 and 17.3 min, respectively. -- Microbial thermal resistance reduced by increasing temperature (55–70°C) and a_w (0.2–0.4 at 25°C). -- D-values of RF-HA were not statistically significantly higher than D-values of hot water treatments in samples with 0.2 a_w.	Zhang et al., 2020

Microstructural category	Product	Treatment	Target	Major Results	Reference
Powder	PIFM	Radio frequency assisted traditional thermal processing (RF assisted-TTP). RF set-up: 6 kW, 27.12 MHz. RF used until target temperature, preheated convection oven holding for specific time periods.	*C. sakazakii*	-- D-values for sample with 0.2 a_w at 65°C was 4.2 h. -- A 5-log reduction was achieved after 21 h oven holding at 65°C	Lin et al., 2020
Powder	WMP SMP	Hot air-assisted radio frequency (HARF). HARF used until target temperature, convection oven holding for specific time periods. Convection oven preheated at target temperature. RF set-up: 6 kW, 27.12 MHz, parallel-plate and 130 mm electrode distance.	5-serotype *Salmonella* cocktail	-- More than 5-log reduction was achieved for both types of milk powder with 0.10 a_w after 15 min at 95°C -- More than 5-log reduction was also achieved in WMP after holding at 90°C for 30 min or 95°C for 10 min	Wei et al., 2021

Microstructural category	Product	Treatment	Target	Major Results	Reference
Powder	WMP	RF used until target temperature, convection oven holding for specific time periods. RF set-up: 27.12 MHz, 6 kW, FRO. Convection oven preheated at target temperature.	*E. faecium* NRRL B-2354 4-serotype *S.enterica* cocktail	-- A 5-log reduction of *Salmonella* was achieved after 2 h oven holding at 75°C -- *E. faecium* NRRL B-2354 can be used as a surrogate for *Salmonella*	Dag et al., 2022
Powder	SMP WMP	RF set-up: 4 kW, 27.12 MHz, 50 Ω RF set-up. Dynamic RF-only heating treatments were used. 2.5 kg of milk powder were first heated to 55°C at a power of 1 kW (0.4 kW/kg), and then heated further at a slower rate at a power of 0.2 kW (0.08 kW/kg) until target temperatures.	*E. faecium* NRRL B-2354 *S.* Typhimurium *L. monocytogenes*	-- A 4-log reduction of *S.* Typhimurium was achieved after 2.3 h and 1.3 h in SMP and WMP, respectively. -- A 4-log reduction of *L. monocytogenes* was achieved after 1.2 h and 1.5 h in SMP and WMP, respectively. -- *S.* Typhimurium was more resistant to the RF treatment than *L. monocytogenes* -- RF inactivation was higher in WMP than in SMP for both microorganisms	Tonti et al., 2024

Microstructural category	Product	Treatment	Target	Major Results	Reference
				-- *E. faecium* can be used as a surrogate for both microorganisms and the specific conditions	
Liquids/ Emulsions	Milk	RF heating under continuous flow conditions RF set-up: lab scale, 2 kW, 27.12 MHz RF	*E. coli* K-12 *L. innocua*	-- 5-log reduction of *L. innocua* and 7-log reduction of *E. coli* were achieved after treating at 1200 W for 55.5 s at temperature of approximately 65°C -- *L. innocua* was more resistant to the RF treatment than *E. coli*	Awuah et al., 2005
Liquids/ Emulsions	Milk	Radio frequency electric field (RFEF), RF set-up: special design, 40 MHz and 1.6 kV/cm electric field strength	Total bacterial count	-- A reduction of bacterial population by treatment at 72.3°C for 2 s by 109260 cfu/ml	Srisuma et al., 2017

Microstructural category	Product	Treatment	Target	Major Results	Reference
Liquids/ Emulsions	Milk	RF heating in combination with steam RF set-up: lab-scale 2.21 kW, 27.12 MHz	Total microbial count	-- 2- log reduction of TMC was achieved after treatment at temperatures above 80°C -- >4.5- log reduction of TMC was achieved after treatment at temperatures above 110°C	Di Rosa et al., 2018
Complex structure	Cheddar Cheese	RF oscillator with a (possible) power output of 750 W. RF treatments at ~47-68°C for 1.5 to 2.7 min. Target temperature maintained at core for 30 min.	Total plate count	-- Total plate count reduced in all treatments by 99.99% -- Storage at ~15°C for 2 months led to an increase in total bacterial count	Kosikowsky et al., 1949
Complex structure	Yogurt	RF water bath RF set-up: pilot scale, 16 kW, 27.12 MHz Treatments: -60 s at 58°C	Lactic acid bacteria	-- Treatment at 72°C for 120 s abandoned due to overheating -- Inactivation of YMC after each treatment	Siefarth et al., 2014

Microstructural category	Product	Treatment	Target	Major Results	Reference
		-90 s at 65°C -120 s at 72°C where holding time was 60 s	Yeast count Mould count	-- Partial survival of LAB after RF heating at 58 and 65°C	

Part II
Research and Publications

Chapter 5

Survival of *S.* Typhimurium and *L. monocytogenes* in dairy model systems: powder

The results presented in this chapter have been published in:

Tonti Maria, Verheyen Davy, Skåra Torstein, Van Impe Jan. Modelling the Behaviour of *Salmonella* Typhimurium and *Listeria monocytogenes* in skimmed and whole milk powder during storage at 20ºC. *Proceedings of FOODSIM 2022* (2022): 54-58.

The contributions of the first author to this work are:

- Literature study
- Co-design of experiments
- Co-performing of experiments
- Co-interpretation of results
- Co-formulation of conclusions
- Text redacting and editing

This chapter includes the referred paper. Part of the content is identical to the content of the submitted paper. However, some adaptations in content and formatting have been made.

Context: In this chapter, the behaviour of *S.* Typhimurium and *L. monocytogenes* in skimmed and whole milk powder is documented, during storage at 20°C for a month. The aim was to verify that the specific microorganisms are relevant in dairy powders, i.e., (i) can survive after their introduction (inoculation) to the powder and (ii) can survive over extended storage. These findings highlighted the necessity of RF processing for the inactivation of *S.* Typhimurium and *L. monocytogenes* in the following chapters.

Abstract

Milk powder is one of the most commonly used food products which can also be utilised as an ingredient in the manufacturing process of other dairy products. In consequence, reassuring the safety of this product is of significance to the food industry. Pathogens can contaminate the powder and survive, leading to recalls and possible outbreaks. This study explores the behaviour of two foodborne pathogens, *Salmonella* Typhimurium and *Listeria monocytogenes,* when inoculated in skimmed and whole Milk powder and stored at 20°C for one month. The Geeraerd Shoulder-Tail (2000) inactivation model was fitted to the data. Out of the two microorganisms, *Salmonella* demonstrated the higher resistance. Additionally, a higher content of fat led to an increase in the inactivation rate for both bacteria, and DNA Damage was observed for the most sensitive microorganism, *L. monocytogenes.*

5.1 Introduction

For decades, low moisture food (LMF) products, i.e. products with water activity (a_w) of 0.85 or below, have been considered microbiologically stable, if not safe. Most of these products undergo different processing methods (e.g., drying) which lead to the loss of water from the product, and subsequently, lower a_w values (<0.60) (Rachon et al., 2016). In this range of a_w, microorganisms are unable to grow and multiply. However, there are certain foodborne pathogens (microorganisms) that can survive the several processing steps and remain viable in the product during prolonged storage (Farakos et al., 2013). So far, there have been numerous outbreaks concerning LMF (Rachon et al., 2016), demonstrating that the food safety of these products has to be taken into consideration.

One of the products implicated in several outbreaks is milk powder (Sekhon et al., 2021). This product is usually produced by spray drying, a process which can be survived by certain pathogens such as *Cronobacter sakazakii*, *Salmonella*, *Listeria monocytogenes*, and pathogenic strains of *E. coli* (Ballom et al., 2020). Even though most dried milk outbreaks are linked to *Salmonella* (Sekhon et al., 2021) , many recalls of *L. monocytogenes* in LMF have been reported, and recent studies demonstrate the microorganism's ability to survive in milk powder (Ballom et al., 2020).

In view of these events and in addition to the ever-possible risk of cross-contamination during the manufacturing process, it is crucial to understand the survival behaviour of foodborne pathogens in LMF products. Additionally, the Covid-19 crisis led consuming habits towards products with long shelf life (e.g., LMF), highlighting the demand to certify the safety of this food category (Bakalis et al., 2020). In this study, two microorganisms with different Gram properties, *Salmonella enterica* serovar Typhimurium (Gram-negative) and *L. monocytogenes* (Gram-positive), were inoculated in skimmed and whole (35% fat) milk powder, and stored for 30 days at 20°C in order to document their behaviour. Afterwards, inactivation models were fitted to the data acquired from the microbiological analyses to generate the inactivation curve and assess the sublethal injury. DNA Damage was investigated for the

microorganism that demonstrated sufficient inactivation in a subsequent experiment.

5.2 Materials and methods

5.2.1 Microorganisms and inoculum preparation

The two pathogenic bacteria used for this study were *Salmonella enterica* serovar Typhimurium ATCC 14028 (isolated from bovine, septicaemic liver) which was obtained from the Gothenburg Bacterial Collection, and *L. monocytogenes* LMG 23356 (isolated from Jalisco Cheese) obtained from the bacteria collection of Ghent University. Stock cultures of the microorganisms were stored at -80°C in 2 ml cryovials. One bead containing one individual colony was transferred to an Erlenmeyer flask containing 20 mL broth, BHI (Merck, Darmstadt, Germany) for *L. monocytogenes* and Tryptone Soy Broth (i.e., TSB, Oxoid, Basingstoke Hampshire, England) for *S.* Typhimurium. The Erlenmeyers were placed at 30° (for *L. monocytogenes*) and 37°C (for *S.* Typhimurium). After 24 hours, a loopful was streaked on a petri dish containing general medium agar, specifically Brain Heart Infusion (i.e., BHI, Merck, Darmstadt, Germany) for *L. monocytogenes* and a Tryptone Soy Broth (i.e., TSA, Merck, Darmstadt, Germany) for *Salmonella*, incubated at 30°C for 48 hours and 37°C for 24 hours, respectively. Afterwards, two consecutive 24-hour precultures were created from individual colonies taken from each plate. The second preculture, after 24 hours with a cell density of approximately 10^9 CFU/mL, was transferred to a sterile 30 mL spray bottle (Glasatelier Saillart, Meerhout, Belgium) which was used for the milk powder inoculation. For each microorganism, three individual replicates were created from single colonies of the purity plate, representing three biological replicates.

5.2.2 Milk powder inoculation procedure

Skimmed and whole milk powder (35% fat) were obtained from TINE SA (Oslo, Norway) and stored in 1 kg bags under vacuum. The microbial

background flora was checked for each bag by conducting random sampling and plating on TSA supplemented with 0.6% yeast extract (i.e., TSAYE, Merck, Darmstadt, Germany).

The inoculation procedure followed was based on the protocol of Michael et al. (2014). Specifically, 10 g of milk powder (skimmed or whole) was weighted in a sterile stomacher bag under sterile conditions (laminar flow cabinet) and inoculated with 0.1 mL of the second preculture by spraying 2 times into the powder. Three individual biological replicates (n = 3) were prepared for each microorganism. The bags were then inserted into a 37ºC incubator for 24 h for the powder to dry and for the bacteria to acclimate to the new environment. After the drying stage, the bags with the inoculated milk powder were placed in a second stomacher bag, which was sealed. The sealed bags were stored inside an incubator set at 20ºC.

5.2.3 Sampling during storage

The storage period lasted 30 days, and 1 g samples were taken from each bag every day, 5 days per week. The reduction of the milk powder from each bag during sampling could result in low numbers towards the end of the storage period and therefore affect the microorganism behaviour. In order to avoid a similar effect on the bacterial survival, for each biological replicate, nine separate inoculated bags were created. Consequently, upon completion, only 2-3 g of the inoculated milk powder was removed out of each bag. Samples were taken under sterile conditions, where 1 g of the inoculated powder was placed in a 15 mL falcon tube containing 9 mL 0.9% saline solution. The rehydrated samples were vortex mixed thoroughly for 1 min and serially diluted. The plate count technique (drops of 20 μL) was used to determine viable cell counts, on nonselective and selective media. For *L. monocytogenes*, BHI Agar and PALCAM (Merck, Darmstadt, Germany) were used as nonselective and selective media, respectively. For *S.* Typhimurium, TSA and Xylose Lysine Deoxycholate Agar (XLD) (Merck, Darmstadt, Germany) were used as a nonselective and selective media, respectively. After plating, the BHI and PALCAM plates were placed in an incubator set at 30ºC and TSA and XLD plates were incubated at 37ºC for 24 hours, after which followed the enumeration.

5.2.4 Mathematical modelling

The Geeraerd Shoulder-tail model (Geeraerd et al., 2000), as represented by Equation 5.1, was fitted to the data using MatLab® version R2021a (The Mathworks Inc., Natick, USA).

$$N_t = (N_0 - N_{res}) \cdot exp(-k_{max} \cdot t) \cdot \left(\frac{exp(k_{max}S_1)}{1 + (exp(k_{max}S_1)-1) \cdot exp(-k_{max}t)}\right) + N_{res} \quad (5.1)$$

Where N_t is the population at time t (CFU/g), N_0 the population at time 0 (CFU/g), N_{res} the residual population density (CFU/g), k_{max} the specific inactivation rate (day^{-1}), and S_l the shoulder phase (day). The Mean Squared Error (MSE) was used as a measure of goodness-of-fit for the model.

5.2.5 Evolution of sublethal injury

When microorganisms are exposed to environmental stress conditions (e.g., low a_w), cell populations can be present as (i) irreversibly damaged (dead) cells, (ii) healthy cells and (iii) sublethally injured (SICs) cells (Akkermans et al., 2021). This last category involves cells that have been injured and even though they can grow on general, nonselective media, are not able to grow on the selective ones. Sublethal injury (SI) depicts the percentage of the cells that have been damaged but retain the ability to grow under favourable environmental conditions (Equation 5.2).

$$SI\ (\%) = \frac{N_{nonselective} - N_{selective}}{N_{nonselective}} \cdot 100\% \quad (5.2)$$

where $N_{nonselective}$ is the cell density (CFU/g) measured on general media and $N_{selective}$ the cell density (CFU/g) measured on selective media (Verheyen et al., 2019a).

The evolution of the percentage of SI with time for the two pathogenic microorganisms was modelled using the model of Akkermans et al. (2021) (Equations 5.3, 5.4).

$$\frac{dN_H(t)}{dt} = k_{H,max} \cdot \left(1 - exp\left(N_{H,res} - N_H(t)\right)\right)$$

$$with\ N_H(t=0) = N_{H0} \quad (5.3)$$

$$\frac{\mathrm{d}N_C(t)}{\mathrm{d}t} = k_{C,max} \cdot \left(1 - exp\left(N_H(t) - N_C(t)\right)\right)$$

$$with\ N_C(t=0) = N_{C0} \quad (5.4)$$

with $N_{H,res}$ and $N_{C,res}$ the resistant subpopulations of the healthy and culturable cells. The obtained parameters $N_{H,res}$, $N_{C,res}$ for each time point (t) were then used in Equation 2 to acquire the SI (%).

Time-averaged Injured Cells Coefficient (TICC) was used to quantify the total amount of SI (equation 5.5) (Miller et al., 2006).

$$TICC = \frac{\int_0^{t_f} SI(t)dt}{t_f} \quad (5.5)$$

with t (days), the sampling time; t_f (days), the total duration of the storage time; and SI (-), the percentage of sublethal injury according to the curve acquired by equation 2.

5.2.6 DNA damage

Following the experimental results for *L. monocytogenes*, further experiments were conducted to assess the DNA damage during storage according to the protocol of Govaert et al. (2020b). SYBR Green I (Fischer Scientific, Merelbeke, Belgium) was used in combination with fluorescence analysis for the detection and quantification of double-stranded DNA (dsDNA). Specifically, three biological replicates of *L. monocytogenes* in skimmed and whole milk powder were created after following the same inoculation

procedure as described above. The inoculated products were placed in the same storage conditions for 30 days and sampling took place at days 0, 1, 9, 19 and 30. Samples were subjected to DNA extraction using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Fluorescence was measured at excitation (485 nm) and emission (525 nm) using a FilterMax F5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). DNA Damage was identified as the destruction of dsDNA led to the formation of single-stranded DNA (ssDNA) and hence a reduction in absorbance. In parallel, plate counts were also performed on nonselective and selective media.

5.2.7 Statistical analysis

Analysis of Variance (ANOVA, single factor) with a confidence level of 95% ($\alpha = 0.05$) was conducted to determine significant differences between model parameters and the DNA Damage analysis. Means that differentiated between each other were established by Fisher's Least Significant Difference test. The analyses were performed using MatLab version R2021a (TheMathworks Inc., Natick, USA). Test statistics were considered as significant when $p \leq 0.05$.

5.3 Results and discussion

5.3.1 Modelling of the bacterial behaviour

In order to compare the parameter values of the two pathogens in the two types of milk powder, the Geeraerd Shoulder-Tail model was used to describe the microorganism inactivation. After fitting the model on the experimental data, the parameter values were identified and shown in Table 5.1. The inactivation curves are illustrated in Figures 5.1 and 5.2, for *L. monocytogenes* and *Salmonella* Typhimurium, respectively.

Table 5.1: Parameter values of the Geeraerd et al. (2000) model for *L. monocytogenes* and *S.* Typhimurium in skimmed (SMP) and whole milk powder (WMP). Significant differences between the microorganisms inoculated in each product for each microorganism are indicated by different uppercase letters. Statistically significant differences between the two products for each microorganism are indicated by different lowercase letters. The lowest values are indicated by 'A' and 'a'.

	L. monocytogenes		*S.* Typhimurium	
Model Parameters	SMP	WMP	SMP	WMP
N_0	6.15 ± 0.10^{aB}	6.33 ± 0.07^{bB}	5.48 ± 0.18^{aA}	5.54 ± 0.079^{bA}
S_l	8.45 ± 3.96^{aA}	14.06 ± 2.06^{aB}	9.61 ± 4.13^{aA}	12.24 ± 1.63^{bA}
k_{max}	0.18 ± 0.07^{aA}	0.23 ± 0.06^{bA}	0.33 ± 0.30^{aB}	0.85 ± 0.95^{bB}
N_{res}	4.59 ± 0.38^{aA}	4.75 ± 0.39^{bA}	4.97 ± 0.08^{aB}	5.07 ± 0.06^{bB}
MSE	0.07	0.06	0.10	0.12

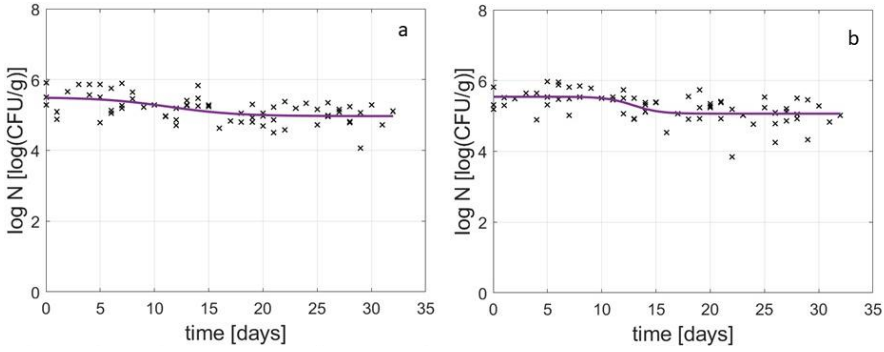


Figure 5.1: Modelling of *L. monocytogenes* in (a) skimmed milk powder and (b) whole milk powder with Geeraerd et al. Shoulder-Tail (2000).

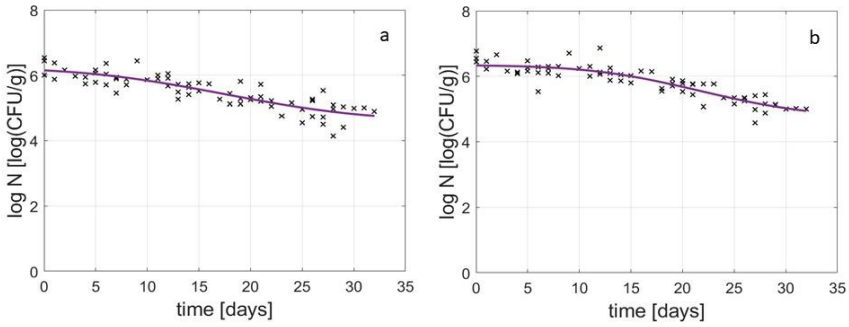


Figure 5.2: Modelling of *S.* Typhimurium in (a) skimmed milk powder and (b) whole milk powder with Geeraerd et al. Shoulder-Tail (2000).

In the skimmed milk powder, the maximum specific inactivation rate k_{max} was almost equivalent for both microorganisms, even though they had an initial cell density difference of 0.5 log (CFU/g). Nonetheless, *S.* Typhimurium exhibited higher resistance and adaptability in milk powder, as shown by the higher residual cell population N_{res}. Comparison of the maximum specific inactivation rate k_{max} between the two products demonstrates that for *S.* Typhimurium, the presence of the fat has an effect on their inactivation. Wei et al. (2021) highlighted the importance of the food matrix in the thermal resistance and survival of *Salmonella* during prolonged storage, where inoculated whole milk powder had higher D values in comparison to skimmed milk powder. Similar results concerning LMF, specifically peanut butter samples (with a_w 0.36-0.47), were reported by Li et al., (2014), with a reduction in fat concentration leading to an increase in *Salmonella* thermal resistance. However, a higher residual population of *L. monocytogenes* in whole milk powder indicates a possible protective effect of the fat on *L. monocytogenes*. Overall, an especially complex relationship between fat content and a structured food matrix is reported. For example, Verheyen et al. (2020) observed that fat had a protective effect on *L. monocytogenes* cells in emulsions and gelled emulsions systems.

Nevertheless, the two products differ not only in fat level, but also for other nutrients. Skimmed milk powder has a higher concentration of carbohydrates and proteins, and these components can also affect the microbial survival. For example, in solutions with low a_w and high sucrose concentration, *L. monocytogenes* and *S.* Typhimurium were more resistant to heat (Sumner et al., 1991). These factors should hence be taken into account in the development

of future models. Additionally, certain model fits demonstrated a very slow inactivation during the 30 days, indicating that a longer storage period is required to gain more insight into the inactivation kinetics of the two microorganisms.

5.3.2 Evolution of sublethal injury

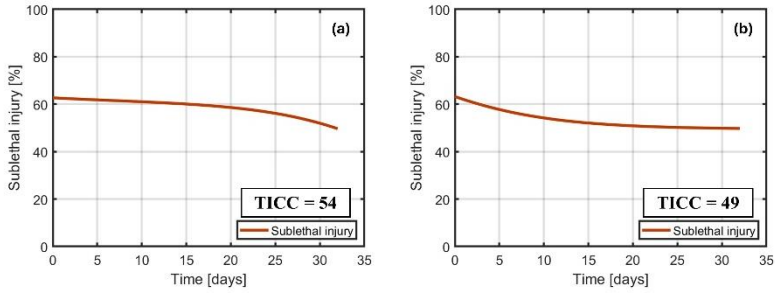


Figure 5.3: SI model of *L. monocytogenes* in (a) skimmed milk powder and (b) whole milk powder.

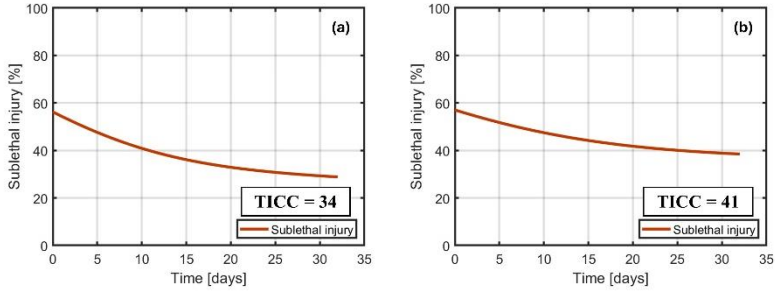


Figure 5.4: SI model of *Salmonella* Typhimurium in (a) skimmed milk powder and (b) whole milk powder.

The SI throughout the storage period is shown in Figure 5.3 for *L. monocytogenes* and 5.4 for *S.* Typhimurium. A significant amount of SI was observed from the beginning of the storage period (day 0). This phenomenon is attributed to the 24 h storage at 37°C during the inoculation protocol. The highest percentage of SI was observed in *L. monocytogenes* in both skimmed

and whole milk powder. *S.* Typhimurium in whole milk powder had also a higher TICC value in comparison to skimmed milk powder. These results suggest that the relationship between the presence of fat in milk powder and cell injury after prolonged storage is rather complex and microorganism-specific.

5.3.3 DNA Damage

Following the results of the inactivation curves and the SI, in order to gain more insight on the type of injury on the microbial cell, assessment of the stability of the structural DNA was performed. The reduction in absorbance in Figure 5.5 indicates the decrease of dsDNA of *L. monocytogenes* in milk powder during storage, hence suggesting that one of the mechanisms that could contribute to the inactivation is DNA damage. The difference in the absorbance in the two powders may stem from the influence of the product ingredients, as in both products, the initial cell density was similar.

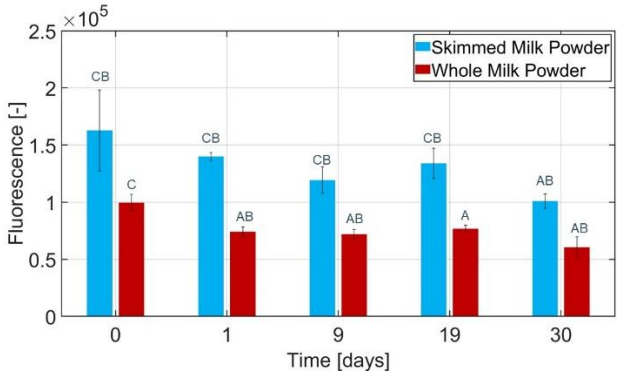


Figure 5.5. Fluorescence measurements (-) demonstrating the DNA Damage of *L. monocytogenes* in skimmed and whole milk powder during storage for 30 days (n = 3). Statistically significantly different values ($p \leq 0.05$) are illustrated by different uppercase letters.

5.4 Conclusions

Both *L. monocytogenes* and *S.* Typhimurium were able to survive in skimmed and whole milk powder during 1-month storage at 20°C, with *Salmonella* being the most resistant. Even though the inactivation model of Geeraerd et al., (2000) was able to describe their behaviour for a time period of 30 days, longer storage is required for the model to characterise the bacterial inactivation more accurately. A critical analysis of the model parameters in addition to the sublethal injury and DNA damage results indicated that the presence of fat influences the maximum inactivation rate. However, both skimmed and whole milk powder are complex products that differ in more than just their fat content, and therefore, more attention has to be drawn to the influence of individual contents separately. The damage to the DNA of microbial cells on milk powder during extended storage was demonstrated in this work. However, other cell components, apart from the DNA, may also become damaged during storage. In a continued research effort, damage to other cell components of both *L. monocytogenes* and *S.* Typhimurium will hence also be investigated. Possible research directions include the elucidation of morphological changes to the cell structure and damage to the cell membranes. In addition, the influence the cell damage occurring during the storage period on the efficacy of subsequent inactivation treatments (e.g., conventional thermal processing, radio frequency processing) will be investigated.

Acknowledgments

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Chapter 6

Development of a RF-only dynamic heating treatment for dairy model systems: powder

The results presented in this chapter have been submitted to *Food Science and Technology International*:

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The contributions of the first author to this work are:

- Literature study
- Co-design of experiments
- Co-performing of experiments
- Co-interpretation of results
- Co-formulation of conclusions
- Text redacting and editing

This chapter includes the referred paper. Some adaptations in formatting have been made.

Context: This chapter focuses on assessing different RF-only dynamic heating treatments using different heating rates and final target temperatures. As the findings of the previous study reported that *S.* Typhimurium was the most resistant microorganism, it was used as a case study to evaluate the efficacy of each treatment. A pilot-scale RF set-up was used for the treatment of milk powders. As foodborne pathogens are not allowed in an open system in food processing halls, the non-pathogenic microorganism *E. faecium* was used to record the temperature profile of each treatment. The effect on two physicochemical properties closely related to the quality of milk powder characteristics, solubility and colour, was also assessed.

Abstract

Radio frequency (RF) is a dielectric heating technology that allows rapid and volumetric heating of milk powder, outperforming the heating uniformity of conventional powder heating methods. Typically, RF milk powder processing consists of a fast RF heating phase, followed by an oven heating phase in temperatures around 90°C. This methodology can result in milk powder quality deterioration due to non-uniform temperature distributions and local overheating. RF-only processes with a more gradual heating rate are alternative solutions to minimise the impact on milk powder quality. This study investigated the effect of the heating rate on the microbial inactivation of *Salmonella* Typhimurium inoculated in skimmed milk powder, as well as the effect of each process on two quality characteristics, colour and solubility. Overall, a slower heating profile resulted in sufficient inactivation rates of *Salmonella* in skimmed milk powder, while still providing a high-quality end product. A 4-log reduction was achieved by treating the skimmed milk powder up to 95°C using a slower, longer heating rate. No statistically significant changes were observed in the solubility of skimmed the milk powder and only the harshest treatment to 95°C led to a slight increase in the yellowness of the skimmed milk powder colour.

6.1 Introduction

Food safety is currently the most pressing issue of the food industry, a result of a dramatic increase in large-scale, intercontinental outbreaks originating from microbial contamination in a variety of food products (EFSA & ECDC, 2019). Many recent studies have often identified the culprit to be a Low Moisture Food (LMF), a food category that is usually considered safe, by both the general population and the industry, on the grounds that the lack of water cannot support microbial growth (Dag et al., 2020). Specifically, these products have a water activity (a_w) < 0.6 and this parameter is even used as a criterium for the identification of critical control point for Hazard Analysis and Critical Control Points (HACCP) programs. However, LMFs can act as Trojan horses, introducing pathogenic bacteria that have survived different processes such as spray drying, later in the production process, and giving them the possibility to grow and increase in number, eventually causing foodborne illnesses (Lucore et al., 2017).

Milk powder is one of the most frequently used LMF ingredients in ready-to-eat foods and desserts. The product was also the causative agent in numerous outbreaks over the last few years. Pathogenic bacteria either survive the spray drying process or are introduced later in the production chain through cross-contamination (Podolak and Black, 2017). Foodborne pathogens associated with most contaminated milk powder cases are *Salmonella* spp., *Cronobacter sakazakii*, pathogenic *Escherichia coli*, and *Listeria monocytogenes* (Ballom et al., 2020). One of the most common pathogens associated with outbreaks or recalls in dairy powders is *Salmonella enterica* (Paswan and Park, 2020). *Salmonella* can survive both the spray drying process and subsequent extended periods of storage (Sekhon et al., 2021). Moreover, as the a_w of the product decreases, the thermal resistance of *Salmonella* increases, highlighting the necessity to develop effective inactivation measures (Wei et al., 2020).

Therefore, to ensure the consumer's safety, additional safety steps are required further in the process. The most commonly used traditional thermal method for powder decontamination is using high heat at 71.06°C for 15 s followed by air drying at inlet temperatures of 135-205°C for 5-6 s. While this thermal treatment ensures microbiological milk powder safety, it requires a

substantial amount of time and energy to reach higher temperatures. Additionally, the treatment exerts a detrimental effect on milk powder quality, concerning organoleptic (e.g., flavour) and nutritional (e.g., vitamin content) factors (Rifna et al., 2019; Wang et al., 2016). Radio frequency (RF) processing is a technology that could provide solutions for the aforementioned challenges related to the safety of milk powder. This technology employs electromagnetic radiation, which is applied to a product through two parallel plates. The generated electromagnetic energy initiates molecular friction, inducing heat throughout the product, i.e., volumetric heating (Altemimi et al., 2019). This heat generation method avoids surplus treatment times and provides a more uniform temperature distribution in the bulk powder, resulting in a milder impact on milk powder quality attributes compared to conventional heating (Rifna et al., 2019).

A common strategy used for the thermal treatment of LMF by RF consists of two stages, i.e., (i) an initial non-isothermal RF heating stage, during which the product temperature is constantly increasing, and (ii) an isothermal oven or air-circulation stage during which the high temperature is maintained (Michael et al., 2014; Wang et al., 2016; Wei et al., 2021). While this approach leads to reduced treatment time, it can also cause non-uniform temperature distribution and, consequently, quality degradation due to local overheating (Dag et al., 2020). A possible prospective solution is to design RF inactivation treatments that employ a low heating rate process without negatively impacting the product quality. Recently, Liu et al. (2022) validated the efficacy of RF-assisted low-temperature long-time (LTLT) treatment of onion powder against *Salmonella enterica* serovar Enteritidis without considerable quality loss.

The objective of this study was to assess the efficacy of a novel RF-only process employing a solely dynamic heating profile, using both high and low heating rates. *Salmonella* Typhimurium inoculated in skimmed milk powder was selected as a case study. This bacterial target was chosen as the presence of *Salmonella* in milk powders is regulated by European legislation (EC 1441/2007). Two of the most important quality characteristics of milk powder, colour and solubility, were also tested to ensure the absence of deteriorative changes, which would render the process unmarketable.

6.2 Materials and methods

6.2.1 Materials and microorganisms

One bag of 25 kg skimmed milk powder (SMP) was purchased from TINE SA (Oslo, Norway). After opening the bag, the content was immediately distributed in portions of 1 kg, vacuum packed (95%) in plastic bags and stored dry at room temperature. The microbial background flora was inspected by random sampling in each bag and plating on TSA (Merck, Darmstadt, Germany) supplemented with 0.6% yeast extract (Merck, Darmstadt, Germany). The plates were then incubated at 37°C for 72 hours.

This case study focused on the pathogenic strain *Salmonella enterica* serovar Typhimurium ATCC 14028, isolated from bovine, septicaemic liver, acquired from the Gothenburg Collection. The non-pathogenic microorganism *Enterococcus faecium* ATCC 8459 was obtained from the NCIMB collection in UK. *E. faecium* and was used as a surrogate only when working with the pathogen was not possible, i.e., to avoid food processing hall contaminations during temperature measurements. *E. faecium* is commonly used as a surrogate for microbial inactivation studies of *S*. Typhimurium in low moisture foods due to its higher heating resistance and its ability to survive in low moisture conditions (Villa-Rojas et al., 2017, Wei et al., 2018, Ozturk et al., 2019, Dag et al., 2022). In this study, the surrogate microorganism *E. faecium* was used to inoculate skimmed milk powder for the temperature measurements, as pathogenic strains such as *Salmonella* are prohibited in processing plants. The microorganisms were stored at -80°C in 2 mL cryovials (Microbank™, Pro-Lab Diagnostics, Canada).

6.2.2 Skimmed milk powder inoculation

Identical procedures were followed for the inoculum preparation of both the pathogen and the surrogate microorganism. One bead from the cryovial stock culture was placed in 20 mL TSB (Oxoid, Basingstoke Hampshire, UK) and incubated at 37°C for 24 h. Subsequently, a purity plate was created on non-selective medium TSA which was then incubated at 37°C for 24 h. Biological replicates of each microorganism were created by taking one individual

colony from the purity plate and performing two consecutive 24-hour precultures. Following the incubation period, the cell density of the preculture was approximately 109 CFU/mL. The skimmed milk powder was inoculated using a protocol based on Ozturk et al. (2019). Initially, 10 g of skimmed milk powder was weighed in a sterile stomacher bag (GRADE, UK) under sterile conditions. Then, 0.1 mL of the second preculture was pipetted in one, swift motion over the powder. The bag was hand-massaged for 5 min and inserted into a desiccator placed in a 37°C incubator for 24 h, for the powder to dry and for the bacteria to acclimate to the new environment (adaptation phase). After the 24 h, the bags were hand-massaged again for one minute to fragment possible clumps and for additional distribution of the bacteria. At this stage, obtained cell densities of *E. faecium* and *S.* Typhimurium were approximately 106 CFU/g and 105 CFU/g, respectively. In order to start with a cell density of 106 CFU/g (i.e., necessary to detect a 4 log reduction in cell counts), an additional concentration step was used to increase the cell density of *S.* Typhimurium. The step involved the centrifugation of 10 mL of the preculture and its resuspension in 1 mL of TSB medium which was used for the powder inoculation. It was crucial for the inoculated samples to (i) have similar a_w to commercial food powders and (ii) also have equivalent a_w values between them. Measurements using the Aqualab 4TE a_w measuring meter (METER Group AG, Germany) at 25°C confirmed that the a_w before and after inoculation showed no statistically significant difference, with a value of 0.256 ± 0.007.

