University of Stavanger

Faculty of Science and Technology

MASTER'S THESIS

Study program/ Specialization:	Spring semester, 2011 Open / Restricted access
Writer: Nataliya Z. Lindås	…… (Writer's signature)
Faculty supervisor: Jan Thomas Rosnes External supervisor(s): Jan Thomas Rosnes, Torstein Skåra and Dagbjørn Skipnes	
Titel of thesis: Inactivation kinetics of *Listeria innocua* in steam surface pasteurisation of fish products	
Credits (ECTS): 60	
Key words: *Listeria monocytogenes*, *Listeria innocua*, heat treatment, steam surface pasteurisation, BugDeath rig	Pages: …… + enclosure: …… Stavanger, …….. Date/year

Abstract

This master project is a part of the Strategic Institute programme, ProSpect, granted by the Norwegian Research Council (NFR). In order to develop safe minimally processed seafood and to increase quality and shelf-life, the project ProSpect combined principles from aseptic packaging with surface pasteurisation with steam. This master project had special focus on thermal steam pasteurisation of fish products. A special designed "BugDeath" test rig has been purchased for the experiments. *Listeria monocytogenes* was selected as target organism for the pasteurisation. To avoid the extra precautions associated with working with pathogenic bacteria, specific strains of *Listeria innocua* were selected as surrogate organisms for *L. monocytogenes*. *L. innocua* is a non-pathogenic organism, but more heat tolerant than *L. monocytogenes*.

The objective of the current study was to determine inactivation kinetics of different *L. innocua* strains (ATCC 33090 from American Type Culture Collection and CCUG 35613 from the Culture Collection, University of Gothenburg) by using two different experimental designs: 1) using classical heat treatment in capillary tubes, 2) using steam on fish product surfaces. Another objective of this study was to investigate and to compare differences in inactivation kinetic in capillary tubes and on fish surface.

In the fist experimental design with heat treatment in capillary tubes two strains of *L. innocua* (ATCC 33090 and CCUG 35613) were used. Both strains were cultivated and heat treated in the tryptone soya broth with yeast extract (TSBYE). In the second experimental design with steam pasteurisation on fish product surfaces *L. innocua* ATCC 33090 was used. This strain was cultivated in the TSBYE and heat treated on surimi "model-product". In both designs *L. innocua* strains after heat treatment were regenerated on the tryptone soya agar with yeast extract (TSAYE).

The D-values were used to determine inactivation kinetics and describe the heat resistance of the microorganisms. The calculations of heat resistance for treatment in capillary tubes were based on the linear first-order kinetics. The D-values were calculated for temperatures 59, 59.5, 60 ° C.

D-values for *L. innocua* ATCC 33090 were D_{59} =2.62 minutes, $D_{59.5}$ =2.1 minutes, D_{60} =1.58 minutes and for *L. innocua* CCUG 35613 were D_{59} =2.54 minutes, $D_{59.5}$ =1.91 minutes, D_{60} =1.50 minutes.

The inactivation of *L. innocua* in steam surface pasteurisation of fish products did not follow log-linear kinetics. Bacterial numbers of *L. innocua* ATCC 33090 declined rapidly during the first 15 s of steam treatment in "BugDeath" rig. This initial rate of decline slowed during the next 45 s. However, after 60 s of steam treatment bacterial numbers declined very slowly, so that bacterial numbers were still present after steam treatment for 4 min. In this experiment steam treatment of samples for 60 s gave total 3-4 $\log_{10}$ CFU reduction, but after 60 s had not considerable reduction of bacterial numbers and most likely had an undesirable effect for quality of the product.

Table of Contents

List of Tables

List of Figures

Acknowledgements

This study was a part of the master's degree in biological chemistry at the University of Stavanger and was performed at Nofima at Måltidets Hus.

Fist off all, I would like to thank my supervisors Jan Thomas Rosnes and Torstein Skåra for good help and support in connection with laboratory work and writing. I am deeply grateful to the staff of the Nofima who gave me an enjoyable working environment and an enthusiastic help during my thesis work.

I would like to express my gratitude to Dagbjørn Skipnes and Åsvald Vågane for their help with different software programs.

I would also send my thanks to the staff at Nofima: Maria Befring Hovda, Trond Løvdal, Karin Tranøy and at Tine Norske Meierier FOU who worked with me in the laboratory for help with some laboratory equipment and positive social cooperation.

Finally, I would like to express lots of thanks to my family for support and understanding.

1. Introduction / Background

Background

This work constitutes a part of the Strategic Institute programme, ProSpect, granted by the Norwegian Research Council (NFR). ProSpect is a project with cooperation between Nofima Norconserv and Nofima Marin (Marine). In order to provide good service to the industry, Nofima has chosen four areas in which the institute has special competence: heat processing, minimal processing, super hygiene and new seafood concepts. The main focus of the research at Nofima is on marine products and relates to quality characteristics, hygiene, preservation, packing technology and analytical instruments.

The full name of the ProSpect programme is "Innovative and safe seafood - processing, hygiene, spectroscopy" and the main objective is to combine aseptic process elements and spectroscopic monitoring systems for production of seafood based convenience foods. The ProSpect project is divided into 2 work packages in order to meet the objectives. The first package takes place at Nofima in Stavanger and works with increasing quality and shelf-life of seafood based convenience products. These products are manufactured from raw materials through thermal processing combined with aseptic technologies. The second package (in Tromsø) is an analytical part of developments of spectroscopic methods.

This master project had special focus in thermal steam pasteurisation of fish products. A specially designed test rig has been purchased for the experiments (Foster *et al.* 2006).The objective of the current study was to determine inactivation kinetics of different *Listeria innocua* strains by using two different experimental designs: 1) using classical heat treatment in capillary tubes, 2) using steam on fish product surfaces. Another objective of this study was to investigate and to compare differences in inactivation kinetic in capillary tubes and on fish surface.

Listeria monocytogenes was selected as target organism. To avoid the extra precautions associated with working with pathogenic bacteria, specific strains of *L. innocua* were selected that are nonpathogenic, but more heat tolerant than *L. monocytogenes.*

Introduction

L. monocytogenes has appeared as an important pathogenic organism during the last 20 years, causing the disease known as listeriosis in animals and humans (Jemmi and Stephan 2006;Schlech 2000). Many epidemiological investigations have shown that this disease may result from the consumption of contaminated foods containing *L. monocytogenes,* and thus it had been recognized as a major food-born illness (Bula *et al.* 1995;Riedo *et al.* 1994). Inactivation of *L. monocytogenes* has been suggested as a criterion for minimum heat treatment by the European Chilled Food Federation (ECFF 1996). This bacteria is a well documented pathogen in fish products and it is the most heat resistant non-spore forming bacteria among the actual fish pathogens. Hence the elimination of *L. monocytogenes* often determines processing parameters for chilled ready to eat meals with short shelf life.

Food poisoning is increasing throughout the EU. Each year in the UK, 4.5 million people (approximately 10 % of the population) suffer from food poisoning (James and Evans 2006).

There is often no terminal step (such as cooking) to eliminate pathogenic organisms from raw products such as meat, fish, fruits and vegetables before they reach the consumer. Several of the pathogens, for example *Listeria*, which are present on such products, are psychotrophic and can grow at refrigeration temperatures. Centralized processing and preparation of these products is growing, increasing the distance and time between initial preparation and the consumption, thus increasing the risk of pathogen growth during this time. Ideally, some form of terminal processing step should be introduced, which would reduce the microbial load of such products and would also be advantageous to public health and would have economic significance to the industry (James and Evans 2006).

But at the same time, a terminal processing step that involves heat treatment can be a problem for many products, especially fish products. The product may change its optimal sensory characteristics and texture. Quality of fish is severely reduced if the thermal process is designed for a shelf life of more than 21 days at chilled conditions, requiring at least a 6 log inactivation of non-proteolytic *Clostridium botulinum* (EC 1999). Designing a thermal process for such a product is challenging as the heat load required for inactivating microorganisms and enzymes may cause undesirable changes such as dry structure and flaking (Ofstad *et al.* 1995). Reduced cooking time at a lower temperature is preferred with respect to the sensory quality of fish

products, but this will reduce the shelf life (Skåra *et al.* 2002). For processing in the temperature range of 60-75 °C, several vegetative microorganisms could pose a threat to product safety.

An alternative method of terminal processing is the use of steam for the decontamination of meat which is now extensively used in the USA, Canada and Australia and also being considered for use in the European Union (EU) (Mccann *et al.* 2006). The effectiveness of surface pasteurisation with steam on beef, pig and sheep carcasses and on poultry has been investigated and the available information and data suggests that these treatments may be effective in combating product contaminated with pathogens (James *et al.* 2000;Mccann *et al.* 2006;Minihan *et al.* 2003;Phebus *et al.* 1997;Whyte *et al.* 2003).

Surface pasteurisation with steam is previously tried for the whole animal carcasses and poultry products, but for fish products it is almost new and in need of targeted research and development. In order to develop safe minimally processed seafood and to increase quality and shelf-life, the project ProSpect combined principles from aseptic packaging with surface pasteurisation with steam. Aseptic packaging is common for liquid dairy products (Brody 2006), and means of sterilization packaging and product, and to pack and seal product under sterile conditions**.** Using (usage) of non-sterile seafood products is new in this technology. The concept Extended-shelf-life (ESL) packaging is probably well suited for many seafood products. ESL does generally, but not necessarily, imply processing under aseptic conditions to achieve sterile products. Treatment of package units is performed to approach sterility. Packaging conditions used are ultra clean, i.e. nearly sterile. The ESL processing will open for the new categories of minimally processed refrigerated shelf stable products. The technology also opens for combinations with modified atmosphere packaging (MAP) that has been unavailable for thermally processed food so far.

Ready-to-eat meals

The consumer wants fresh and healthy food that is easy to prepare. Today many companies are working with developing food products with minimum processing. In light of this development, ready-to-eat food (RTE) and ready meals have become very popular. RTE food is a diverse food category that can be consumed without further processing. This type of food can be in the raw state, or prepared and stored under different conditions. Many RTE have short shelf-life and must be consumed after a short storage period. For food producers, microbial spoilage is one major problem. Studies indicated that outbreaks of food poisoning have been associated with commercially manufactured foods (McLauchlin *et al.* 2004;Rocourt *et al.* 2003)

Some RTE may not undergo thermal or other processing sufficient to inactivate the organism, thus contribute to contamination of food products. In recent years there has been an increase in the consumption of RTE food, such as mixed salads containing raw vegetables and other ingredients such as meat or seafood (EC, 2005b). Pre-packaged mixed salads have a high potential for contamination from *L. monocytogenes* due to extensive handling during preparation or from the cross contamination from the environment. Study Little et al. (2007) of pre-packaged mixed vegetable salads shows that overall contamination of *Listeria* spp. and *L. monocytogenes* found in samples of mixed salads in the UK was 10.8 % and 4.8 %, respectively.

In 1994 *L. monocytogenes* was found in different vacuum-packed meat and fish products (Rorvik 1995b). Rørvik showed that 16 % of the slaughted fishes at slaughter house contained *L. monocytogenes*, and 22% harboured other *Listeria* spp (Rorvik 1995b). The results of molecular examination made by Rørvik et al. (2000) showed that the seafood might be a cause of human listeriosis. Resistance to diverse environmental conditions, ability to grow at refrigeration temperatures and survive in food for prolonged periods under adverse conditions (Ryser 1991) and ability to colonize food processing environments together with the severity of human listeriosis make *L. monocytogenes* of particular concern for manufacturers of RTE food products. The European Commission (EC), Scientific Committee on Veterinary Measures relating to Public Health (SCVPH) concluded that the risk of listeriosis from foods containing less than 100 CFU/g is low (European Commission (EC), 1999). As a result, the EC Regulation on microbiological criteria for foodstuffs (Regulation (EC) No. 2073/2005), in force from January 2006, provides that *L. monocytogenes* should be below 100 CFU/g during the shelf life of RTE foods, and that processing areas and equipment used in the manufacturing of RTE foods must also be monitored for *L. monocytogenes* (EC, 2005a). However, for RTE foods intended for infants or special medical purposes, *L. monocytogenes* should not be present (absent in 25 g) throughout their shelf life (EC, 2005a).

2. Theory

2.1 Listeria monocytogenes

Listeria monocytogenes is one of species in the genus *Listeria*, which includes *L. grayi, L. innocua, L. ivanovii, L. monocytogenes, L. seeligeri, L. muttayi, L. welshimeri.* It is named after the English pioneer of sterile surgery, Joseph Lister in 1940 (Rocourt 1999). Within the genus, only *L. monocytogenes* and *L. ivanovii* have been considered as pathogenic bacteria (Rocourt 1999). *L. ivanovii* is consided as an animal pathogen which is mainly responsible for abortions, but rarely gives disease in man (Rocourt 1999). *L. monocytogenes* is pathogenic to both humans and animals (Swaminathan, 2001; Paoli et al., 2005).

L. monocytogenes is a Gram-positive, non-sporeforming and short rod-shaped bacterium. It is typically 0.5 µm in width and 1-2 µm in length. Cells may either be found a single cell, in short chains, arranged in V or Y forms or in palisades. Under certain growth conditions cells become coccoid and about 0.5 µm in diameter, causing them to be confused with streptococci (Rocourt 1999). *L. monocytogenes* is aerobic or facultatively anaerobic, catalyse positive and oxidase negative. It is motile via a few peritrichous flagellar when cultured at temperatures between 20 °C and 25 °C (Farber 1991). The optimum growth temperature is between 30 °C and 37 °C, and temperature limits for growth were reported to be from 0 °C and 45 °C (Walker *et al.* 1990). *L. monocytogenes* is capable of growing in a wide pH span. In broth, it normally grows from pH 4.4 to 9.6, and the optimum pH for growth is approximately 7 (Thevenot *et al.* 2006). The bacterium also can survive in environment having a salt concentration ranging from 10 % to 30 % (w/v) NaCl (Rocourt 1999). *Listeria* is able to grow at a water activity value (a_w) below 0.93 (WHO, 2004). The ability to be resistant to high salinity or acidity allows the organism to survive longer under adverse conditions than most other non-sporeforming bacteria of importance in foodborne disease (WHO, 2004). The pathogenicity of the bacterium is closely linked to the presence of hemolysin and other virulence factors. Hemolysin is considered the major virulence factor of *L. monocytogenes*, and it helps to differentiate between this bacterium and non-pathogenic *Listeria* species (Swaminathan, 2001).

Human disease caused by *L. monocytogenes* usually occurs in certain well-defined high-risk groups, know as YOPI (young, old, pregnant, immunecompromised) sub-population, including

pregnant women and fetus, newborns, the elderly, individuals with weakened immune systems, drug addicts and the HIV patients (Farber 1991). Classification of illness caused by *L. monocytogenes* is shown in Table 2.1 (EC, 1999).

Table 2. 1 Classification of illness caused by *L. monocytogenes*

Type of Listeriosis	Mode of transmission	Severity	Time to onset
Occupational infection	Primary cutaneous listeriosis after direct contact with infected animal tissues	Usually mild and self-resolving	1–2 days
Neonatal infection	Infection of newborn babies from infected mother during birth or due to cross-infection from one neonate in the hospital to other babies	Can be extremely severe, resulting in meningitis and death.	1–2 days (early onset), usually from congenital infection prior to birth; 5–12 days (late onset), following cross-infection from another infant
Infection during pregnancy (prenatal)	Acquired following consumption of contaminated food	Mild flu-like illness or asymptomatic in the mother, but serious complications for unborn infant, including spontaneous abortion, fetal death, stillbirth and meningitis. Infection is more commonly reported in third trimester	
Infection of non-pregnant adults (nonperinatal)	Acquired following consumption of contaminated food	Asymptomatic or mild illness, which may progress to CNS infections such as meningitis. Most common in immunocompromised or elderly	Illness may occur within 1 day or up to 3 months, but commonly within 20–30 days
Listeria food poisoning (febrile gastroenteritis)	Consumption of food with exceptionally high levels of *L. monocytogenes*, $> 10^7$ cfu/g.	Vomiting and diarrhea, sometimes progressing to bacteraemia but usually selfresolving	<24 h after consumption

2.2 Occurrence in environment, feeds, human and animals

L. monocytogenes was fist isolated from diseased rabbits in 1926. Later the organism has been detected in variety of domestic and wild mammals, including cattle, sheep, pigs, chickens, turkeys and ducks (Schuchat *et al.* 1991). Other common sources of *L. monocytogenes* are soil, plants, fresh and seawater, silage, sewage and in fecal materials (Donald *et al.* 1995).

Fertilized soil may contain decomposing plants, fecal materials, animal waste, and sewage sludge and are well-documented sources of *L. monocytogenes* . That is why soil may be the main source of *Listeria* contamination. Weis and Seeliger (1975) reported that the organism was found in 8.7 - 51.4 % of surface samples of soil and 3.2 - 33.3 % of samples which were collected at a depth of 10 cm. Ho and Ivanek (2007) suggested that *Listeria* contamination of soil may result from land fertilizing practices with sewage sludge, manures from farm animals, and fecal shedding of infected domestic and wild animals, including wild birds which may contain high levels of the bacteria.

Listeriosis in animals has been described to be associated with silage feeding since 1960 (Pauly and Tham 2003). This fermented high-moisture forage made from plants is widely used in feeding ruminants. Pauly and Tham (2003) studied occurrence of *Listeria* spp. in grass and grass silage sample. *L. monocytogenes* or *L. innocua* were isolated from 65 % of the grass samples and 23 % of the silage samples. *L. monocytogenes* was confirmed at least at one occasion in the silage of 34 % of all farms. These numbers show that *Listeria* spp are quite common in fresh forage.

Multiple studies have shown that *L. monocytogenes* is present in fecal specimens from a variety of human populations, including healthy people, pregnant women, patients with gastroenteritis, slaughterhouse workers, laboratory workers handling *Listeria*, food handlers, and patients undergoing renal transplantation or hemodialysis (Schuchat *et al.* 1991). It supposes the human gastrointestinal tract is a potential reservoir of the organism.

2.3 *L .monocytogenes* in foods and food processing factories

One of the main routes of transmission of listeriosis to human begins is food (WHO, 2004). *L. monocytogenes* has been detected regularly in a wide range of food including dairy products, fruits and vegetables, fresh and frozen meat, poultry, seafood and RTE (Farber 1991). Due to the ability of the organism to survive and grow at low temperatures, the bacterium poses a high risk for lightly preserved refrigerated foods stored for long periods of time.

L. monocytogenes has been found in a various types of dairy products. The organism grow well in both naturally and artificially contaminated fluid dairy products at temperatures ranging from 4 °C to 35 °C (Farber *et al.* 1990). Other unpasteurized dairy products such as milk, chocolate milk, butter, ice cream, or cheeses are also high risk causes of *Listeria.* Among these products, cheeses, especially soft cheeses are considered as a common food associated with foodborne listeriosis (Farber 1991).

Meat products such as cooked meat, RTE meat and poultry products have been described as the source of epidemic and sporadic listeriosis on several instances in North America and Europe (Swaminathan, 2001). The growth of *L. monocytogenes* in meat and poultry products depends on the type of meat, the pH, and the type and cell populations of the competitive flora (Swaminathan, 2001). Infection of animal tissue may occur either from symptomatic or asymptomatic carriage of *L. monocytogenes* by the food producing animal before slaughter, or contamination of the carcass after slaughter. Faber and Peterkin (1999) found that the organism was mainly accumulated and multiplied in the kidney, liver and spleen in infected animals, thus eating the processed products from such organ, may be more dangerous than eating muscle tissue. Poultry generally provides better support for the bacterial growth than other meats; roast beef and summer sausage support the growth the least (Swaminathan, 2001).

Various types of seafood have shown a high frequency of *L. monocytogenes* contamination. In the seafood the highest prevalence was found in cold-smoked fish (34- 60 %) while the lowest was found in heat treated and cured seafood (4 - 12 %) (Jorgensen and Huss 1998). Lunestad (2005) found organism in 0 to 50 % of examined samples of unprocessed or frozen fish. These numbers were lower in processed seafood products (0 - 26 %), shrimps (0 - 20 %) and shellfish (0 – 7.5 %), but quite high level of contaminated samples (0 - 75 %) was detected in smoked salmon or trout. Significantly higher numbers of contaminated samples of the smoked fish was explained by studying the incidence of the *L. monocytogenes* in seafood processing factories. A smoked salmon production, including a smokehouse and slaughterhouse was examined for the occurrence of *L. monocytogenes* and other *Listeria* spp. The results revealed that *L. monocytogenes* was often detected in samples from the smokehouse, but in much lower frequencies in the slaughterhouse. That considered the smokehouse as a reservoir for the bacteria (Rorvik 1995a). Huss et al. (2000) have classified four groups of seafood as potential high risk foods for listeriosis: mollusks including fresh and frozen mussels, clams, and oysters; raw fish; lightly preserved fish products

including salted, marinated, fermented, cold-smoked, and “gravd” fish; and mildly heat processed fish products and crustaceans.

Different studies show that the occurrence of *L. monocytogenes* in RTE foods depends on many different factors. Product type and the stage in the production-to-consumption chain at which the sample is taken, products origin (animal or fishfarms, etc.) and geographical distribution (includes climate, food processing environments and health status of workers) are some of them (WHO, 2004). The contamination also occurs at the point of sale, for example due to slicing of processed meats. Other ways of contamination have been demonstrated in domestic environments. Opened packages may be contaminated with *L. monocytogenes* present on surfaces in refrigerators, or in other refrigerated foods, from kitchen environment or from family members (WHO, 2004). Such RTE foods as unpasteurized milk and products prepared from unpasteurized milk, soft cheeses, frankfurters, jelly pork tongue, delicatessen meats and poultry products, smoked mussels, smoked fish, cooked shrimp, raw vegetables and coleslaw are usually preserved by refrigeration and offer an appropriate environment for the multiplication of *L. monocytogenes* (Swaminathan, 2001; WHO, 2004). Bacterial levels in foods are generally low (0 to 10^3 CFU/g with 90 to 99 % being below 10^2 CFU/g and less than 1 % being between 10^3 and 10^4 CFU/g), but the higher concentrations (10^5 to 10^7 CFU/g) also have been reported (Gram, 2001). WHO (2004) documented rather high uncertainty concerning these estimates because the actual level of the pathogen in the serving of food consumed by an infected individual could have varied greatly from that observed in other portions of the food during a subsequent investigation.

L. monocytogenes contaminations of food processing factories may occur through many ways: soil on workers´ and clothing, transport equipment, raw plants, raw foods of animal origin, and possibly healthy human carriers (Swaminathan, 2001). The high humidity and the presence of nutrients create a favourable environment for bacterial growth. Since a large number of healthy animals may be carriers, the contamination of carcasses by fecal matter during slaughter is very usual. The most heavily contaminated working areas are cow dehiding, pig stunning and hoisting. *L. monocytogenes* is also recovered from feather plucker drip water, chilly water overflow, recycling water for cleaning gutters, and mechanically deboned meat. These findings indicate that the importance of the defeathering machine, chillers, and recycled water in product cross contamination (Swaminathan, 2001).

2.4 Factors affecting survival and growth

The growth of *L. monocytogenes* in foods depends on the intrinsic and extrinsic characteristics of the products and processing techniques used in its production. Intrinsic factors may include pH, water activity, and salt concentration and extrinsic factors include storage temperature and relative humidity (FSAI, 2005). The growth limits of *L. monocytogenes* are summarised in Table 2.2.

