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MASTER'S THESIS

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Abstract

This master thesis is a part of the project “KonSpor” (Control of spore-forming bacteria in food with medium and long durability, project number 10415) financed by The Norconserv Foundation. The main objective of this study was to investigate if changes in NaCl concentration would affect the inactivation kinetics of *Clostridium sporogenes* spores in water and different food matrixes.

Clostridium botulinum is a Gram positive, spore forming and anaerobic bacterium that causes botulism. *C. botulinum* is ubiquitous in the environment and can be found in food products that are not properly handled and stored. At suitable environmental conditions *C. botulinum* can grow in foods and produce the dangerous botulinum neurotoxin. *C. sporogenes* is used as a surrogate organism for proteolytic *C. botulinum*. *C. sporogenes* is a spore forming, anaerobic bacterium in the same genus as *C. botulinum*. It is a non-pathogenic bacterium that is often used to investigate the inactivation of *Clostridium* spores in food products.

In this thesis the inactivation kinetics of *C. sporogenes* was investigated by different methods. The capillary tube method allows rapid heating and cooling and was used to find D-values of the spores at different temperatures in water. The effect of NaCl concentrations on the inactivation of spores was also investigated. A self developed method at laboratory scale was used to investigate the inactivation of *C. sporogenes* in minced meat with and without added NaCl. Thermal food processing was used to inactivate *C. sporogenes* spores in liver pate by autoclaving at two different temperatures. The log-reduction of the spores after autoclaving was determined. Germination experiments were also performed using Bioscreen C. Different germinators (L-alanine and Inosine) and NaCl concentrations were applied to *C. sporogenes* spores to investigate the germination process.

The heat tolerance of *C. sporogenes* CIP 104607 in this thesis was lower than the average heat tolerance of *C. sporogenes* reported in other studies. The temperatures used in the experiments were between 91 - 97 °C. The D-value at 97 °C varied from 0.53 min in water to 5.33 min in minced meat as a food matrix with no NaCl added. From 0 to 0.9 % NaCl there was a decrease in the inactivation (increase in D-value) of *C. sporogenes* spores both in water and in the minced meat. NaCl did not increase the inactivation of *C. sporogenes* in

water or in minced meat. The inactivation experiments in water without added NaCl showed decreasing D-values with increasing temperatures. The z-value for *C. sporogenes* CIP 104607 calculated in this thesis was 9.86 °C. In the germination experiments the *C. sporogenes* spores were completely germinated under aerobe conditions when L-alanine as germinator and 2.5 or 5.0 % NaCl were combined.

Table of content

Abbreviations

a_w	Water activity
BoNT	Botulinum neurotoxin
CFU	Colony forming units
DNA	Deoxyribonucleic acid
D-value	Decimal reduction time
F_0	Integrated process lethality
kDa	kilo Dalton
milliQ water	Ultrapure water (trademark)
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
OD	Optical density
PBS	Phosphate buffered saline
PCA	Plate count agar
R^2	Coefficient of regression
RCA	Reinforced clostridial agar
RCM	Reinforced clostridial medium
RNA	Ribonucleic acid
rpm	Rotations per minute
SM	Sporulation medium
TSA	Tryptone soya agar
UV	Ultra violet
z-value	Thermal resistance constant

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1 Introduction

Clostridium botulinum is a pathogenic bacterium that produces one of the most toxic, naturally occurring compounds known, a neurotoxin that causes botulism. A dose of 30 - 100 ng is the estimated amount needed to cause severe illness and even death in humans (Cooksley et al., 2010). All strains of *C. botulinum* can form highly resistant endospores. Under favorable conditions they can germinate to produce vegetative cells that multiply to form the very potent botulinum neurotoxin (Brunt et al., 2016). *C. botulinum* is ubiquitous in the environment and it can also be transferred to food and cause illness if the food is not properly treated (Hill et al., 2007). It is a strict anaerobic bacterium, i.e. it will only grow in the absence of oxygen and with a low redox potential.

C. botulinum is the main pathogen of concern in low-acid canned foods. It is a challenge in the food industry because it may sustain heat -and chemical treatments. The main goal for the low-acid food canning industry is to destroy this pathogen and prevent botulinum toxin formation (Reddy et al., 2016). Proteolytic *C. botulinum* is a mesophilic bacterium that forms highly heat resistant spores. A heat treatment at 121 °C for 3 min called the “botulinum cook” is given to low acid canned food and is constructed to inactivate these heat resistant spores (Brunt et al., 2016).