From each bag with inoculated skimmed milk powder, 1 g was transferred into a small stomacher bag (GRADE, UK) and resuspended at a 1:10 concentration with 0.9% saline solution to measure the initial cell density prior to the RF treatment experiments. Then, 6 g of powder was weighed in a 35x10 mm sterile, polystyrene petri dish with lid (Sarstedt, Oslo, Norway). The closed container was wrapped in thermal resistant, adhesive tape to avert leakage of contaminated powder granules to the RF cavity and processing quarters, and prevent moisture transfer with the environment.

6.2.3 RF processing of skimmed milk powder

The RF treatment was conducted in a 4 kW, 27.12 MHz, pilot scale 50 Ω set-up (SAIREM, Lyon, France), depicted in Figure 6.1. The electrode height for

each treatment was set at 70 mm. A special Polytetrafluoroethylene (PTFE) tray with rounded edges was designed for the RF treatments, as depicted in Figure 6.2. The dimensions were: 430 mm (L) x 330 mm (W) x 50 mm (H). The specific tray included a removable insert at the centre (Figure 6.2i) to position three sample-containing petri dishes symmetrically (Figure 6.2ii). For the temperature measurements and the quality determination experiments, all three petri dishes contained the surrogate microorganism. For the inactivation experiments, two petri dishes contained the pathogenic microorganism and one contained the surrogate microorganism to record the temperature profile during treatments. Initially, 1 kg of non-inoculated skimmed milk powder was dispersed uniformly in the tray with the disk at a depth of 10 mm, after which the petri dishes were placed in their respective positions (Figure 6.2iii). Afterwards, they were covered by 1.5 kg non-inoculated skimmed milk powder, and the product surface was levelled with a 430 mm ruler (Figure 6.2iv). The tray was placed on the grounded electrode, with its centre aligned to the centre of the upper electrode. For the temperature uniformity experiments, three RF process runs were performed. Each process run contained three petri dishes with biological replicates of the surrogate microorganism (three samples). For the quality and inactivation effect experiments, two RF process runs were performed, where two biological replicates of the pathogen (two samples) were used in each process run for the inactivation experiments and three biological replicates of the surrogate microorganism (three samples) were used in each process run for the quality experiments.

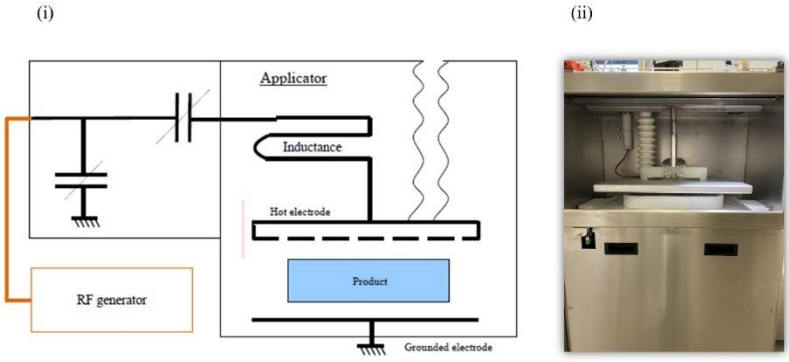


Figure 6.1: Schematic diagram (i) and (ii) picture of the RF set-up

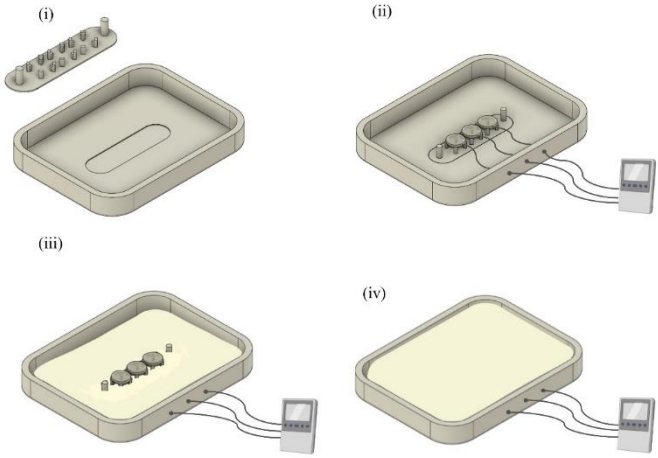


Figure 6.2: Innovative PTFE tray (430 x 330 x 50 mm) with rounded edges and a removable insert with "chair" columns (i), (ii). The milk powder can be placed beneath (iii), and filled above the petri dishes which are nestled in between four "chairs" (iv). The optic fiber sensors are inserted through 2 mm diameter holes inside the containers to measure the temperature during treatment. For the evaluation of temperature consistency experiments, three sensors were used (each sensor in one petri dish). For the inactivation experiments, only one fiber sensor was used in the surrogate-inoculated sample.

6.2.3.1 Temperature measurements

The temperature was recorded in the surrogate-inoculated samples through a multi-channel fiber-optic signal conditioner with calibrated sensors (UMI-8, MAN-00075 R5, FISO Technologies, Smartec SA, Switzerland) throughout the treatment process. Temperature measurements were performed with an automated monitoring system (FISOCommander 2 Control and Analysis Software, FISO Technologies, Smartec SA, Switzerland) taking one measurement/s. A 2 mm hole was drilled in the side of the sealed petri dish containing the surrogate-inoculated SMP and the temperature sensor was positioned in the centre of the sample (Figure 6.2ii).

An Infrared (IR) camera (Testo 871, Maxsievert, Oslo, Norway) was used to evaluate heating uniformity on the surface of each petri dish at the end of each treatment. The IR camera was placed in a fixed position, straight above the centre of the samples and in a distance of 55 cm, and the emissivity level was set to 0.95 (Li et al., 2018). Aluminium foil was used as a reference background to ensure the consistency and accuracy of the thermal imaging data captured. The lid was removed prior to image capturing for the samples containing the surrogate microorganism but was kept intact for the samples inoculated with *S*. Typhimurium following safety regulations. The ironbow HT colour palette was used to display the thermal images of the samples where warm colours represent the hot sections while darker colours are used to show colder sections (Sarawade and Charniya, 2018).

The temperature consistency between the inoculated skimmed milk powder in the different petri dishes after RF treatments was calculated based on the formula uniformity index (λ), described in detail by Wang et al. (2007) (Equation 6.1):

$$\lambda = \frac{\Delta_\sigma}{\Delta_\mu} \quad (6.1)$$

where Δσ is the difference between the initial (t=0) and final standard deviation σ of the sample temperatures and Δμ is the difference between the initial and final mean temperatures of the samples. The uniformity index is a parameter specific to each RF set-up and the treated product in a specific configuration. Small λ values are associated with uniform heating (Hou et al., 2014).

6.2.3.2 RF heating profiles

Four different heating profiles, presented in Table 6.1, were applied to a set sample volume of 2.5 kg uninoculated milk powder. Each profile used a specific input power and a specific target temperature. Initially, all samples were heated using an input power of 1.0 kW until 55°C. Afterwards, the samples were heated further to the target temperatures, either using the same input power of 1.0 kW (treatments 1 and 2, referred to as fast heating), or a

lower input power of 0.2 kW (treatments 3 and 4, referred to as slow heating). In this context, the term "fast heating" indicates a high heating rate with short treatment time, achieved with 1.0 kW, while the term "slow heating" indicates a slower heating rate, achieved with 0.2 kW, towards the higher temperature range. The purpose of the switch at 55°C was to minimise the time that would have been required if the input power entry was 0.2 kW since the beginning of the treatment. The selection of 55°C as the switch temperature was made because no quality deterioration and no inactivation of *Salmonella* spp. are expected below this temperature. Specifically, for mesophilic microorganisms such as *Salmonella*, sublethal temperatures typically refer to the range of 40-50°C, with the lethal temperature threshold generally starting above 55°C (Jay et al., 2006).

Two target temperatures of 85 and 95°C were selected as the goal of the specific RF process design was bacterial inactivation. The specific target temperatures were selected as the most common heat treatments of powders aiming at pasteurisation are performed at temperatures above 80°C (Dag et al., 2020; Wei et al., 2021). High temperatures are more effective in microbial inactivation, but can exert adverse effects on product quality (Chen et al., 2013; Zhong et al., 2017; Dag et al., 2019). Therefore, the two target temperatures were chosen to achieve bacterial lethality, while also assessing the effect on product quality.

After each treatment, the inoculated samples were moved to a set position next to the RF for the IR imaging, and then immersed directly into ice to cool, prior to further quality and microbiological analyses.

Table 6.1: The four different heating profiles using a set sample volume of 2.5 kg uninoculated milk powder. The temperature is recorded on the surrogate-containing sample and the heating rate is the average of the two process runs (n = 2).

RF Treatment	Input power (kW)	Initial temperature (°C)	Target temperature (°C)	Heating rate (°C/min)	Treatment time (min)
Treatment 1	1.0	20.85 ± 1.22	85	4.28 ± 0.47	15.1 ± 0.7

Treatment 2	1.0	21.47 ± 0.18	95	4.80 ± 0.66	15.3 ± 0.2
Treatment 3	1.0	19.26 ± 0.10	55	4.57 ± 0.92	7.5 ± 0.3
	0.2		85	0.39 ± 0.06	83.0 ± 4.0
Treatment 4	1.0	19.76 ± 0.46	55	4.46 ± 0.57	7.8 ± 0.1
	0.2		95	0.30 ± 0.08	139.8 ± 6.1

6.2.3.3 Quality evaluation

To further establish the efficiency of each process and identify possible deteriorative changes to the skimmed milk powder, the most relevant quality aspects were monitored, i.e., solubility and colour. The quality analyses were conducted on uninoculated skimmed milk powder samples. Solubility was tested using the method described by Zhong et al. (2017), based on the ratio of total solids in the supernatant before and after drying in an oven at 105°C for 24 h. The colour was assessed by a digital system for non-contact colour and appearance measurements (DigiEye full system, VeriVide Ltd., Leicester, UK) which provided the measurements black to white (L*), red to green (a*), yellow to blue (b*), as well as an overall measurement of yellowness (Becker et al., 2016). The colour change was quantified by the coefficient ΔE*, calculated in Equation 6.2 (Lang et al., 2017):

$$\Delta E^* = \sqrt{(L^*_{treated} - L^*_{control}) + (a^*_{treated} - a^*_{control}) + (b^*_{treated} - b^*_{control})} \quad (6.2)$$

where *L**, *a**, *b** are the measurements for the (RF) treated and the untreated (control) samples.

6.2.3.4 Inactivation treatments

For the RF inactivation treatments, one petri dish contained the surrogate microorganism to record the temperature profile during the process and the other two contained two biological replicates of *Salmonella*-inoculated-SMP. Each process run was replicated twice. After the treatment and the cooling step, the samples were transferred to a biological safety cabinet.

6.2.3.5 Microbial enumeration

The skimmed milk powder was rehydrated at a 1:10 concentration with 0.9% saline solution and stomached for 1 min. Samples were then plated on non-selective (i.e., TSA) and selective (i.e., XLD Agar, Merck, Darmstadt, Germany) media. Following plating, samples were incubated at 37°C for 24 hours, after which followed the enumeration.

6.2.4 Determination of F-value

In order to accurately compare the different RF heating profiles, the process lethality was calculated using the F-value, which quantifies the cumulative lethal effect of a thermal process on microorganisms (Equation 6.4).

$$F_{90°C} = \int_0^t 10^{\frac{T-T_{ref}}{z}} dt \quad (6.4)$$

where F_{90}°C is the process lethality at the reference temperature of 90°C, T(t) is the temperature at time t, T_{ref} is the temperature set at 90°C, z is the temperature change required for a tenfold reduction in the microbial population. Due to the limited inactivation data points available (only two data points) in this study, it was not feasible to determine D and z values. Hence, the z-value was set at 24°C, based on the study by Michael et al. (2014), which investigated RF inactivation of *Salmonella* spp. in skimmed milk powder. The T_{ref} was chosen as such, as it lies within the range of the target temperatures (85°C and 95°C) used in this study, ensuring the lethality calculations are relevant to the actual process conditions.

6.2.5 Sublethal injury

The percentage of the sublethally injured cells was calculated by Equation 6.5:

$$SI\ (\%) = \frac{N_{non-selective} - N_{selective}}{N_{non-selective}} \cdot 100 \quad (6.5)$$

where $N_{non\text{-}selective}$ is the cell density (CFU/g) measured on non-selective media and $N_{selective}$ the cell density (CFU/g) measured on selective media (Verheyen et al., 2019a).

6.2.6 Statistical Analysis

Significant differences between experimental results were identified by conducting analysis of variance (ANOVA, single factor) with a 95% confidence level (a = 0.05). The Fisher's Least Significant Difference test was used to determine the means that distinguished amongst one another. The studies were carried out with MatLab R2021a (The Mathworks Inc., Natick, USA). When $p \leq 0.05$, test statistics were deemed statistically significant.

6.3 Results and discussion

6.3.1 RF treatment: evaluation of temperature consistency between samples

Temperature monitoring was performed on skimmed milk powder samples inoculated with the non-pathogenic surrogate (i.e., *E. faecium*). The primary goal was to confirm that the temperature across the three samples (i.e., the petri dishes) was consistent. This consistency was crucial to ensure that when pathogens are introduced in the sealed petri dishes, the temperature profile would be similar to that recorded in the surrogate-containing sample.

Therefore, three RF process runs were performed in which the temperature was monitored in each one of the three surrogate-containing samples. A relatively fast heating rate with a target temperature of 65°C was used that aimed to identify any potential temperature differences among the three samples, as rapid heating would likely highlight inconsistencies more clearly.

The averaged temperature evolution of the three monitored samples containing *E. faecium*-inoculated skimmed milk powder in three process iterations is shown in Figure 6.3. The petri dishes had an acceptable temperature variation among them in each replicate and a uniform heating profile. A slight temperature deviation of maximum 5°C between the three replicates was observed which shows a good test repeatability. An ANOVA test showed no statistically significant differences between the three petri dishes ($p > 0.05$). Moreover, similar temperature variations were accepted in other studies using RF heating (Bedane et al., 2021; Kozak et al., 2021).

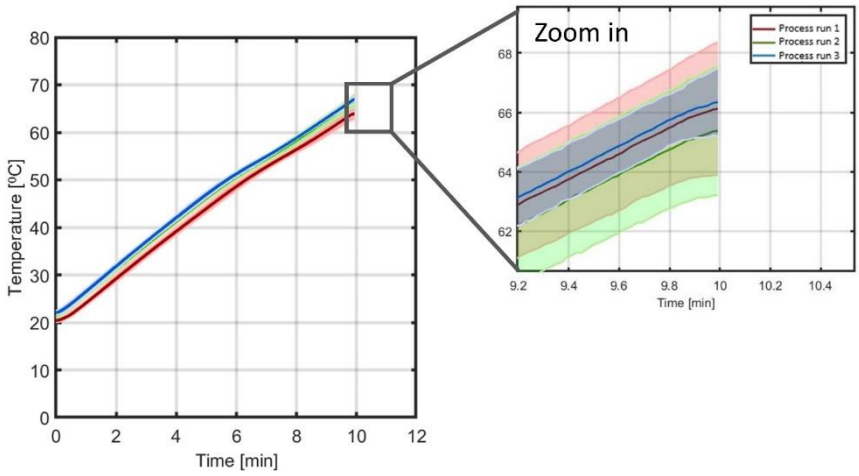


Figure 6.3: Temperature profiles of SMP inoculated with *E. faecium* in three petri dishes located in the centre of the PTFE tray. Three individual process runs (n = 3) are shown in 3 different colours. The average temperature profile of the petri dishes with the standard deviation are shown as a solid line and a shaded area, respectively.

Figure 6.4 illustrates the IR imaging of the three samples in the three process runs. Heating uniformity was achieved between all three petri dishes for every replicate. The temperature variation between each replicate is also visible here, with the hottest spot temperature between the three petri dishes differing by approximately 3-5°C. The observed temperature variations can be attributed to certain limitations regarding the IR measurements. The transfer time from the RF cavity to the designated IR imaging location led to a heat loss in the surface of the treated skimmed milk powder, explaining the difference of the IR temperature values to the optic fiber sensor measurements and between the three process runs. Additionally, powder particles attached in the surface of the petri dishes contributed to lower temperatures measured in the edges of the petri dishes. Moreover, the observed temperature differences between the optical fibers and IR imaging could be attributed to factors such as uneven volumetric distribution of RF energy absorption and the sensor tip's material properties. Temperature uniformity was also evaluated by the heating uniformity index. The uniformity indices after the RF treatments for the three replicates were 0.042, 0.013 and 0.001. Different studies report different λ values, with the lower values corresponding to more uniform heating. Hao et al (2021), using a rotation device, reported values of minimum 0.030 and maximum 0.150 for coix seeds, while Ozturk et al (2017) reported minimum λ values of 0.029 and maximum values 0.044 for the middle surface of RF-treated corn flour. Therefore, the acquired values after the RF treatment of skimmed milk powder in the current study are satisfactory.

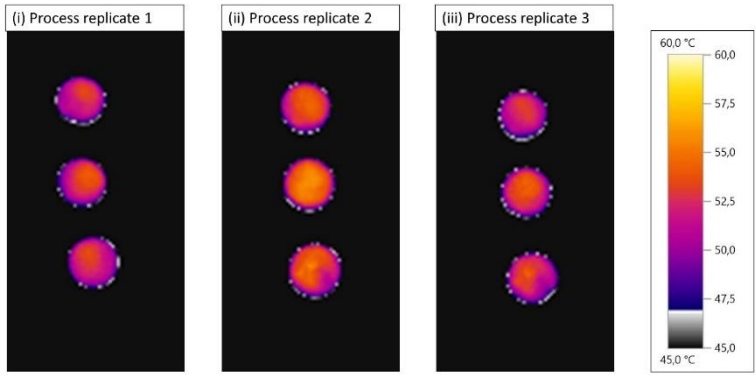


Figure 6.4: Surface temperature distributions of SMP inoculated with *E. faecium* in the three designated petri dishes, in three individual process runs (n = 3) obtained by IR imaging taken directly after RF treatment.

6.3.2 RF treatment: Evaluation of treatments with different heating profiles on safety and quality

Thus far, in RF processing of skimmed milk powder, fast RF heating rates have been favoured, followed by holding the targeted temperature in an oven or using hot air circulation (Wang et al., 2016; Wei et al., 2021; Zhang Y. et al., 2020a). However, holding the temperature in the higher temperature range for more than 5 min can induce degradation of some quality characteristics such as solubility, colour, and wettability (Zhong et al., 2017). A possible approach to overcome this problem and preserve the quality of the product is the use of a slower heating rate during the RF process (Liu et al., 2022a), nullifying the need for a subsequent conventional oven heating phase.

The visual representation of the temperature development of the four treatments used in the current study (detailed in Table 6.1), is shown in Figure 6.5. A rationale for the design of the different treatments is also provided.

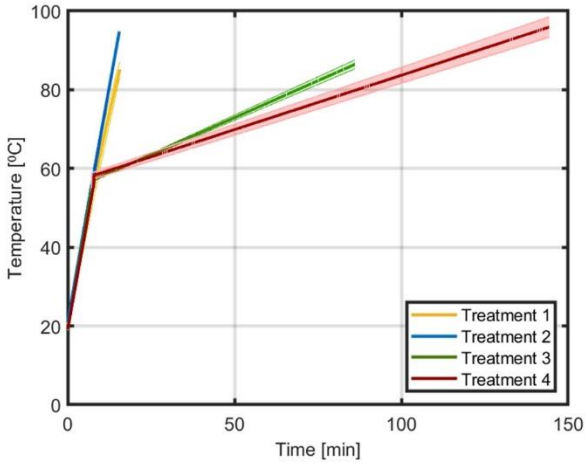


Figure 6.5: Radio frequency heating profiles of skimmed milk powder (sample centre) using the four different input power-temperature profile treatments. The solid lines represent the average temperature profile of two process runs of each treatment and the shaded area represents the standard deviation (n=2). Treatments 1 and 2 use input power of 1 kW until 85°C and 95°C, respectively. Treatments 3 and 4 use input power of 1 kW until 55°C and 0.2 kW until 85°C and 95°C, respectively.

Fast heating: Treatment 1 and 2. First, RF treatments were designed to reach temperatures of 85°C (treatment 1) and 95°C (treatment 2) at a slower rate than for the typical RF treatments that are combined with a subsequent conventional oven heating phase. If these treatments would result in acceptable reductions of *S*. Typhimurium levels, the absence of the oven heating phase in the process would result in less quality deterioration compared to oven phase-containing processes. For both treatments, 1.0 kW input power was used to achieve an approximate heating rate of 4.5°C/min. The time to reach the target temperatures was approximately 15 min. Overall, these times are shorter than typical processes that consist of both a short come-up time (e.g., 2 min to reach 70°C, 10 min to reach 85°C, 12 min to reach 95°C) and an additional oven phase-containing time, which is usually around 15 min (Wei et al., 2021; Zhang H. et al., 2020a).

Slow heating: Treatment 3 and 4. Since fast treatments acquired by treatment 1 and 2 were not suitable to achieve sufficient levels of bacterial inactivation, slower RF treatments to reach 85°C (treatment 3) and 95°C (treatment 4) were designed. The rationale was that maintaining relatively low temperatures (i.e., while heating up the powder) for a longer period of time could result in a sufficiently high total thermal load of the process to achieve sufficient bacterial inactivation, while still staying below the considerable quality deterioration caused by oven phase-containing processes. For both treatments, an initial input power of 1.0 kW was used, followed by 0.2 kW input power (0.35°C/min) after a temperature of 55°C was reached. The high initial input power of 1.0 kW was implemented to (i) minimise the treatment time, ergo decreasing energy consumption, and (ii) prevent bacterial resistance, as it has been confirmed that slow heating rates at sublethal temperatures can trigger stress response mechanisms in microorganisms, which subsequently can lead to increased heat resistance (Zhang L. et al.,

2020). Therefore, by utilising a fast heating rate in the lower temperature range, the ability of bacteria to adapt to the new environment is hindered.

In order to make a comparison of the four treatments and validate their efficiency, the powder quality and microbial inactivation were evaluated.

6.3.3 Effect on quality

The effect of RF on various quality characteristics of skimmed milk powder has been documented in past studies. Specifically, protein denaturation (Chen et al, 2013; Zhang Y. et al., 2020b), in-vitro protein digestibility (IVPD) (Lin et al., 2020), protein structure (Zhang et al., 2023), lactose crystallinity (Zhang et al., 2022, 2023), wettability (Lin et al., 2020; Zhong et al., 2017) have been studied after different RF treatments at different temperatures and treatment times. Colour and solubility are two of the most critical quality attributes of skimmed milk powder that are highly sought after by consumers and the most studied quality attributes (Chen et al., 2013; Dag et al., 2019; Lin et al., 2020; Zhong et al., 2017). Consequently, these properties should be taken into account in the design of RF treatments.

Table 6.2 shows the results of the colour measurements before and after the four different RF treatments. Colour was quantified using the L*, a*, b*, yellowness, and ΔE* values. ΔE* value is commonly used to classify the changes in colour as very distinct ($\Delta E > 3$), distinct ($1.5 < \Delta E < 3$), and small ($1.5 < \Delta E$) (Adekunte et al., 2010). No significant differences in colour with the untreated powder were observed for treatments 1, 2 and 3. There was a small relative change in a* and the yellowness of skimmed milk powder in treatment 4 which was statistically significantly different ($p \leq 0.05$) compared to the untreated sample and the rest of the treatments. This is all shown by the ΔE*, as the 1.93 value falls under the category of distinct changes in colour. Specifically, the increase in yellowness could be attributed to the Maillard reactions taking place in higher temperature ranges (Zhong et al., 2017). Nevertheless, these colour changes were (i) not visually observable, and (ii) smaller than those observed in milk powders treated by conventional oven phase-containing RF processes and other RF only processes. Dag et al. (2022) used a combination of RF heating and 75°C oven holding, resulting in very distinct changes in ΔE* values of whole milk powder at every stage, i.e.,

ΔE* 2.58 ± 0.65 after RF heating, ΔE* 10.89 ± 0.84 after the oven phase. Dag et al. (2019) reported distinct and very distinct changes in ΔE* values during RF-only treatments of skimmed (ΔE* 1.43-1.95) and whole milk powder (ΔE* 2.82-3.71), but only used short treatments with a low thermal load which would not result in sufficient bacterial inactivation (i.e., reaching 80°C after 4-6 min).

Table 6.3 shows the result of the solubility measurements. There were no statistically significant differences in solubility between the untreated sample and any of the treatments. Previous studies on RF heating of milk powder resulted in deteriorated milk powder solubility. For example, Zhong et al. (2017) applied a high RF heating rate for 10 min up to 90°C and observed a 4 % reduction of sample solubility.

Table 6.2: Colour measurements in the untreated milk powder and milk powder treated with the four different heating profiles (n=3). The coefficient ΔE* is utilised to distinguish colour changes between the treated and untreated milk powder.

Sample	Colour measurements				ΔE*
	L*	a*	b*	Yellowness	
Untreated Skimmed Milk Powder	96.14 ± 0.9 [a]	-2.33 ± 0.3 [a]	13.047 ± 1.35 [a]	26.30 ± 1.81 [a]	-
Treatment 1	96.25 ± 1.01 [a]	-2.25 ± 0.18 [a]	13.24 ± 0.49 [a]	26.52 ± 0.74 [a]	0.61
Treatment 2	96.23 ± 1.21 [a]	-2.21 ± 0.10 [a]	13.21 0.41 [a]	26.49 ± 0.65 [a]	0.61
Treatment 3	97.38 ± 0.7 [a]	-2.19 ± 0.2 [a]	11.96 ± 1.56 [a]	24.80 ± 1.56 [a]	0.53
Treatment 4	95.07 ± 0.2 [a]	-2.09 ± 0.2 [b]	17.60 ±1.15 [a]	31.94 ± 1.35 [b]	1.93

Table 6.3: Solubility in the untreated milk powder and milk powder treated with the four different heating profiles (n=3).

Sample	Solubility (%)
Untreated Skimmed Milk Powder	98.54 ± 0.75[a]
Treatment 1	100.41 ± 1.89[a]
Treatment 2	99.41 ± 0.62[a]
Treatment 3	99.63 ± 0.29[a]
Treatment 4	98.67 ± 0.31[a]

Consequently, the selected milk powder quality characteristics (colour and solubility) for the four different treatments surpass those of typical RF treatments involving fast RF heating followed by a conventional oven holding phase, the latter resulting in deteriorative quality characteristics. However, the effect of the designed RF process on a wider range of quality attributes including protein stability, oxidation and functionality, lactose crystallinity, fat oxidation (for whole milk powders) as well as flavour and texture, should be thoroughly investigated in future studies.

6.3.4 Effect on inactivation of *S.* Typhimurium

To establish the validity of the proposed heating profiles, a case study on the inactivation of the pathogen *S.* Typhimurium was conducted. The results of the microbiological analysis after RF treatment are presented in Figure 6.6. For the inactivation experiments, two petri dishes contained biological replicates of *S.* Typhimurium and the third one contained the surrogate microorganism, to measure the temperature evolution in the process. In total, treatment 4 had the best efficacy by achieving a reduction of >4-log CFU/g *S.* Typhimurium cells, as bacterial counts were below the detection limit. Even though there was bacterial inactivation in the other three treatments, it was insufficient. Specifically, heating up to 85°C with a slow heating rate had a small effect on the bacterial cell density, a development verified by Wei et al. (2021) during the treatment of non-fat dry milk at 85°C and holding in an oven for 120 min.

The calculated F-values for each treatment, shown in Table 6.4, provide further insight into the effectiveness of each heating profile. Treatment 4 showed the highest F-value and the most significant microbial reduction, indicating a strong correlation between the cumulative process lethality and the observed inactivation of *S*. Typhimurium. The higher F-values for the slow heating treatments (treatments 3 and 4) reflect a greater cumulative thermal load, which resulted in higher microbial inactivation compared to the fast heating treatments.

Table 6.4: Calculated F-Values for the four RF heating treatments in skimmed milk powder using a *Salmonella* spp. z-value from isothermal treatments (Michael et al., 2014) and calculated by an average temperature profile of each treatment (n=2).

Type of treatment	F-value (min)
Treatment 1	2.2
Treatment 2	4.1
Treatment 3	18.6
Treatment 4	66.1

In order to further assess the effect of the four treatments on the bacteria, sublethal injury was quantified. Sublethally injured cells (SICs) are a category of cells that have been severely damaged after being exposed to stresses. They have received substantial interest for their ability to recover and grow in number when they are placed in a favourable environment (Akkermans et al., 2021). These injured cells can grow on general, non-selective media, but they cannot grow on selective media. Therefore, these cells remain undetected in the selective media commonly used in the food industry, but, if present, they can multiply later in the food chain and cause a health hazard. SICs have been reported following RF heating in other LMF products such as broccoli powder (Zhao et al., 2017), red pepper powder (Zhang H. et al., 2020a), walnut shells (Zhang et al., 2021b), edible seeds (Xu et al., 2022). The sublethal injury (%) was also calculated in this study, and illustrated in Figure 6.7. Interestingly, a low percentage of sublethal injury can be observed in the untreated *S.* Typhimurium cells. Therefore, during the adaptation phase in the inoculation procedure, injured cells were generated due to the new stressful, low a_w environment. Enache et al. (2017) highlighted the effect of previously inflicted stress on the bacterial response to a new stressor. For example, heat resistance is increased when the bacteria have previously been exposed to other stressors such as lower a_w (Wesche et al., 2009). SICs were mostly present in the treatments 1, 2 and 3, as in treatment 4 the bacterial

counts are below the detection limit, indicating a successful reduction. An increasing trend in sublethal injury (%) is noted with longer treatment times.

Overall, treatment 4 was the most optimal treatment concerning microbial inactivation (including the absence of SICs), but also quality characteristics. It is, however, recommended to further investigate the effect of the slow heating rate RF process on the sublethal injury evolution, as there was a clear tendency for SIC formation for the treatment types tested.

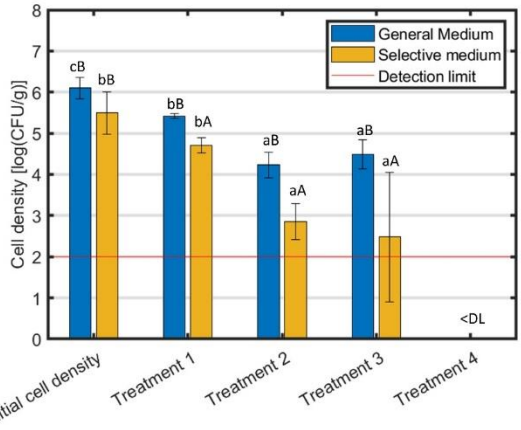


Figure 6.6: Radio frequency heating effect on the cell density of *Salmonella* Typhimurium in skimmed milk powder, plated on general and selective media. Four different input power-temperature profile treatments were used. Treatments 1 and 2 used an input power of 1 kW ("fast heating") until target temperatures of 85°C and 95°C, respectively. Treatments 3 and 4 used an input power of 1 kW until reaching 55°C and then switched to 0.2 kW ("slow heating") until target temperatures of 85°C and 95°C, respectively. Statistically significant differences ($p \leq 0.05$) among the initial cell density and treatments are demonstrated by a different lowercase letter and differences between the two media in each category by an uppercase letter ($p \leq 0.05$) (n

= 4). DL is the detection limit set at 2-log(CFU/g).

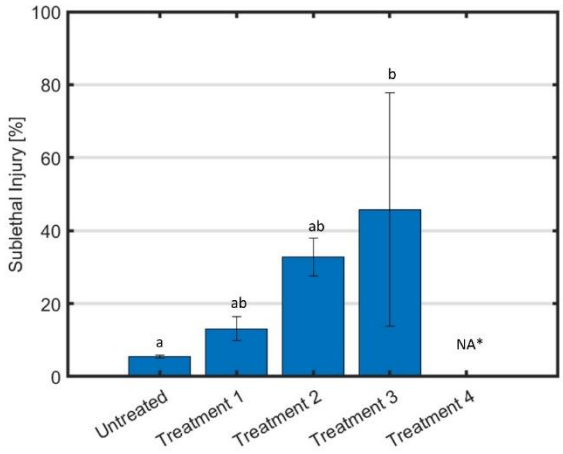


Figure 6.7: Sublethal injury (%) of *Salmonella* Typhimurium in Skimmed Milk Powder after radio frequency treatment with the four different heating profiles. Treatments 1 and 2 used an input power of 1 kW ("fast heating") until target temperatures of 85°C and 95°C, respectively. Treatments 3 and 4 used an input power of 1 kW until reaching 55°C and then switched to 0.2 kW ("slow heating") until target temperatures of 85°C and 95°C, respectively. Different lowercase letters represent statistically significant differences between samples ($p \leq 0.05$) ($n = 4$). NA* denotes 'Not Applicable' due to bacterial counts falling below the detection limit.

6.4 Conclusions

Over the past decade, foodborne outbreaks related to milk powder consumption have proven the need for additional processing to guarantee microbiological safety. However, conventional heat treatments are hindered by the inefficient heat transfer in powders due to low conductivity. While radio frequency (RF) processing is effective at providing powder safety, the typically used methodology of combining fast RF heating with a conventional heating oven phase detrimentally impacts milk powder quality.

In the current study, different RF-only treatments were assessed for their effect on milk powder quality and microbiological safety. With respect to

quality aspects, all treatments resulted in less deteriorative changes to the colour and solubility of skimmed milk powder in comparison to typical RF processes consisting of a fast RF heating followed by a conventional oven holding phase. Concerning safety, a >4-log reduction of *S*. Typhimurium was achieved by using a slow heating rate to the target temperature of 95°C, with no sublethal cell injury occurring for this treatment. Consequently, it was evidenced that tailored slow RF-only processes are suitable to fulfil the necessary safety standards of skimmed milk powder, with quality levels surpassing those achieved by typical RF processes that involve a conventional oven holding phase.

Overall, further studies on the effect of the different heating profiles with different target temperatures and heating rates on bacterial cells in skimmed milk powder are required to determine the most efficient and effective process for optimal safety standards without compromising product quality. These studies should also aim to gather additional data points to precisely calculate inactivation rate constants and develop dynamic models that can depict the RF-only heating process more accurately. Furthermore, since this case study was solely focused on one strain of *Salmonella*, future research should also validate more heat-resistant strains and investigate more foodborne pathogens relevant to LMF products. Introducing different types of milk powders and various LMF products in further studies will determine the broader applicability of the current findings.

Acknowledgment

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Chapter 7

RF inactivation of *S. Typhimurium* and *L. monocytogenes* in dairy model systems: powder

The results presented in this chapter have been published in *International Journal of Food Microbiology*:

Tonti Maria, Verheyen Davy, Kozak Dmytro, Skåra Torstein, Van Impe Jan. Radio frequency inactivation of *Salmonella* Typhimurium and *Listeria monocytogenes* in skimmed and whole milk powder. *International Journal of Food Microbiology, 413*, 110556.