Table 2.2 Growth limits for *L. monocytogenes* (summarised from Ryser (1991) and ICMSF (1996)

Environmental factor	Lower limit	Upper limit
Temperature (°C)	- 2 to + 14	45
Salt (% water phase NaCl)	< 0.5	13-16
(& corresponding a_w)	(0.91–0.93)	(>0.997)
pH (HCl as acidulant)	4.2–4.3	9.4–9.5
Lactic acid (water phase)	0	3.8–4.6 mM, MIC of undissociated acid (800–1000 mM, MIC of sodium lactate)

Temperature

L. monocytogenes is recognized as a psychrophilic microorgamism able to grow slowly at refrigerators temperatures, 0-8 ºC. (Bell and Kyriakides, 2005). The temperature span for grow ranges from 0 to 45 °C with optimum level at approximately 37 °C. Temperature below 0 °C and above 50 °C may inactivate the bacterium (Swaminathan, 2001). Several study suggested that *L. monocytogenes* can survive for several weeks or months in many chilled and frozen food types although viable cell numbers may decrease over time and survival may be poorer in products with a low pH (Bell and Kyriakides, 2005;Lou and Yousef 1999). Temperatures above 70 °C will give considerable reductions in numbers of viable bacterial cells (Bell and Kyriakides, 2005).

pH

The pH range for the growth of *L. monocytogenes* is from 5.6 to 9.6. However, several studies have shown that the minimum pH for the growth of *L. monocytogenes* is 4.3 using HCl as the acidulant (Farber *et al.* 1989;George *et al.* 1996). Swaminathan (2001) reported that at pH values below 4.3 the bacterial cells may survive but do not multiply. For foods which have low pH as a result of metabolic activity such as cheese or fermented meats, the low pH usually contributes to the control of bacterial population growth including any *Listeria* spp. present in food (Bell and

Kyriakides, 2005). Lou and Yousef (1999) reported that survival or growth of *L. monocytogenes* in different food matrices varies with pH values and temperature of storage. Bell and Kyriakides, (2005) reported that *L. monocytogenes* can grow in many types of cheese with pH values > 5.9 such as Camembert, Brick, and white-pickled cheese, but was inactivated quickly in other cheese types with pH value of 4.0 – 5.3 such as Parmesan and Mozzarella.

Water activity and salt concentration

Water activity (a_w) is a measure of the water available to sustain the survival and growth of microorganism in a food. The a_w of a food product is lowered by the addition of sodium chloride, sugars and /or other solutes. Investigations have indicated that *L. monocytogenes* grows optimally at $a_w \geq 0.97$ (Swaminathan, 2001;Bell and Kyriakides, 2005). Farber (1992) reported the minimum a_w (based on NaCl) for growth of *L. monocytogenes* to be 0.91-0.93. However, the bacterium has been reported to survive long periods at water activity values as low as 0.83 (Swaminathan 2001). Sodium chloride is the most common inhibitory to the growth of *L. monocytogenes* (Lou and Yousef, 1999;Bell and Kyriakides, 2005). Lou and Yousef (1999) concluded that survival of *L. monocytogenes* in concentrated salt solutions can be increased dramatically by lowering the storage temperature. The predictions of growth rate of *L. monocytogenes* (model of Tienungoon, 1998) is shown in Table 2.3.

Table 2.3. The predictions of growth rate of *L. monocytogenes* (model of Tienungoon, 1998)

Growth rate (l / generations time (h)			
Temperature	**pH 7.0, a_w : 0.990**	**90 mM total lactate, pH 6.2, a_w : 0.990**	**90 mM total lactate, pH 6.2, a_w : 0.965**
25	1.22	1.03	0.646
10	0.174	0.147	0.092
7	0.078	0.066	0.042
5	0.035	0.030	0.019
0	0.002	0.001	0.001

2.5 *Listeria innocua* as surrogate for *L. monocytogenes*

Listeria innocua is one of the species belonging to the genus *Listeria.* Seeliger and Schoofs discovered *L. innocua* as new microorganism in 1979, and a few years later, Seeliger gave supplementary description of this species (Seeliger 1981). *L. innocua* is non-pathogenic, this also appears from the name, the innocent, which means innocent / harmless.

L. innocua and *L. monocytogenes* are the two *Listeria* species, with many physiological and genetic similarities, and they are evolutionary not far from each other (Schmid *et al.* 2005). There are many common genes between these two species. Only 10.5 and 14 % of the all genome between *L. monocytogenes* strain EDGe and *L. innocua* strain CLIP 11262 are specific (Doumith *et al.* 2004b). Evidences for close relations between these two species, *L. monocytogenes* serovar 4 and *L.innocua,* are in the antigen structure in the flagella (Doumith *et al.* 2004a).

Biochemical characteristics for all *L. innocua* and *L. monocytogenes* strains are also very close. Both *Listeria* specie sare xylose negative, mannitol negative and glucose positive (Chen *et al.* 2010). The same authors selected β-hemolysis as a factor that can distinguish between this two species. All *L. innocua* strains have not hemolysis while *L. monocytogenes* has β-hemolysis. Another difference is the presence of arylamidase in *L. innocua, L. monocytogenes* does not contain this substance.

L. innocua and *L. monocytogenes* also has the same niche, and similar tolerance to pH, temperature and salt. Non-pathogenicity of *L. innocua* gives possibility to avoid the extra precautions associated with working with pathogenic bacteria, when working with bacteria in the laboratory.

In 1993, Foegeding and Stanley proposed to use *L. innocua* as a biological indicator for *L. monocytogenes* in the thermal processing (Fairchild and Foegeding 1993). The reason for this proposal was monitoring of heat resistance characteristic *Listeria* spp. under actual processes without any health risk. One characteristic of a biological indicator is that it must have a thermal resistance equal to or higher than the target. There have been studies which show that *L. innocua* have such properties. Friedly et al. (2008) studied various *L. innocua* and *L. monocytogenes* strains and compared these to confirm that *L. innocua* M1 was an ideal surrogate for heat treatment of *L. monocytogenes.*

2.6 *Listeria* inactivation

2.6.1 Factors affecting heat resistance of *Listeria*

There are several factors that affect the inactivation of bacteria. Incubation temperature and growth media before and after heat treatment are factors that have effect on heat resistance. Composition of the growth medium, pH, salt concentration and water content plays an important role. This applies both to food matrices and laboratory media. In relation to laboratory media it is very important to be in control over the individual growth factors because they have strong influence on the bacteria's heat resistance.

Growth Temperature before heat treatment

Optimal growth temperature for *L. innocua*, and *Listeria* in general, is between 30 and 37 ° C. The temperature during growth affects lipid biosynthesis, the composition of membranes, and protein synthesis. These are factors that affect the thermal properties of *L. monocytogenes* (Juneja and Eblen 1999b). Several studies on *L. monocytogenes* showed that cells are more heat resistant when grown at 35 °C before the heat treatment than when grown at low temperatures (20 °C) (Smith and Marmer 1991).

L. innocua has a higher heat resistance when it is grown at higher temperatures than at low temperatures. In a study it was shown significantly higher D-values for cells grown at 45 °C than at 20 °C. The D-values at 20 °C were up to 5 times higher at 45 °C (Alvarez-Ordonez *et al.* 2009). The same study did not find significant differences in z-value.

Media

Various media are used in heating experiments, for example media for 1) multiplication of bacteria, 2) heat transfer, or 3) the regeneration of damaged bacteria after a heat treatment. There are both selective and non-selective media, and heat resistance will vary with the choice of the media. A selective medium in this context is a growth medium that are favourable for the growth of *Listeria*. A non-selective medium is a medium where the other microflora has similar growth conditions.

Growth Medium (before heat treatment)

The growth medium is a medium that is used for enrichment of bacteria before heat treatment. In the laboratory context it is often a liquid medium (bouillon/broth), containing the necessary nutrients to the bacterium. Some components of food or culture media may protect the bacteria from heat injury by stabilizing membranes and other cell components, or by stimulating the production of stress proteins. The osmotic environment may play an important role for heat resistance to *Listeria. L. monocytogenes* grown in 1.5 M NaCl had an elongated shape and a higher thermal resistance compared with bacteria grown in 0.09 M NaCl (Jorgensen *et al.* 1995). In most cases it is shown that salt has a protective effect of *L. monocytogenes*, resulting in increased heat resistance. Cells from Scott A strain that was grown in a fat medium were 8 times more heat resistant at 60 °C than cells grown in trypsin soy broth (TSB) (Casadei *et al.* 1998). *L. monocytogenes* had significantly higher thermal resistance when there was enough nutrients in the growth medium (Lou and Yousef 1996).

Heat Treatment Medium

A heat treatment medium is the medium used during a heat treatment. Media that is used in the laboratory experiments, e.g. TSBYE, peptone water, distilled water. In food systems the heat treatment medium will have a major impact on the effect on the heat treatment. This is partly due to fat content, water activity and pH, and texture, all of which are effective for bacterial survival (Doyle *et al.* 2001).

Results from various studies indicate that *L. monocytogenes* is more heat resistant in food than in laboratory media (Boyle *et al.* 1990;Casadei *et al.* 1998;Foegeding and Stanley 1991;Jorgensen *et al.* 1999). For *L. monocytogenes* Scott A there is a range in D_{60}-values in food matrices, e.g. liquid egg mass 1.95 min (Muriana *et al.* 1996), crab meat 2.61 minutes (Harrison and Huang 1990), data from Table 2.4.

Regeneration medium

Various selective and nonselective media have been used for regeneration. Many types of media are not satisfactory for the recovery of heat damaged cells. Selective media are designed to promote the growth of a specific bacterium. This comes at the expense of optimal growth conditions and will therefore give a negative impact on the recovery of heat injured bacteria (Patel and Beuchat 1995;Smith and Archer 1988).

The regeneration medium has adverse effect on the recovery of bacteria when the medium contained the ingredients that made the medium selective (phenyletanol, acriflavin, sodium tellurit, polymyxin B sulfate or 5 % salt) (Smith and Archer 1988). It is also found that non-selective media has a higher number of colonies than the selective media after heat treatment (Crawford *et al.* 1989;Golden *et al.* 1988;Heddleson and Doores 1994;Linton *et al.* 1990;Linton *et al.* 1992;Rowan and Anderson 1998).

Growth of *L. innocua* was compared on the non-selective medium tryptone soya agar with 0.6 % yeast extract (TSAYE), and the selective media TSAYE with 5 % salt and Palcam (Miller *et al.* 2006). The result showed that on the non-selective medium bacteria had a higher survival rate than on the two selective media, and among the selective media gave TSAYE with 5 % salt lower survival rates than Palcam.

Water Activity

Lower water activity (a_w) gives a higher heat resistance of *L. monocytogenes.* Scott A strain was subjected to various concentrations of sucrose. At a water activity at 0.98, they demonstrated the $D_{65.6}$ -value is 0.36 and a z-value is 7.6 °C, when water activity was lowered to 0.90 the $D_{65.6}$ -value increased to 3.8 and the z-value increased to 12.9 °C (Sumner *et al.* 1991). In addition to that the lower water activity leads to higher heat resistance, it is also shown that the effect of water activity on heat resistance varies with heat treatment temperature (Fernandez *et al.* 2007). In a medium with a_w 0.90, there was less variation in the increase of the heat treatment temperatures than a corresponding increase in temperature in a heat treatment of bacteria grown in media with a_w 0.93, 0.96 and 0.99.

pH

Experiments with different pH show no clear trends for heat treatment. For acid shock, short exposure to pH 4 gave slight increase of D_{58}-values of *L. monocytogenes* (Farber and Pagotto 1992). However, *L. monocytogenes* is more heat sensitive in the treatment of cabbage juice, when the pH was changed from pH 5.6 to pH 4.6 (Beuchat *et al.* 1986). Juneja og Elben (1999a) found that heat resistance was reduced at pH 4 compared with pH 8.

Smoke components

It is known that wood and liquid smoke treatments influence microbial growth rate, growth limits, and rates of death / inactivation of *L. monocytogenes*. Eklund et al., (1995) found that populations of *L. monocytogenes* inoculated onto the surface of brined salmon portions changed very little during a cold-smoke process at 22.2 to 30.6 °C for 20 h, with or without applied smoke; but when the processing temperature was lowered to 17.2 to 21.1 °C, populations decreased 10- to 25-fold when smoke was applied. In addition, however, they found also that *L. monocytogenes* injected into the interior of these portions increased 2- to 6-fold at 17.2 to 21.1 °C and 100-fold at 22.2 to 30.6 °C, regardless of the presence of smoke. During hot-smoking of salmon the temperature required for inactivation of *L. monocytogenes* without smoke (82.8 °C) was lowered to 67.2 °C by smoke generated from sawdust and to 58.9 °C with a high level of a commercial liquid smoke (Poysky *et al.* 1997).

2.6.2 Kinetic of the microorganism

The growth of microorganisms reproducing by binary fission can be plotted as the logarithm of the number of viable cells versus the incubation time. The resulting curve has four distinct phases: lag phase (A), exponential phase (B), stationary phase (C), death phase (D) and its mathematical expression is called bacterial or microbial kinetics (Figure 2.1).

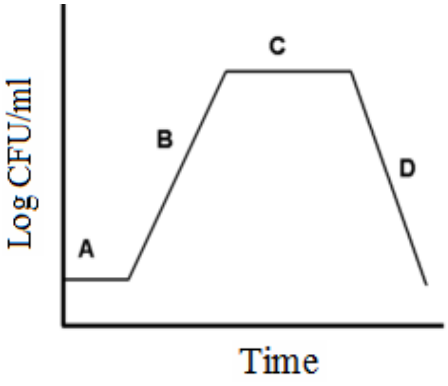


Figure 2.1 Growth is shown as log CFU/ml, where CFU/ml is the number of colony forming units per ml, versus time.

During the lag phase, bacteria adapt themselves to growth conditions. It is the period where the individual bacteria are maturing and not yet able to divide. The lag phase has high biosynthesis rates, as proteins necessary for rapid growth are produced. According to Robinson et al. (1998) concept of the lag time of *L. monocytogenes* two elements determine the lag time: (i) the amount of work required of the cell to adjust to a new environment and/or repair injury due to the shift to the new environment; and (ii) the rate at which those repairs and adjustments can be made.

The exponential phase (sometimes called the log phase or the logarithmic phase) is a period characterized by cell doubling. The number of new bacteria appearing per time unit is proportional to the present population. During the exponential phase each microorganism is dividing at constant rate. Exponential growth cannot continue indefinitely, however, because the medium is soon depleted of nutrients and enriched with wastes.

In the stationary phase, eventually population growth ceases and the growth curve becomes horizontal. During this phase, the growth rate slows as a result of nutrient depletion and accumulation of toxic products. The stationary phase has a constant value as the rate of bacterial growth is equal to the rate of bacterial death. This phase is usually attained by bacteria at a population level of about 10^9 CFU/ml. The cells in the stationary phase are most resistant to the heat treatment (Doyle *et al.* 2000). These authors compared D_{60}-value cells in stationary phases with exponentially phase of *L. monocytogenes* strain 13-249. Cells in the stationary phases were 4 times more heat resistance than those from exponentially phase.

Detrimental environmental changes like nutrient deprivation and the buildup of toxic wastes lead to the decline in the number of viable cells characteristic of the death phase. The death of a microbial population, like its growth during the exponential phase, is usually logarithmic. Beyond the kinetic range, microorganisms will either survive or be inactivated. Inactivation usually follows log-linear kinetics, characterized by D and z- values, although the actual death kinetics of *L. monocytogenes* may be more complex (e.g. sigmoidal) functions and involve several distinct phases, each with its own log-linear rate (Ross *et al.* 2000). Until recently, D and z-values were the primary methods of modeling thermal inactivation of microorganisms. D-value (decimal reduction time) is the time required at a certain temperature to kill 90 % of the organisms being studied. Thus after an organism is reduced by 1 D, only 10 % of the original organisms remain.

For calculation of D-value the first order kinetics approach for inactivation rate usually used. The first order kinetics mean that all bacteria in a population are equally sensitive to heat and will die with a constant rate.

Killing effect can be described by equation (1)

$$N = N_o e^{-kt} \quad (1)$$

where N is the change in bacterial concentration after heat treatment, N_0 is the initial concentration of cells, t is time and k is the rate constant specific to temperature.

To simplify, the equation (1) can be expressed in a logarithm equation (2).

$$\log (N) = \log(N_0) - t/D \quad (2)$$

By plotting the logarithm of the surviving bacteria (CFU/ml) at the time (min), we get a semi-logarithmic linear curve (Figure 2.2). Based on this curve, D-value can be calculated with the formula in equation (3).

$$D=-1/a \quad (3)$$

where a is the slope of the curve, and D is the D-value in minutes

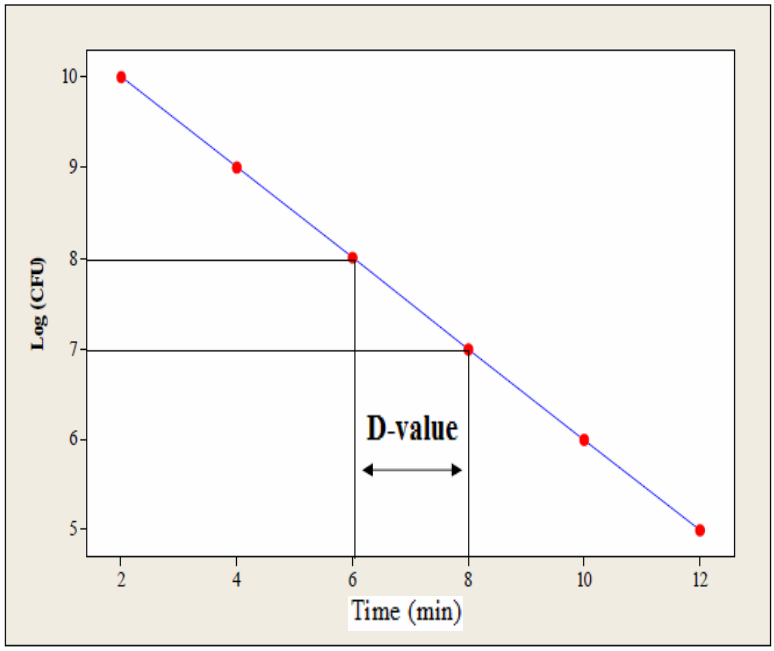


Figure 2.2 Basic inactivation kinetic. Presentation of the D-value

By plotting the logarithm of the various D-values against the corresponding temperature we get a graph where z-value can be calculated from the slope (equation 4).

$$Z=-1/a \quad (4)$$

Z-value (temperature coefficient) is defined as the number of degrees of temperature (°C) necessary to change the D-value with a logarithmic unit (Figure 2.3).

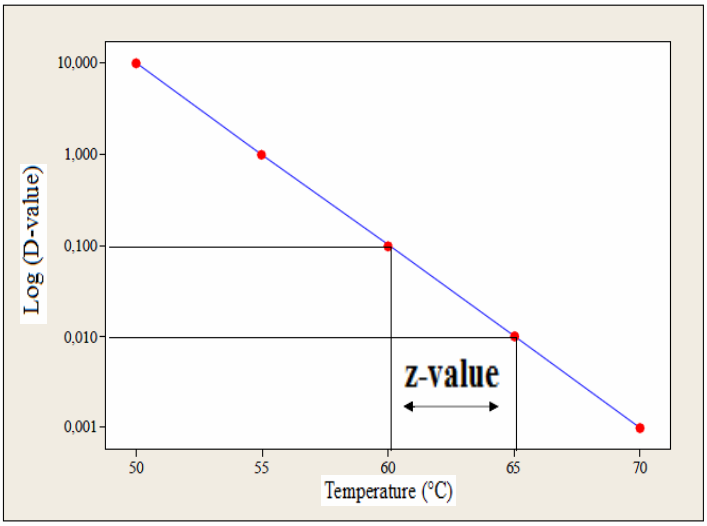


Figure 2.3 Basic inactivation kinetic. Presentation of the z-value.

2.6.3 Thermal and non-thermal inactivation

The use of temperatures above the kinetic range to inactivate microorganisms can be termed 'thermal' processes, while the use of other growth conditions, e.g. high salt concentration or low pH, which result in inactivation has been called 'non- thermal inactivation'.

Thermal inactivation

Several studies do not support the often cited view that *L. monocytogenes* has unusually high thermal tolerance (study of Ryser (1991), lists of thermal inactivation times under different conditions and food types by ICMSF (1996), list of heat resistance of *L. monocytogenes* from Compendium of Fish and Fishery Product Processes, Hazards, and Controls (Table 2.4).

Table 2.4 Heat resistance of *L. monocytogenes*. (Compendium of Fish and Fishery Product Processes, Hazards, and Controls, 2009)

Temp. (°C)	D-Value (min)	Medium	Reference
50	34.48	Blue crabmeat	Harrison and Huang, 1990
50	40.43	Blue crabmeat	Harrison and Huang, 1990
51.6	97.0	Lobster	Budu-Amoako et al., 1992
54.4	55.0	Lobster	Budu-Amoako et al., 1992
55	9.18	Blue crabmeat	Harrison and Huang, 1990
55	12.00	Blue crabmeat	Harrison and Huang, 1990
55	10.23	Crawfish tail meat	Dorsa et al., 1993
56	48.09	Mussels, brine soaked	Bremer and Osborne, 1995
57.2	8.3	Lobster meat	Budu-Amoako et al., 1992
58	16.25	Mussels, brine soaked	Bremer and Osborne, 1995
58	10.73	Salmon	Embarek, 1995
58	7.28	Cod	Embarek, 1995
59	9.45	Mussels, brine soaked	Bremer and Osborne, 1995
60	2.39	Lobster meat	Budu-Amoako et al., 1992
60	1.31	Blue crabmeat	Harrison and Huang, 1990
60	2.61	Blue crabmeat	Harrison and Huang, 1990
60	1.98	Crawfish tail meat	Dorsa et al., 1993
60	5.49	Mussels, brine soaked	Bremer and Osborne, 1995
60	4.48	Salmon	Embarek, 1995
60	1.98	Cod	Embarek, 1995
62	1.85	Mussels, brine soaked	Bremer and Osborne, 1995
62	2.07	Salmon	Embarek, 1995
62	0.87	Cod	Embarek, 1995
62.7	1.06	Lobster meat	Budu-Amoako et al., 1992
65	0.19	Crawfish tail meat	Dorsa et al., 1993
65	0.87	Salmon	Embarek, 1995
65	0.28	Cod	Embarek, 1995
68	0.15	Salmon	Embarek, 1995
68	0.15	Cod	Embarek, 1995
70	0.07	Salmon	Embarek, 1995
70	0.03	Cod	Embarek, 1995

Z-values: lobster meat 5.0 ºC, blue crabmeat 8.40 ºC in trypticase soy agar, crawfish tail meat 5.5 ºC, mussels 4.25 ºC, salmon 5.6 ºC, cod 5.7 ºC.

Heat tolerance of *L. monocytogenes* can be maximised by prior sub-lethal shocks, stress or having reached stationary phase (Ross *et al.* 2000). These effects have been studied and modeled (Augustin *et al.* 1998;Breand *et al.* 1997;Breand *et al.* 1999;Stephens *et al.* 1994). Increased thermal resistance in the presence of fat has been widely reported for other foodborne pathogenic bacteria. Ben Embarek and Huss (1993) concluded that the heat resistance of *L. monocytogenes* was higher (D_{60}-values 4.23–4.48 min, z =6.4) in vacuum-packed sous-vide cooked (58–80 °C) fillets of salmon than in cod fillets (D_{60} -values 1.95–1.98 min, z=5.7).