Different methods are used to control pathogens and bacterial contamination in foods. Some of these methods are heating, freezing, high hydrostatic pressure, fermentation, irradiation, freeze-drying, drying and addition of antimicrobials and chemicals. These treatments may have various impacts on the individual bacteria. Some may be killed, some sub lethally injured, while others may survive without injury (Wu, 2008). To ensure “commercial sterility” low acid shelf stable foods are thermally processed. This requires sufficient heat treatment (above 100 °C) at every point of the container. When products are heat treated in an autoclave the temperature in the autoclave is not equal to the temperature in the food core. Therefore the temperature in the autoclave as well as in the food core is closely monitored to make sure that the food core is sufficiently heated. This results in a total inactivation of vegetative cells and partial or total inactivation of spores (André et al., 2013).

C. sporogenes is commonly used as a non-toxic surrogate for proteolytic *C. botulinum* to validate the effectiveness of different food preservation processes to inactivate *C. botulinum* (Mah et al., 2008). Spores of *C. sporogenes* have sustained as a model organism for proteolytic *C*. *botulinum* (Byun et al., 2011). *C. sporogenes* is a non-pathogenic, putrefactive, spore-forming anaerobe that can cause food spoilage. *C. sporogenes* is in the same genus as *C. botulinum* and they have a high physiological and morphological relatedness, i.e. they are very similar except *C. sporogenes* does not produce the botulinum neurotoxin (Reddy et al., 2016). This means that *C. sporogenes* is a non-pathogenic bacterium and can therefore be used as a test organism at the laboratory. It is particularly important to study the spores because of their high heat tolerance. *C. sporogenes* spores are known to have a slightly higher heat tolerance than *C. botulinum* spores. In this thesis the inactivation kinetics and germination of *C. sporogenes* are further investigated.

1.1 Aim of the study

The purpose of this study was to investigate the inactivation kinetics of *C. sporogenes* variant CIP 104607 from laboratory scale to food production scale and to calculate the D -and z-values, by determining the appropriate inactivation time and temperature. Specific NaCl concentrations were applied to the *C. sporogenes* spores for the inactivation experiments both in water and in meat products. The germination process of *C. sporogenes* spores at different conditions has also been investigated. The inactivation experiments of *C. sporogenes* were performed to achieve a better understanding of the properties of *C. sporogenes* spores in food with specific additives, thereby indicating the inactivation effect on the proteolytic *C. botulinum* spores.

2 Theory

2.1 *C. botulinum*

C. botulinum (non-proteolytic) was first discovered in 1895 by Emile van Ermengem. He isolated the bacterium from a botulism outbreak in Belgium where 31 musicians became ill and three of them died. The organism was first named *Bacillus botulinus*, but it was later discovered that it was an anaerobic spore-forming rod and was therefore assigned to the genus *Clostridium* (Devriese, 1999).

2.1.1 *C. botulinum* characteristics

C. botulinum belongs to the genus *Clostridium* and is a Gram positive, rod shaped, motile and spore forming bacterium. It is strictly anaerobic i.e. the presence of oxygen is harmful for the bacterium. *C. botulinum* is ubiquitous in the environment and is often found in soil and sediments (Lindstrom et al., 2006).

C. botulinum consists of approximately 40 different subtypes and is divided into a total of seven neurotoxin variants (A - G) (Brunt et al., 2016). These variants are again placed into four subgroups, I to IV based on different serological and phenotypic characteristics. Group I and II are known to cause disease in humans (Brunt et al., 2016; Hill et al., 2007; Lindstrom et al., 2006; Lövenklev et al., 2004; Stringer et al., 2013). Group III can cause disease in animals. Group IV are not shown to cause disease in humans or animals (Lindstrom et al., 2006). In this thesis it is focused on group I because *C. sporogenes* is a surrogate for this group and it is human pathogenic.

Group I is the proteolytic variant that consists of type A, B and F neurotoxins. The strains of group I have an optimal growth temperature of 35 - 37 °C, but they do not grow below 10 °C i.e. they are mesophilic (Stringer et al., 2013). The spores of group I are highly heat resistant ($D_{112°C}$ = 1.23 min), the minimum pH of growth is 4.6 and the maximum NaCl concentration of growth is 10 % in brine (Collins and East, 1998).

Group II is the non-proteolytic variant that consists of type B, E and F neurotoxins. The strains of group II have an optimal growth temperature of 18 - 25 °C and a minimum growth temperature of 3.3 °C which makes them psychrotrophic (Collins and East, 1998). The minimum pH for growth is approximately 5 and the maximum NaCl concentration is 5 % in

brine. The spores of group II are less heat resistant ($D_{80°C}$ = 0.6 - 1.25 min) than those of group I, but they may still survive some of the heat processes in the food industry (Table 1) (Collins and East, 1998; Lindstrom et al., 2006).