The contributions of the first author to this work are:

- Literature study
- Co-design of experiments
- Co-performing of experiments
- Co-interpretation of results
- Co-formulation of conclusions
- Text redacting and editing

This chapter includes the referred paper. Part of the content is identical to the content of the submitted paper. However, some adaptations in content and formatting have been made.

Context: After establishing a RF-only dynamic heating of milk powder which successfully caused a 4-log reduction of *S*. Typhimurium (in the previous chapter), the next step was to document the RF inactivation kinetics of both *S*. Typhimurium and *L. monocytogenes* in skimmed and whole milk powder. A log-linear model with a Bigelow-type temperature dependency was used in order to investigate the influence of food composition and the bacterial Gram type on the inactivation. *E. faecium* was used again (similarly to Chapter 6) to record the temperature during RF process, but also to established its efficacy as a surrogate for both microorganisms.

Abstract

Milk powder is a convenient, shelf-stable food ingredient used in a variety of food products. However, pathogenic bacteria can be present and survive during prolonged storage, leading to outbreaks of foodborne diseases and product recalls. Radio frequency (RF) heating is a processing technology suitable for bulk treatment of milk powder, aiming at microbial inactivation. This study investigates the RF inactivation of *Salmonella* Typhimurium and *Listeria monocytogenes* in two types of milk powder; skimmed milk and whole milk powder. Specifically, the aims were to (i) examine the influence of the powder's composition on bacterial inactivation, (ii) evaluate the response of bacteria with different Gram properties (Gram-positive and Gram-negative) and (iii) verify the use of *Enterococcus faecium* as a surrogate for the two microorganisms for the specific RF process. In order to examine exclusively the influence of RF, a non-isothermal temperature profile was used, employing solely different RF energy levels to heat the product to the target temperatures. A log-linear model with a Bigelow-type temperature dependency was fitted to the experimental data. *S*. Typhimurium was less susceptible to RF treatments in comparison to *L. monocytogenes*, demonstrating a higher inactivation rate (k) and higher percentage of sublethal injury. A higher k was also observed for both microorganisms in the whole milk powder, indicating that the increased fat content and decreased levels of lactose and protein in the milk powder had an adverse impact on the microbial survival for both pathogens. The surrogate microorganism *E.*

faecium successfully validated the microbial response of the two microorganisms to RF treatments.

7.1 Introduction

Milk powder is a versatile food ingredient with several benefits. It is highly nutritious and has a long shelf life which facilitates its storage and distribution (Zhong et al., 2017). Milk powder can be used as reconstituted milk or as an additive in various food products, such as puddings, sauces, and baked goods to enhance texture and flavour (Wei et al., 2021). Due to its low water activity (a_w), it is commonly perceived as "safe". The low a_w creates an adverse environment that inhibits bacterial growth. However, several microorganisms can survive for prolonged periods of time, creating challenges with respect to food safety upon rehydration of the product (Dag et al., 2022).

Over the years, several outbreaks have been traced back to contaminated milk powder (EFSA & ECDC, 2019; Michael et al., 2014). One of the most prevalent foodborne pathogens implicated in these outbreaks is *Salmonella*. *Salmonella* is known to survive the spray drying process which is the last critical control point for eliminating microbial contamination (Wei et al., 2021). *Listeria monocytogenes* is another microorganism that can withstand the spray drying process and survive in low moisture foods for an extended period of time, even if it hasn't been linked to any known milk powder outbreaks thus far (Ballom et al., 2020; Ly et al., 2019).

The reduction of microbial contamination in food products is typically addressed through conventional thermal processing technologies (Zhang et al., 2021a). Such technologies have certain limitations regarding the heating rate and uniformity, especially when it comes to the treatment of low moisture food products like milk powder. Radio frequency (RF) heating is an innovative technology which utilises electromagnetic radiation and generates thermal energy within the product (Altemimi et al., 2019). Numerous studies are available on the RF treatment of various low moisture food products, with a particular interest on milk powder (Dag et al., 2020). The milk powder's composition exerts a big influence on the RF treatments (Dag et al., 2019), but the influence on the bacterial survival in milk powders has not been studied yet. Furthermore, RF treatments have been mainly focused on

Salmonella spp., despite the fact that *L. monocytogenes*, if present, can survive storage up to a year (Ballom et al., 2020). In most of the RF inactivation studies, a common approach involves the initial use of RF to elevate temperatures to a specific target, followed by isothermal holding using (i) hot air for a set duration at the target temperature (Michael et al., 2014; Wang et al., 2016; Wei et al., 2021) or (ii) placing products in insulation boxes (Ballom et al., 2021). Oven holding involving hot air is the most commonly applied strategy, since small temperature decreases can often occur during product transfer to insulation boxes (Ballom et al., 2021). However, the stage of isothermal holding needed to allow time for internal heat to mitigate non-uniform RF heating (i.e., to ensure bacteria in the cold zones receive adequate treatments) can result in overtreating (i.e., too long time at high temperature) of the hot zones, consequently leading to a decline in quality (Wang et al., 2016; Dag et al., 2022). RF-only treatments consisting of a slow RF heating phase and no conventional oven holding phase could be used to better preserve product quality, according to a Low Temperature Long Time (LTLT) strategy (Dominguez-Hernandez et al., 2018; Liu et al., 2022a). However, this strategy requires more research prior to industrial application, since (i) RF has not been used as a sole technology aiming at bacterial inactivation thus far, and (ii) the potential of RF-only dynamic heating has not been fully explored yet. In contrast to typical RF processes involving an oven heating phase, RF heating is more significant in the overall process, and hence, the interaction between RF, the powder properties (e.g. composition) and the specific pathogenic microorganisms (e.g., influence of the bacterial gram properties), becomes a critical aspect to consider. The effect of these influences has not been studies so far, not for RF processing in general, and especially not in cases involving a RF-only dynamic heating process.

The main objective of this study was to elucidate the influence of milk powder composition and bacterial properties on RF inactivation kinetics during a RF-only dynamic RF decontamination process. The specific subobjectives were to (i) compare the RF inactivation kinetics of two microorganisms with different Gram-types (i.e., *S.* Typhimurium and *L. monocytogenes*), (ii) unravel the effect of the powder's composition on the microbial survival (i.e., using skimmed and whole fat milk powder), and (iii) validate the use of the non-pathogenic microorganism *Enterococcus faecium* as a surrogate for the aforementioned microorganisms, food products and RF process. In order to

study the influence of these factors solely during RF exposure, a novel, RF-only (non-isothermal) temperature profile was used, not including the typical conventionally heated temperature holding stage. A log-linear model including a Bigelow-type temperature dependency was fitted to the acquired experimental data in order to systematically quantify the effect of the aforementioned factors.

7.2 Materials and methods

7.2.1 Materials

Skimmed milk powder and whole milk powder were purchased from TINE SA (Oslo, Norway) in 25-kg bags. The composition (wt. %) of the milk powders as provided by TINE SA is shown in Table 7.1. After opening each package, the powder was distributed in 1-kg plastic bags which were then vacuumed and sealed. Random sampling was performed in triplicate for each bag, to determine whether microbial background flora is present. The samples were plated on TSA supplemented with 0.6% yeast extract (Merck, Darmstadt, Germany).

Table 7.1: Indicative composition (wt.%) of milk powders, provided by TINE SA

Composition (%)	Skimmed milk powder	Whole milk powder
Fat	0.8	35
Lactose	47.9	33.9
Protein	36.9	23.8
Ash	7.9	5.3
Water	< 5	< 3

7.2.2 Microorganisms and microbial inoculum preparation

The two pathogenic bacteria used for this study were *S*. Typhimurium ATCC 14028 (isolated from bovine, septicaemic liver), which was obtained from the Gothenburg Bacterial Collection and *L. monocytogenes* LMG 23356 (isolated from Jalisco Cheese), which was obtained from the bacterial collection of Ghent University. The non-pathogenic microorganism *E. faecium* ATCC 8459 was obtained from the NCIMB collection in UK. Stock cultures of each microorganism were stored at -80°C in 2 ml cryovials (Microbank™, Pro-Lab Diagnostics, Canada).

To prepare the microbial inoculum, one bead of the stock culture was transferred to an Erlenmeyer flask containing 20 mL broth, BHI (Merck, Darmstadt, Germany) for *L. monocytogenes* and TSB (Oxoid, Basingstoke Hampshire, England) for *S*. Typhimurium and *E. faecium*. Afterwards, the Erlenmeyer flasks were incubated at optimum temperature for 24 h, i.e., 30°C for *L. monocytogenes* and 37°C for *S*. Typhimurium and *E. faecium*. A purity plate was created by streaking on non-selective agar media. *L. monocytogenes* was plated on BHI (Merck, Darmstadt, Germany) with 10% Agar (Merck, Darmstadt, Germany) and the purity plate was then incubated at 30°C for 48 h. *S*. Typhimurium and *E. faecium* were plated on TSA (Merck, Darmstadt, Germany) and the plate was incubated at 37°C for 24 h. For each microorganism, four biological replicates were created by taking one individual colony of the purity plate, placing it in an Erlenmeyer flask containing the relevant broth and incubating at optimum temperature for 24 h. A second 24-h preculture was then created by transferring 20 µL of the first preculture to a new Erlenmeyer flask containing broth and incubating for 24 h at optimum temperature. The viable cell density at the end of the incubation was approximately 10^9 CFU/mL.

7.2.3 Milk Powder inoculation

10 g of the respective milk powder was weighed into a sterile stomacher bag (GRADE, UK) and inoculated by pipetting 0.1 mL of the second microbial preculture on the powder surface. The powder was then hand-massaged for 5 min, taking short breaks to avoid local over-heating caused by friction. The bag was placed inside a desiccator located in a 37°C incubator for 24 h (adaptation phase). The aim was to provide appropriate time for the bacteria to adapt to the new environment whilst maintaining the original water content of the sample. After the adaptation phase, the a_w values measured by a a_w meter (Aqualab 4TE, METER Group AG, Germany) were 0.256 ± 0.007 and 0.276 ± 0.006 (measured at 25°C) for the inoculated skimmed and whole milk powder respectively, both falling within ± 0.02 of the values of the non-inoculated powder. a_w plays a significant role in the RF heating rate and temperature uniformity (Altemimi et al., 2019), as well as the bacterial thermal resistance (Liu et al., 2018a). Thereafter, the bags were hand-massaged again for one minute to ensure sample inoculation uniformity.

By the end of the adaptation phase, the viable cell density of *L. monocytogenes* and *E. faecium* was approximately 10^6 CFU/g and for *S.* Typhimurium it was approximately 10^5 CFU/g. Hence, an extra step was added to increase the viable cell density of *S.* Typhimurium to 10^6 CFU/g, allowing the same initial starting level for all microorganisms. Specifically, 10 mL of the second preculture was centrifuged at 5000×g at 4°C for 10 min. The cell pellet was resuspended in 1 mL of TSB and the concentrated bacterial suspension was used to inoculate the milk powder.

The inoculated milk powder was placed in a 35x10 mm container; a sterile, polystyrene petri dish with lid (Sarstedt, Oslo, Norway). To ensure that the containers were filled to their full volume and eliminate the presence of any air headspace, 6 g of inoculated skimmed milk powder and 5 g of inoculated whole milk powder (i.e., different masses were employed to counteract density differences between the two milk powders) were precisely weighed into the container under sterile conditions for each replicate. The closed container with the inoculated powder was sealed by thermal resistant, adhesive tape to prevent moisture transfer with the environment and leakage of the sample during the RF treatments.

7.2.2 RF processing method of milk powder

A pilot-scale 4 kW, 27.12 MHz, 50 Ω RF set-up (SAIREM, Lyon, France) was used to perform the RF treatments. All samples were subjected to a RF-only treatment process. Specifically, the process aimed at utilising a dynamic RF-only heating profile to inactivate bacteria. The experimental set-up is shown in Figure 7.1. A Polytetrafluoroethylene (PTFE) tray with rounded edges and dimensions: 430 mm (L) x 330 mm (W) x 50 mm (H) was used for the RF treatments (Figure 7.1i). The total sample amount was approximately 2.5 kg of milk powder in each process run. Initially, 1 kg of non-inoculated milk powder was spread uniformly in the bottom of the tray. Then, three sealed containers with inoculated milk powder were placed in fixed positions in a removable insert, in the geometric centre of the PTFE tray (Figure 7.1ii). This position was selected as preliminary experiments had shown more unstable temperature evolution at the edges of the tray compared to the centre, which showed similar temperature profiles. This phenomenon has also been reported in other studies (Liu et al., 2018b; Tiwari et al., 2011a). Two containers enclosed milk powder inoculated with pathogenic microorganisms and the third one enclosed milk powder contaminated with the surrogate microorganism. After the positioning of the containers, 1.5 kg non-inoculated milk powder was added to cover the containers uniformly, and ensuring that the top surface was even over the whole tray area (Figure 7.1iii). The tray was then placed in the centre of the bottom electrode and the electrode height of the movable top electrode was set at 70 mm, which was approximately 30 mm from the sample surface.

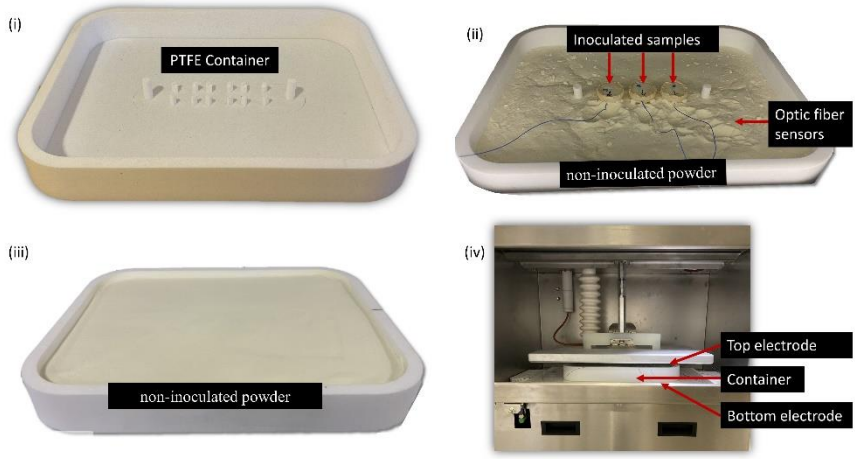


Figure 7.1: (i) PTFE tray (ii) non-inoculated milk powder at the bottom of the tray and samples in the sealed containers (petri dishes) with the optic fibre sensors in the centre of the tray, (iii) covered by non-inoculated milk powder. The container is placed on the bottom electrode in the centre of the RF chamber (iv).

The temperature was logged during the process by mounting an optic fiber sensor (FISO Technologies, Smartec SA, Switzerland) inside the surrogate-inoculated sample (Figure 7.1ii). For the dynamic heating treatments, 2.5 kg of milk powder were first heated to 55°C at a power of 1 kW (0.4 kW/kg), and then heated further at a slower rate at a power of 0.2 kW (0.08 kW/kg) to the different end temperatures. The specific settings allowed for shorter treatment times in the lower temperature range and the switch temperature of 55°C was selected as it did not affect the inactivation of the pathogenic bacteria. The surface temperature uniformity was verified by infrared (IR) imaging (Testo 871, Maxsievert, Oslo, Norway) after each treatment and the samples were submerged swiftly in an ice bath to rapidly cool down and prevent further inactivation.

7.2.3 Sampling and Microbial enumeration

The treated and non-treated samples were placed in a sterile stomacher bag where 0.9% saline solution was added at a 1:10 concentration. The rehydrated samples were stomached for 1 min. To determine viable cell counts, the samples were then plated on non-selective and selective media. For *L. monocytogenes*, BHI Agar and PALCAM (Merck, Darmstadt, Germany) were used as non-selective and selective media, respectively. For *S.* Typhimurium, TSA and XLD Agar (Merck, Darmstadt, Germany) were used as a non-selective and selective media, respectively. For *E. faecium*, TSA and Slanetz and Bartley (Sigma Aldrich, Norway) were used as a non-selective and selective media, respectively. Following plating, the *L. monocytogenes* samples were incubated at 30°C and the *S.* Typhimurium and *E. faecium* samples were incubated at 37°C for 24 h prior to enumeration.

7.2.4 Mathematical modelling

RF processing frequently exhibits temperature variations between different process runs (Bedane et al., 2018, 2021; Saka et al., 2021; Ling et al., 2016). In general, variations between process runs can occur due to differences in the sample's initial temperature, differences in the moisture content of the sample, presence of air gaps, sample inhomogeneity, and sample positioning. In order to eliminate these influencing factors, the RF experimental design established that: (i) the inoculation method ensured similar a_w with the non-inoculated milk powder, (ii) the containers with the contaminated powder were filled to their maximum capacity and each time weighed under sterile conditions to minimise experimental errors, (iii) the containers and the tray itself had a fixed position in the RF cavity, (iv) the surrounding bulk milk powder was weighed. Taking all these measures into account, the authors see two possible reasons because of which variations in temperature profiles still occurred. First of all, initial temperature differences in the sample were observed due to the time necessary to weigh the inoculated samples. However, differences in the heating rate were observed even when the initial temperature between samples differed by only 0.02°C. Another fact that might contribute to this variation is the overheating of the triode tube, which is an important part of the RF setup. According to Awuah et al. (2014), "one of the most important factors affecting system reliability is the cooling system

of the triode tube itself'. The prolonged treatment times using this RF-only approach and multiple experimental repetitions could lead to reduced efficiency of the triode, which subsequently affects the electromagnetic field.

The aforementioned variations in temperature profiles can lead to increased uncertainty in predicting the accurate inactivation rates of microorganisms (Kozak et al., 2021). Therefore, to facilitate accurate data interpretation, the temperature profile of each treated sample as well as the initial and the viable cell density after treatment were independently integrated into the model, avoiding the use of an inaccurate average temperature profile. The model employed was a first order kinetics log-linear model with a Bigelow-type temperature dependency (Stumbo, 1973) (Equations 7.1, 7.2).

$$\frac{dN(t)}{dt} = -k\left(T(t)\right) \cdot N\ ,\ N(t=0) = N0 \qquad (7.1)$$

$$k\left(T(t)\right) = k\left(T_{ref}\right) \cdot 10^{\frac{T(t)-T_{ref}}{z}} \qquad (7.2)$$

with $N(t)$ (CFU/g), the viable cell density at time t; k (1/min), the inactivation rate, T_{ref} (°C), the reference temperature set at 78°C, $k(T_{ref})$ (1/min), the inactivation rate at the reference temperature, and z (°C), the temperature sensitivity parameter. The reference temperature T_{ref} of 78 °C was selected as such since preliminary modelling trials demonstrated that the selection of a reference temperature did not affect parameter estimations, as long as the reference temperature was selected within the 50-95°C temperature range. Therefore, the T_{ref} was set to 78°C in order to provide a temperature which lied in the range of the applicable treatments (Verheyen et al., 2019b; Akkermans et al., 2020).

The lsqnonlin function of the Optimisation Toolbox of MatLab version R2021b (TheMathworks Inc., Natick, USA) was used to calculate the model parameters, minimizing the sum of squared errors. The differential equations of the inactivation model were solved by combining the primary and secondary model, using the MatLab solver ode45. The Jacobian matrix was used to estimate the standard errors of the model parameters.

7.2.5 Sublethal injury

The percentage of the sublethally injured cells (SICs), i.e., a category of cells which have been injured but maintain the ability to grow under favourable conditions was calculated by Equation 7.3:

$$SI\ (\%) = \frac{N_{non-selective} - N_{selective}}{N_{non-selective}} \cdot 100 \quad (7.3)$$

where $N_{non\text{-}selective}$ is the viable cell density (CFU/g) measured on non-selective media and $N_{selective}$ the viable cell density (CFU/g) measured on selective media (Verheyen et al., 2019a)

7.2.6 Statistical analysis

Significant differences between experimental results were identified by conducting analysis of variance (ANOVA, single factor) with a 95% confidence level (a = 0.05). The Fisher's Least Significant Difference test was used to determine the means that distinguished amongst one another. The studies were carried out with MatLab R2021a (The Mathworks Inc., Natick, USA). When $p \leq 0.05$, test statistics were deemed significant.

7.3 Results and discussion

The subobjectives of this study were evaluating the effect of Gram properties and powder composition on the RF inactivation of two pathogens, as well as the suitability of *E. faecium* as a surrogate for these pathogens. To achieve these objectives, both lethal (*k*) and sublethal injury (SI) of all microorganisms present in two milk powders was assessed.

The RF inactivation of *S.* Typhimurium and *L. monocytogenes* in the two types of milk powder is illustrated in Figure 7.2. Overall, a targeted 4 log (CFU/g) reduction was achieved for both microorganisms for the most severe treatment (95°C). While the standard recommended target is a 5 log (CFU/g)

reduction, the detection limit of the microorganisms prevented the identification of a higher level of reduction. The data points acquired by the applied non-isothermal RF treatment indicated absence of residual population and therefore, a log-linear model with a Bigelow-type temperature dependency was fitted to the data. The model parameters are presented in Table 7.2. The temperature dependency of the inactivation rate k is displayed in Figure 7.3. It is important to clarify that all temperature profiles were considered separately for the model fit, along with the respective initial and final viable cell density. Therefore, the model parameters were a result of all individual temperature profiles used for the model fit, as well as the corresponding initial and final viable cell densities. The relatively high standard deviations originated from the variability of the different temperature profiles between different RF process runs (as explained in 7.6). In order to illustrate the model fit on the figure, however, only the average, minimum and maximum temperature profiles were utilised. Figure 7.4 depicts the model fit by assuming these temperature profiles. It should be noted that the employed modelling approach allows for all general findings in the current manuscript to be valid over an entire temperature profile range, ensuring a wide applicability. In future studies, when designing RF processes for any specific setup-product combination, research should, however, focus on obtaining repeatable temperature profiles to guarantee food safety when applying the process in the industry. Furthermore, future research should focus on the optimisation of existing RF equipment to better accommodate the specific characteristics of the product being treated.

The SI after RF treatment to target temperatures in skimmed and whole milk powder for the two pathogens is presented in Figure 7.5. SI is typically defined as any damage that does not result in the organism's death and often leads to stress adaptation as a result (Wesche et al., 2009). When bacterial cells are sublethally injured, they can still survive under certain conditions, but they will not be able to grow in selective culture media (Verheyen et al., 2020).

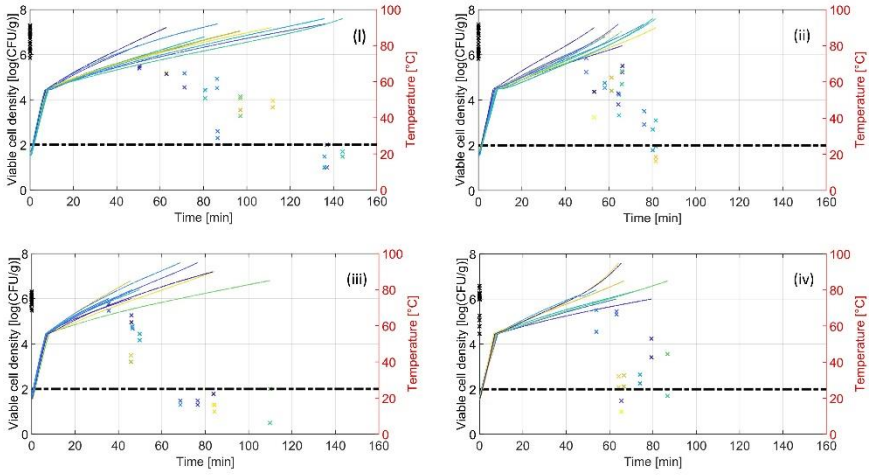


Figure 7.2: Cell density (log(CFU/g)) of *S*. Typhimurium in skimmed (i) and whole (ii) milk powder and *L. monocytogenes* in skimmed (iii) and whole (iv) milk powder after RF treatment to different end temperatures (n = 4). Data points of black colour represent initial cell density (log(CFU/g)). Data points of different colours represent the cell density (log(CFU/g)) after RF treatment to the respective end temperature. The temperature profile of each treatment corresponds to the specific data points of similar colour. The dashed line represents the detection limit set at 2 log(CFU/g).

Table 7.2: Model parameters of the log-linear model with a Bigelow type temperature dependency fitted to experimental data of the RF inactivation of *S.* Typhimurium and *L. monocytogenes* in skimmed and whole milk powder (n = 4). Significant differences ($p \leq 0.05$) between the microorganisms inoculated in each product are indicated by means of a different lowercase letter. Significant differences ($p \leq 0.05$) between the two powders for each microorganism are indicated by means of a different uppercase letter.

Model Parameters	*S.* Typhimurium		*L. monocytogenes*	
	Skimmed milk powder	Whole milk powder	Skimmed milk powder	Whole milk powder
k(78°C) (1/min)	0.035 ± 0.002^{aA}	0.059 ± 0.002^{aB}	0.061 ± 0.003^{bA}	0.068 ± 0.007^{bB}
z (°C)	45.2 ± 13.2^{bA}	34.4 ± 7.6^{aA}	49.8 ± 14.3^{aB}	31.9 ± 9.2^{aA}

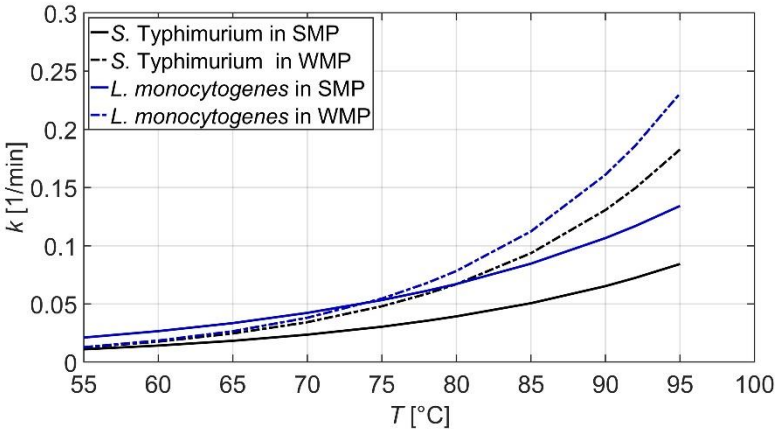


Figure 7.3: The relationship between the inactivation rate k (1/min) and temperature T (°C) for the two microorganisms in the two milk powders as described by the Bigelow-type equation. *S.* Typhimurium is depicted with black colour and *L. monocytogenes* with blue colour while skimmed milk powder (SMP) and whole milk powder (WMP) are depicted with solid and dashed lines respectively.

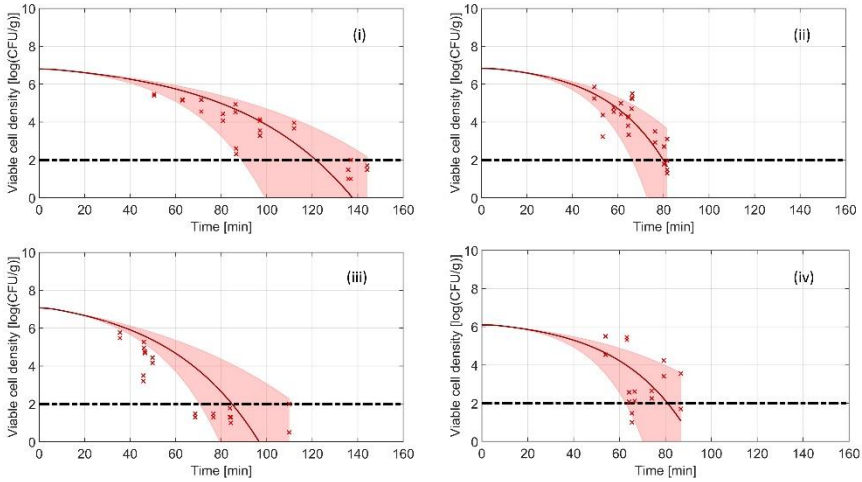


Figure 7.4: Log-linear model with a Bigelow-type temperature dependency fitted to the experimental data of *S*. Typhimurium in skimmed (i) and whole (ii) milk powder and *L. monocytogenes* in skimmed (iii) and whole (iv) milk powder after RF treatment to different end temperatures (n = 4). The 'x' symbols represent the actual data points used to fit the model. An assumed temperature profile was used to generate the model fit. The solid line was generated by taking a profile created using the average heating rate of all the different temperature profiles, while the lower and upper limits of the shaded area were generated by profiles created using the minimum and maximum heating rates, respectively. The dashed line represents the detection limit set at 2 log(CFU/g).

7.3.1 Influence of bacterial Gram-type

S. Typhimurium exhibited a lower *k* and consequently, a higher tolerance to the RF treatments in comparison to *Listeria* in both types of milk powder at temperatures above 60°C A similar trend between the two microorganisms has been documented in different isothermal treatment studies, for various low-moisture food (LMF, products where $a_w < 0.85$ at 25°C) products (Liu et al., 2022b). Rachon et al. (2016) also found *Salmonella* to exhibit varying degrees of higher resistance to thermal treatments than *Listeria* in four different LMFs (confectionery formulation, chicken meat powder, pet food and savoury seasoning). Quinn et al. (2021) reported similar thermotolerances between *L. monocytogenes* and *S*. Typhimurium in wheat flour and in powder

infant formula when treated at 85°C. Conversely, in liquid dairy products, a different pattern has been observed; most species of *Salmonella* are generally more susceptible to heat than *L. monocytogenes* (Mackey and Bratchell, 1989; Rukke et al., 2011). *Salmonella's* acquired resistance to heat appears to be linked to a_w, which has been demonstrated by Liu et al. (2018a) and Xie et al. (2021). These studies showed that D-values for *S. Typhimurium* increased considerably with decreasing a_w values. Dhowlaghar et al., (2021) also verified that a_w is inversely related to the heat tolerance of both *Salmonella* and *L. monocytogenes,* with Salmonella exhibiting higher heat resistance than *L. monocytogenes* during isothermal treatments of desiccated shredded coconut. In the current study, *L. monocytogenes* showed an increase in z value, contrary to *S.* Typhimurium. Similar to the traditional z-value, i.e., the temperature difference required to achieve a tenfold decrease in the D-value, the parameter z in this model represents the sensitivity of k to temperature changes. In skimmed milk powder, *L. monocytogenes* had a higher z in comparison to *S.* Typhimurium, indicating that *L. monocytogenes* could be more resistant in the higher temperature range. *S.* Typhimurium also exhibited higher SICs levels in comparison to *L. monocytogenes* (Figure 7.5). For both microorganisms, a significant standard deviation in SI is observed, likely due to the aforementioned variations in temperature profiles between process runs. While the log-linear model accounts for these variations, the inconsistency in thermal inactivation still leads to fluctuations in SI data since only the final cell densities are considered. Addressing these variations in future studies could lead to more precise determination of SI after RF treatments. Thus far, the generation of SICs after RF treatment has been recorded in various studies with products such as apple cider (Geveke et al., 2009), red pepper powder (Zhang H. et al., 2020b), broccoli powder (Zhao et al., 2017), walnut shells (Zhang et al., 2021b) and edible seeds (Xu et al., 2022). Since SI is predominantly caused by damage in the outer membrane (Zhao et al., 2017), it was crucial to document the differences between bacteria with different cell wall structure i.e., Gram properties, as it is one step closer to establish the inactivation mechanisms.

7.3.2 Influence of milk powder composition

Both the model parameters in Table 7.2 and Figure 7.3 indicate that, for both microorganisms, the inactivation in whole milk powder is significantly higher than in skimmed milk powder. Statistically significant differences ($p \leq 0.05$) between skimmed and whole milk powder are evident above 70°C and 68°C, for *S.* Typhimurium and *L. monocytogenes*, respectively. A comparison of the composition of the two milk powders in Table 7.1 reveals that, along with the difference in fat content, skimmed milk powder contains significantly higher levels of lactose and protein. Therefore, the differences in inactivation rates in the current study were discussed based on the differences in fat, protein and lactose content.

It has commonly been observed that higher fat content results in increased microbial survival in both low and high water activity (a_w) products. Various studies have documented the protective effect of fat in different products (Chhabra et al., 1999; Trimble et al., 2020). Verheyen et al. (2020) documented a more complex relationship between the fat content and the heat resistance of *L. monocytogenes* in fish model systems. Specifically, in emulsions, a positive correlation between fat content and higher inactivation rates was observed, while in gelled systems the opposite relationship occurred. Jin et al. (2018) documented the increased thermal resistance of *S. enterica* in high protein matrices in comparison to high fat matrices, but only at temperatures above 79.5°C. Moreover, Rachon et al. (2016) verified that *Salmonella* shows higher resistance in high protein products than in high sugar products. The protective effect of lactose on the microbial inactivation has also been documented (Ramaswamy et al., 2009; Kim et al., 2017). Ahmad et al., (2022) tested the thermal resistance of *Salmonella* in powders and demonstrated higher D-values in skimmed milk powder in comparison to lactose-free milk powder. The protective effects of lactose and protein, seem to be the most important factor for the observed inactivation behaviour, potentially counteracting the expected influence of fat level based on literature findings. Nevertheless, the complexity of the milk powder's structural environment could lead to different effects of the fat content in comparison to the effects observed in liquid systems.

Even though significantly different, the difference in k values for *Listeria* in the two milk powders at temperatures higher than 68°C was considerably lower than the difference observed in *S.* Typhimurium. At temperatures below 68°C, no significant differences were seen between the types of the milk powder. It is challenging to make a meaningful comparison between the inactivation results in the current study and previous RF inactivation literature results, since most studies on the RF treatment of food products utilise the RF only up to a certain target temperature (come-up-time, CUT) and continue heating either with the assistance of hot air or an oven. Concerning conventional thermal inactivation which primarily relies on heat transfer through conduction, a similar pattern between skimmed and whole milk powder was observed during isothermal treatments using a thermal-death-time sandwich, a dry heat system designed by Lau et al. (2020). Specifically, Wei et al. (2020) isothermally treated *Salmonella*-inoculated skimmed and whole milk powder at 80°C and 0.30 a_w, and reported D values of 9.92 min and 8.11 min, respectively. Additionally, the D values after isothermal treatment at 80°C were slightly higher in skimmed milk powder than whole milk powder for both *Salmonella* and *E. faecium* in the study of Wei et al. (2021).

The complexity of the product's composition i.e., the variety in composition regarding fat, lactose and protein contents poses difficulty in isolating and assessing the individual effect of each component on the bacterial thermal resistance. Moreover, the current study was, to the best of our knowledge, the first to study the effect of milk powder composition on microbial inactivation by a RF-only heating process. The results indicate that a combination of higher lactose and protein content in skimmed milk powder could lead to increased thermal resistance for both microorganisms in the higher temperature range (above 68°C). However, since it is not possible to isolate the effects of each component in the specific powder used in this study, the precise impact of these factors cannot be determined with certainty. Future research should examine the individual effects of lactose and protein concentration on bacterial inactivation after RF treatments by isolating and testing each component separately.

Even though no significant differences were observed between the two milk powders ($p \leq 0.05$), whole milk powder demonstrated an increasing percentage of SI with increasing temperature, whereas high numbers of SI

were already noticed from the beginning of the RF treatments in skimmed milk powder. The rationale for this could be the longer treatment times observed for skimmed milk powder in comparison to the whole milk powder. The microorganisms in skimmed milk powder are exposed to longer periods in the higher temperature range, which may provide an opportunity to activate the stress response adaptation mechanisms. The imminent risk of SICs is that they may undergo morphological changes at a cellular level and strengthen their stress adaptation abilities (Wesche et al., 2009). Therefore the investigation of the acquired traits of recovered SICs should be addressed in future studies. So far, only Jiao et al. (2021) have inspected the recovery and potential cross-protection of sublethally injured *Salmonella* cells in red pepper powder after RF treatment and found no significant differences between regular and recovered cells. Nonetheless, it is vital to verify the lack of acquired resistance to RF treatments in a variety of products, microbial strains and RF processes.