Some scientists (Mackey and Derrick 1987;Quintavalla and Campanini 1991;Thompson *et al.* 1979;Tsuchido *et al.* 1974) have suggested that the heating rate on the thermal inactivation is important, especially when low processing temperatures are employed. Thermotolerances of bacteria due to slow heating rates have increased. Tsuchido et al**.** (1974) reported that the thermotolerance of *Escherichia coli* was increased by raising the temperature of the cell suspension from 0 to 50°C at various rates prior to holding at 50 °C. Mackey and Derrick (1987) reported that the thermotolerance of *Salmonella typhzmurium* was increased by linear heating at rising temperatures before heating at 58 °C. Quintavalla and Campanini (1991) increased the thermotolerance of *L. monocytogenes***,** heated at 0.5 °C min^{-1}, to constant temperatures of 60, 63 and 66 °C in meat emulsion.

More recently, Stephens et al. (1994) investigated the effect of the heating rate on the thermal inactivation of *L. monocytogenes* at the temperature range 50-64 °C. In processes which had rates of heating ≤5.0°C min^{-1} they assumed that the heating rate did significantly affect the inactivation kinetics of a thermal process, at rates of heating between 5.0 °C and 0.7 °C min^{-1} the deviation greatly increased as the rate of heating decreased; approximately a 1.7 x 10^5-fold difference at 0.7 °C min^{-1}. Maximum thermotolerance was induced at rates of heating ≤ 0.7 °C min^{-1} (Figure 2.4). Stephens et al. (1994) also concluded that the increased thermotolerance during slow rates of heating was analogous to the induction of the heat-shock response. The models described by Stephens et al. (1994) allow for confident assessments of safety to be made not only at near instantaneous heating but also when the heating rate varies.

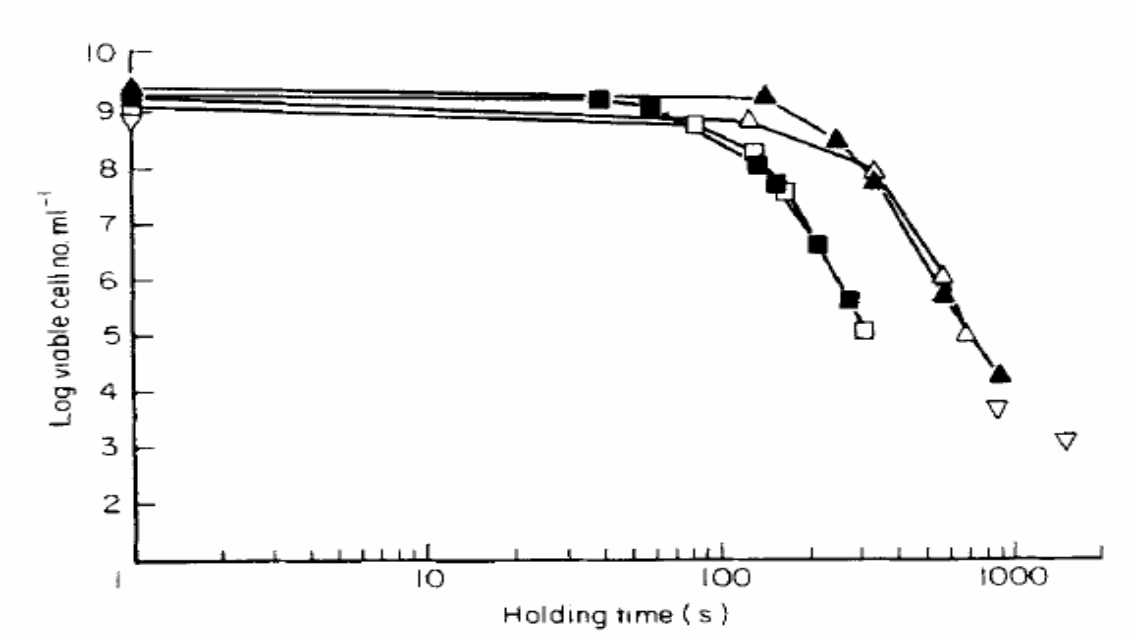


Figure 2.4 The thermal inactivation kinetics of *L. monocytogenes* during the holding period at 60 °C for five heating processes with different rates of heating **(■,** near instantaneous heating; □,5.0 °C min^{-1} ; Δ, 0.7 °C min^{-1} ; **▲,** 0.5 °C min^{-1} ; V,0.3 °C min^{-1}) presented on a modified full-logarithmic plot.

Non-thermal inactivation

It is known that in some products, e.g. heavily salted, marinated, etc., the combination of environmental conditions may prevent growth of microorganisms and ultimately lead to their inactivation. Usually, the more extreme conditions leads to an acceleration of rates of microbial inactivation, but the low temperature shows some deviations to this rule. Lower temperatures reduce the rate of death when other factors prohibit growth; very low temperature is routinely used as a method of culture preservation. Non-thermal inactivation may be very slow (Ross *et al.* 2000). Ross et al. (2000) concluded that the mechanisms of non-thermal inactivation are currently poorly understood but are being studied by several groups around the world, but frequently using pathogenic *E. coli* as the test organism.

In most of *L. monocytogenes* non-thermal inactivation studies, organic acid was considered the main factor causing inactivation (Beuchat *et al.* 1986;Buchanan and Bagi 1997;Buchanan and Golden 1994;Buchanan and Golden 1995). *L. monocytogenes* can also be inactivated with radiation. Data of rates of radiation inactivation are summarised in ICMSF (1996).

2.6.4 Surface heat inactivation

Investigation has indicated that at the time of slaughter the muscle tissue of a healthy animal is essentially sterile (Gill 1979). The surface of the meat is contaminated with pathogenic and spoilage organisms during slaughter and subsequent handling. If bacterial numbers on the surface of raw food products could be eliminated, or substantially reduced, immediately after slaughter or harvest the risk of cross-contamination during processing would be substantially reduced.

Some alternative form of terminal surface processing is the use of steam for decontamination of meat that is now used extensively in the United States of America, Canada and Australia and is also being considered for use in the European Union (EU) (Mccann *et al.* 2006).

McCann et al. (2006) investigated effects of steam pasteurisation on *S. Typhimurium* DT104 and *E. coli* O157:H7 surface inoculated onto beef, pork and chicken. Author concluded that this work provides new information on the dynamic changes, in pathogen numbers on uninjured meat surfaces during steam treatment. Results of pathogen survival after heating showed that, in all cases, pathogen numbers declined rapidly linear during the first 10 s of steam treatment. This initial rate of decline slowed during the next 50 s (period of "tailing"), so that considerable pathogen numbers were still present after steam treatment for 60 s (e.g. Figure 2.5).

McCann et al. (2006) explained "tailing" effect with a few hypotheses: 1) physical protection of the cells from structural changes (the presence of a heat resistant sub-population, which did not decrease over the time course of the experiment); 2) heat shock protein formation; 3) heat resistant sub-populations may persist because they are attached or located in more protected areas of the meat surface; or they may survive by a combination of these factors.

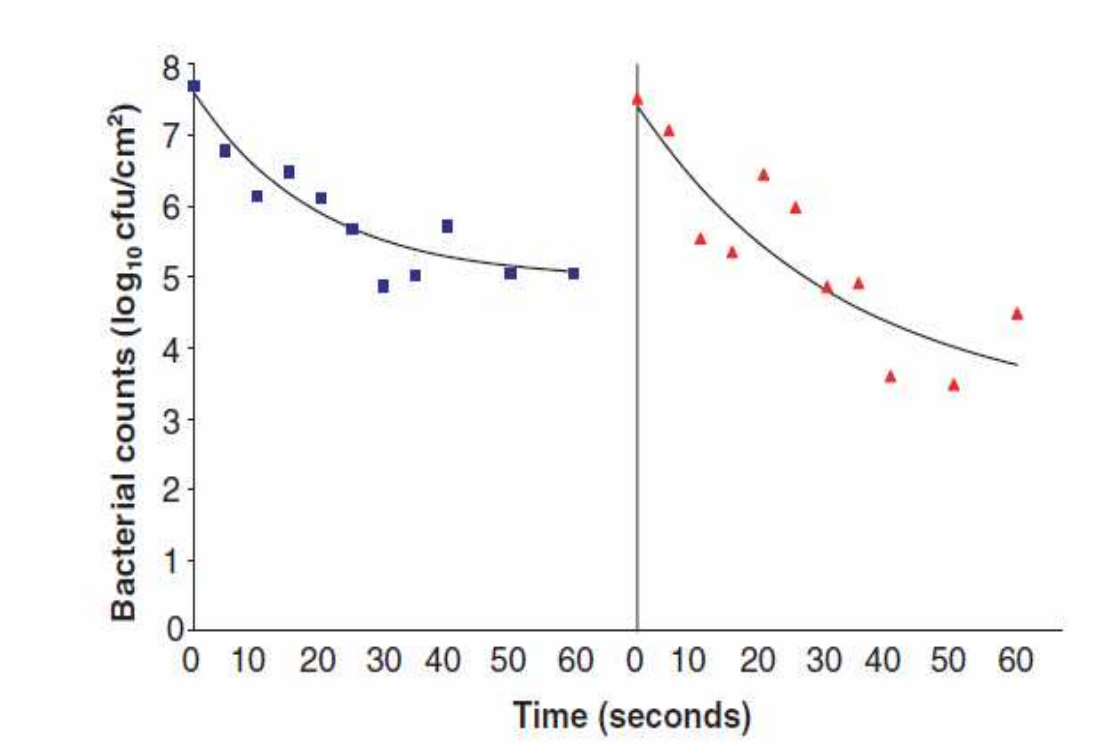


Figure 2.5 Relationship between the survival of (■) *E. coli* O157:H7 and (▲) *S. Typhimurium* DT104 and time on beef surfaces after steam pasteurisation.

Also McCann et al. (2006) compared the results from his study with other investigation. There were difficulties in relation to predicted/calculated surface temperature measurements. In. McCann's study, beef samples reached a maximum of 83 °C after 60 s of heating, Hoke et al. (2003) predicted that beef surfaces heated with steam reached about 70 °C after about 25 s of heating, while James et al. (2000) calculated the surface temperature of chicken to be 95.6 °C after 10 s. These differences are reflections of the heating conditions, substrates and measurements used from which the data for these models were derived.

Differences in the effect of steam treatment are also present in the commercial data. For example, after treatment for 6.0–8.0 s, the data of Nutsch et al. (1997;1998) at steam/air temperatures of 92 and 82 °C, show reductions of about 1.0–1.5 logs for TVCs. These reductions are in contrast to those of Minihan et al. (2003) of only about 0.5 of a log or less after heating at 90 °C for 10 s.

McCann et al. (2006) concluded that the reductions obtained in different studies cannot be compared, because the recorded temperatures refer to different locations, either on the carcass surface or in the chamber. Also differences in the types of carcasses being pasteurised, in particular the surface lean/fat ratios, the weight and the initial levels of contamination.

The same year, (2006), Gaze et al. published the work “Heat inactivation of *Listeria monocytogenes* Scott A on potato surfaces”. Gaze et al. (2006) investigated the death kinetic of *L. monocytogenes* on potato surfaces (with and without skin), when treated with a surface pasteurisation method in a "Bugdeath rig". Gaze et al. (2006) concluded effectiveness of surface pasteurisation in the purpose-designed apparatus. It was possible effectively to decontaminate the surface of potatoes without affecting texture. For example, after heating up to 90 °C within 46 s and holding for a further 20 s, a reduction of 3.8 logs was observed for *L. monocytogenes* Scott A (Gaze *et al.* 2006). It may therefore be calculated that to achieve greater than 6 log reductions, a heat treatment with a 46 s heat up followed by 41 s holding at 90 °C would be adequate. The combination of achieving 90 °C in 1 min and holding for 1 min could be applied to a wide range of food products and would be achievable by many types of heating systems and industrial practices.

James et al. (2002) carried out a short investigation on the applicability of four different heat treatments (hot air, hot water, infra-red radiation, and atmospheric steam) for the surface pasteurization of shell eggs. The reason for this investigation was reduced microbial and visual contamination of eggs during production and processing because pathogens, especially salmonella, on and in eggs are major concern for food safety.

The results show that temperatures sufficient to achieve significant reductions in bacterial numbers can be attained on the outside of an egg without raising interior temperatures to those that would cause coagulation of the egg contents.

The data from Table 2.5 combined with data from Figure 2.6.d are shown that external surface temperature reached 92 °C for 2 s, while internal temperature were 46 °C, when treated with steam (James *et al.* 2002). This is evidence that heat treatment with steam was most effective treatment.

Table 2.5. Time/temperature treatments

Treatment	**External ‘air’ temperature (°C)**	**Exposure time (s)**
Hot air	180	8
Hot water	95	10
Infra-red	210	30
Steam	100	2

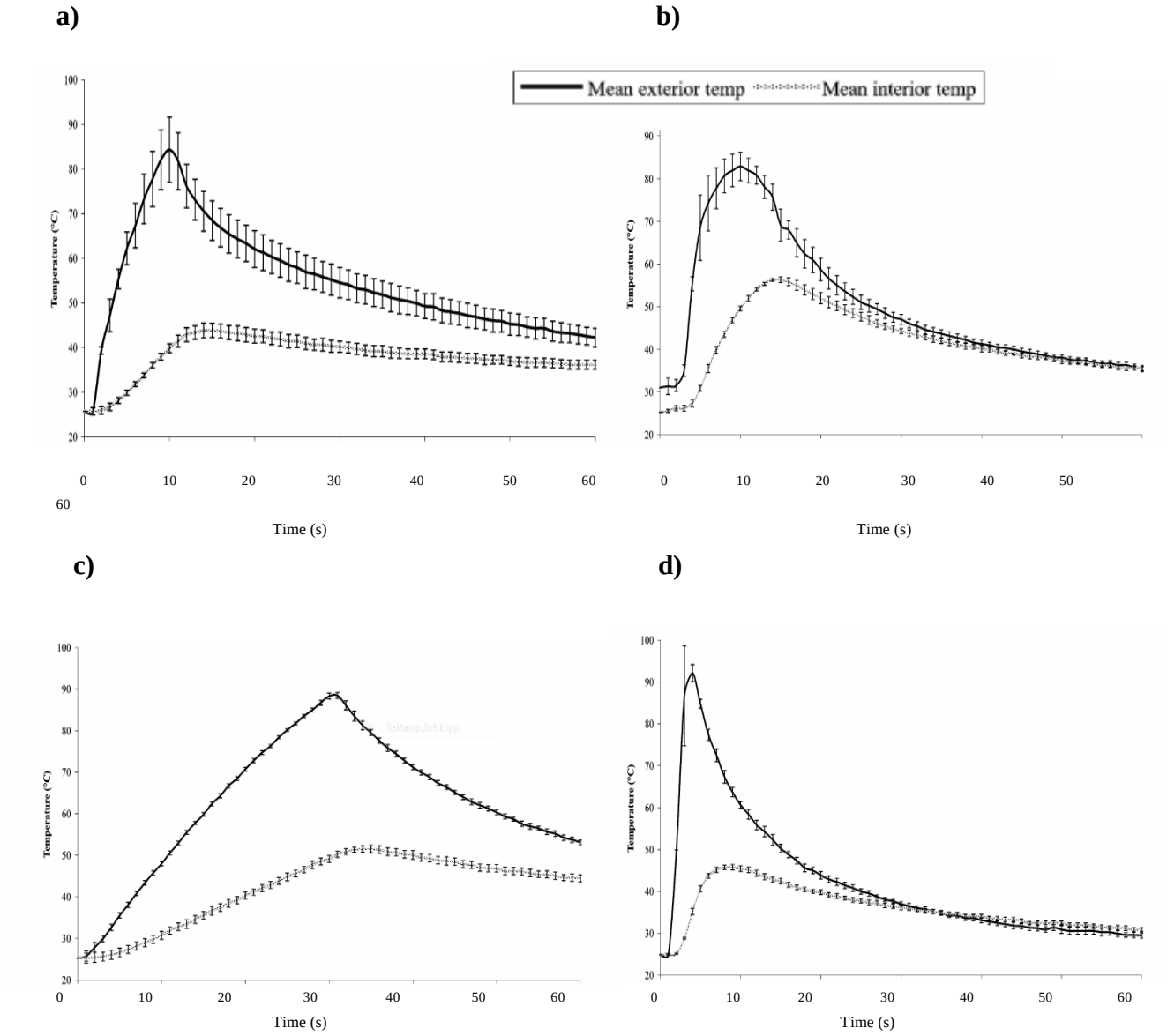


Figure 2.6 Temperature plots of mean (n = 5) exterior and interior temperatures on the shells of eggs subjected: a) to hot air (180 °C) for 8 s; b) to hot water (95 °C) for 10 s; c) to infra-red exposure (210 °C) for 30 s; d) to steam (100 °C) for 2 s. Average maximum external surface temperatures were 85 °C, 86 °C, 88 °C, 92 °C using hot air, hot water, infra-red and steam, respectively. Average maximum internal surface temperatures were 44 °C, 57 °C, 52 °C, 46 °C using hot air, hot water, infra-red and steam, respectively.

The same principle of range distribution of the exterior (T4, upper beef surface temperature) and the interior (T6, temperature at the centre of the beef sample) temperatures on the beef surface are shown on Figure 2.7 (Mccann *et al.* 2006). Also Figure 2.7 showed differences between upper

(T4) and lower (T5) beef surface temperature; differences between steam/air mixture before (T2) and after (T3) sample heating.

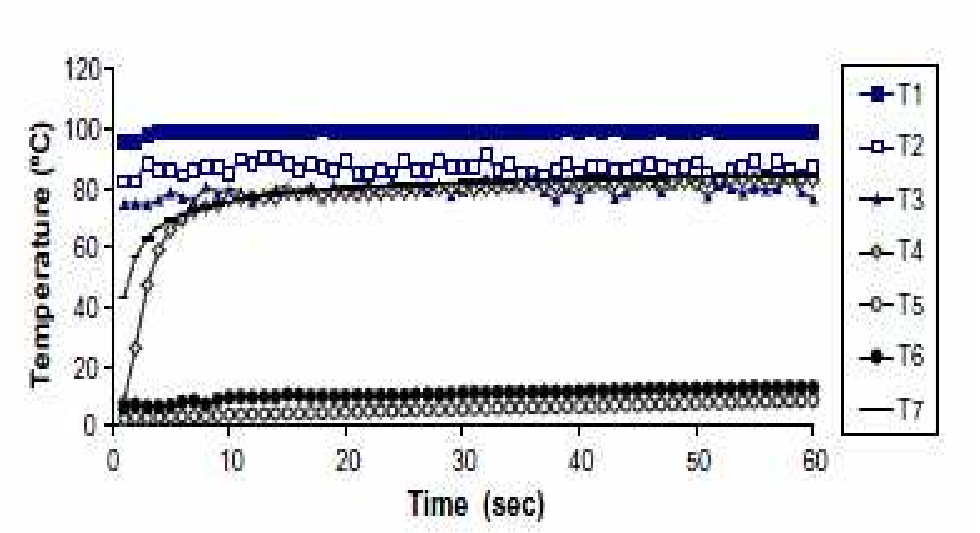


Figure 2.7. Heating profiles recorded during steam pasteurisation for 60 s. T1, initial steam injection temperature; T2, steam/air mixture before sample heating; T3, steam/air mixture after sample heating; T4, upper beef surface temperature; T5, lower beef surface temperature; T6, temperature at the centre of the beef sample and T7, predicted surface temperature of beef surface.

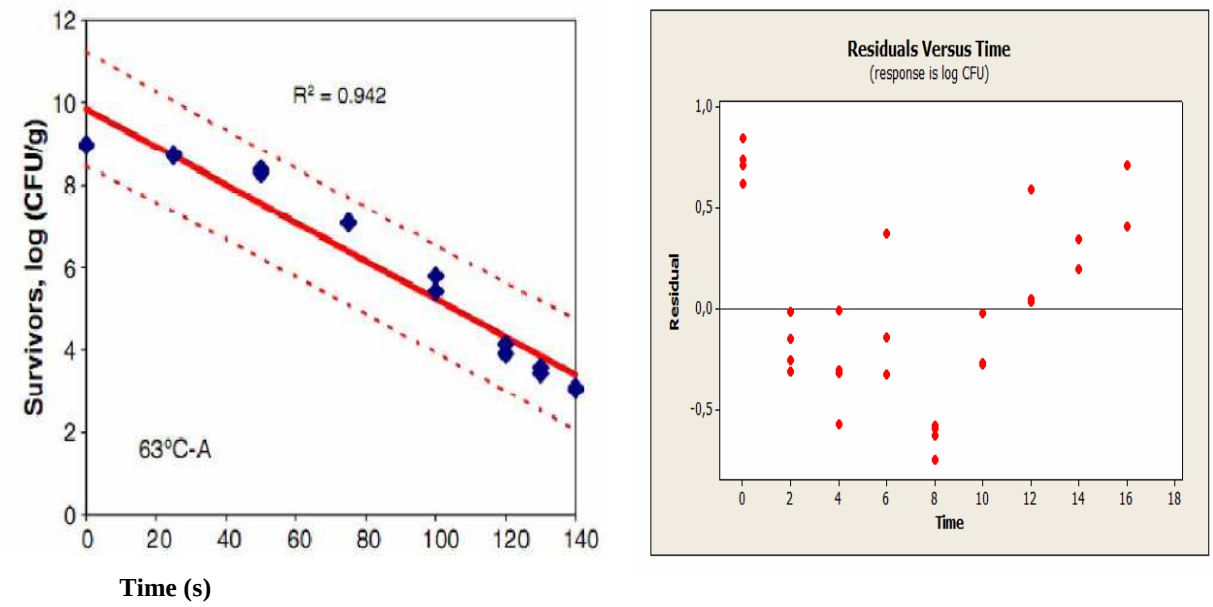
In order to get a better understanding of process temperature uniformity for steam pasteurization, Foster et al. (2006) used a Teflon sample. There was a difference in temperature between the end of the sample and the centre of 4.9 °C at end of the treatment (steam treatment 45 s).

2.7 Statistical analyses

2.7.1 Isothermal heat treatment of *L. innocua* in capillary tubes

Linear regression is used to find the best fit linear line in a set of experimental data on a graph. The slope of the line makes it possible to construct a prediction about how the results have been outside the area where experimental data are collected. Based on the slope, we can also calculate D-value (equation 3) and z-value (equation 4).

Regression coefficient, R^2, is a measure of the spread of the experimental data around trend line. The regression coefficient is best when it is 1, on a scale from 0-1.



a) **b)**

Figure 2.8 a) Logarithm of surviving *L. monocytogenes* plotted against time, and the regression is adapted to the linear model (Huang 2009); b) Regression coefficient, R^2 (as example from current work: isothermal heat treatment of *L. innocua* strain ATCC 33090 in capillary tubes at 60 °C, the linear regression equation is log CFU/ml = 9,18 - 0,455 Time, R^2 = 95,9 %).

The most widely used model for inactivation of bacteria is a linear first-order kinetics, but basically, most of the survival curves are not linear (Ahn and Balasubramaniam 2007;Corradini and Peleg 2007;Peleg and Cole 1998).V.P. Valdramidis et al. (2006) reference to several studies (Devlieghere *et al.* 2004;Huang and Juneja 2001;Valdramidis *et al.* 2005;Xiong *et al.* 1999), concluded that when considering the thermal inactivation of vegetative microorganisms, there are eight commonly observed types of inactivation or survivor curves: linear curves, curves with a shoulder, linear curves with tailing, sigmoidal-like curves, biphasic curves, biphasic curves with a shoulder, concave and convex curves.

Weibull distribution has been presented as an alternative to linear kinetics heat treatment because it can describe the tail and shoulder effects (Corradini and Peleg 2007;Peleg and Cole 1998;van Boekel 2002). The Weibull model can be derived directly from the linear model:

$$\log(N)=\log(N_0) - kt^{\alpha} \quad (5)$$

The model (5) is more general than the traditional linear kinetics, and it allows the curve bends of downward or upward depending on α. If α > 1 amplified the effect of heat treatment time, and the curve bends downward. If α.<1, the curve bending upwards, and the means that the heat treatment time has less effect.