Table 1: Grouping and characteristics of *C. botulinum* strains (Collins and East, 1998).

Characteristics	Group			
	I	II	III	IV
Neurotoxin types	A, B, F	B, E, F	C, D	G
Proteolysis	+	-	-	+
Minimum growth temperature	10 °C	3.3 °C	15 °C	ND*
Optimum growth temperature	35 - 40 °C	18 - 25 °C	40 °C	37 °C
Minimum pH for growth	4.6	Ca. 5	ND*	ND*
Inhibitory [NaCl]	10 %	5 %	ND*	ND*
Minimum a_w for growth	0.94	0.97	ND*	ND*
$D_{100°C}$ of spores	25 min	< 0.1 min	0.1-0.9 min	0.8-1.12 min
$D_{121°C}$ of spores	0.1-0.2 min	< 0.001 min	ND*	ND*
Apathogenic model organism	*C. sporogenes*	No specific organism	*C. novyi*	*C. subterminale*

*ND, not determined

2.1.2 The botulinum neurotoxin

Strains of *C. botulinum* produce highly potent neurotoxins (BoNT) that are known to cause neuroparalytic disease in humans and animals (Hill et al., 2007; Lindstrom et al., 2006). This disease is called botulism and can cause death in as small doses as 30 - 100 ng if not treated appropriately (Peck et al., 2011). Botulism leads to respiratory muscle paralysis. Types A, B, E and F neurotoxins are involved in human botulism. Types C and D neurotoxins are involved in animal botulism (Table 2) (Collins and East, 1998). There are four types of botulism: Food borne botulism, infant botulism, wound botulism and adult infectious botulism.

The botulinum neurotoxin (serotypes A - G) is a protein (150 kDa) that consists of a heavy chain and a light chain. The size of the heavy chain is 100 kDa and the size of the light chain is 50 kDa. They are linked together by a disulfide bond (Kumar et al., 2014). Botulism is rare, but each case is considered a public health emergency (Bassi et al., 2013; Collins and East, 1998; Peck et al., 2011).

Table 2: Diseases and toxin types from *C. botulinum* (Collins and East, 1998).

Toxigenic types	Species mainly affected	Commonest vehicles
A	Man (also wound and infant botulism), chickens ("limber neck")	Home-canned vegetables, fruits, meat and fish
B	Man (also wound and infant botulism), horses and cattle	Prepared meats, especially pork products
C_α	Aquatic birds (western duck sickness)	Rotting vegetation of alkaline marshes, invertebrates
C_β	Cattle (midland cattle disease), horses (forage poisoning)	Toxic food, carrion, pork liver
D	Cattle (lamziekte)	Carrion
E	Man, fish, aquatic birds	Marine products and fish products
F	Man (also infant botulism)	Meat products
G	unknown	Soil

C_α strains produce C_1, a neurotoxin, and C_2, which is not a neurotoxin but is lethal in mice, ducks and geese; congestion and hemorrhage in the lung and dripping of the nares are observed in birds injected with C_2 toxin, but paralytic signs of botulism are absent. C_β strains produce only C_2 toxin.

2.1.3 Food borne botulism

Food borne botulism occurs when preformed neurotoxins in food are ingested which then leads to intoxication (Lindstrom et al., 2006; Peck et al., 2011). In order to minimize the number of botulism cases it is necessary to achieve a better understanding of *C. botulinum* growth and inactivation (Peck et al., 2011).

Food borne illnesses such as botulism have probably been present since foods were first preserved and stored. The traditional preservation methods rely on inactivation of microorganisms. The microbial safety is assured by increasing the process intensity which leads to decrease in nutritional value and sensory properties (Reineke et al., 2013). *C. botulinum* is the main pathogen of concern in low-acid shelf stable foods. It is a challenge in the food industry because it may sustain heat -and chemical treatments (Hassan and Ramaswamy, 2011; Liato et al., 2015; Zhu et al., 2008). Shelf stable foods are foods that have been sufficiently processed and can safely be stored at room temperature in a sealed container, e.g. canned food.

Food borne botulism is rare, but may be a problem in both home -and industrial scale canned food if the thermal food process is insufficient ($< F_0 = 3$) (Liato et al., 2015). Safety during shelf life storage and quality preservation is the main targets of food processing (Hassan and Ramaswamy, 2011; Sevenich et al., 2015). To obtain a fresher appearance of

the food some products are given a lower heat treatment and rely on the packaging and refrigerated storage temperature to prevent potential *C. botulinum* spores from activating and growing. This can lead to botulinum toxin production if temperature abuse occurs (Ishimori et al., 2012).