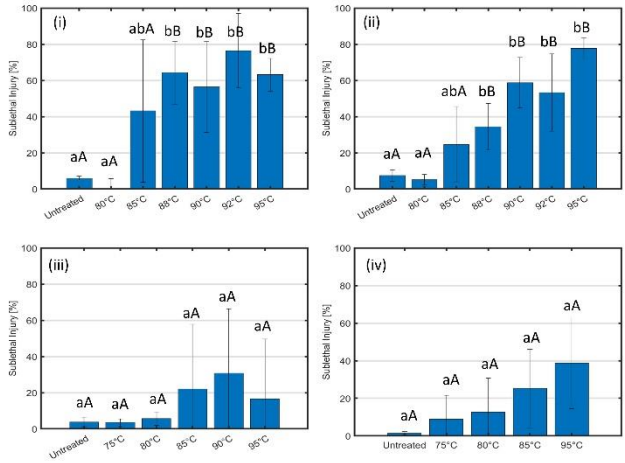


Figure 7.5: Sublethal injury (%) of *S.* Typhimurium in skimmed (i) and whole (ii) milk powder and *L. monocytogenes* in skimmed (iii) and whole (iv) milk powder after radio frequency treatment. Different lowercase letters represent statistically significant differences ($p \leq 0.05$) between RF treated samples in one type of milk powder and different uppercase letters represent statistically significant differences ($p \leq 0.05$) between the two types of milk powder for each microorganism ($n = 4$).

7.3.3 *E. faecium* surrogate validation

Surrogate microorganisms are frequently used for microbial validation studies. Pathogenic strains pose a high risk when introduced to processing plants and draconian safety measures have to be applied upon handling (Ballom et al., 2020). In this study, *E. faecium* was investigated as a surrogate microorganism. The specific *E. faecium* strain has already been successfully used in numerous studies as a surrogate microorganism for *Salmonella* species and *L. monocytogenes* in LMF in both RF and traditional thermal inactivation studies (Dag et al., 2022; Quinn et al., 2021; Liu et al., 2018b; Villa-Rojas et al., 2017). However, the surrogate microorganism should be validated for various microbial strains and industrial processes (Dag et al., 2020). It is vital to verify that it can successfully mirror the behaviour of different pathogenic species under different conditions in order to build safe pasteurisation techniques (Rachon et al., 2016).

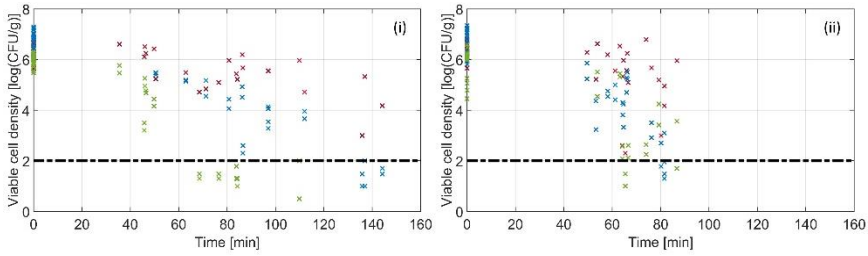


Figure 7.6: The viable cell density (log(CFU/g)) of *E. faecium* (red x), *S.* Typhimurium (blue x), and *L. monocytogenes* (green x) in skimmed (i) and whole (ii) milk powder following RF treatment to various end temperatures. The black dashed line indicates the detection limit set at 2 log(CFU/g).

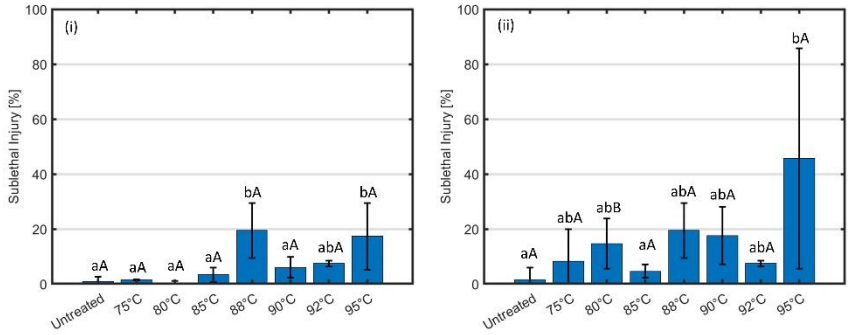


Figure 7.7: Sublethal injury (%) of *E. faecium* in skimmed (i) and whole (ii) milk powder after radio frequency treatment. Different lowercase letters represent statistically significant differences ($p \leq 0.05$) between RF treated samples in one type of milk powder and different uppercase letters represent statistically significant differences ($p \leq 0.05$) between the two types of milk powder ($n = 2$).

Table 7.3: Model parameters of the log-linear model with a Bigelow type temperature dependency fitted to experimental data of the RF inactivation of *E. faecium* in skimmed and whole milk powder. Separate ANOVA tests were performed for each microorganism and each type of milk powder for all model parameters ($n = 2$). Statistically significant differences ($p \leq 0.05$) between the two powders for each microorganism are indicated by means of a different uppercase letter, with 'A' bearing the lowest value.

Model Parameters	*E. faecium*	
	Skimmed milk powder	Whole milk powder
k(78°C) (1/min)	0.009 ± 0.004^{A}	0.020 ± 0.009^{B}
z (°C)	15.8 ± 5.5^{B}	12.3 ± 4.0^{A}

The behaviour of *E. faecium* during RF treatment in skimmed and whole milk powder is illustrated in Figures 7.6 and 7.7. Figure 7.6 depicts the viable cell densities of *E. faecium*, *S.* Typhimurium and *L. monocytogenes* before and after

RF treatments. The temperature profiles to obtain the inactivation data points for *E. faecium* were a combination of the profiles documented in the RF inactivation of both *S.* Typhimurium and *L. monocytogenes* (Appendix A). A log-linear model was fitted to the inactivation data of *E. faecium* and the model parameters are presented in Table 7.3. The SI of *E. faecium* after treatment is presented in Figure 7.7. Overall, *E. faecium* demonstrated higher tolerance to the specific RF treatments than both pathogens and was therefore found to be a reliable surrogate. The trend of higher inactivation rates observed in the whole milk powder for *S.* Typhimurium and *L. monocytogenes* also applies to *E. faecium*. In general, even though there is a slightly higher SI observed in the whole milk powder in comparison to the skimmed milk powder, statistical significant differences ($p \leq 0.05$) are shown only in the treatment up to 80°C. The surrogate also demonstrated considerable lower levels of SICs in comparison to both microorganisms. The standard deviation observed in the SI of *E. faecium* is lower compared to the SI of the pathogens (Figure 7.5). This reduced variability could be attributed to the higher tolerance of *E. faecium*, which makes it less susceptible to temperature fluctuations. Nonetheless, the increasing levels of SI with higher temperatures and the high standard deviation indicate that the current processing method may induce SI, even in the most thermotolerant microorganisms. Furthermore, the long treatment times of this newly designed RF-only process may pose energy and cost limitations for potential industrial applications. The proposed treatment strategy should be compared to conventional RF treatments including a non-RF temperature holding phase for similar products in terms of energy consumption, cost, inactivation efficiency and its impact on product quality. Further research is necessary in order to evaluate its efficacy across a variety of thermotolerant microorganisms and strains, and to investigate the potential for cell recovery during storage.

7.4 Conclusions

In the current study, the influence of bacterial Gram-type (*L. monocytogenes* vs. *S.* Typhimurium) and milk powder (skimmed vs. whole) composition on the inactivation of pathogenic bacteria during a novel RF-only non-isothermal decontamination process of milk powder was investigated. Concerning the influence of bacterial Gram-type, *S.* Typhimurium (Gram-negative) was more resistant to the RF treatment in comparison to *L. monocytogenes*, although this could also be linked to the specific high thermal resistance of *S.* Typhimurium at low a_w. Concerning the influence of milk powder composition, bacteria were more thermally resistant in skimmed milk powder than in whole milk powder, which could be related to protective effects of higher lactose and protein contents in the skimmed milk powder outweighing the (possible) protective effects of higher fat content in the whole milk powder. However, in order to better understand the occurring effects for both the Gram-type and milk powder composition, further studies should be conducted, focusing on (i) including a variety of microorganisms (Gram-negative and Gram-positive, as well as different strains), (ii) isolating each component (protein, lactose, fat) of milk powder to determine their individual impacts on thermal resistance, and (iii)identifying the exact RF inactivation mechanisms. *E. faecium* was also demonstrated to be a suitable surrogate microorganism for this type of RF-only process, showcasing its potential use in future studies aiming to develop similar processes for other food products. Considering the potential of applying RF-only dynamic heating treatments, future research should focus on establishing uniform temperature distribution to ensure product safety and quality.

Acknowledgement

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Chapter 8

RF inactivation of *S.* Typhimurium and *L. monocytogenes* in dairy model systems: liquids and gels

The results presented in this chapter have been published to *Innovative Food Science & Emerging Technologies*:

Tonti Maria, Verheyen Davy, Kozak Dmytro, Coombes Chloe, Hossain Mohammad, Skåra Torstein, Van Impe Jan. Inactivation of *Salmonella* Typhimurium and *Listeria monocytogenes* in dairy systems: effect of fat and food matrix structure under radio frequency heating. *Innovative Food Science & Emerging Technologies*. 103684.

The contributions of the first author to this work are:

- Literature study
- Co-design of experiments
- Co-performing of experiments
- Co-interpretation of results
- Co-formulation of conclusions
- Text redacting and editing

This chapter includes the referred paper. Part of the content is identical to the content of the submitted paper. However, some adaptations in content and formatting have been made.

Context: The RF-only dynamic heating approach was used again for the treatment of dairy liquids and gels. For the experiments of this chapter, a lab-scale RF set-up was used for the treatments. Four dairy systems were used, with two different structures (liquid and gel matrix) and two different fat contents (0.08% and 5.9%). The same skimmed and whole milk powders used in previous chapters were the basis for the formulation of all systems. The RF inactivation kinetics of *S.* Typhimurium and *L. monocytogenes* in the four different systems were acquired. Due to the differences in the inactivation kinetics and as the aim was to compare the effect of two intrinsic factors, i.e., fat content and presence of a gel matrix on the RF inactivation, a specific modelling approach was adopted. A log-linear model with a Bigelow-type temperature relation was used only until the specific treatment point that a linear inactivation behaviour was observed. This enabled the statistical comparison of the model parameter and the behaviour which did not follow the linear trend was discussed qualitatively.

Abstract

The effect of two food intrinsic factors (fat content, matrix structure) on the Radio frequency (RF) inactivation of *Salmonella* Typhimurium and *Listeria monocytogenes* was investigated in dairy model systems: i.e., liquid systems with 0.08% and 5.9% fat and gel systems with 0.08% and 5.9% fat. In liquids, higher fat content resulted in higher inactivation for both microorganisms. In gel systems, inactivation rate was higher at lower fat content, with opposite behaviour occurring for *L. monocytogenes* above 70°C. A gel matrix resulted in a higher inactivation of *S.* Typhimurium, while complex temperature-dependent behaviour was present for *L. monocytogenes*. Sublethal injury was most significant for *L. monocytogenes* at 5.9% fat content and high temperatures. Overall, fat content and matrix structure impacted the microbial inactivation by RF heating, and this different as compared to conventional heating, which should be taken into account when designing novel RF heating food decontamination strategies.

8.1 Introduction

An increasing number of novel technologies are being introduced to and used in the food industry to effectively tackle challenges related to food safety, nutritional quality, and sustainability. For many years, ensuring the safety of food has been a fundamental concern, with both global and regional implications. The most pressing issue of the food industry regarding food safety is the presence of microbial hazards, which can cause serious illnesses and outbreaks leading to severe economic damages with tremendous consequences, not only for the food and other companies, but also the governments (Fung et al., 2018). Currently, the conventional methods of ensuring microbiological safety of food involve thermal treatments like pasteurization and sterilization. However, the conventional methods can have several limitations as they can lead to the loss of nutrients and food attributes, negatively affecting the product's end quality (Chandrasekaran et al., 2013). Additionally, conduction heating can lead to non-uniform temperature distribution, such as the formation of hot and cold spots, and challenges in maintaining consistent temperatures, particularly in dense or bulk products (Ozturk et al., 2017). To address these challenges, the food industry has shifted its focus to innovative heating technologies, such as ohmic heating, microwave heating (MW), and radio frequency (RF) to ensure food safety without compromising the quality of products (Van Impe et al., 2018).

RF has a dielectric heating method similar to MW, as both technologies utilise electromagnetic radiation which penetrates the food products and generates heat. RF has lower frequencies and longer wavelengths compared to MW. The prevailing frequency in the food industry is 27.12 MHz (wavelength of 11.1 m). This leads to a larger penetration depth, and hence, a more uniform heating (Altemimi et al., 2019). Initially, RF has been used for drying, disinfestation and thawing applications, but, in the last decade, numerous studies have been conducted using RF processing for a variety of products aiming at pasteurisation (Di Rosa et al., 2019).

Thus far, the effect of food microstructure on RF treatments has not been explored. Most studies that have investigated RF treatment of liquid products were limited to specific real food products (e.g., Awuah et al., 2005; Geveke

and Brunkhorst, 2004; Ukuku et al., 2008). Furthermore, studies that have investigated RF treatments of solid food products (such as gels) focused on heating uniformity (Birla et al., 2008; Wang S. et al., 2003; Wang Y. et al., 2003), rheological and gelation properties (Ahmed et al., 2007b; Kar et al., 2020), water retention and structural properties (Wang L. et al., 2021; Wang X. et al., 2021). However, food intrinsic complexity influences the type of microbial growth (planktonic cells, submerged colonies, surface colonies), and, in turn, possibly influences the microbial resistance to RF. Hence, it is crucial to analyse and decipher the effect of certain food intrinsic factors on the RF inactivation of foodborne pathogens. The use of relevant food model systems is key as it ensures reproducibility and the absence of interfering background microflora. Additionally, it presents the advantage of allowing altering intrinsic food factors independently from one another, facilitating the isolation of the effect each factor can have on microbial inactivation (Baka et al., 2017b).

One of the targeted food categories for RF processing is dairy products, since conventional heating applications lead to fouling in heat exchangers due to the overheating of the product when it comes into contact with the wall of the heat exchangers (Awuah et al., 2005). These products can contain a variety of foodborne pathogens, among which *Salmonella* species and *Listeria monocytogenes* (EFSA & ECDC, 2019). There have been numerous incidents of recalls and outbreaks implicating these microorganisms in different dairy products such as milk, cheese and dairy powders (Dag et al., 2022; Lindsay et al., 2021). Thus far, the use of RF has been studied for treatment of various dairy products, such as milk (Awuah et al., 2005; Di Rosa et al., 2018; Srisuma et al., 2017), yoghurt (Siefarth et al., 2014), goat's milk (Zhu et al., 2014), skimmed or whole milk powder (Chen et al., 2013; Dag et al., 2022; Michael et al., 2014; Zhong et al., 2017) and powdered infant formula milk (Lin et al., 2020; Zhang Y. et al., 2020b, 2022, 2023). Nevertheless, the role of specific intrinsic factors of dairy products during RF inactivation treatments has yet to be investigated, with the influence of microstructural aspects as a prominent example. Intrinsic factors in dairy products refer to the inherent physical and chemical properties including composition (fat and protein content), water activity (a_w) and pH, while the microstructural aspects describe how these components are intricately arranged. These factors can influence the product's behaviour during processing (Auty, 2018). These

effects have to some extent been established for conventional thermal treatments, but, to the best knowledge of the authors, they have not been studied for RF treatments. For instance, the fat content in dairy can affect microbial resistance to heat, with higher fat levels potentially offering a protective mechanism for pathogens (Bermudez-Aguirre & Barbosa-Cánovas, 2008). Similarly, the protein content plays a crucial role, as protein denaturation during heat treatments can impact the thermal resistance of microbes (Atamer et al., 2010). A_w in dairy products is another critical factor, as it represents the amount of water available for microbial activity, with lower aw often correlating with increased microbial resilience to thermal processes (Wei et al., 2020). The pH also affects microbial heat resistance, where variations in acidity or alkalinity can increase or decrease the effectiveness of thermal inactivation (Blackburn et al., 1997). Finally, the microstructural aspects can influence heat transfer dynamics within the product, affecting the uniformity and efficiency of pathogen inactivation (Verheyen et al., 2020).

The innovative aspect of this study lies in the systematic study of RF treatments by using food-relevant, dairy-based systems with various microstructures. The primary focus was to investigate the effect of two factors, (i) the effects of fat content and (ii) matrix structure on RF thermal inactivation of two pathogenic microorganisms (i.e., *S.* Typhimurium and *L. monocytogenes*). In order to assess the effect of these factors in an objective way (i.e., using inactivation parameters), it was necessary to select a mathematical model that adequately describes the inactivation of the two microorganisms. A log-linear inactivation model, including a secondary model to account for the temperature dependency of the inactivation rate was therefore fitted to experimental data for both microorganisms in the four different systems.

8.2 Materials and methods

8.2.1 Dairy systems preparation

In total four dairy model systems were developed, i.e., two liquid systems, with 0.08% (Liquid System 1/LS1) and 5.9% fat (Liquid System 2/LS2) and two gel systems, with 0.08% (Gel System 1/GS1) and 5.9% fat (Gel System 2/GS2). The model systems were designed as such to represent real food products, i.e., liquid skimmed milk (LS1), liquid whole milk (LS2), reduced-fat milk pudding (GS1) and milk pudding with fat (GS2). The main composition of each of the developed dairy systems is shown in Table 1.

8.2.1.1 Materials and microorganisms

Milk powder was used as basis for the formulation of both liquid and gel systems. Skimmed (0.8% fat) and whole (35% fat) milk powder were purchased from TINE SA (Oslo, Norway). The indicative composition (wt.%) of the two types of milk powder provided by TINE SA was presented in Table 7.1. The presence of background microflora was inspected by sampling in triplicate on TSA supplemented with 0.6% yeast extract (Merck, Darmstadt, Germany). The gel systems were formed by the addition of sodium alginate (Sigma-Aldrich, MO, USA). Alginate has the ability to form gels which are stable during heating, when Ca^{2+}-ions are present in the system (Verheyen et al., 2018a). To serve this purpose, calcium carbonate ($CaCO_3$, Sigma Aldrich, MO, USA) was added to the gel system. Foodborne pathogenic strains *Salmonella enterica* serovar Typhimurium ATCC 14028 (isolated from bovine, septicaemic liver) and *L. monocytogenes* LMG 23356 (isolated from Jalisco Cheese) were used in this study. The *Salmonella* strain was obtained from the Gothenburg Bacterial Collection and the *Listeria* strain was obtained from the bacterial collection of Ghent University. Stock cultures were stored in 2 ml cryovials (Microbank™, Pro-Lab Diagnostics, Canada) at a temperature of -80°C.

8.2.1.2 Dairy systems formulation

To prepare the liquid systems, specific quantities of each milk powder were added to sterile distilled water under sterile conditions, following the manufacturer's instructions. Specifically, 10.3 g of skimmed milk powder was added to 89.7 mL distilled water and 16.8 g of whole milk powder was added to 83.2 mL distilled water to formulate LS1 and LS2, respectively. The mixtures were first thoroughly mixed using a stirring plate at 400 rpm to dissolve the milk powder. Subsequently, they were dynamically heated to 80°C for 17 min while stirring continuously. The purpose of the heating was twofold, first to enhance the solubility of the milk powder and second to eliminate any background microflora present in the solid materials. The systems were then allowed to cool to room temperature.

Table 8.1: Composition of the four systems i.e., liquid system 1 (LS1, 0.08% fat), liquid system 2 (LS2, 5.9% fat), gel system 1 (GS1, 0.08% fat) and gel system 2 (GS2, 5.9% fat), where fat, protein, carbohydrates as well as vitamins and minerals are the components of the respective milk powder.

Main Composition (% w/w)	**LS1**	**LS2**	**GS1**	**GS2**
Fat	0.08	5.88	0.08	5.88
Protein	3.8	3.99	3.8	3.99
Carbohydrates	4.93	5.69	4.93	5.69
Vitamins and minerals	0.75	0.74	0.75	0.74
H_20	90.44	83.7	88.84	82.1
Alginate	/	/	1	1
Calcium carbonate ($CaCO_3$)	/	/	0.6	0.6

The preparation of the gel systems (GS1 and GS2) involved the formulation of two different aqueous solutions. The first solution contained milk powder and $CaCO_3$ diluted in distilled water (aqueous solution 1) and the second was a 5% alginate solution (aqueous solution 2).

For aqueous solution 1 of GS1, a sterile 500 mL Schott bottle equipped with a magnetic stirrer was filled with 256.72 mL of distilled water (dH_2O). For GS2, the bottle was filled with 226.94 mL of distilled water. To ensure sterility, the water was autoclaved in the Schott bottles.

Under a laminar flow cabinet, 41.2 g of SMP and 2.08 g of $CaCO_3$ were added to the autoclaved water for GS1, while 67.2 g of WMP and 1.96 g of $CaCO_3$ were added for GS2, using sterile spoons. The mixtures were then heated dynamically in a designated stirrer at 80°C for 17 minutes while stirring continuously.

For the aqueous solution 2, 5 g of sodium alginate (Sigma Aldrich, St Louis, USA) was mixed with 95 g of distilled water. The mixture was then stirred continuously for a minimum of 3 hours to ensure complete dissolution of the alginate. When dissolved, the stock solution was filter-sterilised using a filter with 0.22 μm pore size membrane (TPP, Trasadingen, Switzerland). Heat sterilisation was avoided as high temperatures can cause depolymerisation of alginate and result in weak gel formation (Daigle and Cotty, 1997).

Solution 1 was inoculated with the bacterial inoculum, mixed thoroughly for 1 min and then 25 g of solution 2 were added to solution 1 under sterile conditions. The mixture was stirred thoroughly for 2 min to ensure complete and uniform integration of the alginate into the solution and to ensure the bacterial inoculum was homogeneously dispersed throughout the mixture. The bacterial inoculation procedure is detailed in 8.2.2.

The model systems were formulated as such to maintain consistent a_w and pH levels between them. The a_w of the liquid and gelled systems were 0.98 ± 0.01 and 0.99 ± 0.01, respectively, as measured by an aw meter (AquaLab 3TE, Decagon Devices Inc., WA, USA). The pH of all systems was 6.64 ± 0.05, as measured by a pH meter (FiveEasy Plus FEP20, Mettler Toledo, Greifensee, Switzerland).

8.2.2 Inoculation and RF treatments

The bacterial inoculum was prepared by transferring one bead of the cryovial to an Erlenmeyer flask with 20 mL appropriate broth for each microorganism. Specifically, BHI (Merck, Darmstadt, Germany) and TSB (Oxoid, Basingstoke Hampshire, England) were used for *L. monocytogenes* for *S*. Typhimurium, respectively. The inoculated cultures were incubated for 24 h at 30°C for *L. monocytogenes* and at 37°C for *S*. Typhimurium. The cultures were then streaked on non-selective agar media to create the purity plate. *L. monocytogenes* was streaked on BHI with 10% Agar (Merck, Darmstadt, Germany) which was then incubated at 30°C for 48 h. *S*. Typhimurium was streaked on TSA and the plate was incubated at 37°C for 24 h. Single developed colonies were used to create two consecutive 24 h precultures by inoculating into Erlenmeyers with the respective broth and incubating at optimum temperature. Three individual biological replicated were made for each microorganism. Each replicate was used to inoculate 100 mL of a dairy system. The dairy systems were placed directly into sterile, glass vials (4 mL, 45.0 x 14.7 mm, BGB Analytik Benelux B.V., Harderwijk, the Netherlands) with a polypropylene screw cap lid with a hole in the middle and a thin glass capillary, also sterile. The capillary was in contact with the sample and served to introduce an optic fiber sensor which measured the temperature during the RF process. The sensor was connected to a Bench Top FOTEMP-PLUS signal conditioner (Weidmann Technologies Deutschland GmbH, Dresden, Germany) and the FOTEMP-Assistant software (Optocon, AG, Dresden, Germany) was used to process the recorded temperature data. For the inoculated liquid systems, the sample volume of each vial was 3 mL. For the gel systems, as mentioned in 8.2.1.2, aqueous solution 1 was inoculated by adding 100 μL of the second bacterial preculture and then mixed with solution 2, creating a viscous solution. Then, 1.5 mL of the system was distributed over the vials. The vials were then incubated at 10°C for 16 hours. Afterwards, they were placed for a short time in a 20°C incubator prior to treatment to acquire the same initial temperature as the liquid systems.

A lab-scale low-power free-running radio frequency (RF) oscillator operating at a frequency of 27.12 ± 0.50 MHz (Espitia et al., 2023; Kozak et al., 2021), suitable for basic microbiological research, was used for the RF treatments. In brief, an input power of 14-28 W was used to generate electromagnetic

waves in approximately around 27.12 MHz. A flyback topology design (involving air-core transformer, i.e., Tesla coil) was used to build the RF generator. The hot end of the high voltage transformer was connected to the electrode terminal (i.e., 16.25 mm diameter copper coin), while the cold end was connected to the base terminal of an NPN bipolar junction transistor (type 2SC2275A, SavantIC Semiconductor, Irvine, CA, USA). The primary and secondary coil consisted of enamelled copper wire winded around glass tubes, with no pinch between turns. A resistor with a resistance of 887 Ω was used. Two power supplies (Aim-TTi EX354RD, 280 W, RS Components, Brussels, Belgium) provided a permanent positive bias at the base-emitter terminal of the transistor (i), and adjustable power (ii). A more detailed description of setup components (e.g., cooling fan) is provided in Kozak et al. (2021).

The samples were treated using a dynamic heating profile to targeted end time points. To ensure a consistent temperature profile for each model system, the power was adjusted manually during the RF process. After treatment, samples were placed immediately in ice cold water to cool down and discontinue the inactivation.

8.2.3 Sampling and microbial enumeration

Liquid systems were diluted and plated immediately, while for the gel systems, a protocol based on Hamoud-Agha et al., (2013) was used. In short, the gel was transferred to a sterile stomacher bag using a sterile loop, and 4.5 mL of 0.90% saline solution was added in two-steps; first to the vial, to wash any remaining of the gel, and then to the bag. The sample was stomached thoroughly for 5 min.

The viable cell counts were determined by plating the samples on non-selective and selective media. Serial dilutions were performed using 0.9% saline solution and the samples were plated using the 3-drop technique (Herigstad et al., 2001). For the undiluted sample, 100 µL were plated uniformly using a spreader, and 1 mL was distributed over 3 plates in order to acquire a detection limit of 1 log(CFU/mL) and 1.6 log(CFU/mL) for liquid and gel systems, respectively. BHI Agar and PALCAM were used for *L. monocytogenes*, while TSA and XLD Agar were used for *S.* Typhimurium.

After plating, the samples were incubated for 24 hours at 30°C and 37°C for *L. monocytogenes* and *S.* Typhimurium, respectively, after which enumeration followed.

8.2.4 Microbial inactivation kinetics modelling and sublethal injury assessment

Two modelling approaches were checked for their suitability to represent the microbial behaviour in the current study, i.e., a log-linear (Equation 8.1) and a log-linear tail (Equations 8.2) model with a Bigelow-type temperature relation (Equation 8.3).

$$\frac{dN_{(t)}}{dt} = -k\,(T(t)) \cdot N(t) \tag{8.1}$$

$$\frac{dN(t)}{dt} = -k(T(t)) \cdot \left(1 - \frac{N_{res}}{N(t)}\right) \cdot N(t) \tag{8.2}$$

$$k\big(T(t)\big) = k\left(T_{ref}\right) \cdot 10^{\frac{T_{(t)} - T_{ref}}{z}} \tag{8.3}$$

with $N(t)$ (CFU/mL), the cell density at time t; N_{res} (CFU/mL), the residual cell density; k (1/min), the specific inactivation rate; $T_{(t)}$ (°C), the average time-dependent core-temperature of the model system over all RF treatments for the respective conditions; T_{ref} (°C), the reference temperature set at 64°C; $k(T_{ref})$ (1/min), the specific inactivation rate at the reference temperature; and z (°C), the temperature sensitivity parameter. The reference temperature T_{ref} at 64°C was selected to fall within the range of inactivation temperatures reached during treatment (Akkermans et al., 2020).

The model parameters were estimated with the Optimisation Toolbox of MatLab version R2021a (The Mathworks Inc., Natick, USA) by the functions patternsearch and lsqnonlin. The Matlab solver ode23 was used to solve the ordinary differential equations of the inactivation model. The parameter standard errors and confidence intervals were computed using the Jacobian matrix and the Fisher information matrix. The initial cell density *n0*

(log(CFU/mL)) was also used as a model parameter in the estimation process. Its inclusion as a parameter allows the model to account for variations in the initial microbial load, which can significantly influence the predicted inactivation dynamics.

While the log-linear tail model was applied to the entire dataset, the log-linear inactivation model (Equation 8.1) was only applied to the (temperature-dependent) log-linear part of the inactivation behaviour. Therefore, the model was fit to subsets of the data with a different final time point, using the Root Mean Squared Error (RMSE) as a measure of goodness-of-fit. Since the RMSE is dependent on dataset size (e.g., if only two data points are considered, RMSE is low and the behaviour linear), subsets included at least four time points. For liquid and gel model systems, subsets had final time points above and including 9 and 10 min, respectively.

Heat treatments can also induce non-lethal injuries to bacterial cells. These sublethally injured cells (SICs) are able to survive and grow in a favourable environment. The percentage of the sublethal injury (SI) can be calculated by Equation 8.4:

$$SI\ (\%) = \frac{N_{non-selective} - N_{selective}}{N_{non-selective}} \cdot 100 \quad (8.4)$$

where $N_{non\text{-}selective}$ is the cell density (CFU/g) measured on non-selective media and $N_{selective}$ the cell density (CFU/g) measured on selective media (Govaert et al., 2020a).

8.2.5 Statistical analysis

Analysis of variance (ANOVA, single factor) was used to identify significant differences between experimental results and the model parameters with a 95% confidence level (a = 0.05). The means that differed from one another were identified by the Fisher's Least Significant Difference test. MatLab R2021a (The Mathworks Inc., Natick, USA) was used to conduct the experimental data processing. Test statistics were regarded significant when $p \leq 0.05$.

8.3 Results and discussion

8.3.1 Modelling approach

The aim of this section was to identify the modelling approach which best characterises the microbial inactivation behaviour, facilitating the accurate comparison of the model parameters among different conditions. The RF inactivation of *S*. Typhimurium in LS1 was used as a case study to demonstrate the step-by-step decision factors regarding the modelling approach. The respective analysis for the inactivation kinetics of *S*. Typhimurium in the other systems, as well as the kinetics of *L. monocytogenes*, can be found in Appendix B. The RF inactivation data of *S*. Typhimurium *and L. monocytogenes* in the four different systems are shown in Figure 1.

First of all, it was determined whether the log-linear tail model could be fitted to the experimental data. Figure 8.2 demonstrates the fitting of the model to the experimental data for *S*. Typhimurium in LS1. Corresponding graphs for the remaining conditions can be found in Appendix B (Figure B.1), accompanied by detailed model parameters (Appendix B, Tables B.1 and B.2). The log-linear tail model cannot effectively represented the inactivation behaviour, which appears to be more complex as two phases can be distinguished in Figure 8.2. Initially, the inactivation follows a temperature-dependent log-linear trend, with the inactivation rate increasing as the temperature increases with time. Towards the end of the treatment, the observed tailing behaviour was characterised by decreasing microbial cell numbers as the treatment progressed. This behaviour suggests a high thermal resistance of the remaining bacterial cells at rising temperatures and extended treatment times. Thus, the second phase can be better characterised by a second inactivation phase than by a constant tail, indicating a biphasic behaviour (Geeraerd et al., 2005). However, a biphasic model would require additional model parameters, and the accuracy of the model for the second inactivation phase would be limited due to the many points below the detection limit that cannot be taken into account for the model.

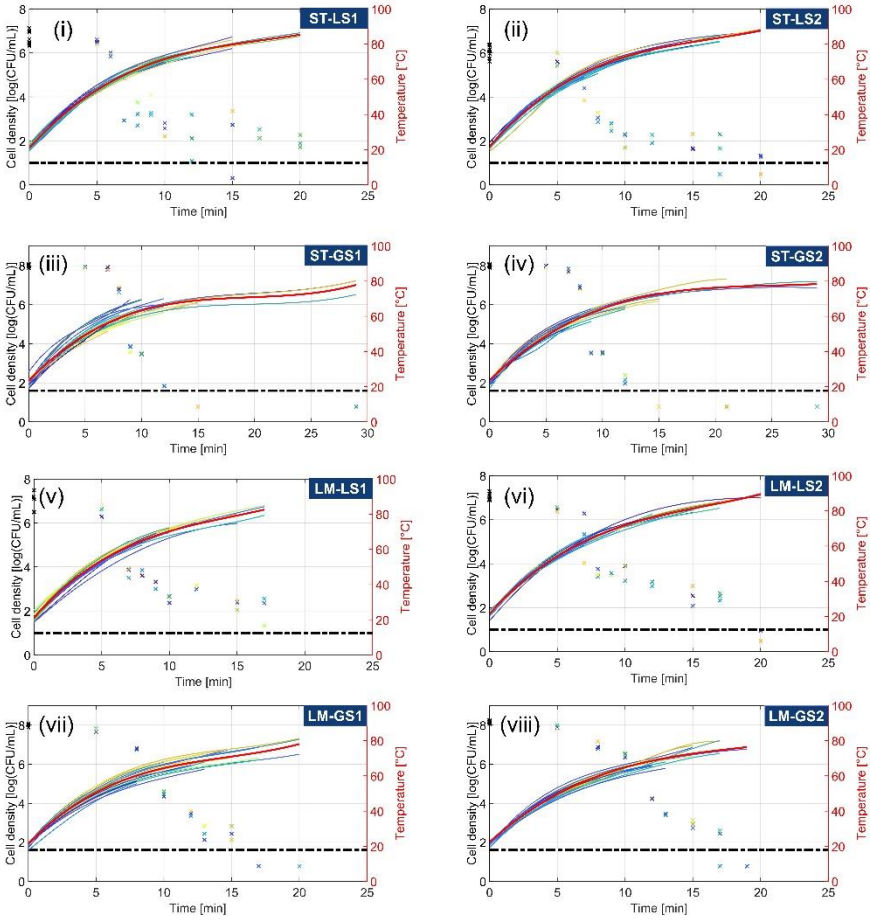


Figure 8.1: Cell density (log(CFU/mL)) of *S.* Typhimurium in (i) liquid system 1, 0% fat (ST-LS1), (ii) liquid system 2, 5.9% fat LS2 (ST-LS2), (iii) gel system 1, 0% fat (ST-GS1), (iv) gel system 2, 5.9% fat (ST-GS2), and *L. monocytogenes* in (v) liquid system 1, 0% fat (LM-LS1), (vi) liquid system 2, 5.9% fat (LM-LS2), (vii) gel system 1, 0% fat (LM-GS1), and (viii) gel system 2, 5.9% fat (LM-GS2) after RF treatment to different end temperatures (n = 3). Data points of black colour represent initial cell density (log(CFU/g)). Data points of different colours represent the cell density (log(CFU/g)) after RF treatment to the respective end temperature. The temperature profile of each treatment corresponds to the specific data points of similar colour. The thick red temperature profile corresponds to a 3rd degree polynomial temperature profile of the average temperature derived by all treatments.