For example, logarithm of the surviving *L. monocytogenes* in minced beef plotted against time and adapted to the Weibull model have higher regression coefficient (R^2 =0.993) than the linear model (R^2 = 0.942). In this case the Weibull model is a better model than the linear model (Huang 2009).

2.7.2 Steam surfaces pasteurisation

McCann et al. (2006) investigated effects of steam pasteurisation on *S. Typhimurium* DT104 and *E. coli* O157:H7 surface inoculated onto beef, pork and chicken. In his work statistical analyses were carried out using SAS statistical package (SAS Institute Inc., Cary, NC, USA). The relationships between pathogen numbers and treatment times (Figure 2.5) were examined using non-linear regression to fit the negative exponential equation:

$$Y = A + BR^t \quad (6)$$

where Y is pathogen counts in $\log_{10}$ units, t is the time in seconds and A, B, and R are parameters that must be estimated. The asymptote of the model is A, the count at time zero is A + B and the parameter R influences the rate of decay.

Predicted counts were used to examine differences between: 1) organisms, where meat type and time remained constant; 2) meat types, where organisms and time remained constant; 3) times, where organisms and meat types remained constant.

An example of estimated standard deviation between replicates (SD_R) and between times (SD_T) for *E. coli* O157:H7 on differences between meat types showed in Table 2.6.

Table 2.6. Estimates of R, B and A and of goodness of fit (% variance) for the negative exponential equation and standard deviation between replicates (SD_R) and between times (SD_T) (Mccann *et al.* 2006)

Organism/substrate	**R**	**B**	**A**	**% Variance**	**SD_R**	**SD_T**
E. coli O157:H7						
Beef	0.95	2.65	4.95	83	1.73	0
Chicken meat	0.94	3.24	4.29	56	0.92	0.42
Chicken skin	0.84	3.53	3.90	88	1.98	0
Pork	0.92	3.89	4.50	81	0.81	0.34

Valdramidis et al. (2006) used to describe the microbial inactivation of *L. monocytogenes* Scott A a reduced version of the dynamic sigmoidal-like model (which includes three parts: shoulder, log-linear inactivation and tailing and was constructed for microbial inactivation by mild heating) of Geeraerd, Herremans, and Van Impe (2000). Kondjoyan and Portanguen (2008) calculated results of the survival of *L. innocua* surface-inoculated onto chicken skin after treatment with superheated steam by two coherent models: the ‘‘Weibull-tail” model (Albert and Mafart 2005) and the ‘‘biphasic-shoulder” model (Geeraerd *et al.* 2005) are compared to measurements in Figure 2.9.

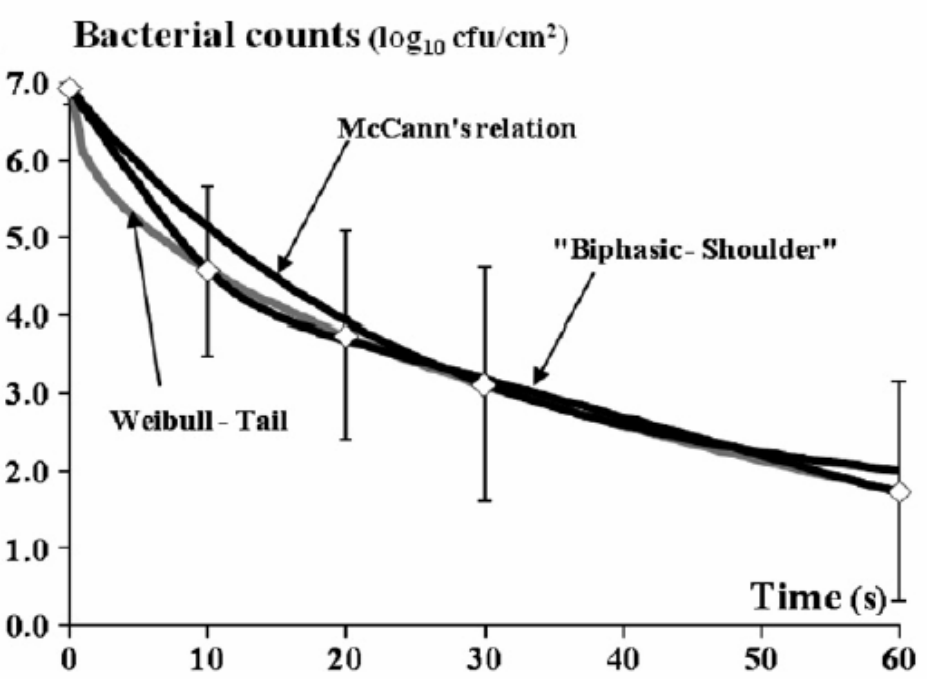


Figure 2.9 Description of the inactivation of *Listeria innocua* CLIP 20595 by relation (6) and by the two basic inactivation models included in the GInaFIT software which agree the best with experimental results in the 96 °C steam treatment (Kondjoyan and Portanguen 2008).

The Weibull plus tail model correspond to the following mathematical relation:

$$N=(N_0-N_{res})10^{-pt/delta} + N_{res} \quad (7)$$

where N is the microbial population at time t (in seconds), N_0 and N_{res} the initial and the residual microbial population respectively; p and delta are parameters which minimize the mean sum of the square error between the results calculated by models and the experimental values.

In the ‘‘biphasic-shoulder” model the shoulder length is not used (shoulder length equals to 0) and the relation reduces to:

$$\log(N)=\log(N_0)(f\, e^{-k1\ max\ t} + (1-f)e^{-k2\ max\ t}) \quad (8)$$

where N is the microbial population at time t (in seconds), N_0 the initial population and *f*, k_{1max} and k_{2max} are parameters which minimize the mean sum of the square error between the results calculated by models and the experimental values.

3. Materials and methods

Recipes for media specified in Chapter 6. Appendix.

3.1 Test organism

L. innocua strains ATCC 33090 and CCUG 35613 were used as surrogates for *L. monocytogenes* for isothermal heat treatment in capillary tubes; *L. innocua* strain ATCC 33090 was used for steam pasteurisation on fish product surfaces.

3.1.1 Freezing of bacterial cultures in Microbank

Microbank® (Prolab Diagnostic) is a freezing system for storage microorganisms. This is a sterile container that consists of porous rings in a cryopreservative solution (glycerol). *L. innocua* strains CCUG 35613 and ATCC 33090 were grown from existing cultures at Nofima. The bacteria were taken from the colonies of the TSAYE Petri dishes with a sterile plastic loop and transferred to the Microbank® tubes. Microbank® was shaken 5 times, so that bacteria would penetrate the pores. After that, the cryopreservative solution was removed and the bacteria stored at -70 °C on the rings. Two Microbank® tubes were made for each bacterial strain (Figure 3.1)

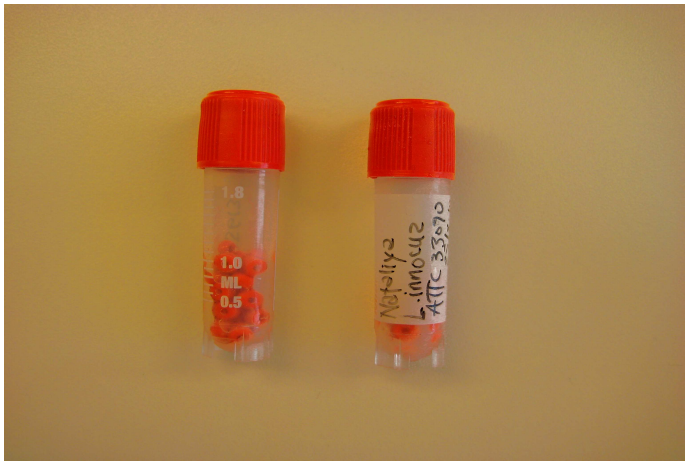


Figure 3.1 Microbank® (Prolab Diagnostic) with *L.innocua.*

At the last step a control was carried out: to see if the transfer had been successful and whether there were signs of contamination. This was done by first incubating each bacterial strain in 10 ml TSBYE at 37 °C over night. Then the bacteria were plated on TSAYE and incubated at 30 °C for 24 hours and checked for colony growth .

3.1.2 Cultivation of bacteria

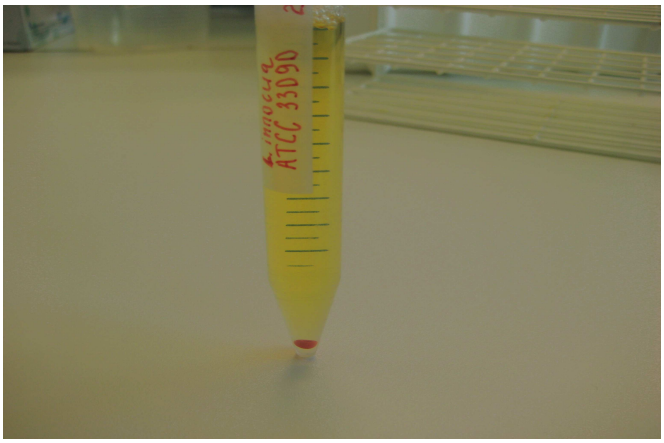


Figure 3.2 Microbank ring in TSBYE.

In order to get unstressed bacterial culture, bacteria were grown in two steps. In step one: the Microbank ring (Figure 3.2) from frozen storage were transferred to 10 ml TSBYE and incubated at 37 °C degrees to the stationary phase, approximately 20 hours. The next step: the bacterial culture of 2 µl from stationary phase was transferred to 20 ml TSBYE in a Erlenmeyer bottle and placed in shaking incubator at 150 RPM and 37 °C for 12 hours. These conditions resulted in a bacterial suspension with approximately 10^9 CFU/ml in early stationary phase.

3.2 Isothermal heat treatment of *L. innocua* in capillary tubes

3.2.1 Calibration of the water bath

To confirm that the temperature distribution in the water bath was stable, it was made validations with temperature loggers. The temperature logging equipment consisted of 10 thermocouples. These thermocouples were spread over 5 racks of capillary tubes where each of the positions was tested in separate experiments (Figure 3.3).

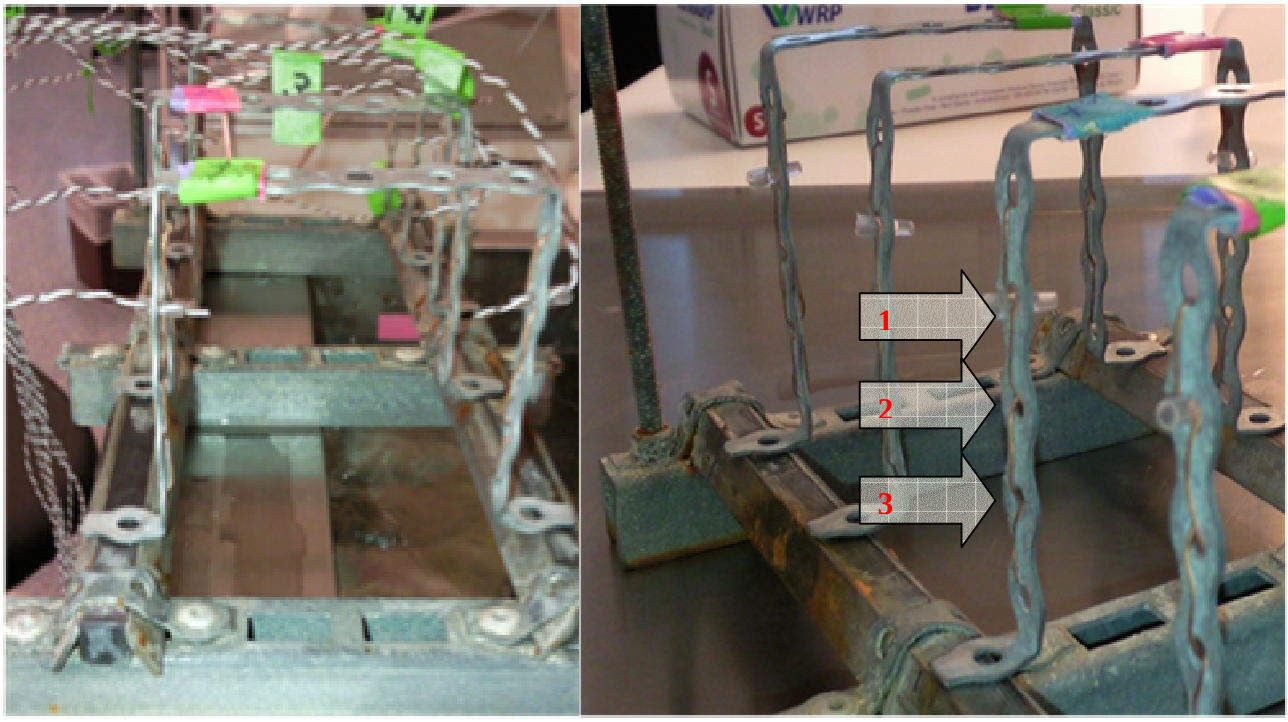


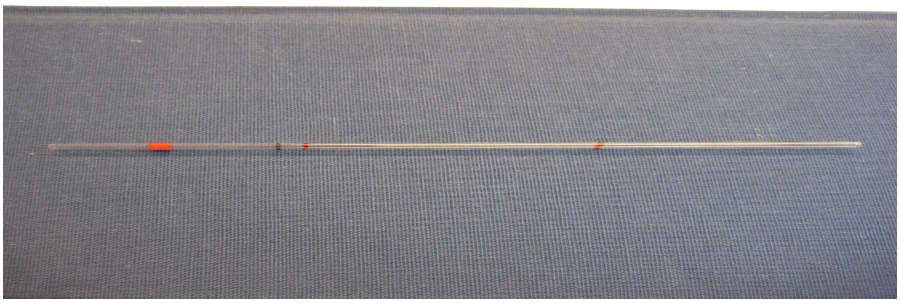
Figure 3.3. Overview of sensor placement. Top and bottom holes were not suitable for the capillary. Position 1 has a plastic tube attached, this was used to keep the sensors in the correct position.

The top position (1) was 7.2 cm from the bottom (0.9 cm from the water surface), in centre (2) was 5.8 cm from the bottom, and the position (3) was 4.2 cm from the bottom (Figure 3.3). Temperature logging was done without the lid on the water bath, and then heat treatment experiments were carried out without lid.

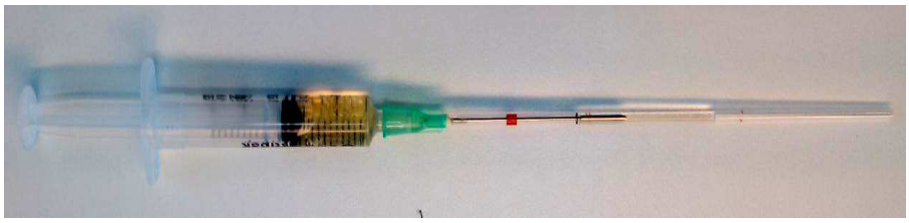
The reference temperature was measured with a calibrated thermometer. The temperature was recorded every 2 second for a total of 15 minutes using the computer program (LabVIEW, Dell Latitude D600). Logging was conducted at 60 °C for water bath Lauda 1 (Lauda Ecoline Staredition E300 O12).

3.2.2 Heat treatment in capillary tubes

A 1 ml volume of a bacterial culture in early stationary phase was transferred to a 1.5 ml sterile Eppendorf tube and centrifuged at 16000 G for 10 minutes. The supernatant was removed and the pellet resuspended in 1 ml TSBYE.



a)



b)

Figure 3.4. a) Sterile capillary tube. b) Pictures shows how the capillary tube was filled with a bacterial suspension using a sterile syringe and needle.

Using a sterile syringe with long needle, 100 µl of *L. innocua* inoculum was transferred to 200 µl sterile capillary tubes (BLAURAND ® intraMARK 708757) (Figure 3.4.b).

In advance, the tubes were marked for volume of 100 µl (Figure 3.4.a). The tubes were then sealed at each end by melting the glass with gas burner. After sealing the tubes were placed vertically to confirm that the tubes were airtight (no movement of the inoculum in the tube).

A capillary tubes assembly was used for heat treatment (Figure 3.5), which consisted of magnetic rails that allowed capillary racks to be easily removed during heat treatment. Each rack could attach 3 capillary tubes.

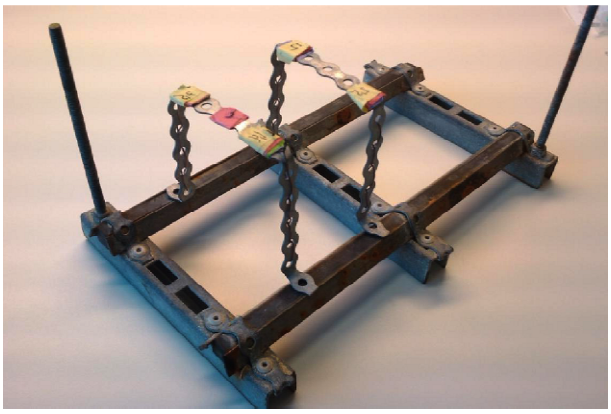
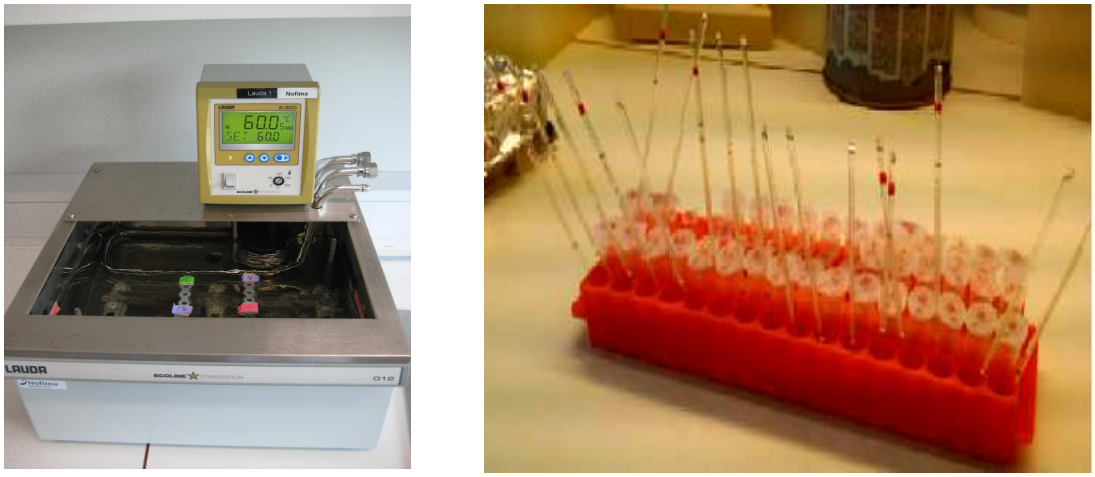


Figure 3.5 Capillary assembly. Capillary racks attached to magnetic rails.

Heat treatment was carried out in a water bath without a lid (Figure 3.6.a). After completing the heat treatment, samples were placed on ice for 20-30 seconds to stop the inactivation of bacterial cells. After cooling, capillary tubes were washed with ethanol and air dried (Figure 3.6.b). The tubes were opened by filing a notch in each end, and then the ends were broken and the contents were transferred to 1.5 ml Eppendorf tubes.



a) **b)**

Figure 3.6 a) Heat treatment in water bath. b) Air drying

Both strains were heat treated at 59 °C, 59.5 °C and 60 °C. Three experiments were carried out for heat treatment at 59 °C and 60 °C and two for heat treatment at 59.5 °C with two parallels for both strains of *L. innocua* to each temperature. Capillary tubes with bacterial suspension that had not been heat-treated were plated for each experiment. This was done to confirm that was nothing wrong with the cultivation factors (pure culture, growth medium and initial concentration of cell). Initial concentration was approximately 10^9 CFU/ml in the bacterial suspension.

3.3. Isothermal heat treatment of *L. innocua* on fish product surfaces by using steam

3.3.1 Fish product sample preparation and inoculation

The surimi "model-product" (surimi, Hake (Pacific Whiting), FDA CFN 3026665, Newport, USA) was transferred from freezer (-30 °C) to refrigerator (0–4 °C) and thawed about 24 h (Figure 3.7.a). Then the surimi "model-product" was cut to approx 10 mm height (level of small Petri dish) with a sterile scalpel. Inoculum (from 3.1.2) of 100 µl was spread over the entire product surfaces of the dish, giving an inoculum concentration of 10^8 CFU per surface (Figure 3.7.b). After inoculation, samples were left to dry in a sterile cabinet for 1 h.

a) **b)**

Figure 3.7 a) Surimi "model-product" . b) Inoculation of surimi "model-product"

3.3.2 Heat treatment apparatus

A detailed description of the experimental test apparatus and analysis of its functioning has been given by Foster *et al*. (2006). Briefly, the apparatus is fully computer controlled and designed to consistently produce known heating time temperature treatments at food surfaces (Figure 3.8).
The BugDeath heat treatment equipment can heat the surface of a food from 8 to 120 °C in 14 s, cool from 120 to 40 °C in 28 s and then to 8 °C in 5 min. There is an average control error of approximately ±1 °C and a temperature variation over the surface of the sample of only 1.2 °C.

The apparatus has four operation units of treatment process: dry heating only, dry heating plus cooling, wet heating (steam) and wet heating plus cooling. The surface temperature of food samples are controlled by software with graphical user interface (GUI).

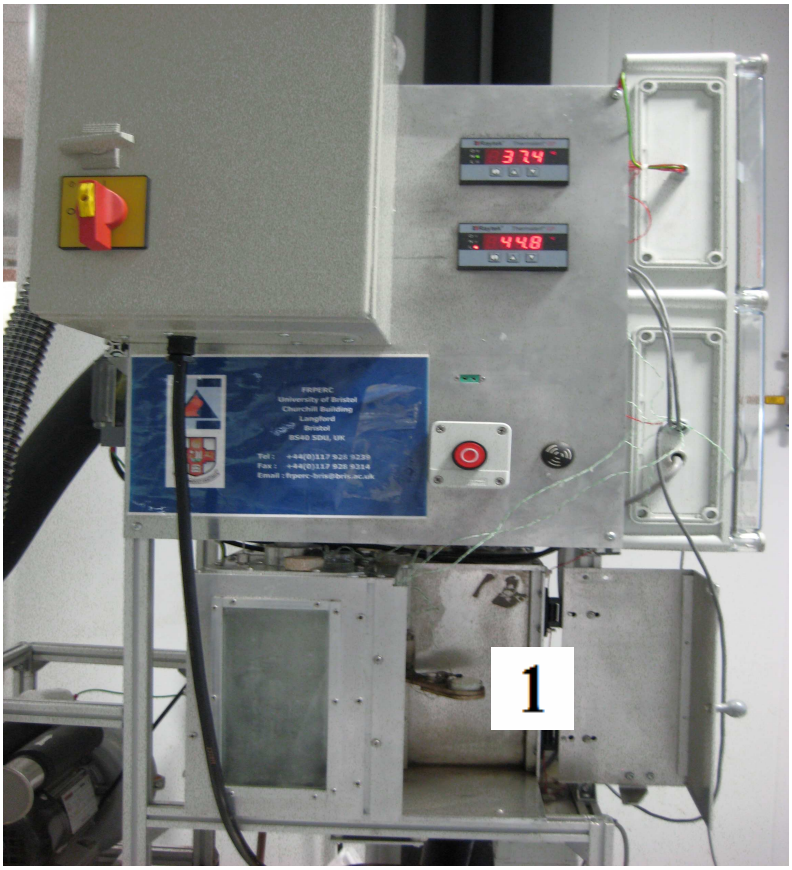


Figure 3.8 BUGDEATH apparatus

The apparatus can be accommodated on a bench top. The steam generator and cooler are separate units, but bench mountable. In order to improve the rate of cooling and uniformity, the apparatus was designed with four chambers (quadrants) and the heating or cooling medium introduced perpendicular to the surface of the sample. The chamber was constructed from aluminium (320 mm x 320 mm x 260 mm high) and split into quadrants for the different processes: dry heating, cooling, wet heating (steam), loading (Figure 3.9.b). One outer side of each quadrant contained toughened glass panels through which the process could be viewed.

A sample was initially placed in the loading section of the chamber. When the start button on the GUI was pressed, the sample was rotated into whichever section of the chamber was required at that time i.e. dry heating, cooling or steam.

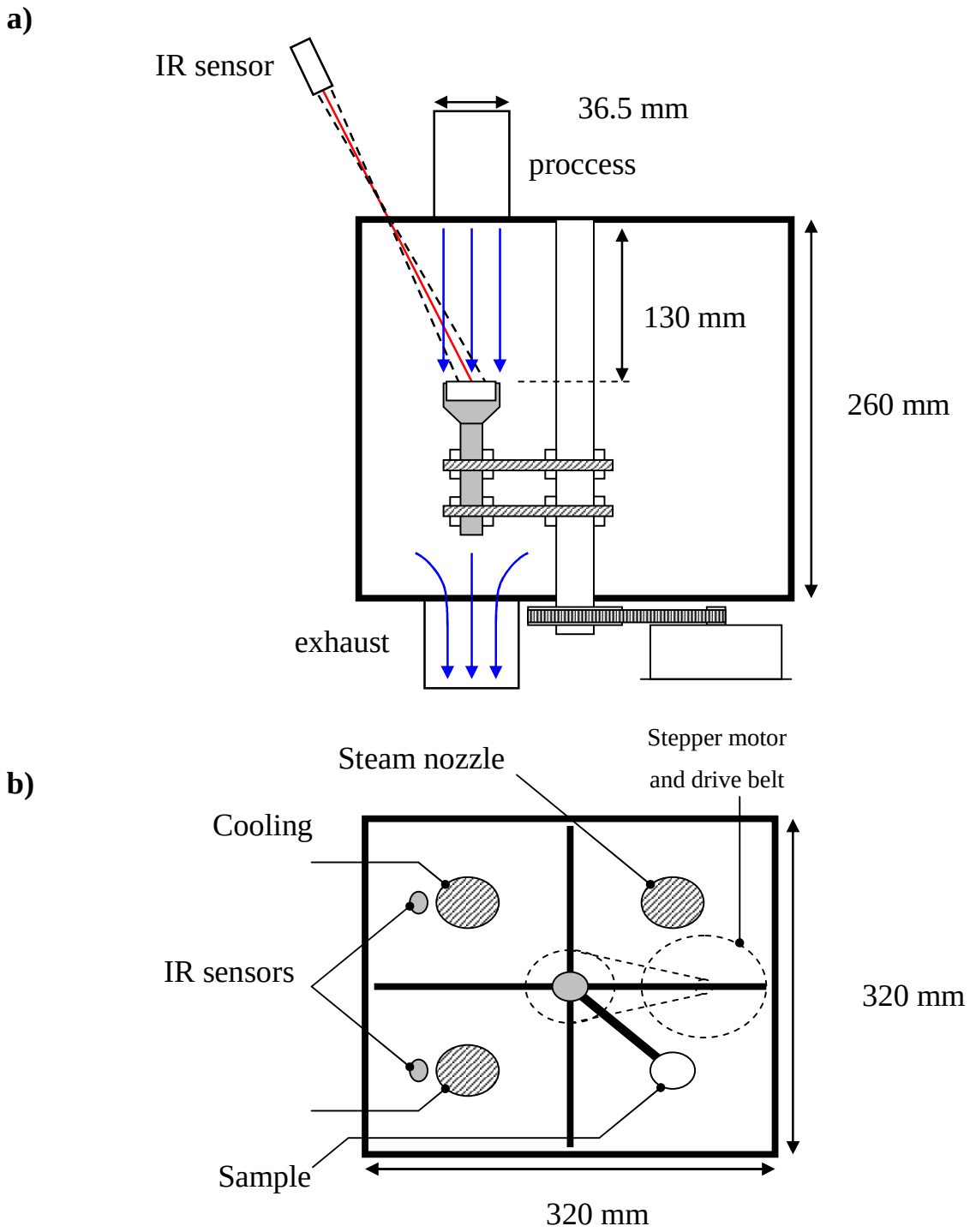


Figure 3.9 Vertical section(a) and plan view (b) of the apparatus (Foster *et al*. 2006).

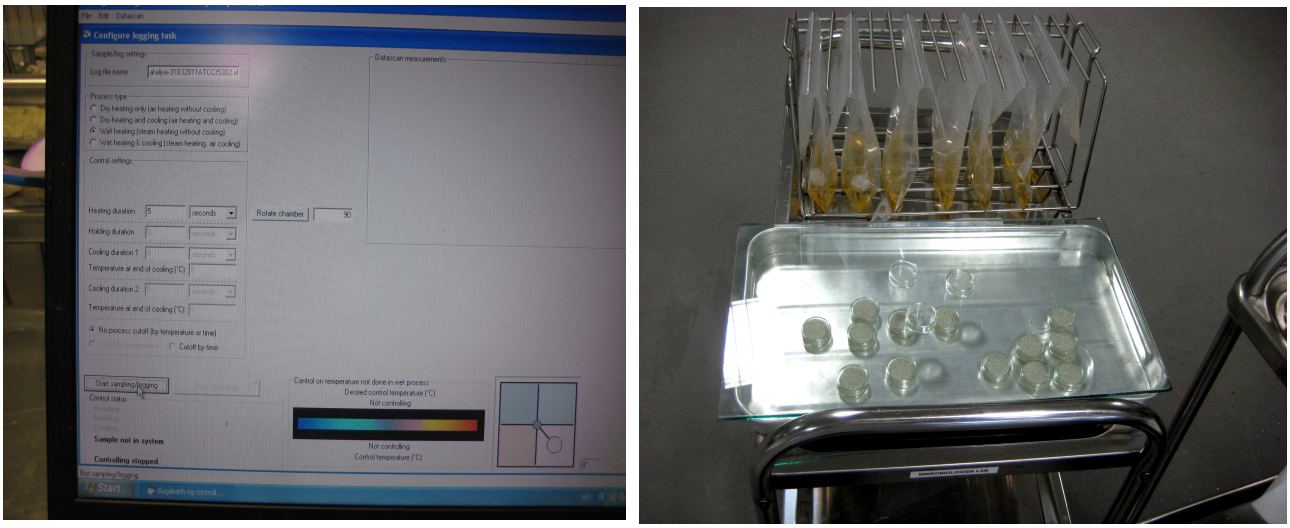
3.3.3 Steam pasteurisation

In advance of pasteurisation, the control software on the computer was run: All programs- Bugdeath Controller- Bugdeath Controller 4.0.2. Then the filename of the log file was inserted. This file recorded temperatures and other information (required for fault finding) during the experiment and it could later be viewed in Microsoft Excel.

At the next step, in the Bugdeath Controller 4.0.2. software (Figure 3.10.a) process type (wet heating) and heat duration (in seconds) was selected for each experiment. A detailed description of the safe operating procedures was given in the "Commercial Bugdeath rig Instructions for Norconserv".

After the drying period, an inoculated sample was placed into a metal sample holder in the apparatus (position 1, Figure 3.8). A pneumatic actuator was moved the sample into a heating chamber, where steam is introduced, using an injection nozzle.

Three setups of experiments were performed: 1) 5 s, 30 s, 2 min; 2) 10 s, 1 min, 3 min; 3) 15 s, 1.5 min, 4 min with two parallels each. Each experiment consisted of three replicates and each was performed on separate days.



a) **b)**

Figure 3.10 a) Screen of the Bugdeath Controller 4.0.2. b) Stomacher bags with samples

Following pasteurisation, samples were removed from the apparatus within 10 s and placed into stomacher bags containing 50 ml of peptone water (Figure 3.10.b). Inoculated sample discs that had not been heat-treated were subjected to the same processing and were plated for each

experiment. This was done to provide control data on the initial bacterial numbers on inoculated discs.

Before examination for the presence of bacterial survivors, each sample was homogenised for 3 min (in 50 ml of peptone water) in a Stomacher Laboratory Blender (Seward, Medical, England)(Figure 3.11.a).

a) **b)**

Figure 3.11 a) Stomacher Laboratory Blender. b) Stomacher bags with samples before and after homogenisering.

3.4 Calculation of results

To determine the effect of heat treatment experiments TSAYE was used as recovery medium.

3.4.1 Dilutions in wells plate

Prior to plating, dilutions were made of the heat treated bacterial suspension (Figure 3.12). In both heat treatments TSBYE was used as medium for dilutions.

Dilutions of bacterial suspensions of *L. innocua* after heat treatment in capillary tubes.

From each Eppendorf tube with bacterial culture 50 µl was transferred to the wells in row A, which contained 450 µl TSBYE. This dilution 1:10 were homogenized with a multichannel pipette, and then 100 µl was transferred to the wells in row B, which contained 900 µl TSBYE.

The procedure to transfer 100 µl to 900 µl TSBYE was continued down the columns until the desired dilution was achieved.

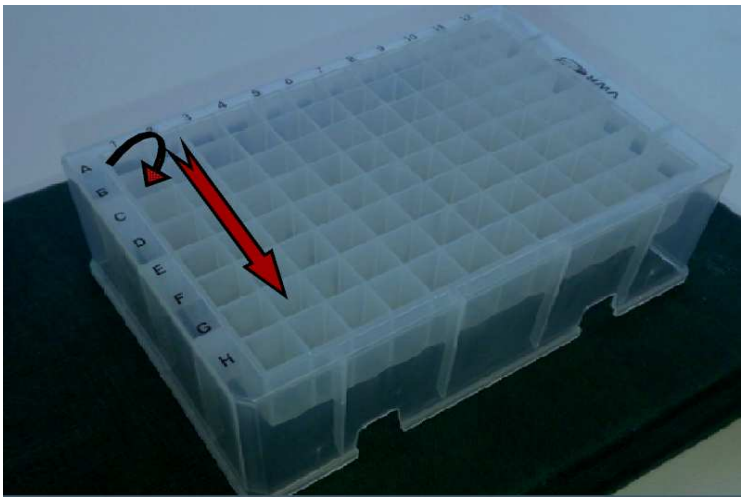


Figure 3.12 A VWR wells plate with 96 wells, each 1.2 ml.

Dilutions of bacterial suspensions of *L. innocua* after heat treatment on fish product surfaces

From each stomacher bag with homogenised bacterial culture 500 µl was transferred to the wells in row A, which contained 500 µl TSBYE. This dilution 1:2 were homogenized with a multichannel pipette, and then the homogenate of 100 µl was transferred to the wells in row B, which contained 900 µl TSBYE, and finally row B giving a dilutions of 1:10 (e.g. with initial concentration of 10^8 CFU dilution will give 10^6 CFU). The procedure to transfer 100 µl to 900 µl TSBYE was continued down the columns until the desired dilution was achieved.

3.4.2 Enumeration after heat treatment

The EDDY JET (Figure 3.13) is plating equipment using a spiral inoculation system. This is an alternative and time saving method compared to traditional plating. It is time saving because one bacterial dish covers up to tree dilutions, e.g. $10^{-1} – 10^{-3}$.

The principle of spiral inoculation system is that a known volume of a sample is placed on agar plate with decreasing volume (Archimedesspiral) from the centre to the edge of the disc. The volume of the liquid, Vs per millimetre of the spiral track is given by (9):

$$Vs = (DF)(A) \qquad (9),$$

where DF is the number of microlitter liquid plated per square millimetre and A is the distance between near spiral in millimetres.

Figure 3.13 EDDY JET.

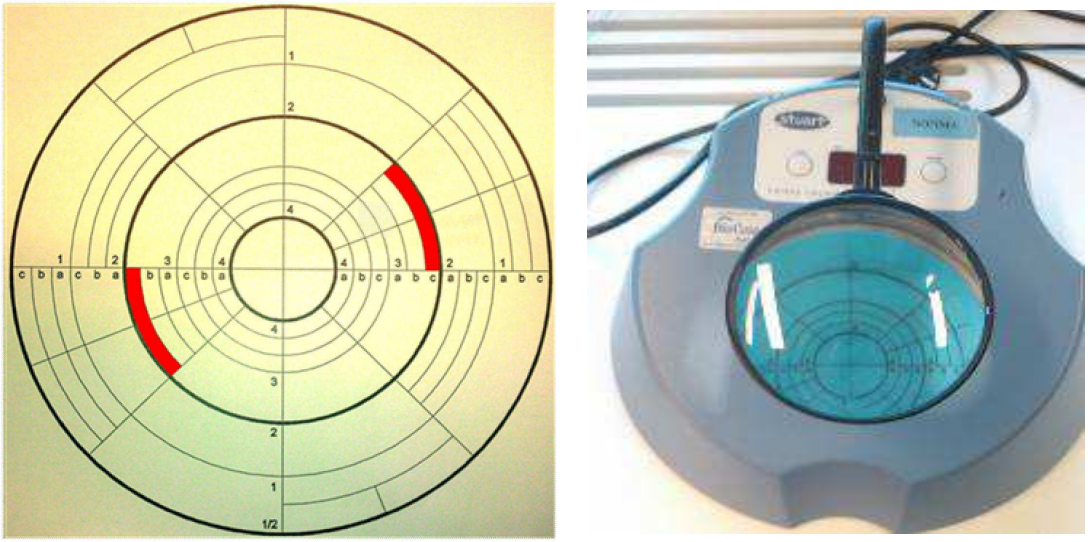
For regeneration after heat treatment, bacterial culture was plated on TSAYE using EDDY JET. In most trials 2 dilutions were used based on which dilutions that could be expected to give countable growth. Each Eppendorf tube was placed manually under the nossle. The same needle was used within dilution series, but was changed for each new sample.

3.4.3 Counting

Since the plated volume on the agar plate was known, it could be used as a count chart (Figure 3.14.a). Area 3 and 4 were adapted to 10 cm Petri dishes. The remaining areas were for dishes with larger diameter.

For an easier counting a colony counter (Stuart-Colony Counter SC6) was used (Fig. 3.14.b).

The result of the count after isothermal heat treatment in capillary tubes was added to the Excel and processed in Minitab to calculate D-values and statistical parameters regression coefficient (R^2 adjust) and standard error (SE).



a) **b)**

Figure 3.14. a) Counting plate for EDDY JET spreader and colony counts. 3c segment (marked in red) corresponded to the outermost part of a 10 cm Petri dish. b) Colony counter.

4. Results and discussion

4.2 Inactivation kinetics of *L. innocua* in capillary tubes

Results of calibration of the water bath are specified in Chapter 6. Appendix. The water bath Lauda 1 was capable to maintain a uniform temperature during the entire thermal process. The variations of the temperature in different parts of the water bath were small (± 0.2 °C).

The experiment with capillary tubes was used for heat treatment at 59 °C, 59.5 °C and 60 °C of *L. innocua* ATCC 33090 and *L. innocua* CCUG 35613. It was carried out three experiments for heat treatment at 59 °C and 60 °C and two for heat treatment at 59.5 °C with two parallels for both strains of *L. innocua* at each temperature. These experiments were designed with a lower detection limit of log 3 (CFU/ml). After heat treatment bacterial solutions were diluted and plated on dish Petri with TSAYE using EDDY JET. The bacteria were incubated at 30 °C and counted after 5 days. The bacterial counts from heat treatment experiments with *L. innocua* ATCC 33090 and *L. innocua* CCUG 35613 were added to a pivot table in Excel and Minitab, and plotted to illustrate the effect of temperature and time. The graphs in Figure 4.1 and Figure 4.2 show the log CFU/ml as a function of time at each heat treatment temperature. Each point on the graphs represent result of one experiment and one parallel; number of the replicates showed on graphs as (1), (2), (3) at each temperature.

The heat treatment in capillary tubes is a classical heat treatment without any modifications. The Figure 4.1 and Figure 4.2 show that inactivation of *L. innocua* ATCC 33090 and *L. innocua* CCUG 35613 follows log-linear kinetics with constant rate inactivation in the specified area (between log 9 and log 3 (CFU/ml). Based on how the data fit to a straight line, regression coefficient (R^2), standard error (SE) and standard error of D-value (SED) were calculated. The D-values were calculated for all experiments with the formula from the theory section (equation 3).

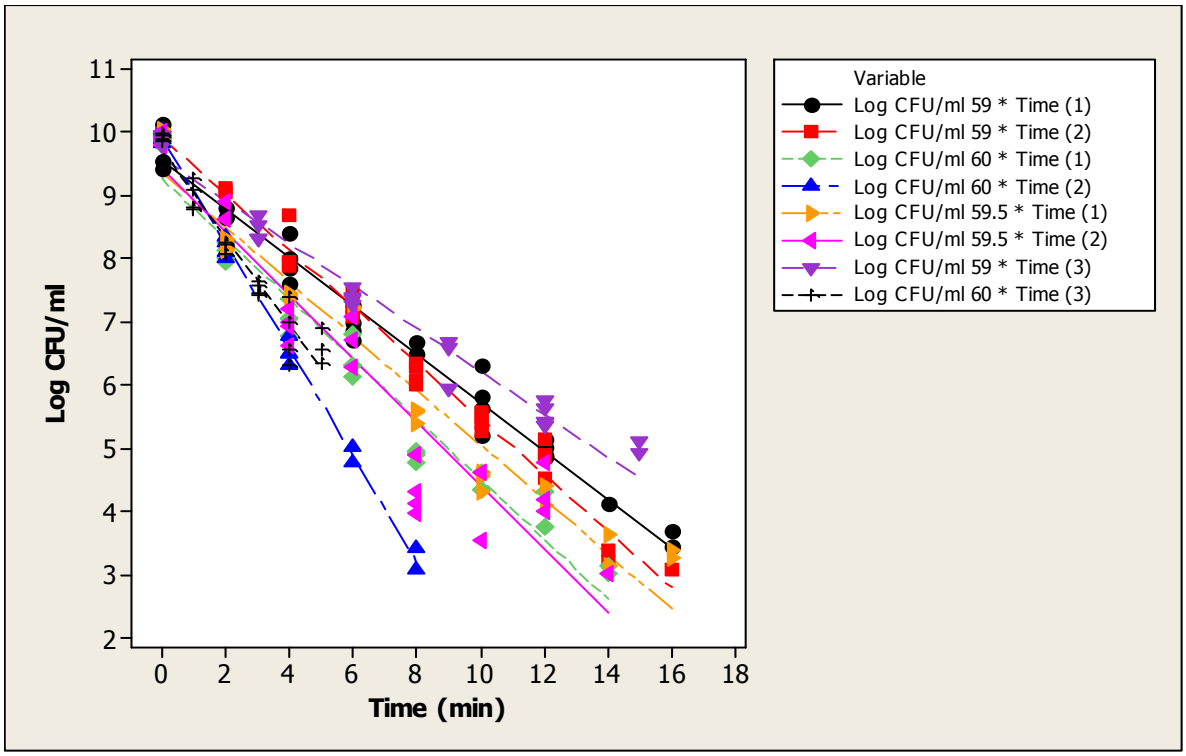


Figure 4.1 Survivors of *L. innocua* ATCC 33090 at different heat treatment temperatures as a function of time.

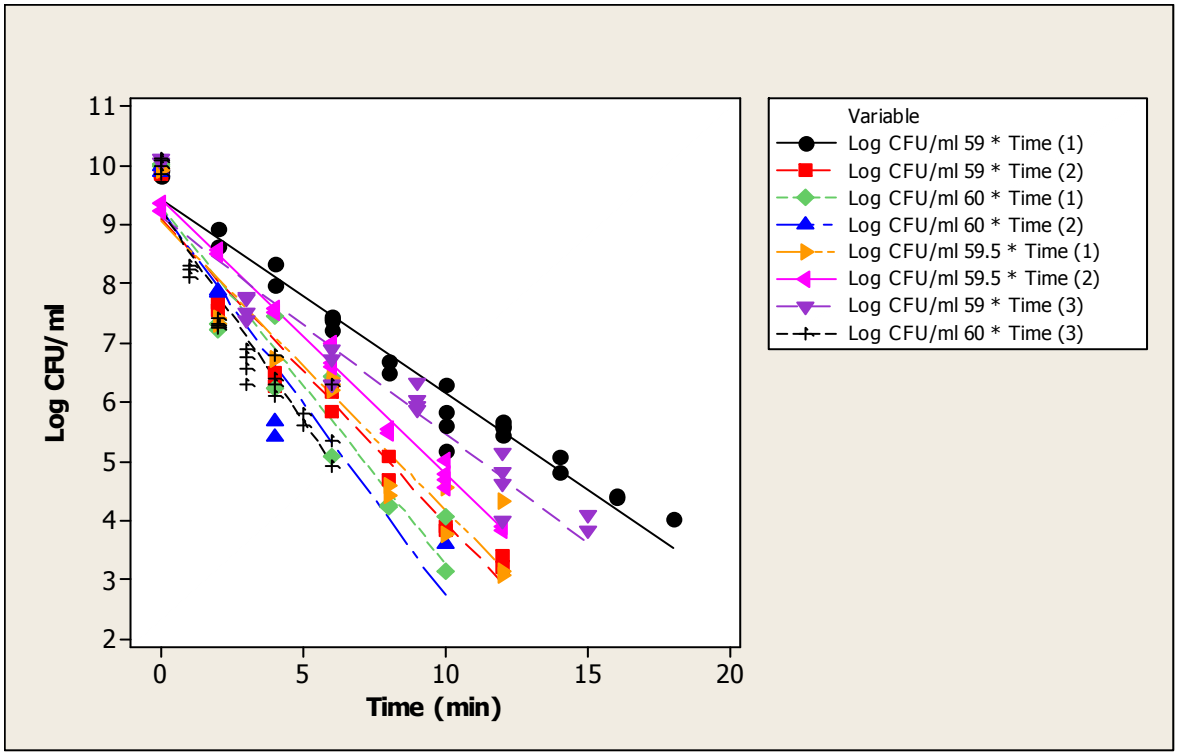


Figure 4.2 Survivors of *L. innocua* CCUG 35613 at different heat treatment temperatures as a function of time

The Table 4.1 and Table 4.2 show the D-values (min), regression coefficient (R^2) standard error (SE) and standard error of D-value (SED) for *L. innocua* ATCC 33090 and *L. innocua* CCUG 35613, respectively.

Some results (Table 4.1 and Table 4.2) showed low regression coefficients: 0.877 at 59.5 °C for *L. innocua* ATCC 33090 and 0.868 at 59.5 °C for *L. innocua* CCUG. In general, for experiments with both strains, regression coefficients were low compared with the literature with similar experimental design, where R^2 were above 0.99 (Alvarez-Ordonez *et al.* 2009;Miller *et al.* 2009). The large spread of the experimental data (R^2) may be explained with theory that the bacteria form clumps before and during a thermal process (Davey 1990). Bacteria in clumps have a different exposure to the heat and especially the bacteria in the centre of the clump will be more protected from the heating. A size and density of the clump have influence on heat resistance. Also, other factors may have influence on these results, e.g. technical skills, handling of the bacteria, problems with enumeration.