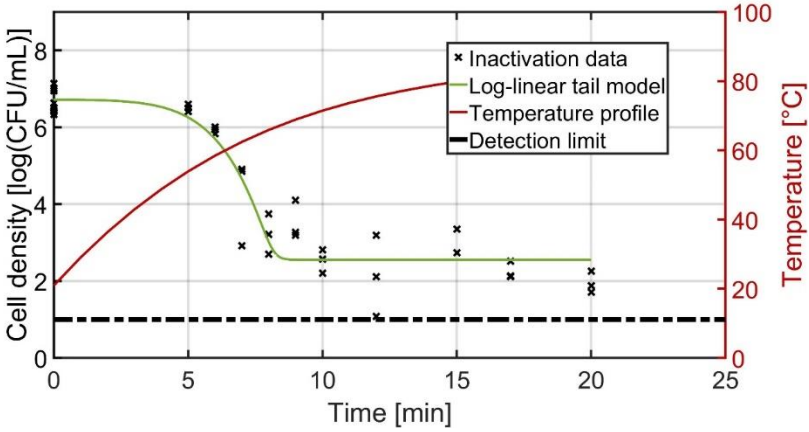


Figure 8.2: Figure 2: Log-linear tail model fitted to the RF inactivation data of *S.* Typhimurium in LS1. The model uses a secondary temperature dependency model (Bigelow-type relation). Data points below detection limit were not considered in the model.

For these reasons, the selected modelling approach was to apply only a log-linear model only for the part of the inactivation that was characterised by (temperature-dependent) loglinear inactivation behaviour, and qualitatively assess the behaviour of the resistant cells (not accounted for in the model) during results interpretation. Figure 8.3 shows the model of *S.* Typhimurium in LS1 fitted for specific subsets of data separately (i)-(vii) and all the generated model lines corresponding to each of these subsets (viii). The estimated model parameters are presented in Table 8.2. Based on the lowest RMSE criterium and interpretation of the graphical representation, the data subset with final treatment time point of 10 min best demonstrated the linear behaviour. Furthermore, selecting longer treatment times, such as the 15, 17, and 20 min treatment points, resulted in a significant increase in the z-value closer to the model bounds, further supporting the decision to opt for shorter time points.

The described method was employed for both microorganisms in all four systems (Appendix B, Figures B.2-B.8 and Tables B.3-B.9). The optimal final times for the liquids systems of both microorganisms were 10 and 9 min for LS1 and LS2, respectively. For the gel systems, the time points of 10 min and 13 min were selected for *S.* Typhimurium and *L. monocytogenes*, respectively.

All generated models are presented in Figure 8.4 and the model parameters are shown in Table 8.3.

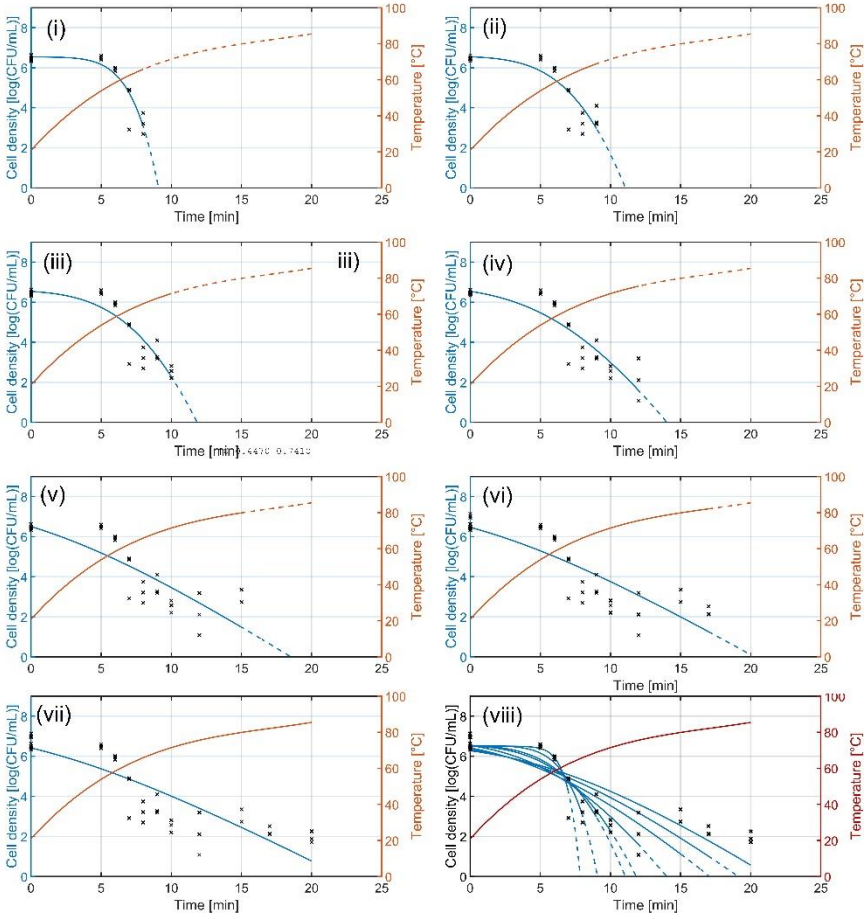


Figure 8.3: Log-linear models with a Bigelow-type temperature dependency fitted to the RF inactivation data of *S.* Typhimurium in LS1 for subsets with the specific final time points (min): (i) 9, (ii) 10, (iii) 12, (iv) 15, (v) 17, (vi) 20 shown in solid blue line. The average temeprature profile is depicted in red solid line and the extended prediction of each model is depicted in dashed lines. A combination of all possible model iterations can be seen in (viii).

Table 8.2: Model parameters of the Log-linear model with a Bigelow type temperature dependency fitted to experimental data of the RF inactivation of *S*. Typhimurium in liquid system 1 (LS1) (n = 3) with their standard deviations.

Treatment time point (min)	Model Parameters			RMSE
	$k(T_{ref})$ (1/min)	z (°C)	$n0$ (log(CFU/mL))	
9	0.73 ± 0.14	47.84 ± 21.33	6.83 ± 0.38	1.05
10	0.69 ± 0.10	51.64 ± 18.82	6.81 ± 0.32	0.96
12	0.47 ± 0.05	119.11 ± 64.94	6.85 ± 0.31	1.12
15	0.35 ± 0.04	600 ± 1807.80	6.76 ± 0.51	1.53
17	0.32 ± 0.04	600 ± 2078.00	6.59 ± 0.66	1.52
20	0.28 ± 0.03	600 ± 1439.40	6.33 ± 0.41	1.63

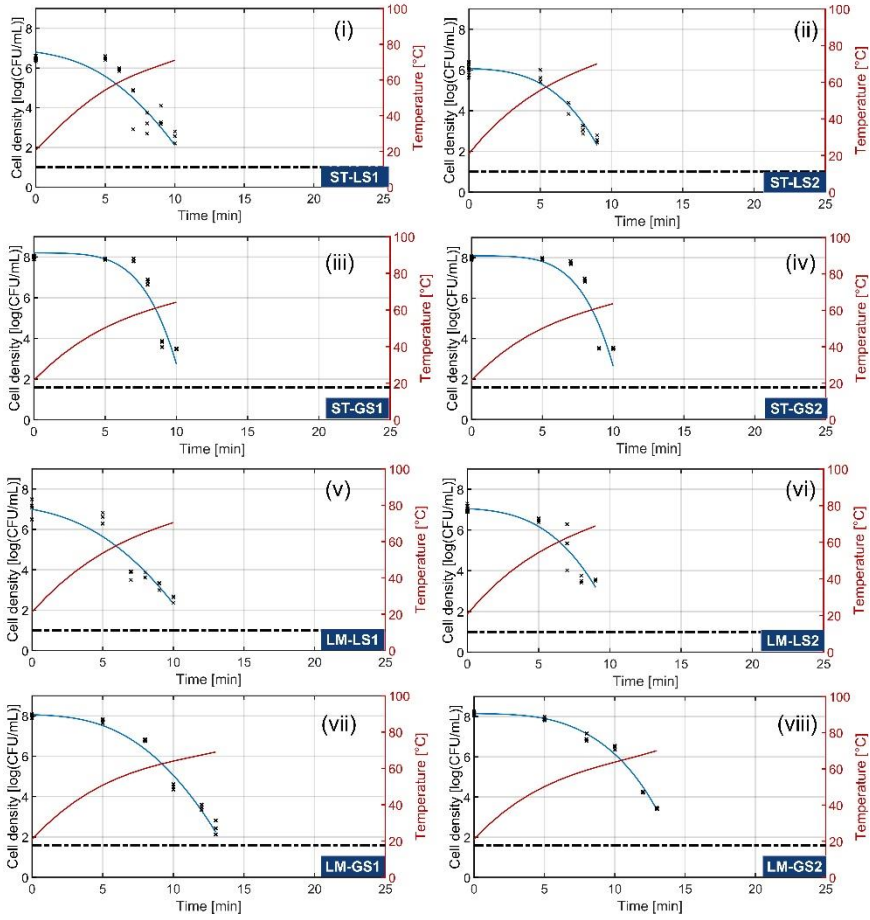


Figure 8.4: Log-linear model with an average temperature profile fitted to the experimental data of *S.* Typhimurium in (i) LS1, (ii) LS2, (iii) GS1, (iv) GS2, and *L. monocytogenes* in (v) LS1, (vi) LS2, (vii) GS1, and (viii) GS2 after RF treatment (n = 3).

Table 8.3: Model parameters of the log-linear model with a Bigelow type temperature dependency fitted to experimental data of the RF inactivation of *S*. Typhimurium and *L. monocytogenes* in the four systems (n = 3), with their standard deviations. Lowercase letters denote statistically significant differences ($p \leq 0.05$) in fat content within each microorganism. Uppercase letters indicate significant differences ($p \leq 0.05$) in structure. LS1, liquid system 1 (0.08% fat); LS2, liquid system 2 (5.9% fat); GS1, gel system 1 (GS1, 0.08% fat); GS2, gel system 2 (5.9% fat).

Model Parameters	*S*. Typhimurium				*L. monocytogenes*			
	LS1	LS2	GS1	GS2	LS1	LS2	GS1	GS2
$k(Tref)$ (1/min)	0.69 ± 0.10^{aA}	0.72 ± 0.07^{aA}	2.56 ± 0.15^{bB}	2.20 ± 0.32^{aB}	0.68 ± 0.22^{aA}	0.81 ± 0.16^{aB}	0.74 ± 0.05^{bA}	0.61 ± 0.02^{aA}
z (°C)	51.64± 18.82^{aB}	32.34 ± 6.71^{bB}	14.50 ± 1.30^{aA}	16.75 ± 3.05^{bA}	57.55 ± 15.38^{bB}	33.00± 12.92^{aB}	28.72 ± 5.23^{bA}	19.46 ± 0.85^{aA}
$n0$ (log (CFU/mL))	6.81 ± 0.32^{bA}	6.11 ± 0.13^{aA}	8.34 ± 0.25^{aB}	8.33 ± 0.32^{aB}	7.00 ± 0.20^{aA}	7.05 ± 0.21^{aA}	8.07 ± 0.28^{aB}	8.06 ± 0.06^{aB}

8.3.2 Investigation of the influence of food intrinsic factors on RF heating inactivation

The log-linear model was used to investigate the influence of food intrinsic factors on the RF heating inactivation, i.e., fat content and the matrix structure. The inactivation rate k was used to compare the effects of each factor, along with the behavioural trend of k in the higher temperature range, presented in Figure 8.5 (effect of the fat), Figure 8.6 (effect of the matrix

structure) and Table 8.3 (model parameters). The behaviour of the resistant cells which do not follow the log-linear trend was discussed qualitatively. Additionally, the SI (%) is presented in Figure 8.7 for each condition in each data point.

8.3.2.1 Influence of the fat content

Early studies with conventional heating treatments established that the presence of fat in different products can have a protective role in the inactivation of microorganisms (Ahmed et al., 1995; Chhabra et al., 1999; Fain et al., 1991; Senhaji, 1977; Senhaji & Loncin, 1977). However, more recent studies document an increase in inactivation with increasing fat content (Oliveira et al., 2018; Schultze et al., 2007) or no effect at all (Byelashov et al., 2010). The impact of the presence of fat on the microbial inactivation appears to be linked to several factors, including the temperature range, the specific product, and the type of microorganism. However, the effect of fat content has not yet been documented in RF inactivation studies.

For the current study, Figure 8.5 demonstrates the effect of fat presence on the inactivation of the two pathogenic microorganisms in the two systems, i.e., liquids and gels, including the temperature-dependency of the inactivation rate k.

In liquid systems, for both microorganisms, there was no statistically significant ($p > 0.05$) effect of fat on k at the reference temperature T_{ref} of 64°C (Table 8.3). Similar behaviour was observed at lower temperatures. However, at temperatures higher than 70°C, the inactivation rate was higher in LS2 for both microorganisms. Hence, the presence of the fat only increases the RF inactivation rate in the higher temperature range. This behaviour is different compared to the inactivation by conventional thermal heating observed in previous studies. In this regard, only studies in which the effect of fat content could be adequately isolated were taken into account (i.e., not from studies on real food products in which results may have been influenced by compositional, physicochemical, and microstructural variations among the systems with different fat content, offering a possible explanation for the above-mentioned contradicting results among studies, as discussed in Verheyen et al., 2020). As a first example, Kim and Kang (2015) studied the

influence of milk fat content on the conventional thermal inactivation (maximum temperature of 70°C) of *L. monocytogenes* and *S.* Typhimurium (along with *E. coli* O157:H7), and observed no statistically significant differences in inactivation rate in fat contents of 0, 3 and 7%. As a second example, Verheyen et al. (2020) studied the influence of fat content on the conventional thermal inactivation (maximum temperature of 69°C) of *L. monocytogenes* in emulsion systems containing 1, 5, 10, and 20% fat, and observed that inactivation rate decreased with increasing fat content. Nevertheless, a significant effect was only observed starting at a fat content of 10%, while only fat contents of 0.08 and 5.9% were taken into account in the current study. In general, the increased inactivation at increased fat contents at relatively high temperatures (i.e., above 64°C) which was observed for RF inactivation in the current study, is already present at lower fat contents than for conventional heating. The difference in bacterial inactivation between RF and conventional heating is possibly related to the specific dielectric nature of RF heating. Even though fat exhibits relatively low dielectric properties, the low heat capacity of fat causes RF to heat faster at higher fat content (Piyasena et al., 2003). Hence, if the fat content is higher, at a microscale, microorganisms can be exposed to higher temperatures than those experimentally measured. This effect would become relevant at lower fat content than for conventional heating. Verheyen et al. (2020) linked the fat content influence on *L. monocytogenes* inactivation during conventional thermal treatments to the direct interaction between fat and the bacterial cells, an effect which hence only kicks in at higher fat contents, but could theoretically also become apparent for RF. If this hypothesis is true, RF heating at fat contents higher than 5.9% should result in even higher bacterial inactivation rates than those observed for 5.9% fat.

In gel systems, there were notable differences in the behaviour of *S.* Typhimurium and *L. monocytogenes*. At T_{ref}, a higher k was observed for both microorganisms in GS1 than in GS2, indicating that a higher fat content causes lower RF inactivation rates. However, at temperatures above 70°C, *L. monocytogenes* showed a higher inactivation rate at higher fat content, a behaviour that was not observed for *S.* Typhimurium. The increased inactivation rate with the increased fat content observed in the higher temperature range aligns with previous observations regarding the effect of fat content on conventional thermal inactivation of *L. monocytogenes* in gel

emulsion systems containing 1 and 5% fat (Verheyen et al., 2020). These findings were linked to cells immobilised at the fat-water interface experiencing slower local temperature increases due to the lower thermal conductivity and water activity of fat. Since similar findings were observed for RF heating, the faster heating of the fat at microscale (as assumed as a possible explanation in liquid systems) is not sufficient to counteract the aforementioned effects that lower the inactivation rate at higher fat content in gel systems. It should be noted that Verheyen et al. (2020) did observe a more complex relationship in gel emulsion systems when fat contents of 10 and 20% were also taken into account.

Survivors that do not follow the log-linear trend were in the current study not considered in the model, and hence discussed qualitatively. For *L. monocytogenes,* higher resistance was observed in both liquid and gel systems with 5.9% fat content than in systems with 0.08% fat. On the contrary, *S.* Typhimurium exhibited higher resistance in LS1 (0.08% fat) in comparison to LS2 (5.9% fat), while no differences were observed in the gel systems. *L. monocytogenes* resistance is seemingly increased by high fat content, whereas *S.* Typhimurium shows the opposite effect, but it is only noticeable in the liquid systems. For *L. monocytogenes*, these effects are not identical to those observed for conventional heating by Verheyen et al. (2020): in liquid systems, resistance decreased with increasing fat content, while in gel systems, resistance increased with increasing fat content. Verheyen et al. (2020) linked this phenomenon to the direct effects of fat on bacterial cells. The difference with RF heating can possibly again be linked to the explanation provided for the inactivation rate. In liquids, the faster heating of the fat at microscale would reduce the amount of resistant cells during RF heating of high fat content systems. In gels, the faster heating of fat at a microscale is not sufficient to counteract the thermal resistance increasing effects of a higher fat content.

Interestingly, *S.* Typhimurium shows no statistically significant differences in the SI percentage in the liquid systems, whereas a higher percentage of generated SICs is observed in GS1 in comparison to GS2, but only in the early time points (Figure 8.7). On the contrary, *L. monocytogenes* shows higher SI in systems with 0.08% fat after 10 minutes of treatment in both liquids and gels. This SI behaviour for RF inactivation is the current study is more complex than for the conventional heating of *L. monocytogenes*, where

increasing fat content resulted in increased SI, for both liquid and gel systems (Verheyen et al., 2020). This observation indicates the different effects of RF and conventional heating on bacterial cells. However, to verify this speculation, future experiments using conventional dynamic heating treatments with the same samples should be conducted.

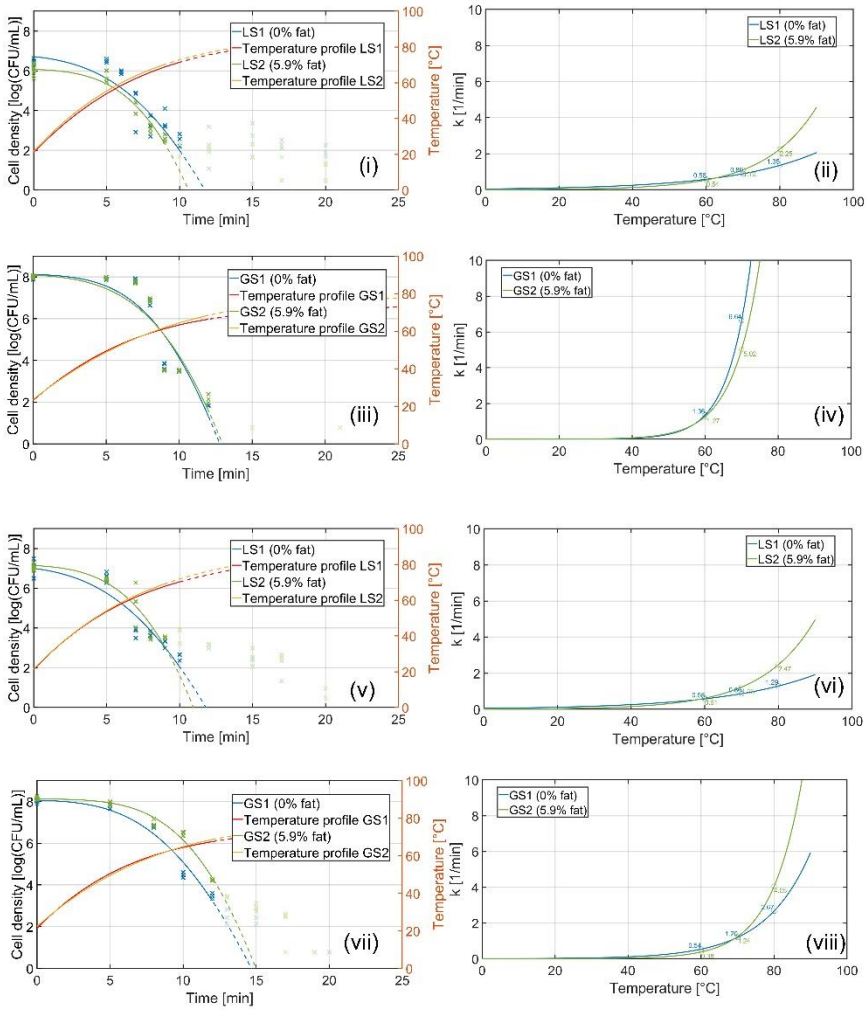


Figure 8.5: Comparison of the fat effect of *S.* Typhimurium in (i), (ii) LS and (iii), (iv) GS and *L. monocytogenes* in (v), (vi) LS and (vii), (viii) GS after radio frequency treatment, including the temperature-dependency of the inactivation rate k, with emphasis on points at 60, 70 and 80°C.

8.3.2.2 Influence of the matrix structure

In the current study, the food microstructural influence was compared for each microorganism in each system of similar fat content i.e., LS1 and GS1, LS2 and GS2 (Figure 8.6 and Table 8.3). For *S*. Typhimurium, k was higher in gel than in liquid systems, for both fat levels, and at all temperatures. For *L. monocytogenes*, the influence of a gel matrix was dependent on the temperature. At temperatures below 70°C, no significant differences were observed at low fat content (i.e., LS1 vs. GS1), while inactivation was lower in gel systems at high fat content (i.e., LS2 vs. GS2). At temperatures above 70°C, the *S*. Typhimurium inactivation rate is higher in gel systems than in liquids at temperatures above 70°C, as seen in *L. monocytogenes*.

The RF heating behaviour was again compared to conventional heating. Velliou et al. (2013) reported contradicting findings compared to the current study: the inactivation rate of *S*. Typhimurium in gel systems was lower than in liquids. For *L. monocytogenes*, Verheyen et al. (2019) also observed a higher inactivation rate k in liquid than in gel matrices at 59, 64, and 69°C after conventional heating treatments of fish model systems. Hence, the effect of a gel matrix on the bacterial inactivation rate could be different for RF and conventional heating. Nonetheless, to accommodate an accurate comparison between a RF-only dynamic heating treatment and conventional treatments, future studies should involve the use of conventional heating using similar temperature profiles in the same dairy model systems. Regarding the RF inactivation of *L. monocytogenes,* a complex behaviour was observed at low temperatures. For conventional heating, the decreased inactivation rates in gel matrices are possibly related to the distribution of microorganisms and/or a protective effect of the food matrix. Specifically, in gel systems microorganisms grow as colonies, whereas in liquids they are present as planktonic cells (Wilson et al., 2002). Concerning colony formation, apart from causing additional stress during microbial growth that can affect subsequent microbial inactivation due to a cross-protection mechanism, colonies could be more heat-resistant due to the cells protecting each other (Velliou et al., 2013; Verheyen et al., 2019b). Concerning the protective effect of a gel matrix, cells in a gel matrix may be more heat resistant due to induced changes in cell development, membrane permeability, surface tension, osmotic pressure and metabolism (Noriega et al., 2013; Verheyen et al.,

2019b; Wilson et al., 2002). Since the inactivation rate for RF heating was higher in gel than in liquid matrices in the current study, it is speculated that thermal inactivation of bacteria is also affected by the heating mechanism. RF heating is characterised by a large penetration depth, allowing the heat to directly reach to microorganisms, possibly nullifying any protective effects of the gel matrix that are unrelated to the changes to the cell itself. This direct heating would allow the cells to be heated faster than by the conduction and convection mechanisms inherent to conventional heating. In this regard, Espitia et al. (2023) have reported that *S*. Typhimurium and *L. monocytogenes* cause RF heating in pure water, indicating that bacterial cells may indeed be directly heated by the RF energy. However, the reason for the reduced RF inactivation rates in liquids, where cells would also be heated by RF energy, still remain unclear. Possibly, heat generated in bacteria is dissipated more easily in a liquid than in a gel matrix.

The acquired model parameters also indicate that *L. monocytogenes* displays a statistically significant higher level of resistance in the gel systems compared to *S*. Typhimurium. On the contrary, in the liquid systems no statistically significant differences are observed between the two microorganisms. Interestingly, most often in literature, *Salmonella* species are less heat resistant than *L. monocytogenes* in liquid dairy systems (Rukke et al., 2011). Consequently, the thermal resistance of bacteria is again confirmed to be dependent on the heating mechanism, with differences occurring between conventional thermal heating and electromagnetic methods (e.g., RF and ohmic heating).

Another confirmation of the above hypotheses is the observation that the resistant viable cells which were not considered in the model were present at levels above detection limit in the liquid systems even during longer treatment times (usually starting after approximately 15-17 min), while these were not present in the gels at the same time points. The behaviour again contradicts the findings of Noriega et al. (2013), Velliou et al. (2013), and Verheyen et al. (2019) for conventional heating of *S*. Typhimurium and *L. monocytogenes*. They documented a tailing phenomenon during conventional heating treatments for *S*. Typhimurium when the cells where present in the matrix structure in a colony form in a gel matrix, contrary to the log-linear behaviour of the planktonic cells.

SI (%) was also compared between liquids and gels for each system and each microorganism. The SI (%) of *S*. Typhimurium in the treated sample shows no statistically significant differences in system with 0.08% fat (i.e., LS1 vs. GS1), whereas an increasing number of SICs are observed in *L. monocytogenes* in gel systems, but only in treatment times between 8 and 12 minutes. In systems with 5.9% fat (i.e., LS2 vs. GS2), both microorganisms show statistically significant differences where, for *S*. Typhimurium an increased SI is observed in LS2 in comparison to GS2, whereas in *L. monocytogenes* the increased SI is observed in GS2. For conventional thermal treatments, the same trend was observed for *Salmonella* by Wang et al. (2017), while an opposite trend was observed by Noriega et al. (2013), although the relation was dependent on colony density for the latter. An opposite trend was observed for conventional thermal treatments of *L. monocytogenes* both at low and high fat content (Verheyen et al., 2020). SI was always higher in liquid than in gel systems. In this case, it seems that the effect of the heating mechanism is again influencing the bacterial inactivation mechanisms, although the trend is complex, possibly due to the different colony densities and food matrices used in different studies.

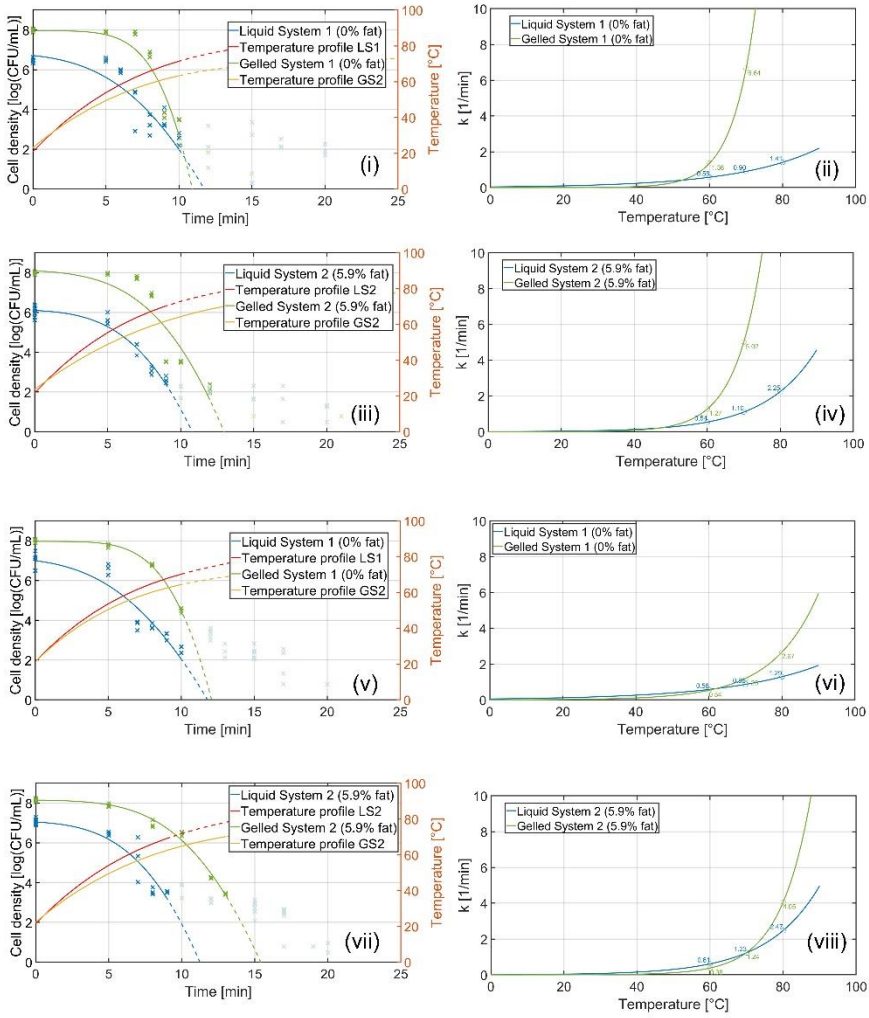


Figure 8.6: Comparison of the effect of structure presence in the RF inactivation of *S*. Typhimurium in systems with (i), (ii) 0.08% fat and (iii), (iv) 5.9% fat and *L. monocytogenes* in systems with (v), (vi) 0.08% fat and (vii), (viii) 5.9% fat, including the temperature-dependency of the inactivation rate *k*, with emphasis on points at 60, 70 and 80°C.

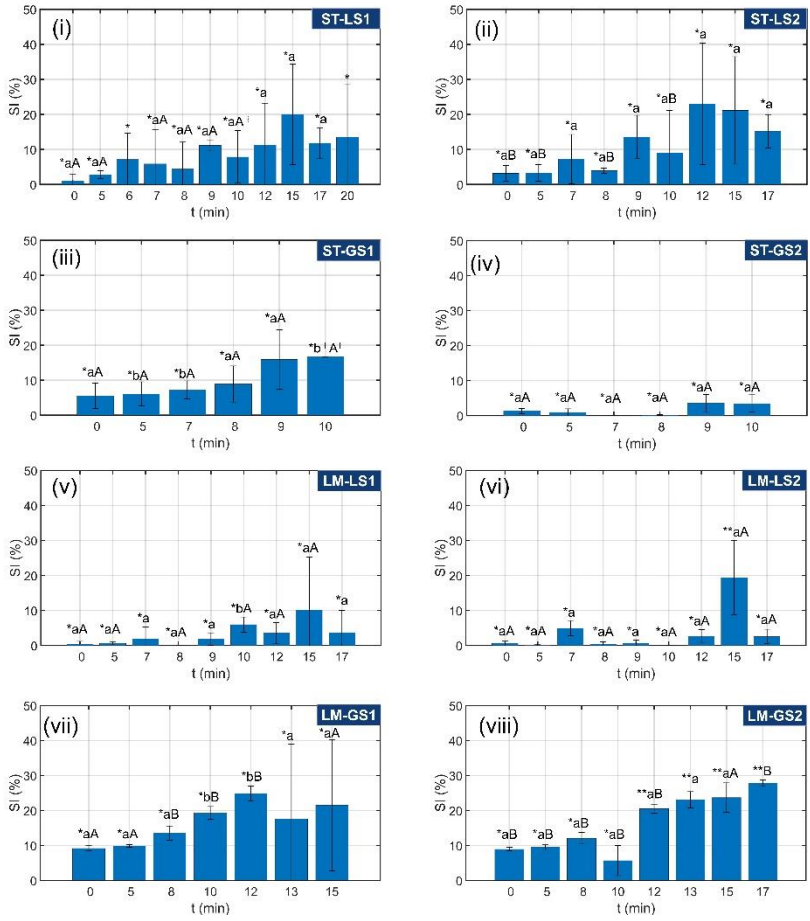


Figure 8.7: Sublethal injury (%) of *S.* Typhimurium in (i) liquid system 1, 0% fat (ST-LS1), (ii) liquid system 2, 5.9% fat LS2 (ST-LS2), (iii) gel system 1, 0% fat (ST-GS1), (iv) gel system 2, 5.9% fat (ST-GS2), and *L. monocytogenes* in (v) liquid system 1, 0% fat (LM-LS1), (vi) liquid system 2, 5.9% fat (LM-LS2), (vii) gel system 1, 0% fat (LM-GS1), and (viii) gel system 2, 5.9% fat (LM-GS1) after radio frequency treatment. The * symbol represents statistically significant differences ($p \leq 0.05$) between the different time samples. Different lowercase letters represent statistically significant differences ($p \leq 0.05$) in fat content within each microorganism for each corresponding time point, when applicable. Different uppercase letters represent significant differences ($p \leq 0.05$) in structure within each microorganism for each corresponding time point, when applicable. The symbol ǂ denotes instances

where one of the groups contained only a single data point, as the remaining data points fell below the detection limit.

8.4 Conclusions

In contrast to conventional heating, the influence of food microstructural aspects on RF inactivation of foodborne pathogens has not been systematically studied to date. In the current study, the impact of two intrinsic food factors (i.e., fat content and matrix structure) on the RF inactivation of *S*. Typhimurium and *L. monocytogenes* in dairy model systems was investigated. For fat content, the effect on microbial inactivation varied with system type and microorganisms, but there was a general trend towards increased fat content leading to increased inactivation at high temperatures. This phenomenon was assumed to be related to the fast dielectric heating of fat compared to water, possibly exposing microorganisms to higher temperatures at the microscale when the fat content is higher. For matrix structure, RF inactivation was most often higher in gel matrices than in liquid matrices, a behaviour which is completely opposite to conventional heating. This phenomenon was assumed to be related to the direct heating of microorganisms by RF and the slower dissipation of this heat in gel matrices.

Overall, it was demonstrated that fat content and matrix structure exert a significant effect on inactivation rates of microorganisms by RF treatment,. These microstructural effects are in part specific for RF heating, and not the same as for conventional heating. These findings are important to take into account when designing RF heating food decontamination strategies, since common knowledge concerning the influence of temperature on bacterial inactivation for conventional heating strategies cannot be simply extrapolated to RF. In future studies, the effect of more different food intrinsic factors (e.g., composition) on RF heating inactivation of should be investigated to get a complete view on the aforementioned issues.

Acknowledgement

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Chapter 9

Exploring the RF inactivation mechanisms of *S.* Typhimurium and *L. monocytogenes*: liquid broth

The results presented in this chapter have been submitted to *Journal of Applied Microbiology*:

Tonti Maria, Verheyen Davy, Løvdal Trond, Kozak Dmytro, Skåra Torstein, Van Impe Jan. Radio frequency thermal inactivation mechanisms of *Salmonella* Typhimurium and *Listeria monocytogenes* in liquid broth: comparison to conventional thermal treatments. *Journal of Applied Microbiology*. (submitted)

The contributions of the first author to this work are:

- Literature study
- Co-design of experiments
- Co-performing of experiments
- Co-interpretation of results
- Co-formulation of conclusions
- Text redacting and editing

This chapter includes the referred paper. Some adaptations in formatting have been made.

Context: The RF-only dynamic heating approach was used to inactivate the two microorganisms in a simple, laboratory medium (liquid broth). The aim was to gain insights in the RF inactivation mechanisms. For the experiments of this chapter, the average temperature profile from the treatments of Chapter 8 was used for the RF treatments. Additionally, conventional thermal treatments were conducted in a water bath, replicating the same temperature profile of the RF treatments. The inactivation kinetics and the sublethal injury were calculated after each treatment, in order to identify the RF effect on a macroscopic level. The Geeraerd et al. (2000) model with a Bigelow-type temperature dependency was fitted to the experimental data and the inactivation rate was used to compare the two treatments. Then, for selected treatment time points, the effect of RF on a microscopic level was evaluated. Specifically, the effect of both treatments on membrane and DNA integrity was evaluated.