Table 4.1 D-values (min), regression coefficient (R^2), standard error (SE) and standard error of D-value (SED) for *L. innocua* ATCC 33090

Temperature, °C	D-value, min	R^2	SE	SED
59	2.65	0.980	0.01	0.15
59	2.96	0.964	0.01	0.25
59	2.25	0.986	0.01	0.11
59.5	2.32	0.956	0.02	0.20
59.5	1.88	0.877	0.04	0.30
60	1.20	0.996	0.01	0.04
60	2.10	0.958	0.02	0.17
60	1.45	0.942	0.04	0.16

Table 4.2 D-values (min), regression coefficient (R^2), standard error (SE) and standard error of D-value (SED) for *L. innocua* CCUG 35613

Temperature, °C	D-value, min	R^2	SE	SED
59	3.06	0.964	0.01	0.25
59	2.72	0.919	0.03	0.38
59	1.85	0.957	0.03	0.18
59.5	1.98	0.921	0.03	0.24
59.5	1.83	0.868	0.05	0.31
60	1.45	0.899	0.07	0.31
60	1.66	0.921	0.04	0.24
60	1.40	0.869	0.59	0.30

The average D-values for *L. innocua* ATCC 33090 were D_{59} =2.62 minutes, $D_{59.5}$ =2.1 minutes, D_{60} =1.58 minutes. The average D-values for *L. innocua* CCUG 35613 were D_{59} =2.54 minutes, $D_{59.5}$ =1.91 minutes, D_{60} =1.50 minutes.

It was a special background for the design this experiment with the range of heat treatment temperature 59 °C, 59.5 °C and 60 °C. In a previous experiment with capillary tubes at Nofima some inconsistent results were found (Olsen, 2010) (Table 4.3).

Table 4.3 D-values (min) for *L. innocua* ATCC 33090 and *L. innocua* CCUG 35613 (from master thesis by Olsen (2010).

Temperature, °C	D-value, min	
	L. innocua ATCC 33090	*L. innocua* CCUG 35613
59	2.19	2.82
59	3.70	2.80
60	2.91	1.74
60	3.11	2.45

Inconsistent results were found for the average D-values for *L. innocua* ATCC 33090. The average D-value at 59 °C was lower (2.95 minutes) than the D-value at 60 °C (3.0 minutes). Also for *L. innocua* CCUG 35613 small differences were found between the D-values at 59 °C and 60 °C. As opposed to Olsen (2010), results from the current work showed difference between

average D_{59}–values, $D_{59.5}$–values and D_{60}-values. The difference between D_{59}–values and $D_{59.5}$–values was 0.52 minutes; between D_{59}–values and D_{60}–values was 1.04 minutes for *L. innocua* ATCC 33090. The difference between D_{59}–values and $D_{59.5}$–values was 0.63 minutes; between D_{59}–values and D_{60}–values was 1.04 minutes for *L. innocua* CCUG 35613.

In the experiment of Ahn and Balasubramaniam (2007) *L. innocua* ATCC 33090 was cultivated in TSBYE at 30 ° C for 20 hours (early stationary phase), heat treated at 60 °C in TSBYE and regenerated on TSAYE at 30 °C. This gave a D_{60}=2.43 minutes. In other similar experiment Miller *et al.* (2006) used *L. innocua* NCTC 10528 at the same conditions, he found that D_{60}=2.73 minutes. In the current work the D_{60}-values of *L. innocua* ATCC 33090 was 1.58 minutes, that is for 1.05 and 1.15 minutes less that in the works of Ahn and Balasubramaniam (2007) and Miller *et al.* (2006), respectively.

Both strains of *L. innocua* will be inactivated with the normal pasteurisation: 70 °C for 2 minutes or 72 °C for 15 seconds. This treatment is also the minimum requirement for products that will have shelf life for up to 10 days, e.g. milk pasteurisation (FAIR programme, EU, 1997).

4.2 Inactivation kinetics of *L. innocua* on fish products surfaces with using steam

Listeria innocua strain ATCC 33090 was used for steam pasteurisation on fish product surfaces. It was carried out three experimental designs: 1) 5 s, 30 s, 2 min; 2) 10 s, 1 min, 3 min; 3) 15 s, 1.5 min, 4 min with two parallels for each heat treatment time. Each experiment consisted of three replicates and each was carried out at separate days.

After heat treatment bacterial solutions were diluted and plated on dish Petri with TSAYE using EDDY JET. The bacteria were incubated at 30 °C and counted after 3 days. Data from heat treatment experiments (bacterial counts) was added to a pivot table in Excel and plotted to illustrate the effect of steam pasteurisation. The change in pathogen numbers on the fish product surfaces during steam treatments for 4 min is presented in Figure 4.3.

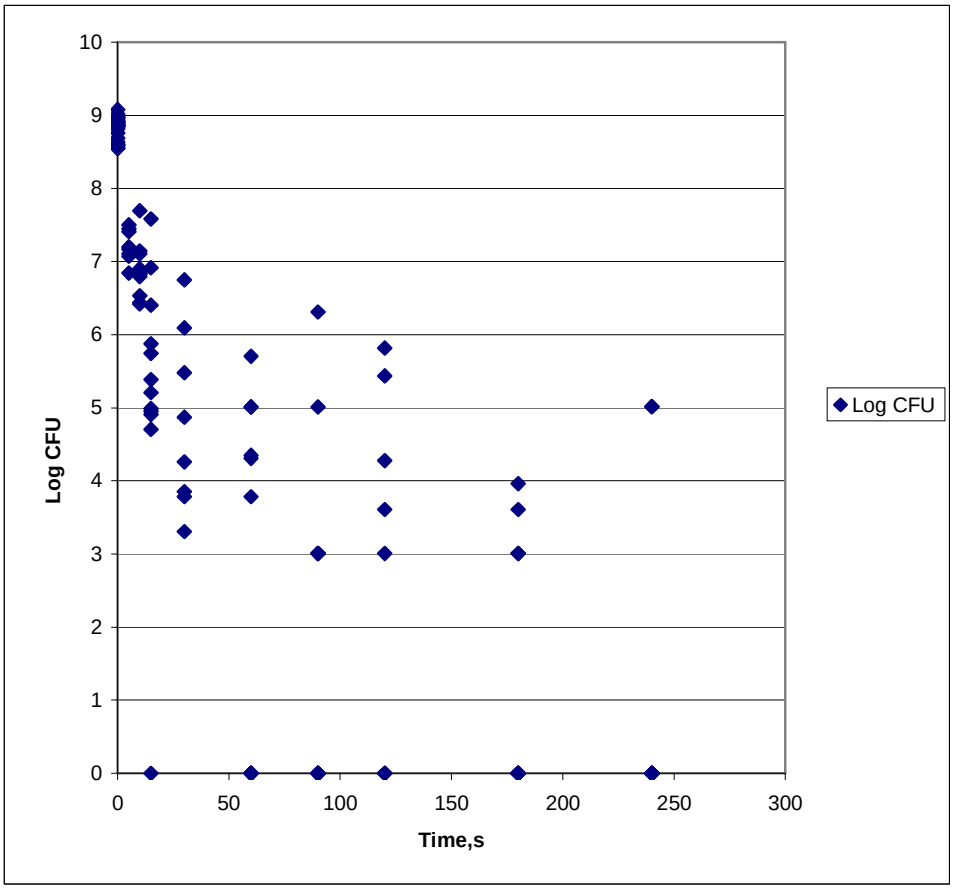


Figure 4.3 Survivors of *L. innocua* ATCC 33090 on fish product surfaces during steam treatments as a function of time.

The average survivors *L. innocua* ATCC 33090 on fish product surfaces during steam treatments as a function of time are presented in Figure 4.4

The graphs in Figure 4.3 and Figure 4.4 show that a bacterial numbers declined rapidly during the first 15 s of steam treatment with 2.2 $\log_{10}$ CFU reduction, the first 5 s was 1.6 $\log_{10}$ CFU reduction, the next 10 s was only 0.6 $\log_{10}$ CFU. This initial rate of decline slowed down during the next 45 s with 1.5 $\log_{10}$ CFU reduction. After 60 s of steam treatment bacterial numbers declined very slowly, so that bacterial numbers were still present after steam treatment for 4 min. The reduction in bacterial numbers between 60 s and 4 min of steam treatment was very low with 1 $\log_{10}$ CFU for 3 min. A similar effect of bacterial reduction was shown by McCann et al. (2006).

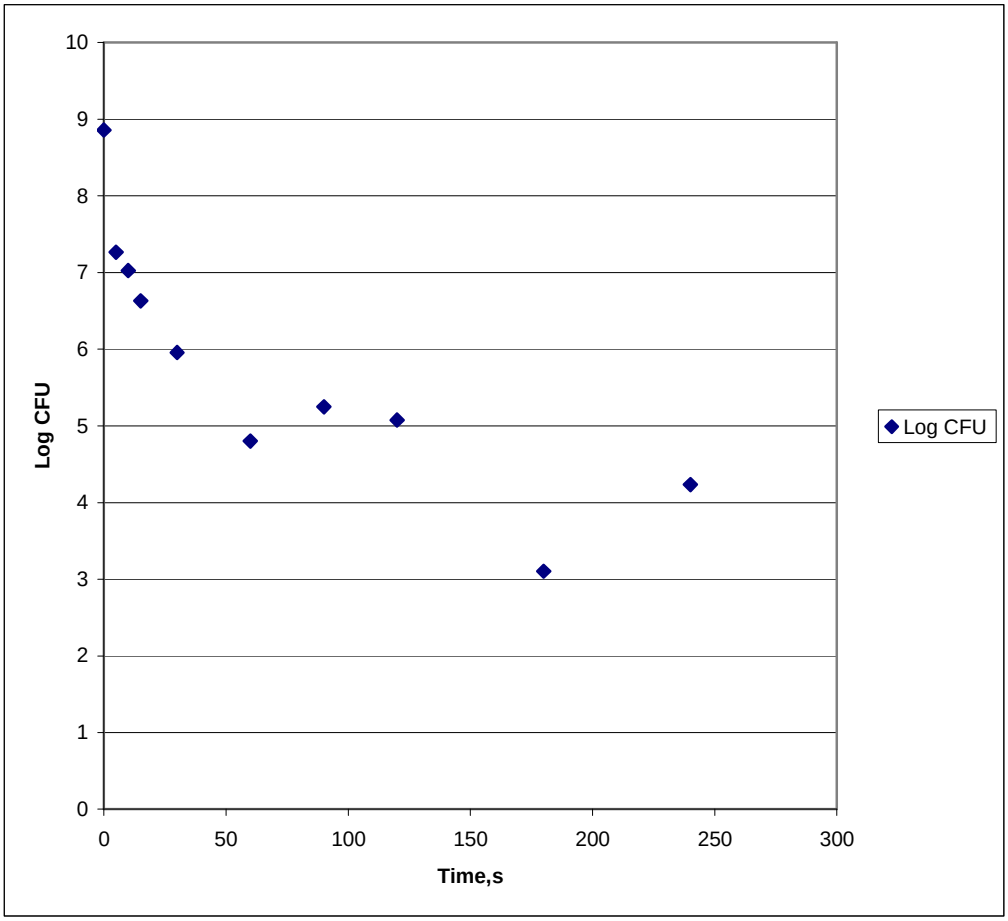


Figure 4.4 The average numbers between the replicated experiments for *L. innocua* ATCC 33090 on fish product surfaces during steam treatments as a function of time

The results of pathogen survival after heating (Mccann *et al.* 2006) showed that, in all cases, pathogen numbers declined rapidly linear during the first 10 s of steam treatment, initial rate of decline slowed during the next 50 s (period of "tailing") and considerable pathogen numbers were still present after steam treatment for 60 s (Figure 2.5).

The inactivation of *L. innocua* in steam surface pasterisation of fish products did not follow log-linear kinetics, because several factors had influence on heat resistance of bacteria (Figure 4.3 and Figure 4.4). These figures showed a pattern of biphasic inactivation of *L. innocua* in which there was an initial rapid (15 s) linear decrease in bacterial numbers, followed by period of “tailing”(225 s), during which no considerable reduction occurred.

The non-linear inactivation kinetics of *L. innocua* may be explained with a few hypotheses. First, the steam surface pasteurisation of fish products is more complicated system, because the surface constitutes a two-dimensional food structure. The water content and water activity may change

quickly in the two-dimensional food structure. Also, the bacteria can be attached or located in more protected areas (channels and microchannels of issue) of the fish products surface. These channels and microchannels may harbour reservoirs which a rapid surface treatment will not inactivate. Specifically, steam heating can fill such channels and microchannels of issue with water, during steam condensation on cold product surfaces (Kim et al., 1996). The water in these structures may not reach high temperatures to kill the enclosed cells. This water reaches temperatures of 50–60 °C, differences in cell survival could arise, due to variation in the production of heat shock (Mccann *et al.* 2006). An examination with an electron microscope could have revealed attachment or location *L. innocua* in channels and microchannels of issue of the surimi surface, but this was out of the scope of this thesis.

McCann et al. (2006) explained non-linear inactivation kinetics ("tailing" effect) with the presence of a heat resistant sub-population, which did not decrease over the time of the experiment. Heat resistant sub-populations may persist because they are attached or located in more protected areas of the product surface. Also, they may represent those cells capable of mounting an effective heat shock response or they may survive by a combination of these two factors (Mccann *et al.* 2006).

Several investigations indicated that rapid responses in heat shock protein formation of less than 1 min have been observed in *Salmonella Enteriditis PT4* and *Pseudomonas aeruginosa* (Allan *et al.* 1988;Humpheson *et al.* 1998). Frisk and Ison (1998) reported that in experiments with *Haemophilus ducreyi* the presence of heat-shock proteins was associated with binding of cells to the host and to each other.

In conclusion, it is likely that the non-linear inactivation kinetics of *L. innocua* ATCC 33090 with increasing variation in experimental data after 15 s of steam treatment ("tailing" effect) is a reflection of the potentially different conditions caused by microenvironments on the surface.

In order to describe the microbial inactivation of *L. innocua* ATCC 33090 several parameters must be estimated, e.g. A, B, C - parameters from formula (6). In the current study was not enough experimental data for the mathematical description. For thermal inactivation of vegetative microorganisms, there are eight commonly observed types of inactivation or survivor curves: linear curves, curves with a shoulder, linear curves with tailing, sigmoidal-like curves, biphasic curves, biphasic curves with a shoulder, concave and convex curves (Valdramidis *et al.* 2006). As an example, Kondjoyan and Portanguen (2008) calculated results of the survival of *L. innocua*

surface-inoculated onto chicken skin after treatment with superheated steam by two coherent models, the ‘‘Weibull-tail” model (Albert and Mafart 2005) and the ‘‘biphasic-shoulder” model (Geeraerd *et al.* 2005) (Figure 2.9).

In the current laboratory experiments it was created optimal conditions for growth and regeneration for *L. innocua*. It was also used high initial levels of surface contamination (10^8 CFU). Incubation temperature and growth media before and after heat treatment are factors that have major effect on heat resistance. The temperature during growth affects lipid biosynthesis, the composition of membranes, and protein synthesis (Juneja and Eblen 1999b). In the current study incubation and regeneration temperatures were 37 °C and 30 °C, respectively. This was optimal growth temperature for *L. innocua*, and *Listeria* in general. Several studies have shown that *L. monocytogenes* has a higher heat resistance when it is grown before heat treatment at higher temperatures than at low temperatures (Juneja and Eblen 1999b;Smith and Marmer 1991). In addition, composition of the growth medium, pH, salt concentration and water content plays an important role. TSBYE was used as growth medium and TSAYE as regeneration medium. Compositions of these media have positive influence on cultivation of the bacteria. Miller et al. (2006) showed that *L. innocua* incubated on the non-selective medium tryptone soya agar with 0.6 % yeast extract (TSAYE) gave higher number after heat treatment than on two selective media, and among the selective media gave TSAYE with 5 % salt lower survival rates than Palcam.

In practical application in the industry, surimi or other fish products (with specific water activity, pH and texture) will be the food surface matrix for attachment, heat treatment and regeneration. Here will be more difficult to have control over the individual growth factors of *Listeria*. Also the bacteria can be attached or located in more protected areas (channels and microchannels of issue) of the fish products surface. However, in real fish products the number of *Listeria* is much lower than the contamination levels (10^8 CFU) used in this study. *L. monocytogenes* levels in foods are generally low (0 to 10^3 CFU/g with 90 to 99 % being below 10^2 CFU/g and less than 1 % being between 10^3 and 10^4 CFU/g)(Swaminathan, 2001). Also, steam pasteurisation will reduce the numbers of *Listeria* and in combination with aseptic packaging and refrigerator temperature will increase the shelf-life of seafood. However, it is difficult to achieve a 6 log reduction using surface pasteurisation. Many studies suggested that a variety of treatment temperatures and times

are used to decontaminate meat, with results from almost no effect to 1.0 to 4.0 log reductions (James *et al.* 2000;Minihan *et al.* 2003;Phebus *et al.* 1997;Whyte *et al.* 2003)

In this experiment steam treatment of samples for 60 s gave total 3-4 $\log_{10}$ CFU reduction. But it must be noted that in this experiment steam treatment of samples for 60 s or longer had not considerable reduction of bacterial numbers compared to the goal of 6 log reduction (Figure 4.3, Figure 4.4), and after 60 s most likely had an undesirable effect for quality of the product. McCann et al. (2006) reported that meat surfaces in the samples had a cooked appearance after 10 s or longer.

For further work, it can be interesting to investigate superheated steam as an alternative form of treatment on fish product surface. Kondjoyan and Portanguen (2008) investigated effect of superheated steam on the inactivation of *L. innocua* (CLIP 20595) surface-inoculated onto chicken skin. They reported that superheated steam was clearly more bacterial inactivation-efficient than non-superheated steam, leading to an average reduction of more than 5 $\log_{10}$ CFU/cm^2 after 30 s of treatment. However, Kondjoyan and Portanguen (2008) added that large variations in surviving cell numbers of *L. innocua* were observed between replicates which cannot be explained by variations in the heat treatment.

5. Conclusion

The thermotolerance of *L. monocytogenes* has been a widely studied, but the results are often conflicting. Some of the differences can be explained by inaccuracies in the method of data analysis (Cole *et al.* 1993). In order to get better understanding of the inactivation kinetics of *L. innocua* two different experimental designs in the current study were used: 1) using classical heat treatment in capillary tubes; 2) using steam on fish product surfaces.

The heat treatment in capillary tubes is a classical heat treatment without any modifications. The inactivation of *L. innocua* ATCC 33090 and *L. innocua* CCUG 35613 followed log-linear kinetics with constant rate inactivation. D-values for both strains of *L. innocua* were low compared to literature D-values (Ahn and Balasubramaniam 2007;Miller *et al.* 2006). The D_{60}-values of *L. innocua* ATCC 33090 from current work was 1.58 minutes, that is for 1.05 minutes less that in the work of Ahn and Balasubramaniam (2007) and for 1.15 minutes less that in the works of Miller *et al.* (2006). The results of D-values for both strains of *L. innocua* from current work fit into the data-set of Olsen (2010) in the range 59-60 °C.

L. innocua strain ATCC 33090 was used for steam pasteurisation on fish product surfaces. This experiment provides new information on the dynamic changes, in bacterial numbers of *L. innocua* on fish product surfaces during steam treatment. Although steam is widely used for the reduction of pathogens on the surface of animal carcasses and poultry products, limited investigations of fish product surface pasteurisation have been carried out.

The inactivation of *L. innocua* in steam surface pasteurisation of fish products did not follow log-linear kinetics. Bacterial numbers of *L. innocua* ATCC 33090 declined rapidly during the first 15 s of steam treatment in “BugDeath” rig. This initial rate of decline slowed during the next 45 s. However, after 60 s of steam treatment bacterial numbers declined very slowly, so that bacterial numbers were still present after steam treatment for 4 min. In this experiment steam treatment of samples for 60 s gave total 3-4 $\log_{10}$ CFU reduction, but after 60 s had not considerable reduction of bacterial numbers and most likely had an undesirable effect for quality of the product.

6. Appendix

6.1 Media

Preparation of the TSAYE (Tryptone Soya Agar with 0.6 % Yeast Extract)

20g TSA [Oxoid CMO131]
3g yeast [Merck 1.03353.0500]
500 ml Elix water

- Mixed with a magnetic stirrer
- Autoclavered at 121 °C for 15 min.
- Poured in 10 cm Petri dishes.
- Store at 4 °C.

Preparation of the TSBYE (Trypsin Soya Broth with 0.6 % Yeast Extract)

15g TSB [Oxoid CMO129]
3g yeast [Merck 1.03353.0500]
500 ml Elix water

- Mixed with a magnetic stirrer
- Autoclavered at 121 °C for 15 min.
- Store at 4 °C.

Preparation of the peptone water (buffered)

25.5 g peptone [Merck 1.07228.0500]
1000 ml Elix water

- Mixed with a magnetic stirrer
- Autoclavered at 121 °C for 15 min.
- Store at 4 °C.

6.2 Calibration of the water bath

The water bath Lauda 1 was calibrated at 60 °C. The reference temperature was measured with a calibrated thermometer, deviation was -0.1 °C. At the first step the 10 thermocouples (T01-T10, Figure 6.1.a) were measured in top position (Figure 3.3, position 1).

The temperature distribution in top position in the water bath is shown on Figure 4.1.a

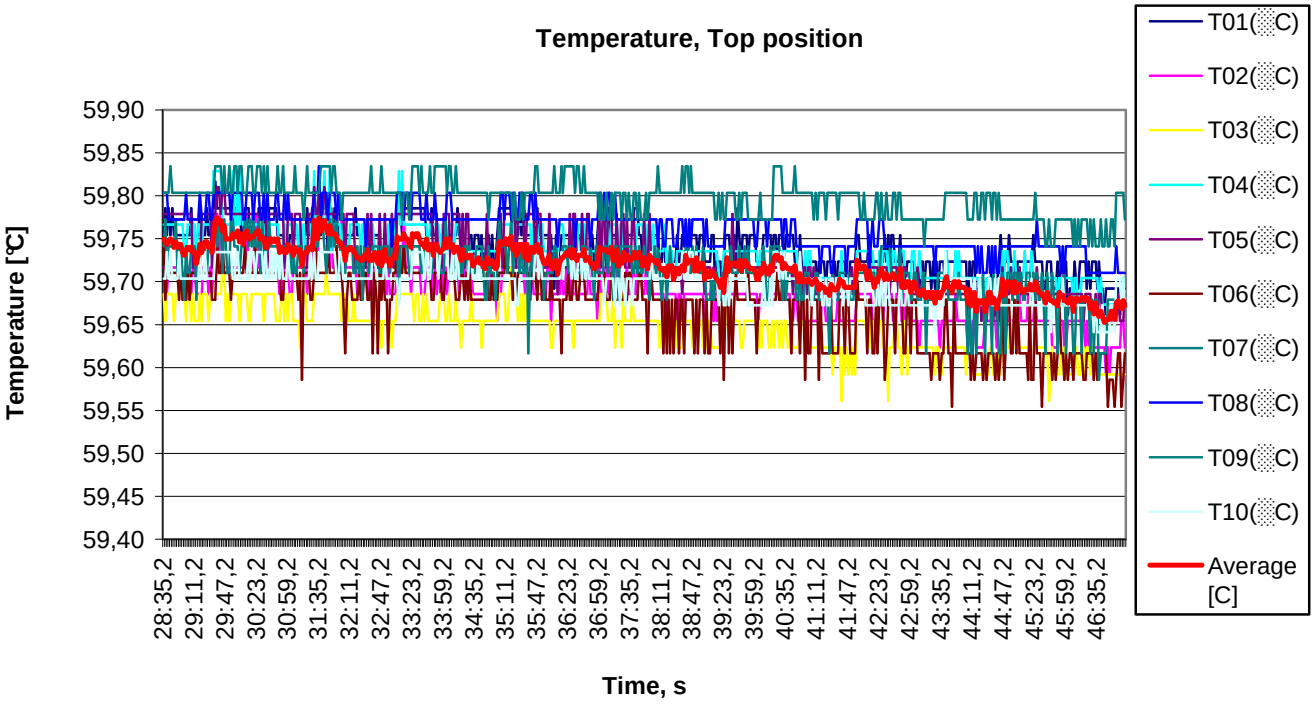


Figure 6.1.a The temperature distribution in the water bath Lauda 1 in the top position.

The measurement for water bath, illustrated in Figure 6.1.a, Figure 6.1.b and Figure 6.1.c showed the evidence that temperature chances every 2 sec in the all 10 thermocouples. The temperature maximum in the top position was showed thermocouple T09 (59.83 °C); the temperature minimum in the top position was showed thermocouple T06 (59.55 °C). The difference between maximum and minimum temperature (Max-Min) °C in the top position are shown on Figure 6.2.a

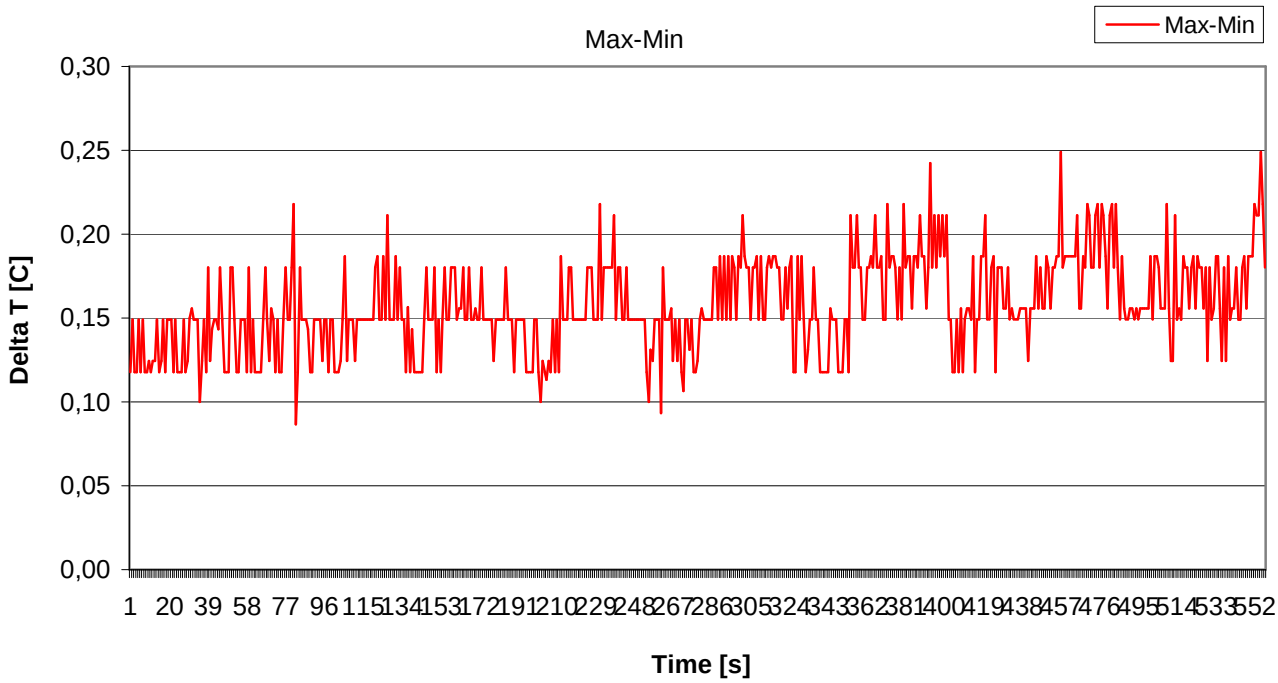


Figure 6.2.a The difference between maximum and minimum temperature (Max-Min) °C .

At the second step the 10 thermocouples (T01-T10, Figure 6.2.b) were measured in the centre position (Figure 3.3, position 2). The temperature distribution in centre position in the water bath is shown on Figure 6.2.b. The temperature maximum in the centre position was showed thermocouple T09 (59.77 °C); the temperature minimum in the centre position was showed thermocouple T03 (59.40 °C). The difference between maximum and minimum temperature (Max-Min) °C in the centre position are shown on Figure 6.2.b

At the last step the 10 thermocouples (T01-T10, Figure 6.2.c) was measured in the bottom position (Figure 3.3, position 3). The temperature distribution in bottom position in the water bath is shown on Figure 6.2.c. The temperature maximum in the bottom position was showed thermocouple T09 (59.71 °C); the temperature minimum in the bottom position was showed thermocouple T03 (59.34 °C). The difference between maximum and minimum temperature (Max-Min) °C in the bottom position are shown on Figure 6.2.c.

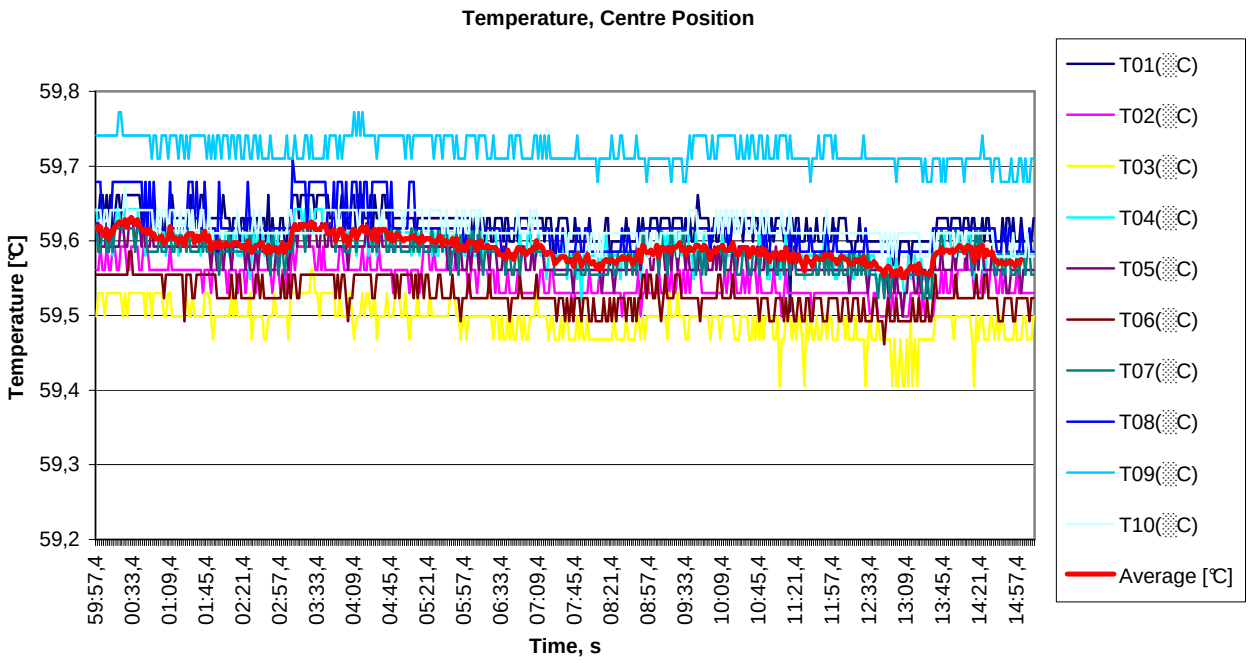


Figure 6.1.b The temperature distribution in the water bath Lauda 1 in the centre position.

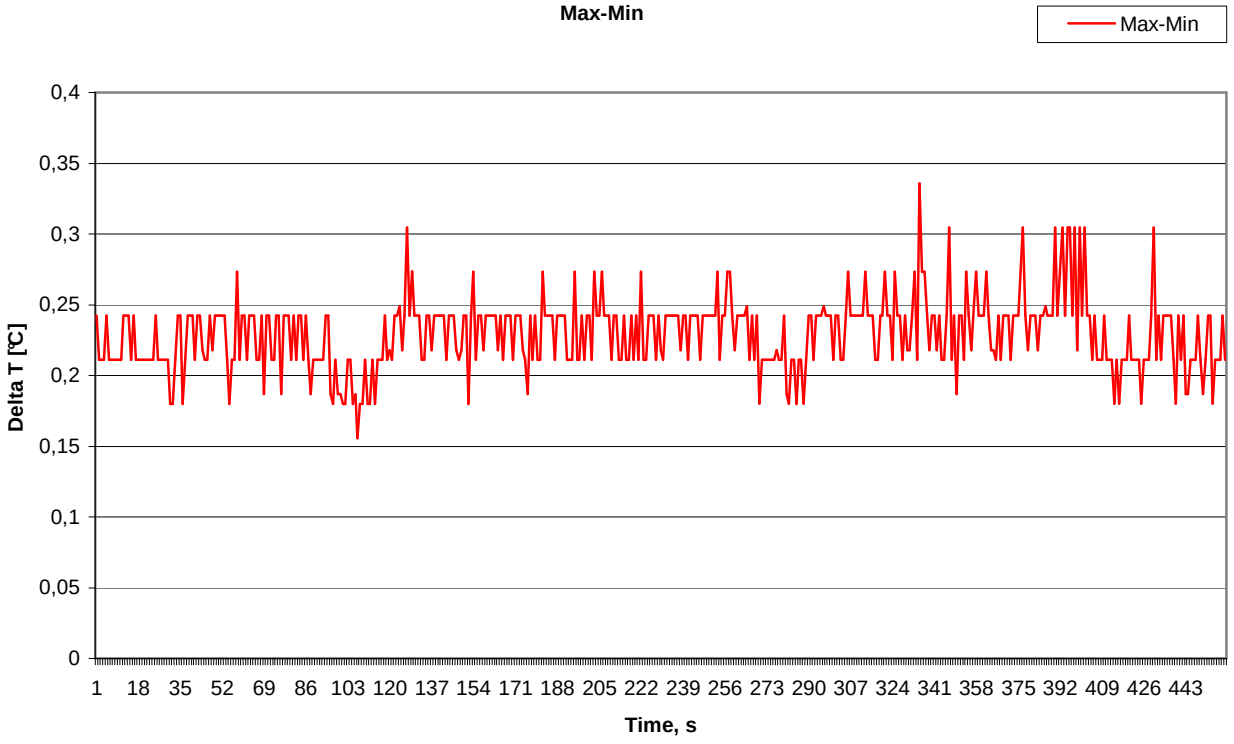


Figure 6.2.b The difference between maximum and minimum temperature (Max-Min) °C1 in the centre position.

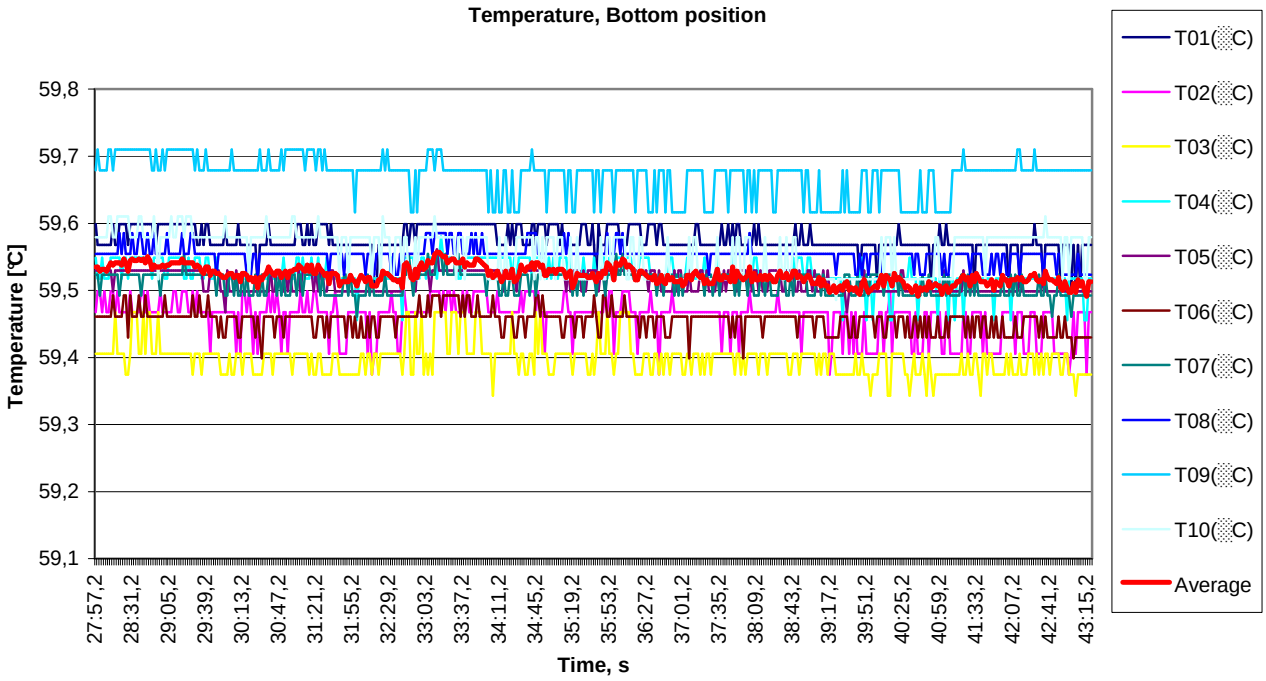


Figure 6.1.c The temperature distribution in the water bath Lauda 1 in the bottom position.

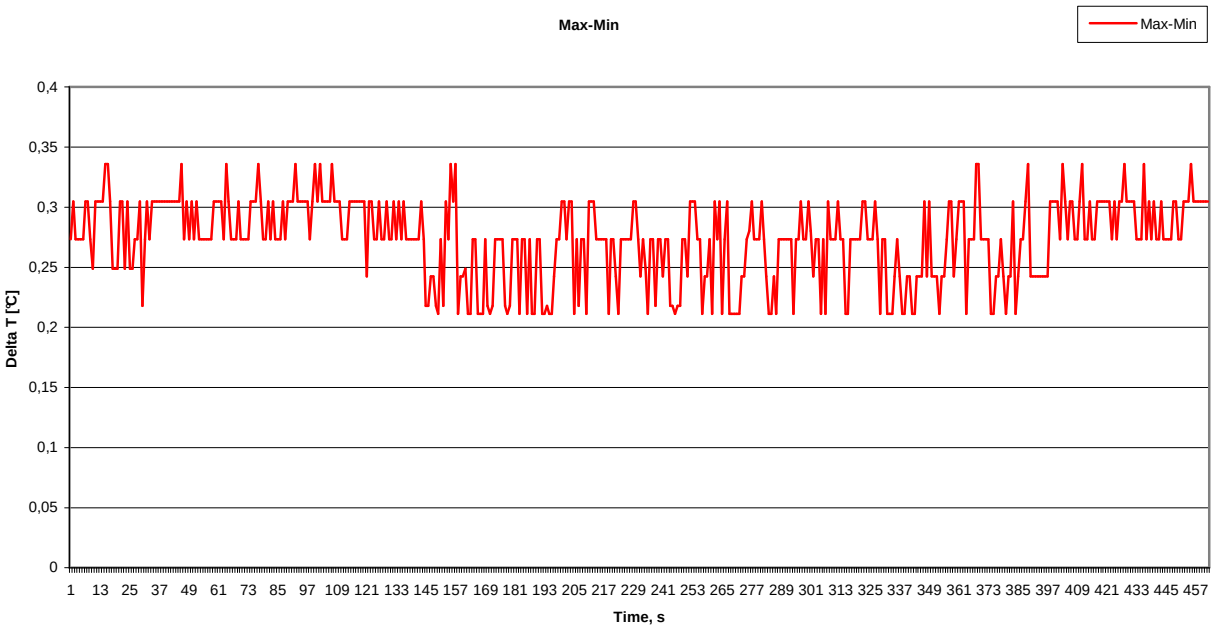


Figure 6.2.c The difference between maximum and minimum temperature (Max-Min) °C in the bottom position.

7. References

Ahn,J. and Balasubramaniam,V.M. (2007) Inactivation kinetics of *Listeria innocua* ATCC 33090 at various temperature heating-up and pressure building-up rates. *Food Science and Biotechnology* 16, 255-259.

Albert,I. and Mafart,P. (2005) A modified Weibull model for bacterial inactivation. *International Journal of Food Microbiology* 100, 197-211.

Allan,B., Linseman,M., Macdonald,L.A., Lam,J.S. and Kropinski,A.M. (1988) Heat-Shock Response of Pseudomonas-Aeruginosa. *Journal of Bacteriology* 170, 3668-3674.

Alvarez-Ordonez,A., Fernandez,A., Bernardo,A. and Lopez,M. (2009) Heat-Resistance Prediction of *Listeria Innocua* Grown at Different Temperatures. *Journal of Food Safety* 29, 474-483.

Amanatidou,A., Smid,E.J. and Gorris,L.G.M. (1999b) Effect of elevated oxygen and carbon dioxide on the surface growth of vegetable-associated micro-organisms. *Journal of Applied Microbiology* 86, 429-438.

Amanatidou,A., Smid,E.J. and Gorris,L.G.M. (1999a) Effect of elevated oxygen and carbon dioxide on the surface growth of vegetable-associated micro-organisms. *Journal of Applied Microbiology* 86, 429-438.

Augustin,J.C., Carlier,V. and Rozier,J. (1998) Mathematical modelling of the heat resistance of *Listeria monocytogenes*. *Journal of Applied Microbiology* 84, 185-191.

Bell,C. and Kyriakides, A. (2005). Factors affecting the growth and survival of *Listeria monocytogenes*. In: *Listeria:* A practical approach to the organism and its control in foods. 2nd ed, Iowa State Press, pp. 62-74

Benembarek,P.K. and Huss,H.H. (1993) Heat-Resistance of *Listeria-Monocytogenes* in Vacuum-Packaged Pasteurized Fish Fillets. *International Journal of Food Microbiology* 20, 85-95.

Beuchat,L.R., Brackett,R.E., Hao,D.Y.Y. and Conner,D.E. (1986) Growth and Thermal Inactivation of *Listeria-Monocytogenes* in Cabbage and Cabbage Juice. *Canadian Journal of Microbiology* 32, 791-795.

Boyle,D.L., Sofos,J.N. and Schmidt,G.R. (1990) Thermal-Destruction of *Listeria-Monocytogenes* in A Meat Slurry and in Ground-Beef. *Journal of Food Science* 55, 327-329.

Breand,S., Fardel,G., Flandrois,J.P., Rosso,L. and Tomassone,R. (1997) A model describing the relationship between lag time and mild temperature increase duration. *International Journal of Food Microbiology* 38, 157-167.

Breand,S., Fardel,G., Flandrois,J.P., Rosso,L. and Tomassone,R. (1999) A model describing the relationship between regrowth lag time and mild temperature increase for *Listeria monocytogenes. International Journal of Food Microbiology* 46, 251-261.

Brody, A. (2006) Aseptic and Extended-Shelf-Life Packaging. Food Technology 60(2), 66-68.

Buchanan,R.L. and Bagi,L.K. (1997) Microbial competition: Effect of culture conditions on the suppression of Listeria monocytogenes Scott A by Carnobacterium piscicola. *Journal of Food Protection* 60, 254-261.

Buchanan,R.L. and Golden,M.H. (1994) Interaction of Citric-Acid Concentration and Ph on the Kinetics of Listeria-Monocytogenes Inactivation. *Journal of Food Protection* 57, 567-570.

Buchanan,R.L. and Golden,M.H. (1995) Model for the Nonthermal Inactivation of *Listeria-Monocytogenes* in A Reduced Oxygen Environment. *Food Microbiology* 12, 203-212.

Bula,C.J., Bille,J. and Glauser,M.P. (1995) An Epidemic of Food-Borne Listeriosis in Western Switzerland - Description of 57 Cases Involving Adults. *Clinical Infectious Diseases* 20, 66-72.

Casadei,M.A., de Matos,R.E., Harrison,S.T. and Gaze,J.E. (1998) Heat resistance of *Listeria monocytogenes* in dairy products as affected by the growth medium. *Journal of Applied Microbiology* 84, 234-239.

Chen,B.Y., Pyla,R., Kim,T.J., Silva,J.L. and Jung,Y.S. (2010) Antibiotic resistance in *Listeria* species isolated from catfish fillets and processing environment. *Letters in Applied Microbiology* 50, 626-632.

Cole,M.B., Davies,K.W., Munro,G., Holyoak,C.D. and Kilsby,D.C. (1993) A Vitalistic Model to Describe the Thermal Inactivation of *Listeria-Monocytogenes. Journal of Industrial Microbiology* 12, 232-239.

Compendium of Fish and Fishery Product Processes, Hazards, and Controls (2009). Seafood Network Information Center. Chapter 15 *Listeria monocytogenes.* Available at: http://seafood.ucdavis.edu/haccp/compendium/chapt15.htm

Corradini,M.G. and Peleg,M. (2007) A Weibullian model for microbial injury and mortality. *International Journal of Food Microbiology* 119, 319-328.

Crawford,R.G., Beliveau,C.M., Peeler,J.T., Donnelly,C.W. and Bunning,V.K. (1989) Comparative Recovery of Uninjured and Heat-Injured *Listeria-Monocytogenes* Cells from Bovine-Milk. *Applied and Environmental Microbiology* 55, 1490-1494.

Davey,K.R. (1990) Equilibrium Temperature in A Clump of Bacteria Heated in Fluid. *Applied and Environmental Microbiology* 56, 566-568.

Devlieghere,F., Francois,K., Vereecken,K.M., Geeraerd,A.H., Van Impe,J.F. and Debevere,J. (2004) Effect of chemicals on the microbial evolution in foods. *Journal of Food Protection* 67, 1977-1990.

Devlieghere,F., Geeraerd,A.H., Versyck,K.J., Vandewaetere,B., Van Impe,J. and Debevere,J. (2001) Growth of *Listeria monocytogenes* in modified atmosphere packed cooked meat products: a predictive model. *Food Microbiology* 18, 53-66.

Donald,A.S., Fenlon,D.R. and Seddon,B. (1995) The Relationship Between Ecophysiology, Indigenous Microflora and Growth of *Listeria-Monocytogenes* in Grass-Silage. *Journal of Applied Bacteriology* 79, 141-148.

Doumith,M., Cazalet,C., Simoes,N., Frangeul,L., Jacquet,C., Kunst,F., Martin,P., Cossart,P., Glaser,P. and Buchrieser,C. (2004a) New aspects regarding evolution and virulence of *Listeria monocytogenes* revealed by comparative genomics and DNA arrays. *Infection and Immunity* 72, 1072-1083.

Doumith,M., Cazalet,C., Simoes,N., Frangeul,L., Jacquet,C., Kunst,F., Martin,P., Cossart,P., Glaser,P. and Buchrieser,C. (2004b) New aspects regarding evolution and virulence of *Listeria monocytogenes* revealed by comparative genomics and DNA arrays. *Infection and Immunity* 72, 1072-1083.

Doyle,E.M., Mazzotta,A.S., Wang,T., Wiseman,D.W. and Scott,V.N. (2000) Heat resistance of *Listeria monocytogens. Journal of Food Protection* 64, 410-429.

Doyle,M.E., Mazzotta,A.S., Wang,T., Wiseman,D.W. and Scott,V.N. (2001) Heat resistance of *Listeria monocytogenes. Journal of Food Protection* 64, 410-429.

Eklund,M.W., Poysky,F.T., Paranjpye,R.N., Lashbrook,L.C., Peterson,M.E. and Pelroy,G.A. (1995) Incidence and Sources of *Listeria-Monocytogenes* in Cold-Smoked Fishery Products and Processing Plants. *Journal of Food Protection* 58, 502-508.

ECFF (European Chilled Food Federation), (1996) "Guideline for the hygienic manufacture of chilled foods" (Chilled Food Association, London).