Abstract

Radio frequency (RF) is a novel dielectric heating technology recently gaining increasing attention for microbial inactivation. Nonetheless, inactivation mechanisms have not been deciphered, especially concerning RF-specific (non)thermal effects. This study adds valuable insight to the inactivation mechanisms of (Gram-negative) *Salmonella* Typhimurium and (Gram-positive) *Listeria monocytogenes* by comparing RF heating to conventional thermal treatments with similar dynamic temperature profiles. Inactivation kinetics parameters were estimated, and DNA and membrane integrity were evaluated. For both microorganisms RF demonstrated a higher inactivation rate and higher sublethal injury than conventional treatments. RF treatments caused more membrane damage than conventional treatments in both microorganisms, although differences were possibly linked to viable cell density differences. There were no significant DNA damage differences between RF and conventional treatments for both microorganisms, indicating that occurring DNA damage was purely thermal. Agarose gel electrophoresis also demonstrated no considerable differences in DNA integrity between RF and conventional thermal treatments. In conclusions,

while the existence of RF-specific inactivation mechanisms is possible, based on the results of this study are probably only related to membrane damage. Future research using additional techniques (e.g., flow cytometry, next-generation sequencing) is necessary to gain deeper insights into the RF inactivation mechanisms of the two foodborne pathogens.

9.1 Introduction

Food Safety has always been a global concern which touches every aspect of daily life, from farm to table. Paramount to this issue for the food industry is handling microbial hazards and especially foodborne pathogens which are known to cause outbreaks and trigger product recalls (Rivera et al., 2018; Schirone et al., 2019).

So far, the primary strategies of the food industry for protecting food from microbiological hazards have relied on heat-based treatments like pasteurisation and sterilisation, which, despite their effectiveness, often lead to reduced nutritional value and freshness, adversely affecting the overall quality of food (Zhang H. et al., 2020a). There is a growing consumer demand for a diverse range of food products that are minimally processed but rich in nutrients and naturalness (Roman et al., 2017). Consequently, the food industry is driven to explore and implement alternative technologies such as cold atmospheric plasma, pulsed light, microwave heating, and ohmic heating, to maintain food safety standards while providing consumers with high quality products (Roohinejad et al., 2018).

Radio frequency (RF) heating is another emerging processing technology which has gathered much of interest as of late. This dielectric heating technology involves the conversion of electrical energy to electromagnetic radiation, which, when applied to food products, is subsequently converted to thermal energy (Tong et al., 2022). In contrast, conventional thermal treatments rely on heat transfer via conduction and convection, which can lead to surface overheating or hot/cold spots of food products. The frequency most commonly used for RF applications is 27.12 MHz, a frequency which results in a long penetration depth and a relatively uniform field distribution and hence, temperature uniformity (Altemimi et al., 2019). RF has been successfully used commercially in drying, thawing and

disinfestation applications (Jiao et al., 2018), but as of late, the focus turns to bacterial inactivation. Specifically, there has been an increase in the number of studies on microbial inactivation in a variety of products (Geveke et al., 2009), such as apple juice (Geveke & Brunkhorst et al., 2004; Ukuku et al., 2008), milk (Awuah et al., 2005; Di Rosa et al., 2018; Srisuma et al., 2017), scrambled eggs (Luechapattanaporn et al., 2005), mashed potatoes (Wang et al., 2007), whey protein gel (Wang et al., 2009) and low-moisture products such as red pepper, in-shell almonds and milk powder (Chen et al., 2013; Michael et al., 2014; Zhong et al., 2017; Jiao et al., 2019; Zhang H. et al., 2020b; Dag et al., 2022; Tonti et al., 2024).

However, a systematic analysis of the RF inactivation mechanisms on a biological level is still lacking (Zhang et al., 202a1). Thus far, most available investigations of inactivation mechanisms were limited to specific real food products (e.g., Geveke and Brunkhost, 2004; Ukuku et al., 2008; Kou et al., 2918; Zhang et al., 2021b) with the exception of Cui et al. (2022). Additionally, the ongoing debate over the existence of possible RF-specific (thermal or non-thermal) effects and their influence on inactivation remains unresolved. On the one hand, Geveke et al. (2002) and Ukuku et al. (2008) have demonstrated the existence of non-thermal effects at a lower frequency range, indicating the possibility of non-thermal effects at higher RF frequencies. On the other hand, Michael et al. (2014), Kou et al. (2018), Zhang et al. (2020), and Cui et al. (2022) concluded that there were no significant differences between the effects on bacteria of RF and conventional thermal heating, with the exception of differences on the gene level, where a higher number of differentially expressed genes was observed after RF treatments. However, some uncertainty may exist concerning the conclusions of these studies, since (i) the use of real food products in some studies possibly caused limitations in identifying significant effects, or (ii) the occurrence of possible non-thermal effects was only assessed during the holding time at the target temperature without considering the (realistic) dynamic heating profiles inherent to RF treatments. Concerning the latter issue, dynamic heating should also be investigated for RF, since dielectric heating causes the food product and the bacterial cells to, in addition to the holding phase, also absorb energy during the come-up time (as traditionally interpreted for conventional heating), possibly already resulting in RF-

specific effects on the cells during this stage (Altemimi et al., 2019; Espitia et al., 2023).

The main aim of this research was to identify the RF-specific inactivation mechanisms of two representative foodborne pathogens (high pathogenicity and virulence) of different Gram properties i.e., *Listeria monocytogenes* (Gram-positive) and *Salmonella* Typhimurium (Gram-negative). Experiments were conducted in a homogeneous liquid broth medium to avoid the clouding of the RF effects on the bacteria by the interference of food compositional and microstructural aspects. In order to explore RF-specific (thermal or non-thermal) effects, dynamic heating RF heating profiles realistic for RF-only inactivation processes were employed, and mimicked by conventional thermal treatments in a water bath. The effects of each treatment were assessed, first on a macroscopic level through plating on microbiological media, and then on a microscopic level by investigating membrane and DNA damage. This research presents a novel approach to examining the RF-only effect on *L. monocytogenes* and *S.* Typhimurium and it is a first step in the unravelling of the underlying inactivation mechanisms.

9.2 Materials and methods

9.2.1 Materials and microorganisms

Salmonella enterica serovar Typhimurium CCUG 31969 isolated from bovine, septicaemic liver was obtained from the Culture Collection at the University of Gothenburg (CCUG). *L. monocytogenes* LMG 23356 isolated from Jalisco Cheese was obtained from the bacterial collection of Ghent University. Bacterial stock cultures were stored in 2 mL cryovials (Microbank™, Pro-Lab Diagnostics, Canada) at a temperature of -80°C. One bead from the stock culture was transferred into an Erlenmeyer flask containing 20 mL BHI broth and incubated for 24 h at 30°C and 37°C for *L. monocytogenes* and *S.* Typhimurium, respectively. To create the working preculture, an inoculation loop was used to streak the sample on a BHI agar plate. Following streaking, the plate was incubated 30°C for 48 h and 37°C for 24 h for *L. monocytogenes* and *S.* Typhimurium, respectively. A biological replicate for each microorganism was created by transferring one individual colony using an

inoculation loop from the working preculture in an Erlenmeyer flask containing BHI broth (1st preculture). The inoculated Erlenmeyer flasks were placed in an incubator for 24 h at 30°C and 37°C for *L. monocytogenes* and *S.* Typhimurium, respectively. After 24 h, a 2nd preculture was created by transferring 20 µL of the 1st preculture to fresh BHI broth in a new Erlenmeyer flask which was then subjected to another round of incubation at the appropriate temperatures for 24 h. In total, three biological replicates were prepared for each microorganism and for each treatment.

9.2.2 RF and thermal treatments

Sterile, glass vials (4 mL, 45, 14.7 mm, BGB Analytik Benelux B.V., Harderwijk, the Netherlands) with a polypropylene screw cap lid were used as the sample containers for the RF and thermal treatments. Initially, 2.7 mL of sterile BHI were placed in the glass vials and 300 µL of the preculture was added to each vial and thoroughly mixed by vortexing. The purpose was to achieve a total sample volume of 3 mL and an initial cell density of approximately 10^8 CFU/mL. In order to measure the temperature throughout the treatments, the lids were modified by adding a hole in the centre and attaching a sterile thin glass capillary which came in contact with the sample (Figure 9.1(i), (ii)). Then, an optic fiber sensor was inserted into the capillary to record the temperature and connected to a Bench Top FOTEMP-PLUS signal conditioner (Weidmann Technologies Deutschland GmbH, Dresden, Germany). The temperature data were processed by the FOTEMP-Assistant software (Optocon, AG, Dresden, Germany). The temperature uniformity throughout the sample during RF and water bath treatments was evaluated in a separate experiment by using a special lid equipped with three capillaries. Each capillary was precisely positioned within the sample at intervals of 0.5 cm, at the bottom, the middle and the top of the sample (Figure 9.1(iii)). Prior to the treatments, the vials were placed in a 20°C incubator for a short time to reach the same initial temperature.

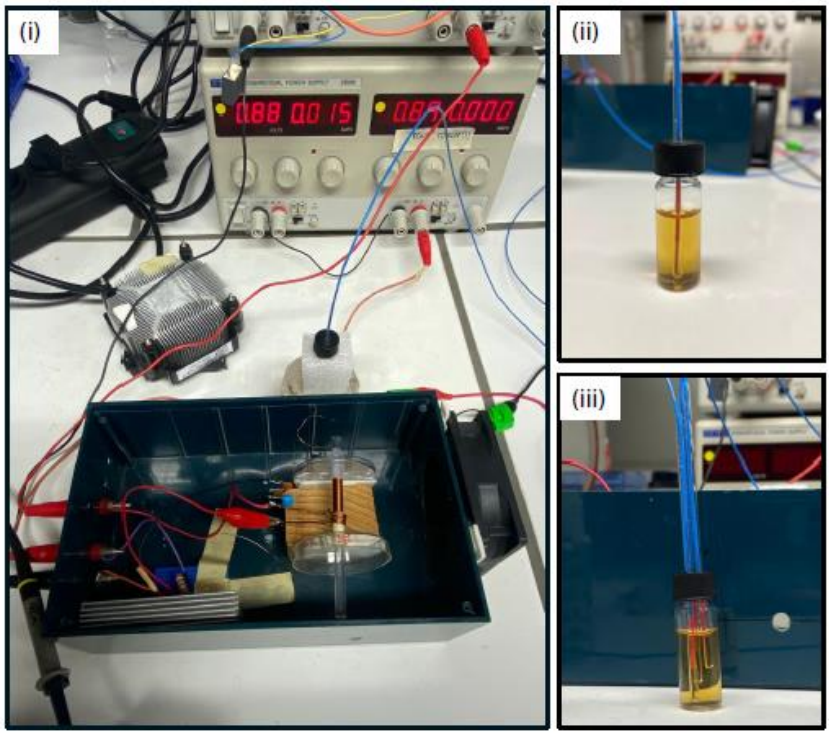


Figure 9.1: RF lab-scale experimental set-up (i) and sample containing liquid BHI broth with a lid which facilitates (ii) one optic fiber sensor positioned at the bottom of the sample and (iii) three optic fiber sensors positioned at intervals of 0.5 cm.

The RF treatments were conducted using a lab-scale low-power free-running radio frequency (RF) oscillator described in detail in Kozak et al. (2021). Overall, an input power of 14-28 W was used to generate electromagnetic waves in a frequency range of 27.12 ± 0.50 MHz. A dynamic heating profile was used to treat samples to targeted end time points.

Thermal treatments were conducted in water baths. In order to replicate the temperature profile acquired by RF treatments, two water baths were used. Firstly, the samples were placed in the water bath set at 43.5°C, after which the set point was immediately changed to 85°C. After exactly 4 min, the samples were moved to the second water bath, initially set at 58.5°C and the set point was promptly shifted to 85°C, at which it was kept until the end

time point of each treatment. After treatments, samples were immediately immersed in ice cold water until they reached 10°C to stop further inactivation.

9.2.3 Sampling and microbial enumeration

The enumeration of viable cells was performed by plating serial dilutions of the samples using 0.9% saline solution on both non-selective and selective agar, using the 3-drop technique (Herigstad et a l., 2001). A detection limit of 2 log CFU/mL was achieved by plating 100 µL of the undiluted samples evenly across the plate surface. BHI Agar was used as non-selective medium for both microorganisms. PALCAM and XLD Agar (Merck, Darmstadt, Germany) were used as selective media for *L. monocytogenes* and *S.* Typhimurium, respectively. Enumeration was performed after incubation for 48 h at 30°C and 37°C for *L. monocytogenes* and *S.* Typhimurium, respectively.

9.2.4 Microbial inactivation kinetics modelling

The Geeraerd et al. (2000) log-linear tail model and the Geeraerd et al. (2000) log-linear model were fitted to the inactivation data acquired by RF and conventional thermal treatments, respectively (Equations 9.1, 9.2). For both models, a Bigelow-type temperature relation was used to describe the dependency of the inactivation to the temperatures (Equation 9.3).

$$\frac{dN(t)}{dt} = -k(T(t)) \cdot \left(1 - \frac{N_{res}}{N(t)}\right) \cdot N(t) \quad (9.1)$$

$$\frac{dN(t)}{dt} = -k(T(t)) \cdot N(t) \quad (9.2)$$

$$k(T(t)) = k\left(T_{ref}\right) \cdot 10^{\frac{T(t)-T_{ref}}{z}} \quad (9.3)$$

with $N(t)$ (CFU/mL), the cell density at time t; N_{res} (CFU/mL), the residual cell density; k (1/min), the specific inactivation rate; $T(t)$ (°C), the average time-dependent core-temperature of the model system over all treatments for

the respective conditions; T_{ref} (°C), the reference temperature set at 64°C; $k_{(T_{ref})}$ (1/min), the specific inactivation rate at the reference temperature; and z (°C), a parameter which evaluates the sensitivity to temperature changes.

The Optimisation Toolbox of MatLab version R2021a (The Mathworks Inc., Natick, USA) was used to calculate the model parameters, with the functions patternsearch and lsqnonlin. Odinary differential equations of the inactivation model were solved by Matlab solver ode23. The standard errors and confidence intervals for the model parameters were calculated with the Jacobian matrix and the Fisher information matrix. Root Mean Squared Error (RMSE) was used to evaluate the goodness-of-fit.

9.2.5 Sublethal injury assessment

Sublethal injury (SI) is a phenomenon in which bacterial cells have been damaged by external stressors (e.g., heat, radiation, chemical agents), but they are not considered dead as they may have the ability to maintain metabolic activity and pathogenicity. These sublethally injured cells (SICs) can multiply and grow on non-selective media but not on selective ones (Hansen & Knøchel, 2001; Suo et al., 2014). The percentage of SI can be calculated by Equation 9.4:

$$SI\ (\%) = \frac{N_{non-selective} - N_{selective}}{N_{non-selective}} \cdot 100 \quad (9.4)$$

where $N_{non\text{-}selective}$ is the cell density (CFU/g) measured on non-selective media and $N_{selective}$ the cell density (CFU/g) measured on selective media (Verheyen et al., 2019a).

9.2.6 Cell membrane damage

The integrity of cell membranes was evaluated based on the protocol of Zhang et al. (2020), using the LIVE/DEAD® BacLightTM Bacterial Viability Kit (L7012 from molecular probes) per manufacturer's instructions. Specifically, a mixture of two dyes was used: (i) green fluorescent nucleic acid stain (SYTO® 9) which can penetrate all bacteria, and (ii) red-fluorescent

nucleic acid stain (propidium iodide) which penetrates only bacteria with damaged membranes. The ratio of green to red fluorescence emission (Ratio G/R) is proportional to the relative numbers of bacteria with intact membranes. Following treatments, 1 mL of sample was centrifuged at 10000 x g for 10 min, the supernatant was discarded, and the pellet was washed once with 0.9% saline solution. 100 µL of the resuspended pellet were mixed with 100 µL working solution of the LIVE/DEAD BacLight staining reagent mixture on a black-walled, clear-bottom 96-well microplate and measured at the excitation/emission of 485/535 nm and 495/625 nm by using a FilterMax F5 plate reader (Molecular Devices, LLC, California, United States).

9.2.7 DNA integrity analysis

The evaluation of the intracellular DNA integrity was performed by two different analyses. Initially, the DNA integrity was accessed by means of DNA denaturation i.e., the dissolution of the double-stranded helix into two separate single strands. Furthermore, intracellular DNA was extracted, quantified and subjected to agarose gel electrophoresis to further assess its integrity. Agarose gel electrophoresis is an established procedure which separates DNA fragments based on their molecular sizes.

DNA denaturation was assessed using the protocol of Govaert et al. (2020b). SYBR Green I (2-[N-(3-dimethylaminopropyl)-N-propylamino]-4-[2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene]-1-phenylquinolinium) was used for the detection and quantification of intracellular double-stranded DNA (dsDNA). SYBR Green has the ability to bind to dsDNA and emit fluorescence at 525 nm upon excitation at 485 nm. A decrease in the fluorescence signal indicates a decrease in the amount of dsDNA present. After treatments, 1 mL of sample was centrifuged at 10000 x g for 10 min. The supernatant was discarded, the pellet was washed with 0.9% saline solution and centrifuged again at 10000 x g for 10 min. The supernatant was again discarded and the pellet was used for to perform cell lysis with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), following the protocol described by Govaert et al. (2019) for all subsequent steps.

For the agarose gel electrophoresis, the total cellular DNA extraction was performed using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) with the additional step of RNase treatment to prevent RNA contamination. The extracted DNA was stored in TE Buffer at -20°C. To quantify the DNA and assess its purity, 100 µL were diluted with 900 µL of TE Buffer and the mixture was measured at 260 nm, 280 nm and 320 nm using an UV–Vis VWR® spectrophotometer, model UV-6300PC. The ratio of the absorbance at 260 nm and 280 nm was used to establish DNA purity, with a ratio of approximately 1.8-2.1 considered acceptable. The samples which fitted those criteria were used for the agarose gel electrophoresis. Specifically, 10 µL of sample was mixed with 2 µl loading dye buffer (50% glycerol, 50% sterile water and a spatula tip each of xylene cyanol and bromophenol blue) and loaded on a 1% agarose gel (Bio-Rad Laboratories, Inc.) and electrophoresed for 30 min at 100 V at room temperature. A 100 base pair DNA ladder (TrackIt™ 100bp DNA ladder, 0.1 µg/µl, 10488-058, Invitrogen Life Technologies) was used in the first cell of each gel. After electrophoresis, the gel was stained with x10000 SYBR Green I solution for 15 min and visualised using the GelDoc Go Gel Imaging System (Bio-Rad Laboratories, Inc..). The imaging and the intensity of the DNA bands was analysed by Image Lab 6.1 2020 Software (Bio-Rad Laboratories, Inc.).

9.2.8 Statistical analysis

The experimental data processing was performed with MatLab R2021a software (The Mathworks Inc., Natick, USA). Significant differences for the model parameters and experimental results were identified by Analysis of variance (ANOVA, single factor), with a 95% confidence level ($\alpha = 0.05$) and Fischer's Least Significant Difference test was used to identify means which differed from each other.

9.3 Results and discussion

The overall objective of this study was to identify the RF-specific mechanisms of bacterial inactivation. A comparative analysis of RF and conventional thermal water bath treatments of similar temperature profiles on the inactivation of *S*. Typhimurium and *L. monocytogenes* was conducted, both at

macroscopic and microscopic levels. Initially, macroscopic inactivation data were collected to identify differences between the two treatments. Subsequently, DNA and membrane damage were assessed at selected time points, representing a range of different viable cell densities and applied temperatures. To ensure a valid comparison between RF and thermal treatments, specific experimental conditions were established considering the medium in which the bacteria were inactivated and the used temperature conditions, described in detail as follows.

First of all, a simple liquid broth medium was used, providing the benefits of (i) the food compositional and microstructural aspects not interfering with the effects to be observed, (ii) homogeneity of the intrinsic factors (e.g., nutrient concentration, pH) throughout the sample, and (iii) a relatively uniform temperature distribution throughout the sample.

Secondly, specific efforts were undertaken to ensure the use of relevant temperature conditions during the RF and conventional thermal treatments by (i) employing dynamic temperature profiles and (ii) by focussing heavily on the temperature distribution for the two heating methods. The temperature profile of the conventional thermal treatments corresponded to the profile of a RF-only dynamic heating (Kubo et al., 2020), and the temperature measurements were taken using the same (calibrated) optic fibre sensors for both treatments, ensuring accuracy and consistency in the collected data. A small sample size of 3 mL was also used to minimise the impact of hot and cold spots. The temperature distribution within the sample was also monitored, with a maximum of 1.0°C and 0.6°C difference being recorded for RF and conventional thermal treatments, respectively (Supplementary materials). To account for any slight variations in inactivation kinetics due to temperature differences, the Geeraerd et al. log-linear tail inactivation model was used to acquire temperature-dependent inactivation mechanisms for RF and conventional thermal heating inactivation.

9.3.1 Inactivation kinetics

Figure 9.2 illustrates the RF and conventional thermal inactivation data of *S.* Typhimurium and *L. monocytogenes* in BHI broth. For the RF treatments, the recorded temperature profiles were unique for each sample, and presented in

the same colour as the corresponding post-treatment sample cell density. For the conventional thermal treatments, one general temperature profile was used for all samples, designed based on the average temperature profile of all RF treatments. This methodology ensured that bacterial cells were exposed to identical time-temperature exposures as for RF treatments. The fitting of the Geeraerd inactivation models (Equations 9.1, 9.2, 9.3) to the data is depicted in Figure 9.3. The estimated model parameters can be found in Appendix C.

For both microorganisms, the inactivation rate (k) was significantly higher for the RF treatments (13.11 min^{-1} and 2.52 min^{-1} for *S.* Typhimurium and *L. monocytogenes*, respectively) as compared to the conventional thermal treatments (0.51 min^{-1} and 0.43 min^{-1} for *S.* Typhimurium and *L. monocytogenes*, respectively). The temperature variation within the sample, at 1°C for RF treatments, did not significantly impact inactivation. Even when accounted for the higher temperature observed at the top of the RF sample (1°C), k for RF remained higher than that for thermal treatments for both microorganisms. The difference in inactivation rate was slightly larger for *S.* Typhimurium (i.e., approximately 95%) than for *L. monocytogenes* (i.e., approximately 83%). Additionally, no statistically relevant residual cell population N_{res} was observed for any of the conditions, since N_{res} was below the detection limit for the model with tail, and the inactivation reached levels below the detection limit for the log-linear model. Consequently, RF was in the current study more effective at inactivating the bacteria than conventional thermal heating, with the effects being strongly microorganism-specific.

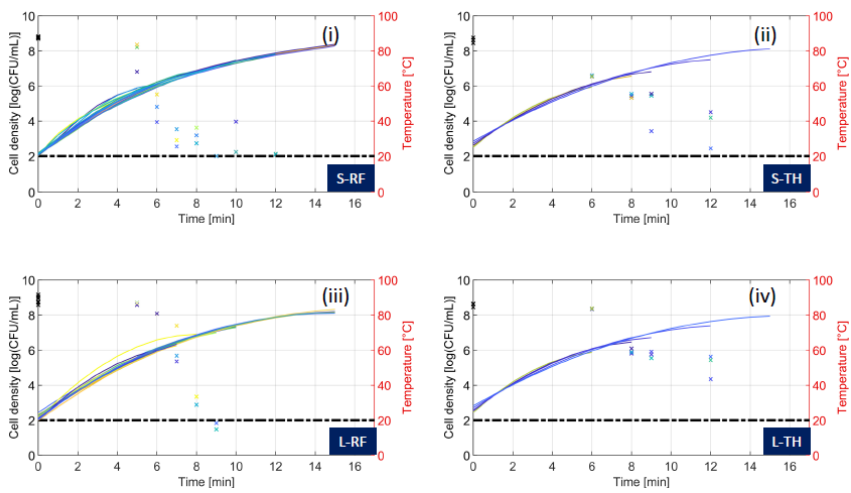


Figure 9.2: Cell density (log(CFU/mL)) of *S*. Typhimurium in liquid BHI broth after (i) RF treatments and (ii) conventional thermal treatments and *L. monocytogenes* after (iii) RF treatments and (iv) conventional thermal treatments (n = 3). Data points of black colour represent initial cell density (log(CFU/mL)). Data points of different colours represent the cell density (log(CFU/mL)) after RF treatment to the respective end temperature. The temperature profile of each treatment corresponds to the specific data points of similar colour. The detection limit (DL) is represented by a dotted black line.

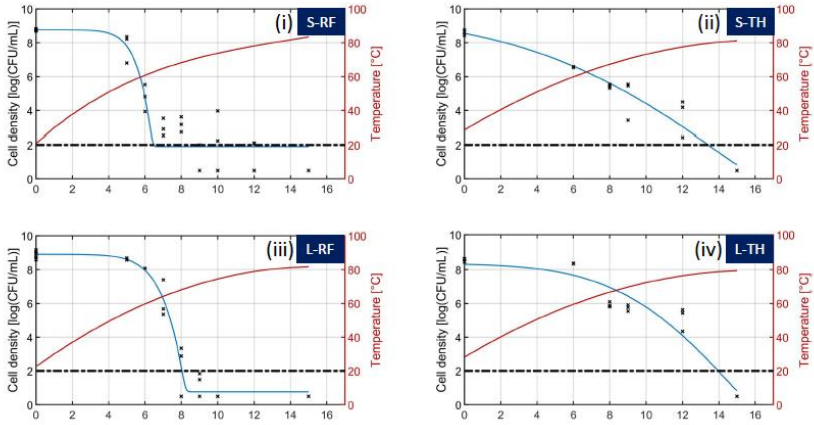


Figure 9.3: Log-linear tail model with a Bigelow-type temperature dependency fitted to the inactivation data of *S*. Typhimurium in liquid BHI

broth after (i) RF treatments and (ii) conventional thermal treatments, and *L. monocytogenes* after (iii) RF treatments and (iv) conventional thermal treatments (n = 3). The red temperature profile corresponds to a 2nd degree polynomial temperature profile of the average temperature derived by all treatments. The detection limit (DL) is represented with a dotted black line. Data points below DL were not considered in the model.

These findings contradict the studies of Michael et al. (2014), Kou et al. (2018), Zhang et al. (2020) and Cui et al. (2022), that reported the general absence of significant inactivation differences between RF and conventional thermal heating. First of all, the different findings in the current study are possibly related to the different target-microorganisms as compared to the four aforementioned studies (i.e., *L. monocytogenes* and *S.* Typhimurium vs. *Cronobacter sakazakii*, *Salmonella* spp., *Escherichia coli*, *Staphylococcus aureus*, and/or *Saccharomyces cerevisiae*), as also indicated by the differences between the two microorganisms in the current study. Different microorganisms demonstrate varied responses to different types of inactivation with their thermal tolerance being influenced by characteristics such as strain type, physiological state, and environmental conditions (Whiting et al., 2002; Rodríguez-Calleja et al., 2006; Lianou et al., 2013). Secondly, most of the aforementioned studies were conducted in real food products (e.g., milk powder, apple juice, mashed potato). These products consist of a complex food matrix that possibly influences and increases the variability of the inactivation kinetics. Different food components could offer a protective effect on the bacterial inactivation or have no effect at all (Kubo et al., 2020; Verheyen et al., 2021; Tonti et al., 2024). While it should be noted that the study of Cui et al. (2022) was, similar to the current study, also conducted in a simple medium, the study did in fact identify differences in inactivation mechanisms between RF and conventional heating (i.e., gene expression), and used non-dynamic RF heating profiles (as mentioned above).

Microwave and ohmic heating processing treatments, which follow similar principals of dielectric heating as RF, have also demonstrated increased inactivation rates in comparison to conventional thermal treatments (Shamis et al., 2011; Siguemoto et al., 2018; Inmanee et al., 2019; Shao et al., 2019; Schottroff et al., 2019). The enhanced inactivation observed for dielectric heating methods could be attributed to the energy absorption of the bacterial

cells present in the medium (Ponne & Bartels, 1995; Kozempel et al., 1998; Cui et al., 2022; Espitia et al., 2023). The applied electromagnetic field causes the stimulation of ions and the rotation of dipoles inherent to the cell, which subsequently leads to molecular friction. While validity of these hypotheses cannot be identified at a macroscopic level, the investigation of sublethal injury and inactivation mechanisms (e.g., membrane and DNA damage) could shed more light on the issue.

9.3.2 Sublethal injury assessment

Exposure to stress factors such as heat or radiation can damage the bacterial cells, affecting their ability to grow and function (Wesche et al., 2009). The nature of sublethal injury can be (a combination of) structural (i.e., membrane-related) and metabolic (i.e., functional cell components) aspects (Brashears et al., 2001). The food industry shows a particular concern on the existence of sublethally injured cells (SICs) due to their potential to acquire adaptive mechanisms and grow and multiply under suitable conditions at later stages of the food chain (Wang et al., 2018).

The SI (%) of *S*. Typhimurium and *L. monocytogenes* following RF and conventional thermal treatments is presented in Figure 9.4. RF generated more SICs in comparison to the conventional thermal treatments for both microorganisms. The differences in SI between RF and conventional thermal treatments were similar for both microorganisms. Specifically, a statistically significant SI was observed after 8 min and 9 min of treatments for microorganisms. However, after 9 min of RF treatment all microbial counts were below detection limit for *L. monocytogenes*, whereas the same phenomenon occurred to *S*. Typhimurium after 15 min of RF treatment.

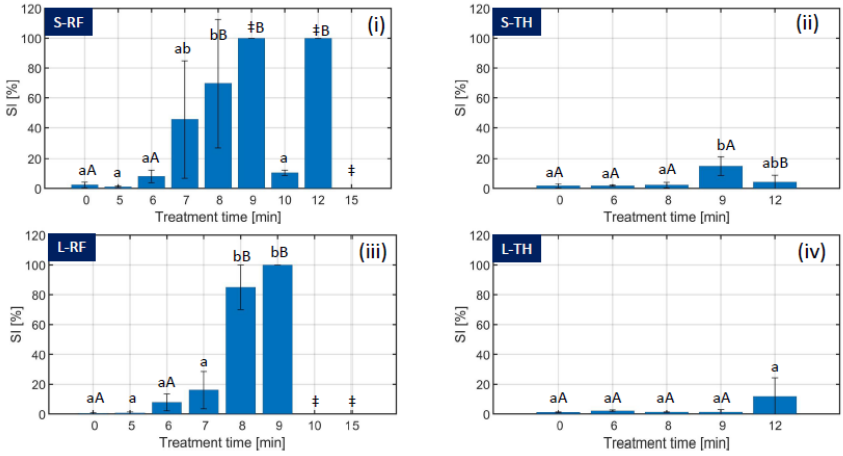


Figure 9.4: Sublethal injury (%) of *S.* Typhimurium in liquid BHI broth after (i) RF treatments and (ii) conventional thermal treatments and *L. monocytogenes* after (iii) RF treatments and (iv) conventional thermal treatments (n = 3). Different lowercase letters represent statistically significant differences ($p \leq 0.05$) between the treatment points, when applicable. Different uppercase letters represent statistically significant differences ($p \leq 0.05$) between the two treatments, when applicable. The symbol ‡ denotes instances where one of the groups contained only a single data point or data points which fell below the detection limit.

Overall, RF can induce the formation of SICs in *Salmonella* species and *L. monocytogenes* (Ozturk et al., 2019; Zhang H. et al., 2020a; Jiao et al., 2021; Tonti et al., 2024) and other microorganisms (Geveke et al., 2009; Zhao et al., 2017). Interestingly, heat treatments are known to generally only cause sublethal injury in the lower temperature range (below 60°C) in non-stressing environments (Wuytack et al., 2003; Shen et al., 2014). Therefore, the increased SI observed for the RF treatments could be attributed to the dielectric heating mechanism and localised heating effects within the cells (Espitia et al., 2023). Nonetheless, it should be noted that there is a considerable variability in the experimental data after RF treatments (Figure 9.4), which in turn could be attributed to the unique temperature profile experienced by each individual sample.

The observed differences in SI between the RF and conventional thermal treatments in the current study confirm the different effects on bacteria of the two heating mechanisms, as already identified in Section 9.3.1. In the following sections, the nature of the (sublethal or lethal) injury inflicted on the cells is investigated by evaluating cell membrane integrity and DNA degradation.

9.3.3 Evaluation of cell membrane integrity

In liquids, the primary mechanisms of cell death in vegetative cells due to heat treatments are membrane damage and protein denaturation (Smelt and Brul, 2014). Earlier studies regarding the effect of electromagnetic radiation also report the effect on bacterial cell membrane integrity (Ponne et al., 1996). Ukuku et al. (2008) assessed membrane integrity by measuring the leakage of intracellular biological compounds of *E. coli*. Similarly, Cui et al. (2022) reported similar findings after RF treatment of *S. aureus*, although not significantly different to the conventional thermal treatments.

Figure 9.5 illustrates the assessment on membrane integrity of *S.* Typhimurium and *L. monocytogenes* after RF and conventional thermal treatments in the current study, with a decrease in the green/red ratio demonstrating the existence of damaged membranes. In general, membrane damage was increasing with RF and conventional thermal treatment time for both microorganisms. For *S.* Typhimurium, more membrane damage was observed for RF treatments than for conventional thermal treatments at treatment times of 6 and 9 min, while the opposite relation was observed at 15 min. For *L. monocytogenes*, no significant differences in membrane damage between RF and conventional thermal treatments were observed at 6 min, membrane damage was higher for RF at 9 min, and membrane damage was higher for conventional thermal treatments at 15 min. It must be acknowledged that, following 15 min of both treatments, the cell densities for both microorganisms fell below detectable levels, meaning that the differences between RF and conventional thermal treatments were probably statistically insignificant at this treatment time. Similarly, following 6 and 9 min of treatment, the viable cell density after conventional thermal treatments significantly exceeded that of RF, suggesting the higher fluorescence signal after conventional treatments may have been the result of the higher number

of viable cells. Considering these aspects, non-thermal effects of RF on the membrane integrity of both microorganisms were observed, but more research is needed to attribute membrane damage exclusively to non-thermal effects.

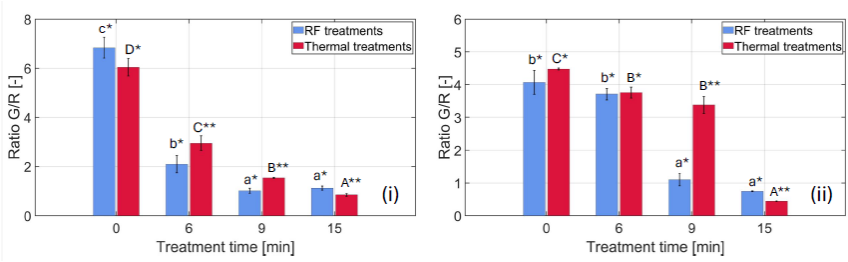


Figure 9.5: Membrane damage of (i) *S.* Typhimurium and (ii) *L. monocytogenes* in liquid BHI broth after radio frequency (blue) and conventional thermal (red) treatments (n=3). Statistically significant differences ($p \leq 0.05$) between the different treatments times are represented by different lowercase and uppercase letters, for RF and conventional thermal treatments, respectively. Significant differences for each specific time point between the two inactivation methods are represented by the symbol *.

Zhang et al. (2021) performed a transcriptomic analysis on the inactivation of *S. aureus* in walnut shells by RF and reported damage to the cell membrane transport function and cell wall biosynthesis. Cui et al. (2022) also found significant differences in gene expression after the RF treatments in comparison to conventional thermal treatments, which likely impact membrane structure and function. Thus, integrating these methodologies after RF and conventional thermal treatments may yield a clearer understanding regarding the presence or absence of non-thermal effects on membrane integrity.

9.3.4 Detection of DNA degradation

No prior studies were conducted on the influence of RF on the bacterial DNA. DNA damage has only been demonstrated in other research fields like radiation biology (e.g., Lai, 1996). In the current study, two methods were used to evaluate the integrity of the total bacterial DNA after RF and conventional thermal treatments i.e., (i) identification of DNA denaturation

using fluorescence and (ii) agarose gel electrophoresis to assess the general quality of the DNA.

Figure 9.6 demonstrates the fluorescence absorbance before and after treatments where a reduction in the fluorescence signal implies the dissolution of the DNA double helix. After 6 and 9 min of treatment, there were no statistical differences in DNA damage between RF and conventional thermal heating. After 15 min, there was statistically significantly higher DNA damage caused by RF in comparison to thermal treatments for both microorganisms. However, the viable cell counts after 15 min of treatment were below detection limit. Additionally, inflicted membrane damage after both treatments possibly led to differences in the DNA concentration of each sample. Specifically, due to the compromised cell membranes, DNA may flow out the cells and be removed along with the supernatant during the centrifugation and washing steps (Section 9.2.7). Therefore, it was essential to determine the DNA concentration of each sample.

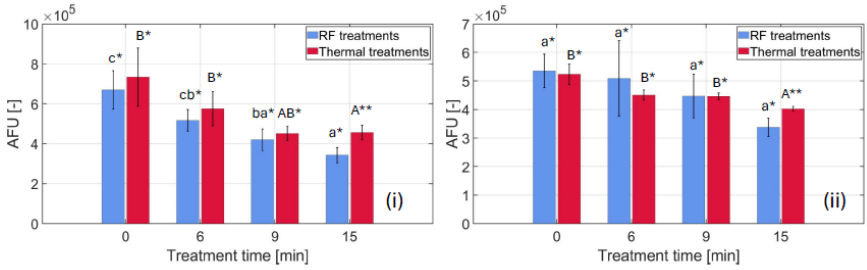


Figure 9.6: DNA denaturation of (i) *S*. Typhimurium and (ii) *L. monocytogenes* in liquid BHI broth after radio frequency (blue) and conventional thermal (red) treatments (n=3). Significant differences ($p \leq 0.05$) between the different treatments times are represented by different lowercase and uppercase letters, for RF and conventional thermal treatments, respectively. Significant differences for each specific time point between the two thermal processes are represented by the symbol *. Results are expressed in arbitrary fluorescence units (AFU).

The concentration and purity of the extracted DNA is provided in Table 9.1. In order to standardise the initial DNA concentrations for the agarose gel electrophoresis, all samples were hence diluted to align with the lowest measured concentrations, i.e., 2.45 ng/µL and 7.85 ng/µL for *L. monocytoses*

and *S.* Typhimurium, respectively. Agarose gel electrophoresis results are depicted in Figure 9.7.

Table 9.1: DNA concentration and purity ratio (A260/A280)

Microorganism	Treatment type	Treatment time (min)	DNA Concentration (ng/μL)	Ratio A260/280
Listeria monocytogenes	Water bath	0	10.35	2.0
		6	6.25	2.0
		9	8.80	1.8
		15	5.60	2.0
	RF	0	9.55	2.1
		6	3.90	2.1
		9	3.40	1.9
		15	2.45	2.2
***Salmonella* Typhimurium**	Water bath	0	14.3	1.8
		6	9.75	1.9
		9	8.85	2.0
		15	9.55	2.1
	RF	0	13.7	2.0
		6	13.65	2.0
		9	10.35	2.1
		15	7.85	2.1

For *S.* Typhimurium there was no discernible effect on the integrity of the DNA for either the RF or the conventional thermal treatments. For *L. monocytogenes*, there was an indication of a small influence of RF on the DNA integrity after 9 and 15 min of treatment. Consequently, a small RF-specific effect on DNA strand breakage was detected for *L. monocytogenes* (i.e., microorganism-specific) in the current study.

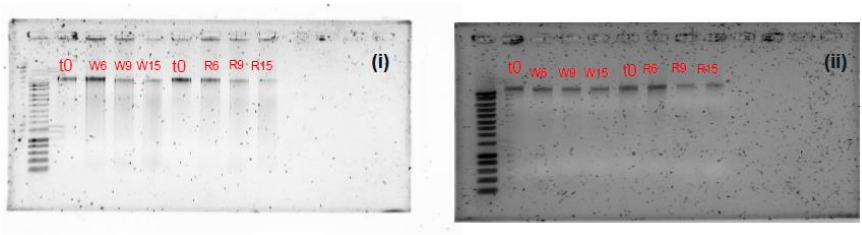


Figure 9.7: DNA agarose gel electrophoresis of (i) *S.* Typhimurium and (ii) *L. monocytogenes* in liquid BHI broth after conventional thermal (W) and RF (R) treatments where the number next to each symbol stands for the minutes of treatment, i.e., W6 for 6 min of conventional thermal treatments. t0 corresponds to the untreated samples.

Generally, for most bacterial DNA, the temperature of which DNA denaturation occurs can fall within a broad range, often between 70°C to 95°C and varies for each microorganism. Specifically, the DNA denaturation temperature depends on the GC (Guanine-Cytosine) content, as DNA sequences with higher GC content have more hydrogen bonds holding the strands together and thus, they require higher temperatures (Marmur & Doty, 1962). Since after 15 min of both RF and conventional thermal treatments, the temperature reached 80°C, it is likely that DNA denaturation occurred. However, agarose gel electrophoresis has a limited sensitivity and can detect only substantial alterations in DNA fragments, rendering it inadequate for identifying minor damages such as genetic variations or mutations. Therefore, more research and the application of different techniques are needed to conclude the effects of RF on the DNA integrity. Besides DNA denaturation, DNA damage could relate to single-strand breaks or double-strand breaks of the helix, damage to individual nucleotide bases and structural abnormalities or modifications in the DNA molecule (Purschke et al., 2010). Future research could involve techniques such as flow cytometry (for detecting DNA breaks using fluorescent markers), Single Cell Gel Electrophoresis (for identifying DNA damage at the individual cell level), Next-Generation Sequencing (for sequencing the entire genome or specific regions of interest to assess changes in mutation rates, sequence alterations, or coverage depth), and enzymatic assays such as DNA repair enzyme activity assays, which offer insights into the type and extent of DNA damage. These techniques can aid in drawing conclusions regarding the impact of RF on the bacterial DNA.

Finally, different methods should also be investigated to validate the findings in broth and in real food products.

9.4 Conclusions

The current study aimed to identify the RF-specific inactivation mechanisms of two microorganisms with different Gram properties, *S.* Typhimurium (Gram-negative) and *L. monocytogenes* (Gram-positive) in liquid broth. This was achieved by comparing the inherent RF-only dynamic heating to conventional thermal treatments with identical temperature profile. The assessment of inactivation kinetics and sublethal injury, brought to light the existence of certain RF-specific inactivation effects on the cell membrane of both microorganisms, additional to conventional thermal heating. A possible hypothesis is that enhanced inactivation is caused by energy absorption of the bacterial cells, by intracellular heating due to stimulation of ions and rotation of dipoles. These RF-specific effects were demonstrated to be possibly related to cell membrane integrity and DNA denaturation. This research acquired insights on the inactivation mechanisms of a RF-only dynamic heating treatment and the possible influence or not of non-thermal effects in the inactivation. Nonetheless, further studies should focus on additional techniques which could provide further understanding of the impact on DNA, cellular structure, and membrane integrity.

Acknowledgement

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Part III

Conclusions

Chapter 10

General conclusions & future perspectives

Radio frequency (RF) heating is a promising, novel technology, particularly for the inactivation of foodborne pathogens in a variety of food products. The effectiveness of RF inactivation treatments is different in different products, influenced by factors such as moisture content, composition, and physical properties. These factors can either promote or inhibit foodborne pathogen survival (Piyasena et al., 2003; Sprong et al., 2002). In the meantime, the ongoing debate on the nature of the RF inactivation mechanisms continues, with some studies suggesting the existence of non-thermal effects and others identifying inactivation mechanisms attributed only to the effect of heat (Zhang et al., 2021a). Additionally, the use of complex matrices (i.e., real food products) complicates the understanding of the findings so far. Finally, most studies have ignored the inherent, dynamic nature of RF heating and its potential and effect on microbial inactivation.

The main objective of this dissertation was to investigate the influence of a RF-only dynamic heating treatment on foodborne pathogen inactivation in different dairy model systems. The two target microorganisms were *Salmonella* Typhimurium and *L. monocytogenes,* a Gram-negative and a Gram-positive microorganism, respectively. The dairy model systems corresponded to three main food structural categories (powders, liquids, gels) and two different fat level contents (0.8% and 35% fat for the powders, and 0.08% and 5.9% fat for the liquids and gels). Skimmed and whole milk powders were the basis for the development of the dairy liquid and gel systems. Therefore, the three sub-objectives were to analyse: (i) the RF inactivation in powders, (ii) the RF inactivation in liquids and gels, and (iii) the RF inactivation mechanisms in a simple, minimalistic medium.

10.1 General conclusions

The key findings and conclusions of this study are discussed below based on the three established sub-objectives.

Sub-objective 1: Explore RF-only treatments in the microbial inactivation in dairy powders

In **Chapter 5,** the behaviour of the two target microorganisms in the two types of milk powder (i.e., skimmed and whole milk powder) was monitored for a month, during storage at 20°C. The Geeraerd Shoulder-Tail (2000) and the Akkermans et al. (2021) model were fitted to the experimental data to describe the inactivation kinetics and the evolution of the sublethal injury (SI), respectively. Experiments were also conducted to detect the existence of DNA damage in *L. monocytogenes*. The main findings of this chapter can be summarised as follows.

- *Salmonella* demonstrated higher resistance and higher amount of SI in comparison to *L. monocytogenes* in both types of milk powder.
- The inactivation rate and the amount of SI in whole milk powder was higher than in skimmed milk powder for both bacteria.
- DNA damage was detected for the most sensitive microorganism, *L. monocytogenes*.

L. monocytogenes and *S.* Typhimurium survived in skimmed and whole milk powder over a month at 20°C, with *Salmonella* showing higher resistance. Even though the Geeraerd et al. (2000) log-linear tail model described their behaviour in milk powder for 30 days, longer storage periods are necessary to accurately evaluate the microbial kinetics. Due to the inherent complexity of the milk powder composition, it is challenging to deduct a conclusion on the effect of the fat on the survival of the two microorganisms. While this study indicated the existence of DNA denaturation of *L. monocytogenes* cells after a month of storage, this observation could also be attributed to the decreasing number of viable cells. Future research should therefore explore the effect of a desiccated environment to cell components and how storage-induced damage can impact the effectiveness of inactivation treatments like thermal and radio frequency processing.

The aim of **Chapter 6** was to design an effective RF-only dynamic heating process for the treatment of milk powder. In this chapter, only skimmed milk powder was used for the RF treatments. Four different heating profiles were designed, two with a fast heating rate and two with a slow heating rate, until target temperatures 85°C and 95°C. As *Salmonella* Typhimurium was more resistant in skimmed milk powder during the storage experiments (Chapter 5), it was used as a case-study for the inactivation experiments. Two quality characteristics, colour and solubility, were also evaluated after each treatment. The main findings of this chapter can be summarised as follows.

- Treating the skimmed milk powder up to 95°C using a slow heating rate led to a 4-log reduction of *Salmonella.*

- No significant changes were observed in the solubility of skimmed milk powder in any of the treatments.

- Treatment to 95°C led to an increase in the yellowness of the skimmed milk powder colour.

A RF-only slow heating profile effectively inactivated *Salmonella* in skimmed milk powder. The quality properties were not affected significantly, contrary to the common RF processes which involve dynamic heating until a target temperature and a conventional oven holding phase at the target temperature. Nonetheless, the effect on other quality attributes (e.g., organoleptic properties) and physicochemical characteristics should be conducted to ensure the efficacy of this method. Future studies should also verify the effectiveness of the developed RF-only method for other relevant microorganisms, such as *L. monocytogenes* and *Cronobacter sakazaaki.*

In **Chapter 7,** the established RF-only dynamic heating profile from Chapter 8 was used to acquire the inactivation kinetics of *S.* Typhimurium and *L. monocytogenes* in skimmed and whole milk powder. *E. faecium* was used as a surrogate microorganism for the RF treatments. A log-linear model with a Bigelow-type temperature dependency was fitted to the inactivation kinetics of the three microorganisms and the percentage of SI was also calculated. The main findings of this chapter can be summarised as follows.

- *S.* Typhimurium was more resistant to RF treatments in comparison to *L. monocytogenes* based on the inactivation rate *k*.
- *L. monocytogenes* had a higher percentage of SI in comparison to *S.* Typhimurium.
- The inactivation was higher in whole milk powder in comparison to skimmed milk powder.
- *E. faecium* can be used successfully as a surrogate for both microorganisms.

Gram-negative bacterium *S.* Typhimurium was more resistant to the RF treatment in comparison to *L. monocytogenes*, a phenomenon which could be attributed to the increase high thermal resistance of *Salmonella* at low a_w environments. However, further studies should evaluate further microorganisms and strains to draw a concrete conclusion of the specific effect of the Gram-type on the microbial resistance after RF treatments. As concluded in Chapter 5, the inherent composition of milk powder complicates the isolation of the effect a single factor (i.e., fat, carbohydrate, protein content). In this regard, the lower inactivation rates observed in skimmed milk powder could be attributed to the increased lactose and protein content. In order to isolate the influence of each factor, further research can be conducted separately on each component i.e., lactose powder, whey protein powder and total milk protein powder, originating from the same milk source, to reduce variability.

Sub-objective 2: Investigate RF-only treatments on the microbial inactivation in dairy liquids and gels

In **Chapter 8,** the focus turns on the RF-only dynamic heating inactivation of the two foodborne pathogens in dairy liquid and gel systems. Four dairy systems were developed and specifically, two liquid systems with 0.08% and 5.9% fat and two gel systems with 0.08% and 5.9% fat. The dairy model systems were formulated as such, so that their nutritional composition differs primarily in the fat content levels and 1% alginate was used to formulate the gels. A log-linear model with a Bigelow-type temperature dependency was used to fit to the inactivation kinetics data. In order to accurately isolate and compare the two intrinsic food factor (i.e., fat content and presence of gel structure), the log-linear model was fitted only until the time point which demonstrated a log-linear inactivation behaviour. The SI (%) after each RF treatment was also calculated. The main findings of this chapter can be summarised as follows.

- In liquids systems, higher fat content led to higher inactivation rates for both microorganisms.

- On the contrary, in gel systems, the inactivation rate was higher at lower fat content for *S*. Typhimurium.

- *L. monocytogenes* followed the same trend but only until 70°C. For temperatures above 70°C, the inactivation rate was higher at higher fat content.

- A gel matrix led to higher inactivation rates of *S*. Typhimurium

- Once more, a more complex, temperature-dependent behaviour was observed for *L. monocytogenes*.

- Sublethal injury was significant for L. *monocytogenes* at 5.9% fat content and high temperatures.

Fat content and matrix structure may have an influence on the RF-only inactivation of *S*. Typhimurium and *L. monocytogenes*. These effects seem to be different than the typical microbial behaviour observed after conventional heating. However, to effective compare the effect between RF-only and conventional studies, further experiments with conventional dynamic heating treatments in the same samples should be considered. Regarding the RF-only treatments, even though the effect of each factor was relatively complex depending on the microorganism and the applied temperatures, a general trend indicates that increased fat content led to increased inactivation rates at high temperatures. This phenomenon could possibly be attributed to the higher heat absorption of fat in comparison to water. The presence of a gel matrix also led to higher inactivation rates. A hypothesis could be that the gels have higher dielectric properties (due to the presence of alginate) and/or the slow heat dissipation of the RF energy absorbed by the microorganisms. Therefore, further research should identify the dielectric properties and the heating absorption and uniformity of each system.

Sub-objective 3: Evaluate the RF inactivation mechanism in a minimalistic medium

Finally, **Chapter 9** explores the inactivation mechanisms of the RF-only dynamic heating process in comparison to conventional thermal treatments. The two microorganisms were treated in a minimal laboratory medium (BHI liquid broth) using the same dynamic heating profile as the one acquired in Chapter 8, for both treatment types. The inactivation kinetics and the SI (%) were documented after the RF and the conventional thermal treatments. Then, for selected treatment times (6, 9 and 15 min) three techniques were used to investigate the impact of the treatments on membrane and DNA integrity. The main findings of this chapter can be summarised as follows.

- RF caused higher inactivation and SI (%) than conventional treatments for both microorganisms.

- RF treatments led to more membrane damage than conventional treatments in both microorganisms, although this could also be attributed to the lower viable cell density.

- No significant differences on the DNA damage observed by fluorescence techniques (denaturation) was shown between RF and conventional treatments for both microorganisms.

- No considerable differences in DNA integrity (as seen by agarose gel electrophoresis) were observed after either treatment type.

RF-only dynamic heating demonstrated a more efficient inactivation effect in comparison to conventional thermal treatments, for both microorganisms. The higher inactivation could be attributed to the energy absorption by the cells of the microorganisms, due to the stimulation of ions and rotation of dipoles in their membranes and cytoplasm. However, it proved challenging to attribute with certainty the specific, (possibly non-thermal) RF effects on the bacterial membrane and DNA. In order to conclude the existence or not of non-thermal effects, future studies should conduct more advanced analyses such as electron microscopy (to identify damages to the cell structure and transcriptomics (to establish the gene response mechanisms). Nonetheless, this study uses an inherent RF-only dynamic heating and conventional thermal treatments of an identical, uniform temperature profile and it can act as a stepping stone for the accurate comparison of the two treatments.

10.2 Perspectives of future research

This dissertation focused on the RF inactivation of foodborne pathogens in different dairy model systems. The innovative aspect of this project was the use of the inherent dynamic heating of the RF process in the treatments of all dairy systems. While the efficiency of the proposed RF method was validated for the two foodborne pathogens, it is only an initial step at exploring the possibilities of RF-only dynamic heating applications.

Initially, future research should aim to establish uniform temperature distribution during RF treatments. The observed variability in the RF processes of this dissertation, mainly due to factors such as equipment design (i.e., set-up configuration, electrode position, quality and connection of coaxial cables), highlights the need for equipment optimisation. Additionally, as RF is a dielectric heating technology, the dielectric properties of the sample can highly influence and be influenced by the distribution of the electromagnetic field (Altemimi et al., 2020). Therefore, the design and optimisation of the equipment should take into account the targeted product and the specific application.

Additionally, a crucial factor is the uniform temperature distribution in the sample during RF heating process, specifically if a dynamic heating profile is adopted with prolonged processing times. Computational modelling can aid in understanding the factors which influence the temperature uniformity by simulating the RF energy distribution within the food. (Marra et al., 2009; Tiwari et al., 2011b; Chen et al., 2017). Specifically, these simulations help to identify uneven heating areas, enabling adjustments to the RF heating parameters. This approach can simplify the current method of conducting multiple trial-and-error experiments.

The validation of the developed RF-only dynamic heating approach should also be extended to other microorganisms and food products such as trending plant-based beverages or lactose-free products. Future studies could explore the inactivation efficacy on foodborne pathogens of interest. Specifically, the efficiency of this RF-only dynamic heating should also be evaluated against *Cronobacter sakazakii* in dairy powders. Other foodborne pathogens could include *Escherichia coli* and *Staphylococcus aureus*, given their prevalence in foodborne outbreaks (Kim et al., 2012; Michael et. al., 2014; Kou et al., 2018). Moreover, heat-resistant bacterial toxins and also spore-forming bacteria constantly pose a threat to the food industry and therefore, further investigation under RF treatment conditions should be conducted (Jiao et al., 2019; Akhila et al., 2021).

Finally, RF heating should be considered as a component of hurdle technology. The combination of RF treatments with other food preservation methods (e.g., hot air or plasma) presents a viable strategy for increasing the efficiency of processing treatments. This integrated approach can be a robust solution for the inactivation of foodborne pathogens in various food products, with minimal quality loss (Choi et al., 2018; Guo et al., 2019).

Part IV

Exploitation of research

Chapter 11

Valorisation plan

Context: This chapter outlines a valorisation plan that uses the results of this dissertation to explore opportunities in contract research. The plan aims to enable external parties to use and build upon the knowledge produced by this PhD research. By doing so, the research can have practical applications and benefits beyond the academic environment. The goal is to establish a system where the results of this study contribute to real-world solutions and advancements.

11.1 Introduction

Dairy products, especially low-moisture products like milk powder and powdered infant formula milk, pose significant microbial safety challenges. These challenges arise due to the ability of certain foodborne pathogens to survive in low-moisture environments. Even though these products usually undergo a spray-drying process, pathogens such as *Salmonella*, *Bacillus cereus,* and *Cronobacter sakazakii* can survive and then persist for extended periods of storage, even under adverse conditions. Currently, there are no other critical control point implemented following the spray drying process to ensure the safety of dairy powders (Becker et al., 1994; Lang & Sant'Ana, 2021).

Other dairy products with higher moisture content can also present food safety concerns. Dairy products such as milk, cream, and certain types of cheese provide a favourable environment for microbial growth if not handled and stored properly. Most commonly, foodborne pathogens such as *Salmonella*, *Listeria monocytogenes*, and *Escherichia coli* can contaminate these

products during production, processing, or even at the retail level (Paramithiotis et al., 2017).

When it comes to ensuring the safety of high-moisture dairy products, traditional methods typically include pasteurisation and sterilisation. These technologies involve heating the products to specific target temperatures for a set period of time in order to inactivate foodborne pathogens. However, despite their effectiveness, these methods may exhibit certain drawbacks (Lindsay et al., 2021). The most common drawback is the degradation of the nutritional value of the product. Conventional heat treatments can cause some degradation of nutrients and bioactive compounds present in the dairy foods. For instance, certain vitamins like vitamin C may be destroyed during pasteurisation, affecting the nutritional quality and potential health benefits of the product (Bezie, 2019). Additionally, another disadvantage is the high energy consumption (Feil et al., 2020). Conventional pasteurisation methods require significant energy inputs to heat products to the required temperature. This can contribute not only to higher operational costs, but also to environmental impacts associated with energy consumption. Moreover, traditional heat treatments can sometimes lead to changes in the organoleptic profile of dairy products. High temperatures can cause caramelisation or Maillard reactions, which subsequently, can alter the taste, texture, or colour of the product (e.g., the slight yellow discoloration of UHT milk) (Rozhkova & Olentsova, 2020). Finally, the heat transfer mechanisms of conventional thermal treatments are conduction and convention. Specifically, there is a direct contact between the product and the equipment's heated surfaces, which usually leads to fouling. Fouling is a phenomenon which causes the accumulation of deposits on heat exchanger surfaces, compromising the efficiency of heat transfer. This buildup not only increases energy consumption, but also requires frequent cleaning and maintenance of the heat exchangers in order to restore the performance levels. This leads to interruptions in production and high costs which in turn are linked to equipment repairs which impacts the profits of the dairy market (Wilson, 2018; Flint et al., 2020).

To address these challenges, the industry is exploring innovative technologies that can offer safer and more efficient alternatives to traditional pasteurisation and sterilisation methods.

11.2 Value proposition

Radio frequency (RF) technology is a promising solution to the challenges of the dairy industry, offering a pasteurisation alternative that could enhance the safety and shelf-life of these products without compromising their nutritional and sensory qualities (Dag et al., 2022). RF heating offers the following benefits (Piyasena et al., 2003; Orsat & Raghavan, 2005; Jiao et al., 2018):

- Efficient energy transfer
- Short processing time
- Uniform heating
- Reduced equipment dimension
- Reduced thermal losses to the environment
- Better preservation of organoleptic and physicochemical properties of the product
- Low maintenance costs

Given the many benefits of RF technology, this project aimed to delve deeper into the RF applications in the dairy industry, focusing on food safety. Specifically, this dissertation investigates the efficacy of RF treatments targeted at the inactivation of foodborne pathogens in dairy model systems. The primary objective was to understand how RF treatments influence the inactivation of pathogens in different model systems which represent (and are) real food products. The dairy model systems included dairy powders (skimmed and whole milk powder), dairy liquids (reconstituted skimmed milk and milk with 5.9% fat) and dairy gels (which correspond to dairy puddings and creams). By systematically studying the inactivation of a RF-only dynamic heating treatment on the different dairy model systems, the research offers insights into innovative food safety strategies which can by easily used by the dairy industry.

Specifically, the results of this project correspond to two Technology Readiness Levels (TRL). First, the technology was tested and validated in a laboratory setting (TLR 4), providing a solid foundation for potential real-world applications. This covered the work conducted in lab scale RF set-up with dairy liquid and gel model systems. This phase is essential, as it confirms

the technology's effectiveness for the inactivation of foodborne pathogens in these products and sets the stage for the next level of development, where the technology can be validated in the future in an industry-scale equipment (TRL 5). Second, the RF treatment of milk powder was successfully conducted in a pilot-scale, industrial RF set-up. Specifically, a novel, RF-only dynamic heating method was developed and used to successfully achieve a 4-log reduction of *Salmonella* and *L. monocytogenes* in skimmed and whole milk powder, while maintaining the quality characteristics of the product.

11.3 Societal and economic significance

The results of this project can significantly aid two sectors: the food industry and the government and social healthcare sector.

The Food industry is the largest manufacturing sector in Europe with a turnover of €1.2 trillion and the leading employer, offering jobs to 4.82 million people (Eurostat, 2020). Especially in Belgium, the food sector is at the forefront of economy, with a strong financial position (Figure 11.3). Moreover, the dairy industry holds the position of the second-largest agricultural sector in EU, contributing to more than 12% of the total agricultural production.

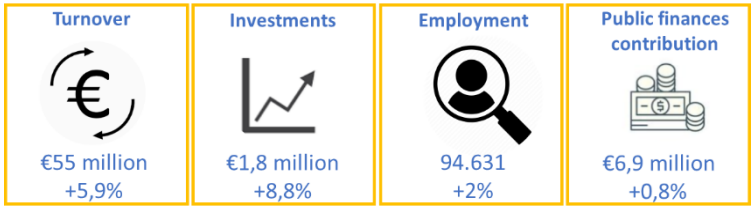


Figure 11.3: Overview of economic numbers of the Belgian food industry in 2019-2020 (Fevia, 2020)

The main challenge of the food sector is to guarantee the safety of the food while offering the consumer a high quality product. In 2018, pathogenic microorganisms were declared as 1st hazard for health and food safety with

979 notifications in Europe. In Belgium, two of the top five hazards notified were *Listeria* and *Salmonella*, with *Salmonella* ranking 1st (RASFF, 2018), creating challenging problems for not only the food industry, but also governmental healthcare and social security services. Infected people sometimes get hospitalised as a result of their illness, resulting in loss of work time due to the severity of their symptoms and therefore significant costs to employers, that are above and beyond medical spending. Specifically, in 2022 there were 29,003 and 1,386 hospitalisations out of which 81 and 286 led to deaths for salmonellosis and listeriosis respectively (EFSA, 2022). In the past, the widespread of numerous outbreaks attributed to microbial contamination has caused severe economic damages with tremendous consequences, not only for food and other companies, but also governments.

One these grounds, the European Union is investing in food safety in the next years (2021-2027) by providing 1.68 billion euro to projects concerning food safety. Similarly, Belgium also invests in food safety, as noted in Flander's Vision 2050, a long-term strategy for Flanders. Furthermore, Flanders' FOOD, an innovative platform, currently operates 12 projects about food safety and food processing (Flanders' FOOD, 2024).

Furthermore, the EU constantly keeps applying new measures and policies to promote the concept of "Sustainable Food Systems". Currently, the conventional heat processing methods consume tremendous amount of water and they are responsible for 28% of the total energy consumption of the food industry in Europe, resulting in a detrimental environmental impact (Monforti et al., 2015). RF, due to its longer penetration depth, is capable of processing food products faster, leading to less energy and water consumption and hence, operational costs. This project developed and applied an innovative, RF-only dynamic heating method, which when compared to conventional thermal treatments, reduces the time necessary to inactivate foodborne pathogens in dairy liquids and gels. Future studies can further investigate the sustainable aspect and consequently, the societal impact, of the specific RF technology.

Another economic and also societal aspect is food waste. Overall, the food retail business has a responsibility to verify that their own retail units are following the most current science to prevent food safety hazards during retail food production. Contaminated batches get dispatched or recalled,

causing major economic losses to the industry and unnecessary food waste. Additionally, food waste has a huge environmental impact, contributing to approximately 16% of the total greenhouse gas emissions in the EU. In the EU, more than 58 million tonnes of food waste are generated annually and around 10% are generated by the processing and manufacturing sector (Eurostat, 2021). The total food losses in the Flemish food supply chain are estimated at approximately 2 million tonnes out of which half are attributed to the food industry. Hence, Flanders has designated a goal to reduce food losses 30% by 2025 (VARIO, 2014).

The systematic study of the RF technology targeted at pathogen inactivation, in this project, supported the development and optimisation of a novel food process and thus, leads to improved food safety and less food waste, profiting a more stable and sustainable economy. Furthermore, the research results provided information on the RF inactivation mechanism, knowledge that will directly contribute to the optimisation of RF as a food pasteurisation and sterilisation process.

Moreover, the Covid-19 crisis led consuming habits towards products with long shelf life (e.g., low moisture food), highlighting the demand to certify the safety of this food category (Bakalis et. al., 2020). This project (i) identified the behaviour (survival) of two foodborne pathogens on two types of milk powder, and (ii) developed an efficient RF heating process, achieving a 4-log microbial reduction without compromising the colour or solubility of the milk powders.

Apart from food applications, RF has also some applications in the pharmaceutical sector, where the technology can be used for drying and sanitisation of medicinal plants, spices, nutraceuticals and natural hair (Stalam, 2017). Moreover, recent studies conducted on the medicine sector, propose RF coupled with magnetic nanoparticles as a treatment against multiple drug resistant bacteria (Chaurasia et al., 2016). The results (e.g., set-up parameters, temperature-time profiles) of this project can act as an initial step to use a RF dynamic heating profile for these applications.

11.4 Market analysis and partnership opportunities

The dairy industry is an important sector in the global food market and it is expanding quickly. It is expected that by 2025, world milk production will go up by 177 million tonnes, growing at a rate of 1.8% each year. In the same timeframe, people in developing countries will drink 0.8% to 1.7% more dairy each year, while those in developed countries will consume 0.5% to 1.1% more (OECD/FAO, 2016).

It is predicted that the global dairy sector will consistently grow over time. Specifically, market analysis projections estimate that by 2030, the Compound Annual Growth Rate which measures the investments' average annual growth over time, will increase by approximately 6%. Additionally, the growth rate is expected double in its current size. So far, the Asia–Pacific region is the largest in the dairy sector, a trend that is expected to continue (Figure 11.4). Moreover, the dairy sector in also a key player in the EU, as it is the second biggest in the food sector. The EU also ranks as the second-highest milk-producing region globally (Augère-Granier, 2018).

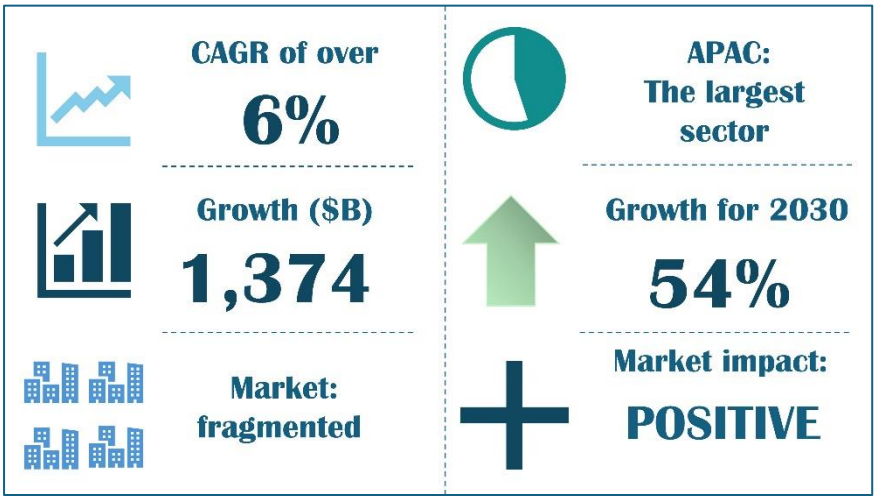


Figure 11.4: Dairy market projections by 2030. Information retrieved by Fortune Business Insights, accessed 4 Mar. 2024

The dairy market is fragmented and currently the major global players are Nestle, Dairy Farmers Of America, Fonterra, Danone and Arla Foods. These companies continually seek and invest on innovative technologies to enhance food safety and quality of their products. The application of RF technology, as explored in this dissertation, presents a significant opportunity within this context. By focusing on the inactivation of foodborne pathogens in dairy products, the research aligns with industry priorities around food safety and regulatory compliance. Market analysis indicates a growing need for novel preservation methods that maintain product quality while ensuring safety.

The development of an efficient RF treatment method of various categories of dairy products, both in laboratory and pilot-scale settings, positions it as a promising solution for dairy producers. The market's readiness for such innovations, driven by the industry's demand for efficient, cost-effective safety solutions, suggests a favourable environment for the introduction and adoption of the developed RF treatment method.

11.5 Market strategy and communication

In order to enhance the integration of the developed RF-only treatments in the dairy sector, forming partnerships with key industry players is crucial. Collaborating with dairy producers (e.g., Norwegian company TINE- constructed the milk powder purchased for the conduction of the RF treatments of dairy), technology providers (e.g., French manufacturing company SAIREM- developed the RF set-up used for the RF treatments of dairy), and research institutions (e.g., NOFIMA, the Norwegian Institute of Food, Fisheries and Aquaculture Research which provided funding and research facilities for a part of this dissertation) can lead to knowledge exchange, technology optimisation, and practical implementation on real-case industry scenarios. Establishing pilot projects with different dairy companies can demonstrate the RF effectiveness and benefits on different products, encouraging its integration into existing systems.

In order to commercialise the results of this PhD, it's essential to identify and target dairy industry segments most likely to benefit from RF technology. As

discussed in 11.1, dairy powders constitute the predominant food product target as there is no current step after spray-drying which certifies the safety of the product. A post-drying treatment step such as RF, can aid in efficient thermal inactivation without influencing the quality of the product. Additionally, products such as milk, can also benefit from RF treatments. RF is a dielectric heating technology which means that there is no need for the product to come in contact with a material in order to heat up as in conventional thermal treatments which eventually lead to the undesirable fouling phenomena.

Communicating clear messages which highlight the aforementioned RF advantages over traditional thermal processing is the most effective strategy to draw in potential partners. Communication efforts should focus on industry conferences or fairs, such as the bi-annual Anuga Food Fair in Germany, trade publications, and direct outreach to the decision-makers in dairy companies. Moreover, engaging with regulatory bodies (e.g., EU commission, FAO, EFSA) and institutes (e.g., NOFIMA, Flanders' Food, EIT Food) to ensure the RF technology meets regulations and acquire support can also aid its integration and acceptance to the market.

11.6 Risk analysis

Identifying potential risks, such as challenges in adopting the technology, market competition and regulatory challenges are crucial. The adoption of Radio frequency (RF) technology in the dairy industry, while promising, is not without its potential risks. One significant challenge is the initial investment and cost associated with implementing RF technology on an industrial scale. Dairy producers may be hesitant to adopt this new technology due to the high capital expenditure required for purchasing and installing RF equipment. Additionally, the industry must ensure that there is sufficient training for personnel to operate and maintain the RF systems effectively. The complexity of transitioning from traditional pasteurisation methods to RF technology might also pose operational risks, potentially disrupting existing production processes.

A considerable challenge is upscaling RF technology from a lab-scale to an industrial-scale setup. Technical issues include achieving uniform heating and

optimising equipment design for larger volumes. Energy efficiency becomes crucial as power requirements increase, necessitating effective heat distribution and minimising thermal losses. Integrating RF technology into existing production lines requires compatibility with other processes and maintenance protocols. Another aspect to consider is ensuring worker safety during the process. Additionally, the investment must be justified by a favourable return on investment through improved product quality and reduced waste. Thus far, most companies in the food industry accept the risk of potential contamination, acknowledging that it is minimal and that the safety measures already in place significantly reduce this likelihood and hence, consider the post treatment of products unnecessary. Furthermore, they would need to maintain consistent product quality and adapt the technology to various dairy products, which requires extensive testing and quality control measures.