European Commission, (1999). "Harmonization of safety criteria for minimally processed foods"

European Commission (EC), (1999). Opinion of the scientific committee on veterinary measures relating to public health on *Listeria monocytogenes,* 23 September 1999. Available at: http://ec.europa.eu/food/fs/sc/scv/out25_en.pdf . Accessed on 27 November 2006.

European Commission (EC), (2004). Regulation (EC) No 852/2004 of the European parliament and of the council of 29 April 2004 on the hygiene of foodstuffs. Off. J. Eur. Union L 139, 1–54.

European Commission (EC), (2005a). Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Off. J. Eur.Union L 338, 1–26.

European Commission (EC), (2005b). Commission recommendation of 1 March 2005 concerning a coordinated programme for the official control of foodstuffs for 2005 (2005/175/EC). Off. J. Eur. Union L 59, 27–39.

Fairchild,T.M. and Foegeding,P.M. (1993) A Proposed Nonpathogenic Biological Indicator for Thermal Inactivation of Listeria-Monocytogenes. *Applied and Environmental Microbiology* 59, 1247-1250.

Farber,J.M. (1991) *Listeria monocytogenes*, a food-borne pathogen. *Microbiological-Review; 55 (3) 476-511, 445 ref.*

Farber,J.M., Coates,F. and Daley,E. (1992) Minimum Water Activity Requirements for the Growth of Listeria-Monocytogenes. *Letters in Applied Microbiology* 15, 103-105.

Farber,J.M. and Pagotto,F. (1992) The Effect of Acid Shock on the Heat-Resistance of *Listeria-Monocytogenes*. *Letters in Applied Microbiology* 15, 197-201.

Farber,J.M. and Peterkin,P.I. (1999). Incidence and behaviour of *Listeria monocytogenes* in meat products. In: Ryser,E.T. and Marth,E.H.(eds). *Listeria*, Listeriosis and Food Safety, 2nd ed. Marcel Dekker, New York, USA, pp. 505-564.

Farber,J.M., Sanders,G.W., Dunfield,S. and Prescott,R. (1989) The Effect of Various Acidulants on the Growth of *Listeria-Monocytogenes*. *Letters in Applied Microbiology* 9, 181-183.

Farber,J.M., Sanders,G.W. and Speirs,J.I. (1990) Growth of *Listeria-Monocytogenes* in Naturally-Contaminated Raw-Milk. *Lebensmittel-Wissenschaft & Technologie* 23, 252-254.

FAIR programme - European Commission. Harmorization of safety criteria for minimally processed food - Inventory report - FAIR concerted action FAIR CT96-1020. (1997)

Fernandez,A., Lopez,M., Bernardoa,A., Condon,S. and Raso,J. (2007) Modelling thermal inactivation of *Listeria monocytogenes* in sucrose solutions of various water activities. *Food Microbiology* 24, 372-379.

Foegeding,P.M. and Stanley,N.W. (1991) Listeria-Innocua Transformed with An Antibiotic-Resistance Plasmid As A Thermal-Resistance Indicator for *Listeria-Monocytogenes*. *Journal of Food Protection* 54, 519-523.

Foster,A.M., Ketteringham,L.P., Purnell,G.L., Kondjoyan,A., Havet,M. and Evans,J.A. (2006) New apparatus to provide repeatable surface temperature-time treatments on inoculated food samples. *Journal of Food Engineering* 76, 19-26.

Friedly,E.C., Crandall,P.G., Ricke,S., O'Bryan,C.A., Martin,E.M. and Boyd,L.M. (2008) Identification of *Listeria innocua* surrogates for *Listeria monocytogenes* in hamburger patties. *Journal of Food Science* 73, M174-M178.

Frisk,A., Ison,C.A. and Lagergard,T. (1998) GroEL heat shock protein of *Haemophilus ducreyi:* Association with cell surface and capacity to bind to eukaryotic cells. *Infection and Immunity* 66, 1252-1257.

FSAI (2005). The control and management of *Listeria monocytogenes* contamination of food. Food Safety Authority of Ireland (http://www.fsai.ie).

Gaze,J.E., Boyd,A.R. and Shaw,H.L. (2006) Heat inactivation of *Listeria monocytogenes* Scott A on potato surfaces. *Journal of Food Engineering* 76, 27-31.

Geeraerd,A.H., Herremans,C.H. and Van Impe,J.F. (2000) Structural model requirements to describe microbial inactivation during a mild heat treatment. *International Journal of Food Microbiology* 59, 185-209.

Geeraerd,A.H., Valdramidis,V. and Van Impe,J.F. (2005) GInaFiT, a freeware tool to assess non-log-linear microbial survivor curves. *International Journal of Food Microbiology* 102, 95-105.

George,S.M., Richardson,L.C.C. and Peck,M.W. (1996) Predictive models of the effect of temperature, pH and acetic and lactic acids on the growth of *Listeria monocytogenes*. *International Journal of Food Microbiology* 32, 73-90.

Gill,C.O. (1979) Intrinsic Bacteria in Meat - Review. *Journal of Applied Bacteriology* 47, 367-378.

Golden,D.A., Beuchat,L.R. and Brackett,R.E. (1988) Evaluation of Selective Direct Plating Media for Their Suitability to Recover Uninjured, Heat-Injured, and Freeze-Injured *Listeria-Monocytogenes* from Foods. *Applied and Environmental Microbiology* 54, 1451-1456.

Gram,L. (2001). Potential hazards in cold-smoked fish: *Listeria monocytogenes. Journal of Food Science* 66(7): 1072-1080.

Harrison,M.A. and Huang,Y.W. (1990) Thermal Death Times for *Listeria-Monocytogenes* (Scott-A) in Crabmeat. *Journal of Food Protection* 53, 878-880.

Heddleson,R.A. and Doores,S. (1994) Factors Affecting Microwave-Heating of Foods and Microwave-Induced Destruction of Foodborne Pathogens - A Review. *Journal of Food Protection* 57, 1025-1037.

Ho,A.J., Ivanek,R., Groehn,Y.T., Nightingale,K.K. and Wiedmann,M. (2007) L*isteria monocytogenes* fecal shedding in dairy cattle shows high levels of day-to-day variation and includes outbreaks and sporadic cases of shedding of specific *L-monocytogenes* subtypes. *Preventive Veterinary Medicine* 80, 287-305.

Hoke,K., Houska,M., Kyhos,K., Landfeld,A. and Pipek,P. (2003) Modelling of beef surface temperatures during steam decontamination. *Journal of Food Engineering* 58, 95-102.

Huang,L.H. (2009) Thermal inactivation of *Listeria monocytogenes* in ground beef under isothermal and dynamic temperature conditions. *Journal of Food Engineering* 90, 380-387.

Huang,L.H. and Juneja,V.K. (2001) A new kinetic model for thermal inactivation of microorganisms: Development and validation using *Escherichia coli* O157 : H7 as a test organism. *Journal of Food Protection* 64, 2078-2082.

Humpheson,L., Adams,M.R., Anderson,W.A. and Cole,M.B. (1998) Biphasic thermal inactivation kinetics in *Salmonella enteritidis* PT4. *Applied and Environmental Microbiology* 64, 459-464.

Huss,H.H., Jorgensen,L.V. and Vogel,B.F. (2000) Control options for *Listeria monocytogenes* in seafoods. *International Journal of Food Microbiology* 62, 267-274.

ICMSF (International Commission on Microbiological Specifications for Foods), 1996. Micro-organism in Foods 5: Microbiological Specifications of Food Pathogens. London, Blackie Academic and Professional, 514 pp.

James,C., Goksoy,E.O., Corry,J.E.L. and James,S.J. (2000) Surface pasteurisation of poultry meat using steam at atmospheric pressure. *Journal of Food Engineering* 45, 111-117.

James,C., Lechevalier,V. and Ketteringham,L. (2002) Surface pasteurisation of shell eggs. *Journal of Food Engineering* 53, 193-197.

James,S.J. and Evans,J.A. (2006) Predicting the reduction in microbes on the surface of foods during surface pasteurisation - the 'BUGDEATH' project. *Journal of Food Engineering* 76, 1-6.

Janssen,M., Geeraerd,A.H., Cappuyns,A., Garcia-Gonzalez,L., Schockaert,G., Van Houteghem,N., Vereecken,K.M., Debevere,J., Devlieghere,F. and Van Impe,J.F. (2007) Individual and combined effects of pH and lactic acid concentration on *Listeria innocua* inactivation: Development of a predictive model and assessment of experimental variability. *Applied and Environmental Microbiology* 73, 1601-1611.

Jemmi,T. and Stephan,R. (2006) *Listeria monocytogenes:* food-borne pathogen and hygiene indicator. *Revue Scientifique et Technique-Office International des Epizooties* 25, 571-580.

Jorgensen,F., Hansen,T.B. and Knochel,S. (1999) Heat shock-induced thermotolerance in *Listeria monocytogenes* 13-249 is dependent on growth phase, pH and lactic acid. *Food Microbiology* 16, 185-194.

Jorgensen,F., Stephens,P.J. and Knochel,S. (1995) The Effect of Osmotic Shock and Subsequent Adaptation on the Thermotolerance and Cell Morphology of *Listeria-Monocytogenes*. *Journal of Applied Bacteriology* 79, 274-281.

Jorgensen,L.V. and Huss,H.H. (1998) Prevalence and growth of *Listeria monocytogenes* in naturally contaminated seafood. *International Journal of Food Microbiology* 42, 127-131.

Juneja,V.K. and Eblen,B.S. (1999b) Predictive thermal inactivation model for *Listeria monocytogenes* with temperature, pH, NaCl, and sodium pyrophosphate as controlling factors. *Journal of Food Protection* 62, 986-993.

Juneja,V.K. and Eblen,B.S. (1999a) Predictive thermal inactivation model for *Listeria monocytogenes* with temperature, pH, NaCl, and sodium pyrophosphate as controlling factors. *Journal of Food Protection* 62, 986-993.

Kim,K.Y., Frank,J.F. and Craven,S.E.(1996). Three-dimensional visualisation of *Salmonella* attachment to poultry skin using confocol scanning laser microscopy. *Letters in Applied Microbiology*, 22, 280-282.

Kondjoyan,A. and Portanguen,S. (2008) Effect of superheated steam on the inactivation of *Listeria innocua* surface-inoculated onto chicken skin. *Journal of Food Engineering* 87, 162-171.

Linton,R.H., Pierson,M.D. and Bishop,J.R. (1990) Increase in Heat-Resistance of *Listeria-Monocytogenes* Scott-A by Sublethal Heat-Shock. *Journal of Food Protection* 53, 924-927.

Linton,R.H., Webster,J.B., Pierson,M.D., Bishop,J.R. and Hackney,C.R. (1992) The Effect of Sublethal Heat-Shock and Growth Atmosphere on the Heat-Resistance of *Listeria-Monocytogenes* Scott-A. *Journal of Food Protection* 55, 84-87.

Little,C.L., Taylor,F.C., Sagoo,S.K., Gillespie,I.A., Grant,K. and McLauchlin,J. (2007) Prevalence and level of *Listeria monocytogenes* and other *Listeria* species in retail pre-packaged mixed vegetable salads in the UK. *Food Microbiology* 24, 711-717.

Lou,W.Q. and Yousef,A.E. (1996) Resistance of *Listeria monocytogenes* to heat after adaptation to environmental stresses. *Journal of Food Protection* 59, 465-471.

Lou,Y and Yousef,A.E. (1999). Characteristics of *Listeria monocytogenes* important to food processors. In: Ryser,E.T. and Marth,E.H.(eds). *Listeria*, Listeriosis and Food Safety, 2nd ed. Marcel Dekker, New York, USA, pp. 131-224.

Lunestad,B.T. (2005). Compendium in Seafood Microbiology, NIFES, Bergen

Mackey,B.M. and Derrick,C.M. (1987) Changes in the Heat-Resistance of *Salmonella-Typhimurium* During Heating at Rising Temperatures. *Letters in Applied Microbiology* 4, 13-16.

Mccann,M.S., Sheridan,J.J., McDowell,D.A. and Blair,I.S. (2006) Effects of steam pasteurisation on *Salmonella Typhimurium* DT104 and *Escherichia coli* O157 : H7 surface inoculated onto beef, pork and chicken. *Journal of Food Engineering* 76, 32-40.

McLauchlin,J., Mitchell,R.T., Smerdon,W.J. and Jewell,K. (2004) *Listeria monocytogenes* and listeriosis: a review of hazard characterisation for use in microbiological risk assessment of foods. *International Journal of Food Microbiology* 92, 15-33.

Miller,F.A., Brandao,T.R.S., Teixeira,P. and Silva,C.L.M. (2006) Recovery of heat-injured *Listeria innocua*. *International Journal of Food Microbiology* 112, 261-265.

Miller,F.A., Gil,M.M., Brandao,T.R.S., Teixeira,P. and Silva,C.L.M. (2009) Sigmoidal thermal inactivation kinetics of *Listeria innocua* in broth: Influence of strain and growth phase. *Food Control* 20, 1151-1157.

Minihan,D., Whyte,P., O'Mahony,M. and Collins,J.D. (2003) The effect of commercial steam pasteurization on the levels of *Enterobacteriaceae* and *Escherichia coli* on naturally

contaminated beef carcasses. *Journal of Veterinary Medicine Series B-Infectious Diseases and Veterinary Public Health* 50, 352-356.

Muriana,P.M., Hou,H.Y. and Singh,R.K. (1996) A flow-injection system for studying heat inactivation of *Listeria monocytogenes* and *Salmonella enteritidis* in liquid whole egg. *Journal of Food Protection* 59, 121-126.

Nutsch,A.L., Phebus,R.K., Riemann,M.J., Kotrola,J.S., Wilson,R.C., Boyer,J.E. and Brown,T.L. (1998) Steam pasteurization of commercially slaughtered beef carcasses: Evaluation of bacterial populations at five anatomical locations. *Journal of Food Protection* 61, 571-577.

Nutsch,A.L., Phebus,R.K., Riemann,M.J., Schafer,D.E., Boyer,J.E., Wilson,R.C., Leising,J.D. and Kastner,C.L. (1997) Evaluation of a steam pasteurization process in a commercial beef processing facility. *Journal of Food Protection* 60, 485-492.

Paoli,G.C., Bhunia,A.K. and Bayles,D.O. (2005). *Listeria monocytogenes*. In: Fratamico,P.M., Bhunia,A.K. and Smith,J.L. Foodborne pathogens. Caister Academic Press. UK, pp. 295-325

Patel,J.R. and Beuchat,L.R. (1995) Evaluation of Enrichment Broths for Their Ability to Recover Heat-Injured *Listeria-Monocytogenes. Journal of Applied Bacteriology* 78, 366-372.

Pauly,T.M. and Tham,W.A. (2003) Survival of *Listeria monocytogenes* in wilted and additive-treated grass silage. *Acta Veterinaria Scandinavica* 44, 73-86.

Peleg,M. and Cole,M.B. (1998) Reinterpretation of microbial survival curves. *Critical Reviews in Food Science and Nutrition* 38, 353-380.

Phebus,R.K., Nutsch,A.L., Schafer,D.E., Wilson,R.C., Riemann,M.J., Leising,J.D., Kastner,C.L., Wolf,J.R. and Prasai,R.K. (1997) Comparison of steam pasteurization and other methods for reduction of pathogens on surfaces of freshly slaughtered beef. *Journal of Food Protection* 60, 476-484.

Poysky,F.T., Paranjpye,R.N., Peterson,M.E., Pelroy,G.A., Guttman,A.E. and Eklund,M.W. (1997) Inactivation of *Listeria monocytogenes* on hot-smoked salmon by the interaction of heat and smoke or liquid smoke. *Journal of Food Protection* 60, 649-654.

Ofstad,R., Kidman,S., Myklebust,R., Olsen,R.L. and Hermansson,A.M. (1995). *Academic Press Limited* 173-183.

Olsen,Ø. (2010). Isothermal heat treatment of *Listeria innocua* in capillary tubes. Master thesis. University in Stavanger, Norway, 43 p.

Quintavalla,S. and Campanini,M. (1991) Effect of Rising Temperature on the Heat-Resistance of *Listeria-Monocytogenes* in Meat Emulsion. *Letters in Applied Microbiology* 12, 184-187.

Riedo,F.X., Pinner,R.W., Tosca,M.D., Cartter,M.L., Graves,L.M., Reeves,M.W., Weaver,R.E., Plikaytis,B.D. and Broome,C.V. (1994) A Point-Source Foodborne Listeriosis Outbreak -

Documented Incubation Period and Possible Mild Illness. *Journal of Infectious Diseases* 170, 693-696.

Robinson,T.P., Ocio,M.J., Kaloti,A. and Mackey,B.M. (1998) The effect of the growth environment on the lag phase of *Listeria monocytogenes. International Journal of Food Microbiology* 44, 83-92.

Rocourt, J. (1999) The Genus *Listeria* and *Listeria monocytogenes*: Phylogenetic Position, Taxonomy, and Indentification. In *Listeria, Listeriosis and Food Safety* ed. Ryser,E.T. and Marth,E.H. pp. 1-20.

Rocourt,J., BenEmbarek,P., Toyofuku,H. and Schlundt,J. (2003) Quantitative risk assessment of *Listeria monocytogenes* in ready-to-eat foods: the FAO/WHO approach. *Fems Immunology and Medical Microbiology* 35, 263-267.

Rorvik,L.M. (1995b) Contamination pattern of *Listeria monocytogenes* and other *Listeria* spp. in a salmon slaughterhouse and smoked salmon processing plant. *International-Journal-of-Food-Microbiology; 25 (1) 19-27, 17 ref.*

Rorvik,L.M. (1995a) Contamination pattern of *Listeria monocytogenes* and other *Listeria* spp. in a salmon slaughterhouse and smoked salmon processing plant. *International-Journal-of-Food-Microbiology; 25 (1) 19-27, 17 ref.*

Rørvik,L.M., Aase,B., Alvestad,T. and Caugant,D.A. (2000) Molecular epidemiological survey of *Listeria monocytogenes* in seafoods and seafood-processing plants. *Applied and Environmental Microbiology* 66, 4779.

Ross,T., Dalgaard,P. and Tienungoon,S. (2000) Predictive modelling of the growth and survival of *Listeria* in fishery products. *International Journal of Food Microbiology* 62, 231-245.

Rowan,N.J. and Anderson,J.G. (1998) Effects of above-optimum growth temperature and cell morphology on thermotolerance of *Listeria monocytogenes* cells suspended in bovine milk. *Applied and Environmental Microbiology* 64, 2065-2071.

Ryser,E.T. (1991) *Listeria*, listeriosis, and food safety. *vii + 632pp. ISBN 0-8247-8480-4, many ref.*

Schlech,W.F. (2000) Foodborne listeriosis. *Clinical Infectious Diseases* 31, 770-775.

Schmid,M.W., Ng,E.Y.W., Lampidis,R., Emmerth,M., Walcher,M., Kreft,J., Goebel,W., Wagner,M. and Schleifer,K.H. (2005) Evolutionary history of the genus *Listeria* and its virulence genes. *Systematic and Applied Microbiology* 28, 1-18.

Schuchat,A., Swaminathan,B. and Broome,C.V. (1991) Epidemiology of Human Listeriosis. *Clinical Microbiology Reviews* 4, 169-183.

Seeliger,H.P.R. (1981) Non-Pathogenic *Listeriae* - *Listeriae-Innocua* Sp-N (Seeliger et Schoofs, 1977). *Zentralblatt fur Bakteriologie Mikrobiologie und Hygiene Series A-Medical Microbiology Infectious Diseases Virology Parasitology* 249, 487-493.

Skåra,T., Rosnes,J.T. and Sivertsvik,M. (2002) *Food Technology International* 2, 75-76.

Smith,J.L. and Archer,D.L. (1988) Heat-Induced Injury in *Listeria-Monocytogenes*. *Journal of Industrial Microbiology* 3, 105-110.

Smith,J.L. and Marmer,B.S. (1991) Temperature Shift Effects on Injury and Death in *Listeria-Monocytogenes* Scott-A. *Journal of Food Safety* 11, 73-80.

Stephens,P.J., Cole,M.B. and Jones,M.V. (1994) Effect of Heating Rate on the Thermal Inactivation of *Listeria-Monocytogenes*. *Journal of Applied Bacteriology* 77, 702-708.

Sumner,S.S., Sandros,T.M., Harmon,M.C., Scott,V.N. and Bernard,D.T. (1991) Heat-Resistance of *Salmonella-Typhimurium* and *Listeria-Monocytogenes* in Sucrose Solutions of Various Water Activities. *Journal of Food Science* 56, 1741-1743.

Swaminathan,B. (2001). *Listeria monocytogenes.* In: Doyle,M.P., Beuchat,L.R. and Montville,T.J. (eds). Food Microbiology: Fundamentals and Frontiers, 2nd ed, ASM Press, Washington, pp. 383-409.

Tienungoon,S. (1998). Some aspects of the ecology of *Listeria monocytogenes* in Salmonid Aquaculture, Ph.D. Thesis. University of Tasmania, Hobart, Australia, 286 p.

Thevenot,D., Dernburg,A. and Vernozy-Rozand,C. (2006) An updated review of *Listeria monocytogenes* in the pork meat industry and its products. *Journal of Applied Microbiology* 101, 7-17.

Thompson,W.S., Busta,F.F., Thompson,D.R. and Allen,C.E. (1979) Inactivation of *Salmonellae* in Autoclaved Ground-Beef Exposed to Constantly Rising Temperatures. *Journal of Food Protection* 42, 410-415.

Tsuchido,T., Takano,M. and SHIBASAK.I (1974) Effect of Temperature-Elevating Process on Subsequent Isothermal Death of Escherichia-Coli K-12. *Journal of Fermentation Technology* 52, 788-792.

Valdramidis,V.P., Belaubre,N., Zuniga,R., Foster,A.M., Havet,M., Geeraerd,A.H., Swain,M.J., Bernaerts,K., Van Impe,J.F. and Kondjoyan,A. (2005) Development of predictive modelling approaches for surface temperature and associated microbiological inactivation during hot dry air decontamination. *International Journal of Food Microbiology* 100, 261-274.

Valdramidis,V.P., Geeraerd,A.H., Gaze,J.E., Kondjoyan,A., Boyd,A.R., Shaw,H.L. and Impe,J.F.V. (2006) Quantitative description of *Listeria monocytogenes* inactivation kinetics with temperature and water activity as the influencing factors; model prediction and methodological validation on dynamic data. *Journal of Food Engineering* 76, 79-88.

van Boekel,M.A.J.S. (2002) On the use of the Weibull model to describe thermal inactivation of microbial vegetative cells. *International Journal of Food Microbiology* 74, 139-159.

Walker,S.J., Archer,P. and Banks,J.G. (1990) Growth of *Listeria-Monocytogenes* at Refrigeration Temperatures. *Journal of Applied Bacteriology* 68, 157-162.

Weis,J. and Seeliger,H.P.R. (1975) Incidence of *Listeria-Monocytogenes* in Nature. *Applied Microbiology* 30, 29-32.

Whyte,P., McGill,K. and Collins,J.D. (2003) An assessment of steam pasteurization and hot water immersion treatments for the microbiological decontamination of broiler carcasses. *Food Microbiology* 20, 111-117.

WHO, (2004). Risk Assessment of *Listeria monocytogenes* in Ready-to-Eat Foods. In: Microbiological Risk Assessment Series, No.5. Technical Report ISBN 92 4 1562625 (http://www.who.int/foodsafety).

Xiong,R., Xie,G., Edmondson,A.E. and Sheard,M.A. (1999) A mathematical model for bacterial inactivation. *International Journal of Food Microbiology* 46, 45-55.