Another considerable risk lies in market competition and regulatory challenges. As RF technology is still relatively novel in the food industry, gaining regulatory approval for its widespread use could be a lengthy and complicated process. Different countries have varying regulatory standards, which could affect the global adoption of RF technology. Moreover, the dairy industry is highly competitive, with established companies continually seeking innovative technologies. There is a risk that competing technologies, especially non-thermal novel technologies could potentially offer more cost-effective or efficient solutions, thereby limiting the market penetration and adoption of RF technology.

11.7 Mitigation strategies

Ensuring that RF technology meets all safety, quality, and regulatory standards is crucial to mitigate these risks and facilitate its integration into the dairy industry. Mitigation strategies to the aforementioned risks include:

- ongoing research and optimisation of the technology
- building strong cases on the economic benefits
- staying informed about regulatory changes

Currently, in Europe there are at least 6 companies that have invested in RF equipment manufacturing including Sairem, Stalam and Keysight, manufacturing not only large scale industrial installations, but also pilot-scale installations suited more for scientific research. Additionally, between 2000-2019, there were 24 published research studies on the RF pasteurisation and in the last four years this number almost doubled (41 studies, from 2020 until March 2024). Therefore, there is already an increasing considerable effort on investigating the applications of RF pasteurisation.

These (already established and ongoing) strategies are key to the successful integration of the RF technology to the food sector. By focusing on these areas, companies can acquire information on how to adopt the technology, spur innovation, and stay competitive. The rise in investments by European companies and also the increase in research studies highlight the growing interest in RF. Addressing risks will be instrumental for the efficient implementation of RF in improving food safety and quality in the food sector.

11.8 Conclusions and future perspectives

This dissertation has drawn attention to the potential of a RF-only dynamic heating method as a novel technology targeted at microbial inactivation. The results can aid in enhancing the food safety and quality of different products in the dairy industry. This valorisation plan illustrates how RF technology can become a key player in addressing microbial safety challenges, particularly in dairy powders and liquids, offering a viable alternative to conventional thermal pasteurisation methods. The successful application of the developed RF method in lab and pilot-scale settings lays a solid foundation for future industry-scale implementations.

The role of RF technology in food safety and quality within the dairy industry can become increasingly significant. Continued research and development should focus on refining and adapting RF treatments for broader applications across different food product categories. Moreover, efforts should concentrate on scaling the technology, optimising process parameters, and ensuring that it is applicable and efficient for various processing scenarios.

Collaboration with industry stakeholders is essential to the efficient incorporation and acceptance of RF applications. Building partnerships with dairy producers, equipment manufacturers, and regulatory bodies can facilitate the integration of RF treatments into the existing dairy processing landscape. These collaborations are vital in complying with regulations and ensuring that these applications align with industry standards and consumer expectations.

The increasing research and interest in RF technology its potentials in food safety. Using novel technologies such as RF is vital in tackling food safety challenges and reaching sustainability goals. The efficient integration of RF can set new standards for food safety and quality in the food industry, benefiting producers, consumers, and stakeholders across the food supply chain.

Part V

Appendices

Appendix A: Supplementary materials

Chapter 7

Context: This appendix contains additional information for Chapter 7. Specifically, Figure A1 shows the inactivation kinetics of the surrogate microorganism *E. faecium* in skimmed and whole milk powder after RF heating treatments.

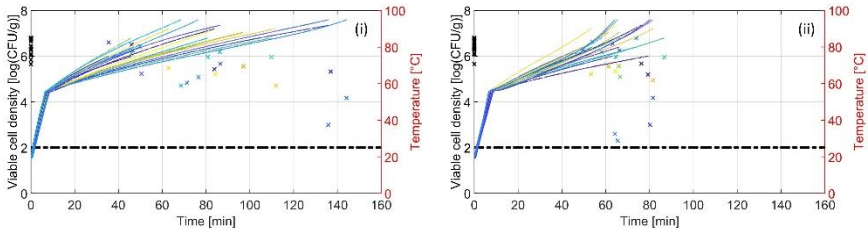


Figure A1: Cell density (log(CFU/g)) of *E. faecium* in skimmed (i) and whole (ii) milk powder after RF treatment to different end temperatures (n = 2). Data points of black colour represent initial cell density (log(CFU/g)). Data points of different colours represent the cell density (log(CFU/g)) after RF treatment to the respective end temperature. The temperature profile of each treatment corresponds to the specific data points of similar colour. The dashed line represents the detection limit set at 2 log(CFU/g).

Appendix B: Supplementary materials Chapter 8

Context: This appendix provides additional information for Chapter 10. Specifically, the comparison between log-linear and log-linear tail inactivation models and estimated model parameters are shown in Figure B1 and Tables B1 and B2, respectively. In Figures B2-B8 and Tables B3- B.9, the unique modelling approach discussed in 8.3.1 was used for all microorganisms in all different conditions. Specifically, the log-linear model was fitted only to the part of the inactivation that was characterised by (temperature-dependent) loglinear inactivation behaviour.

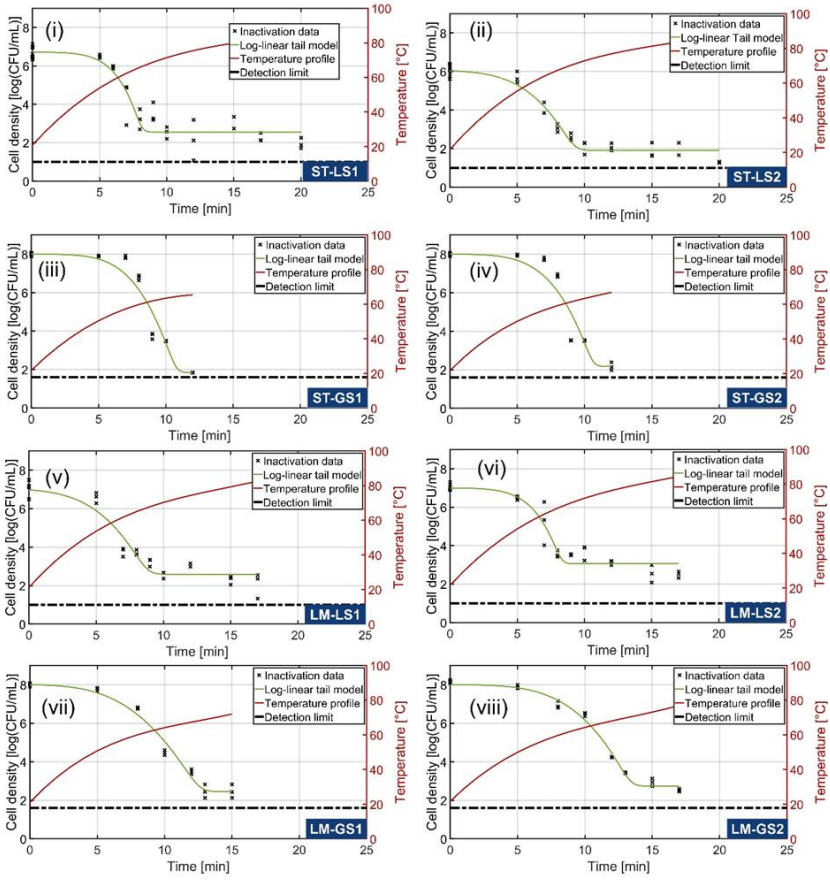


Figure B.1: Log-linear tail model fitted to the RF inactivation data of *S.* Typhimurium in (i) liquid system 1, 0% fat (ST-LS1), (ii) liquid system 2, 5.9% fat LS2 (ST-LS2), (iii) gelled system 1, 0% fat (ST-GS1), (iv) gelled system 2, 5.9% fat (ST-GS2), and *L. monocytogenes* in (v) liquid system 1, 0% fat (LM-LS1), (vi) liquid system 2, 5.9% fat (LM-LS2), (vii) gelled system 1, 0% fat (LM-GS1), and (viii) gelled system 2, 5.9% fat (LM-GS1). The model uses a secondary temperature dependency model (Bigelow-type relation). Data points below detection limit were not considered in the model.

Table B1: Model parameters of the log-linear model with a Bigelow type temperature dependency fitted to experimental data of the RF inactivation of *S.* Typhimurium and *L. monocytogenes* in the four systems (n = 3). LS1, liquid system 1 (0.08% fat); LS2, liquid system 2 (5.9% fat); GS1, gelled system 1 (GS1, 0.08% fat); GS2, gelled system 2 (5.9% fat).

Log-linear model parameters	*S.* Typhimurium				*L. monocytogenes*			
	LS1	LS2	GS1	GS2	LS1	LS2	GS1	GS2
$k(Tref)$ (1/min)	0.26 ± 0.10	0.26 ± 0.20	1.29 ± 0.28	1.56 ± 0.09	0.34 ± 0.03	0.31 ± 0.02	0.54 ± 0.03	0.41 ± 0.02
z (°C)	100.00 ± 12.57	100.00 ± 50.81	19.62 ± 3.67	23.05 ± 3.43	100.00 ± 12.52	100.00 ± 6.00	49.08 ± 9.88	52.16 ± 5.04
$n0$ (log(CFU/mL))	6.28 ± 0.17	5.64 ± 0.16	8.00 ± 0.14	8.00 ± 0.17	6.67 ± 0.17	6.80 ± 0.13	8.00 ± 0.12	8.00 ± 0.10
RMSE	0.96	0.81	0.64	0.74	0.86	0.72	0.49	0.48

Table B.2: Model parameters of the log-linear tail model with a Bigelow type temperature dependency fitted to experimental data of the RF inactivation of *S.* Typhimurium and *L. monocytogenes* in the four systems (n = 3). LS1, liquid system 1 (0.08% fat); LS2, liquid system 2 (5.9% fat); GS1, gelled system 1 (GS1, 0.08% fat); GS2, gelled system 2 (5.9% fat).

Log-linear tail model parameters	*S.* Typhimurium				*L. monocytogenes*				
	LS1	LS2	GS1	GS2	LS1	LS2	GS1	GS2	
k(Tref) (1/min)	1.75 ± 0.22	0.77 ± 0.07	2.00 ± 0.29	2.00 ± 0.57	1.05 ± 0.27	1.44 ± 0.26	0.82 ± 0.05	0.65 ± 0.04	
z (°C)	15.45 ± 2.30	28.49 ± 5.50	13.78 ± 2.39	14.87 ± 4.41	32.15 ± 11.60	16.69 ± 3.23	23.47 ± 2.92	17.78 ± 0.72	
n0 (log(CFU/mL))	6.72 ± 0.16	6.04 ± 0.12	8.00 ± 0.23	8.00 ± 0.28	6.98 ± 0.21	7.00 ± 0.19	8.00 ± 0.13	8.00 ± 0.07	
RMSE	0.63	0.36	0.68	0.73	0.56	0.52	0.34	0.23	

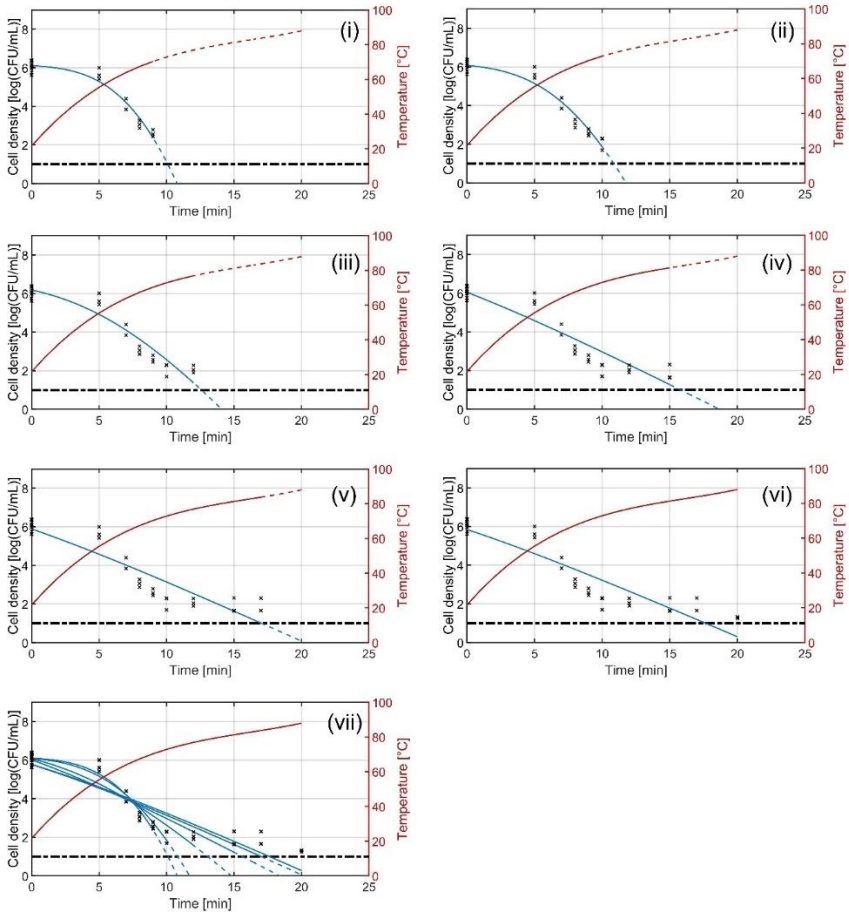


Figure B.2: Log-linear models with a Bigelow-type temperature dependency fitted to the RF inactivation data of *S.* Typhimurium in LS2 for subsets with the specific final time points (min): (i) 9, (ii) 10, (iii) 12, (iv) 15, (v) 17, (vi) 20 shown in solid blue line. The average temperature profile is depicted in red solid line and the extended prediction of each model is depicted in dashed lines. A combination of all possible model iterations can be seen in (vii).

Table B.3: Model parameters of the Log-linear model with a Bigelow type temperature dependency fitted to experimental data of the RF inactivation of *S.* Typhimurium in liquid system 2 (LS2) (n = 3).

Treatment time point (min)	Model Parameters			RMSE
	k(Tref) (1/min)	z (°C)	*n0* (log(CFU/mL))	
9	0.72 ± 0.07	32.34 ± 6.71	6.11 ± 0.13	0.25
10	0.59 ± 0.08	42.78 ± 2.89	6.08 ± 0.13	0.31
12	0.45 ± 0.04	90.04 ± 8.73	6.19 ± 0.18	0.55
15	0.32 ± 0.02	599.96 ± 80.43	6.05 ± 0.22	0.80
17	0.29 ± 0.01	600 ± 125.46	5.89 ± 0.17	0.93
20	0.27 ± 0.04	600 ± 27.75	5.85 ± 0.17	0.96

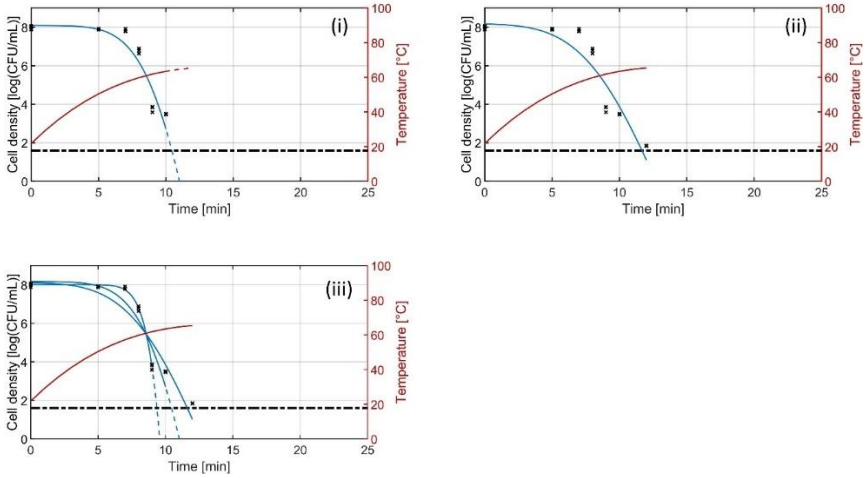


Figure B.3: Log-linear models with a Bigelow-type temperature dependency fitted to the RF inactivation data of *S.* Typhimurium in GS1 for subsets with the specific final time points (min): (i) 10, (ii) 12 shown in solid blue line. The average temperature profile is depicted in red solid line and the extended prediction of each model is depicted in dashed lines. A combination of all possible model iterations can be seen in (iii).

Table B.4: Model parameters of the Log-linear model with a Bigelow type temperature dependency fitted to experimental data of the RF inactivation of *S.* Typhimurium in gelled system 1 (GS1) (n = 3).

Treatment time point (min)	Model Parameters			RMSE
	k(Tref) (1/min)	*z* (°C)	*n0* (log(CFU/mL))	
10	2.56 ± 0.15	14.50 ± 1.30	8.34 ± 0.25	0.69
12	1.30 ± 0.10	25.99 ± 3.32	8.43 ± 0.28	1.06

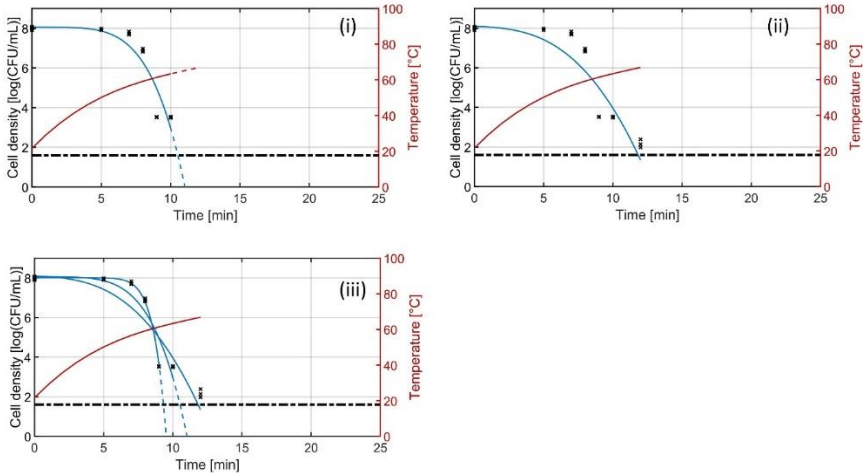


Figure B.4: Log-linear models with a Bigelow-type temperature dependency fitted to the RF inactivation data of *S.* Typhimurium in GS2 for subsets with the specific final time points (min): shown in solid

blue line. The average temperature profile is depicted in red solid line and the extended prediction of each model is depicted in dashed lines. A combination of all possible model iterations can be seen in (iii).

Table B.5: Model parameters of the Log-linear model with a Bigelow type temperature dependency fitted to experimental data of the RF inactivation of *S.* Typhimurium in gelled system 2 (GS2) (n = 3).

Treatment time point (min)	Model Parameters			RMSE
	k(Tref) (1/min)	*z* (°C)	*n0* (log(CFU/mL))	
10	2.20 ± 0.32	16.75 ± 3.05	8.33 ± 0.32	0.89
12	1.09 ± 0.21	35.59 ± 2.92	8.42 ± 0.21	1.34

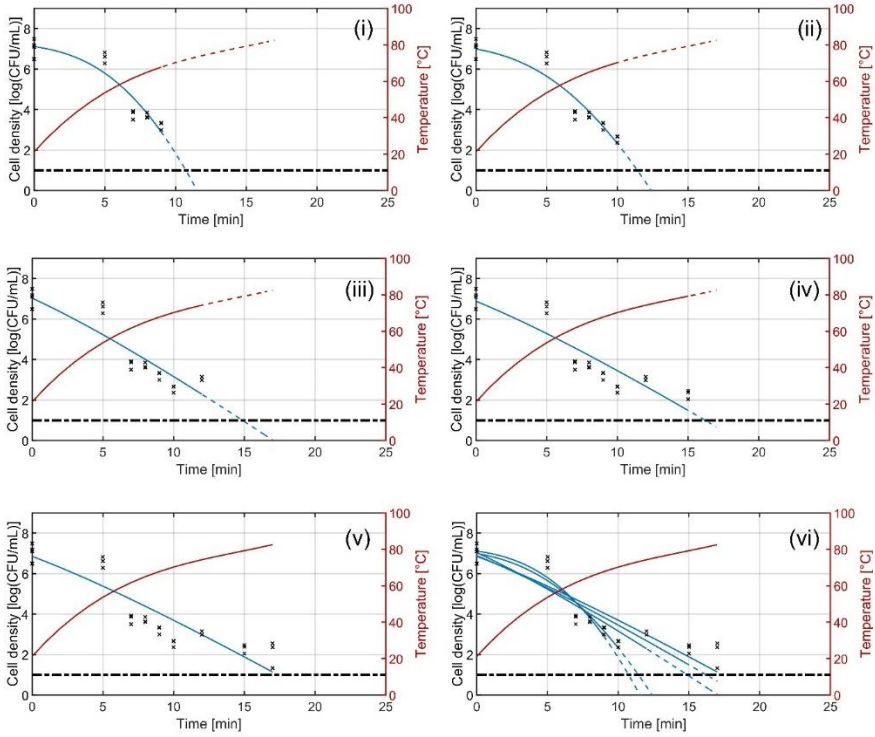


Figure 5: Log-linear models with a Bigelow-type temperature dependency fitted to the RF inactivation data of *L. monocytogenes* in LS1 for subsets with the specific final time points (min): (i) 9, (ii) 10, (iii) 12, (iv) 15, (v) 17 shown in solid blue line. The average temperature profile is depicted in red solid line and the extended prediction of each model is depicted in dashed lines. A combination of all possible model iterations can be seen in (vi).

Table B.6: Model parameters of the Log-linear model with a Bigelow type temperature dependency fitted to experimental data of the RF inactivation of *L. monocytogenes* in liquid system 1 (LS1) (n = 3).

Treatment time point (min)	Model Parameters			RMSE
	k(Tref) (1/min)	*z* (°C)	*n0* (log(CFU/mL))	
9	0.81 ± 0.12	46.56 ± 18.41	7.11 ± 0.24	0.83
10	0.68 ± 0.22	57.55 ± 15.38	7.04 ± 0.20	0.80
12	0.42 ± 0.00	400.00 ± 4.54	6.89 ± 0.00	0.03
15	0.37 ± 0.03	400.00 ± 31.05	6.89 ± 0.20	0.99
17	0.34 ± 0.05	400.00 ± 303.99	5.89 ± 0.20	1.05

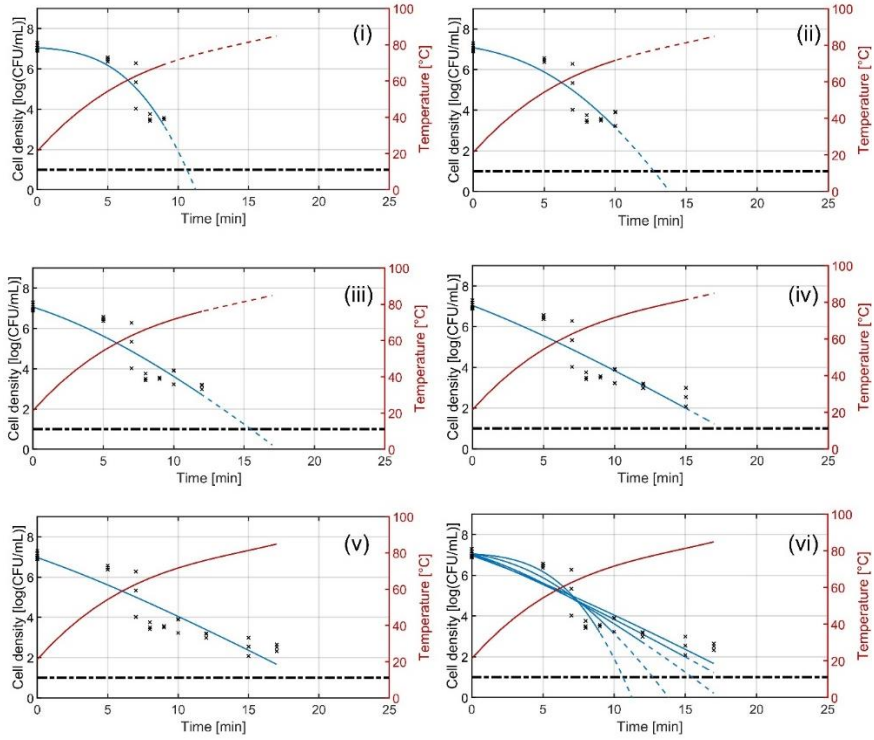


Figure B.6: Log-linear models with a Bigelow-type temperature dependency fitted to the RF inactivation data of *L. monocytogenes* in LS2 for subsets with the specific final time points (min): (i) 9, (ii) 10, (iii) 12, (iv) 15, (v) 17 shown in solid blue line. The average temperature profile is depicted in red solid line and the extended prediction of each model is depicted in dashed lines. A combination of all possible model iterations can be seen in (vi).

Table B.7: Model parameters of the Log-linear model with a Bigelow type temperature dependency fitted to experimental data of the RF inactivation of *L. monocytogenes* in liquid system 2 (LS2) (n = 3).

Treatment time point (min)	Model Parameters			RMSE
	k(Tref) (1/min)	*z* (°C)	*n0* (log(CFU/mL))	
9	0.81 ± 0.16	33.00 ± 12.92	7.05 ± 0.21	0.73
10	0.54 ± 0.20	65.86 ± 6.34	7.08 ± 0.20	0.81
12	0.40 ± 0.07	173.67 ± 208.13	7.07 ± 0.19	0.80
15	0.34 ± 0.04	400.00 ± 146.20	7.04 ± 0.17	0.81
17	0.31 ± 0.03	400.00 ± 44.72	6.97 ± 0.17	0.85

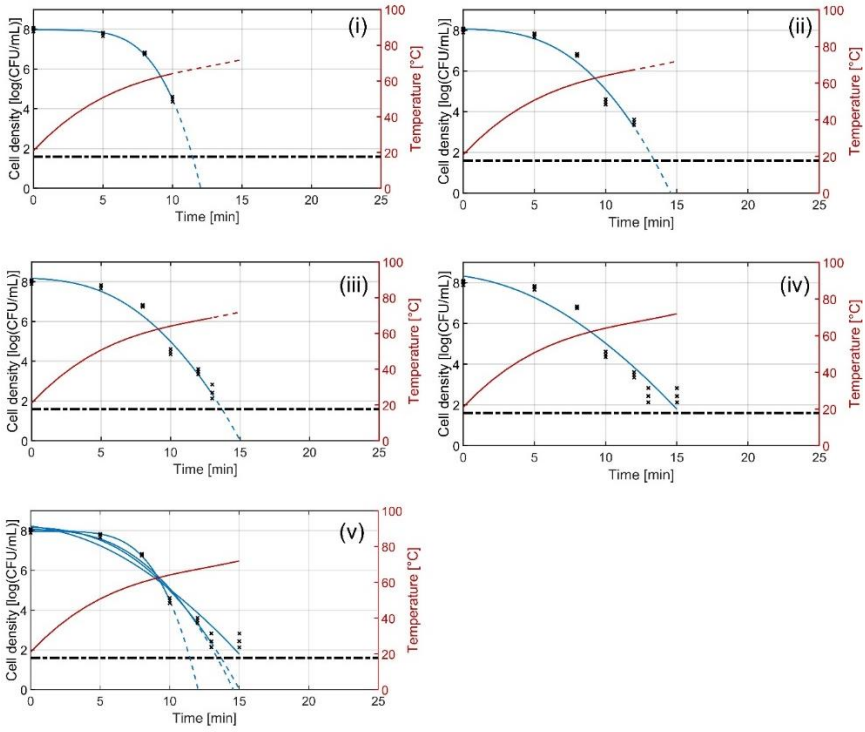


Figure B.7: Log-linear models with a Bigelow-type temperature dependency fitted to the RF inactivation data of *L. monocytogenes* in GS1 for subsets with the specific final time points (min): (i) 10, (ii) 12, (iii) 13, (iv) 15 shown in solid blue line. The average temperature profile is depicted in red solid line and the extended prediction of each model is depicted in dashed lines. A combination of all possible model iterations can be seen in (v).

Table B.8: Model parameters of the Log-linear model with a Bigelow type temperature dependency fitted to experimental data of the RF inactivation of *L. monocytogenes* in gelled system 1 (GS2) (n = 3).

Treatment time point (min)	Model Parameters			RMSE
	k(Tref) (1/min)	z (°C)	*n0* (log(CFU/mL))	
10	1.48 ± 0.03	12.50 ± 0.70	7.97 ± 0.03	0.02
12	0.77 ± 0.03	26.34 ± 3.08	8.04 ± 0.09	0.25
13	0.74 ± 0.05	28.72 ± 5.23	8.07 ± 0.28	0.23
15	0.55 ± 0.11	50.53 ± 25.97	8.09 ± 0.42	0.56

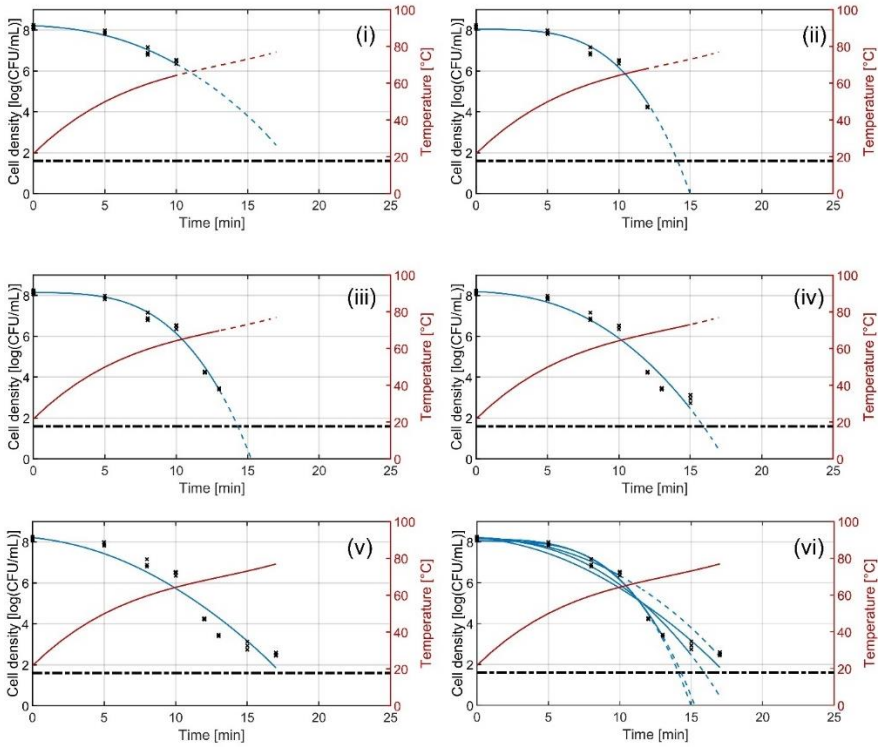


Figure B.8: Log-linear models with a Bigelow-type temperature dependency fitted to the RF inactivation data of *L. monocytogenes* in GS2 for subsets with the specific final time points (min): (i) 10, (ii) 12, (iii) 13, (iv) 15, (v) 17 shown in solid blue line. The average temperature profile is depicted in red solid line and the extended prediction extended prediction of each model is depicted in dashed lines. A combination of all possible model iterations can be seen in (vi).

Table B.9: Model parameters of the Log-linear model with a Bigelow type temperature dependency fitted to experimental data of the RF inactivation of *L. monocytogenes* in gelled system 2 (GS2) (n = 3).

Treatment time point (min)	Model Parameters			RMSE
	k(Tref) (1/min)	*z* (°C)	*n0* (log(CFU/mL))	
10	0.38 ± 0.03	42.50 ± 1.33	8.22 ± 0.06	0.04
12	0.61 ± 0.02	19.64 ± 0.85	8.06 ± 0.06	0.09
13	0.61 ± 0.03	19.46 ± 0.77	8.05 ± 0.07	0.08
15	0.50 ± 0.02	36.54 ± 9.87	8.19 ± 0.40	0.28
17	0.42 ± 0.02	56.50 ± 1.31	8.21 ± 0.17	0.44

Appendix C: Supplementary materials Chapter 9

Context: This appendix contains additional information for Chapter 9 about the RF inactivation mechanisms. The dynamic temperature profiles after RF and thermal treatments are illustrated in Figure C1. The model parameters acquired from the Geeraerd (2000) log-linear and log linear and tail models with a Bigelow type temperature relation fitted to the microbial inactivation data after conventional thermal and RF treatments, respectively are presented in Table C1.

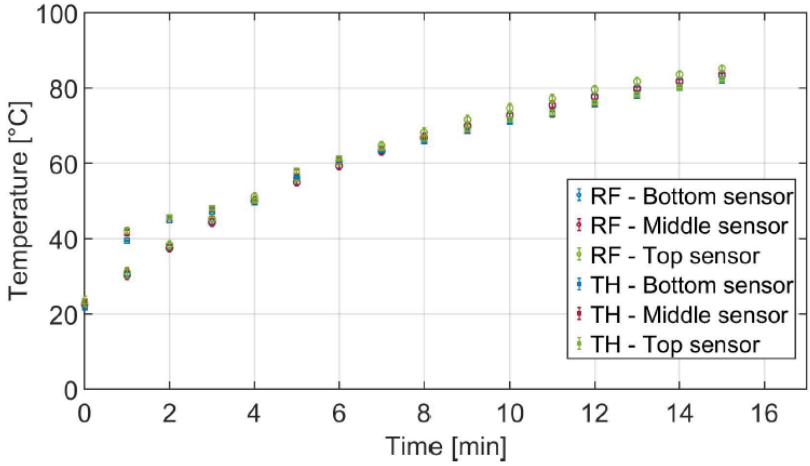


Figure C1: Temperature uniformity of samples containing BHI broth during RF (represented by a circle symbol "○") and thermal (represented by a square symbol "□") treatments. Three different optic fibre sensors were placed in 0.5 cm intervals at the bottom (blue), the middle (red) and at the top (green) of the sample. Three individual replicates were conducted for each treatment (n=3).

Table C1: Model parameters and RMSE for *S.* Typhimurium and *L. monocytogenes* inactivation in liquid BHI broth. For both microorganisms, the log-linear tail model with a Bigelow type temperature dependency was used to fit the experimental data for the RF treatments, whereas the log-linear model with a Bigelow type temperature dependency was fitted to experimental data for the conventional thermal treatments (n = 3). Different lowercase letters denote statistically significant differences ($p \leq 0.05$) between the two treatment types. 'NA' indicates that the tail model was not applied to conventional treatments.

Model parameters	*S.* Typhimurium		*L. monocytogenes*	
	RF	Thermal	RF	Thermal
k_{ref} (T_{ref}) (min^{-1})	13.11 ± 13.61[b]	0.51 ± 0.00[a]	2.52 ± 0.17[b]	0.43 ± 0.01[a]
z (°C)	8.28 ± 4.75[a]	87.05 ± 0.11[b]	11.03 ± 1.89[a]	35.09 ± 0.34[b]
N_0 (log(CFU/mL))	8.76 ± 0.44[a]	8.59 ± 0.03[a]	8.91 ± 0.24[b]	8.57 ± 0.05[a]
N_{res} (log(CFU/mL))	1.91 ± 0.25	NA	0.75 ± 0.22	NA
RMSE	1.08	0.78	0.65	0.89

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Curriculum Vitae

Maria obtained a Master's degree in Agriculture Science from Aristotle University of Thessaloniki in Greece, with a specialisation in Food Science and Technology. During her studies, she followed an Erasmus exchange program in Lebensmitteltechnologie in Technische Universität Berlin in Germany and a 3-month Erasmus+ internship program in the BioTeC+ research team in Belgium. In 2019, she started her PhD within BioTeC+ under the supervision of Prof. Jan Van Impe. Part of her PhD research was conducted at Nofima in Stavanger, Norway, a leading institute for applied research within the fields of fisheries, aquaculture and food research. During her PhD, Maria was the first author of 4 international peer-reviewed journal papers, was co-author of 2 more, and contributed to 5 publications at international conferences.

Publications

Peer reviewed articles in international journals

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