2.2 Growth factors of proteolytic (group I) *C. botulinum*

Proteolytic *C. botulinum* requires certain physical and environmental conditions for survival and growth. These conditions are divided into intrinsic and extrinsic factors.
Intrinsic factors include low salt concentration, fat content, high water activity (a_w) and pH. Extrinsic factors on the other hand are moderate temperature and absence of oxygen.

2.2.1 Temperature

C. botulinum are known to be mesophilic with a minimum growth temperature of 10 °C and an optimum growth temperature of 35 - 40 °C (Collins and East, 1998). Growth of *C. botulinum* slows down at 47.5 °C and at 50 °C no growth is found (Ohye and Scott, 1953; Plowman and Peck, 2001).

Wachnicka *et al.* (2016) has reported large differences in the D -and z-values for the different strains of *C. sporogenes* and *C. botulinum*. There are differences in the inactivation temperature of the different spores from different strains and also differences within the same strain. According to a meta study performed by Diao *et al.* (2014) the D-value at 121.1 °C of *C. botulinum* is reported to be 0.19 min, a little lower than for *C. sporogenes*.

2.2.2 pH

The minimum pH of growth for proteolytic *C. botulinum* is 4.6. A pH of 4.6 has been set as a boundary between high acid and low acid foods in the industrial food processing (FDA, 2017). The maximum pH for growth of *C. botulinum* is 9 (FDA, 2012). The optimum pH for germination is 5.5 - 8 (Plowman and Peck, 2001). The optimum pH for sporulation is approximately 7 (Stumbo, 1973).

2.2.3 Water activity (a_w)

The water activity of a product is closely connected to its salt concentration. When the salt concentration increases the water activity decreases. As the water activity decreases, the inhibition of *C. botulinum* increases (Baird-Parker and Freame, 1967). Proteolytic *C.*

botulinum has a minimum water activity of 0.94 for growth (Graham et al., 1997). The lower limit for toxin production for *C. botulinum* is 0.93 - 0.95 (McClure et al., 1994). The maximum NaCl concentration for growth of proteolytic *C. botulinum* is 10 % in brine (Collins and East, 1998). Other methods to reduce the water activity in food is to dry the food. Drying has been used as a preservation method for hundreds of years. To add sugar is also used to reduce the water activity. This works in the same manner as adding salt to the food; it will bind to the water making the water unavailable for the bacteria.

2.2.4 Atmosphere

C. botulinum is a strict anaerobic bacterium, i.e. it cannot grow in the presence of oxygen. A redox potential of -300 mV is considered strictly anaerobic conditions, but some anaerobic spore forming bacteria may sporulate when the redox potential is higher than -300 mV. At 100 % CO_2 *C. botulinum* has a slower growth rate and grows over a narrower range of conditions compared to a hydrogen/nitrogen or a hydrogen/nitrogen/carbon dioxide atmosphere. E.g. at 10 °C with 1.5 % NaCl at pH 6, the time variation to achieve visible turbidity is 21 - 29 days under 100 % CO_2, 14 days under hydrogen/ nitrogen (10:90) and 7 days under hydrogen/nitrogen/carbon dioxide (10:85:5) (Gibson et al., 2000).

2.2.5 Preservatives

There are different preservatives that are added to food today to extend the shelf life of food products. Preservatives can be divided into natural (e.g. salt, sugar, alcohol and vinegar) and chemical (e.g. nitrites, sorbates, benzoates and sulphites) compounds.

It is getting more and more common to reduce the NaCl content in food because of the unfortunate effect an excessive sodium intake can have on the body. This can be done by reducing the NaCl content or partially replace it by other chloride salts (e.g. potassium chloride). The addition of nitrates and nitrites to cured meat products reinforces the preservative effect of salts which effectively inhibits the growth and toxin formation of *C. botulinum* (Armenteros et al., 2012; Jackson et al., 2010). Most of the sodium nitrite in meat is in the form of nitric oxide or bound to large components like myoglobin, lipids or proteins, while the rest can be detected as residual nitrite in the meat product. It is uncertain if the microbial stability and safety of these products is a function of the initial or the residual nitrite (Redondo-Solano et al., 2013).

2.3 *C. sporogenes* as a test organism

C. sporogenes was first discovered by Metchnikoff (1908) as a part of the healthy environment of the human intestines and was considered non-pathogenic (Brown et al., 2011). The originally isolated strain PA 3679 is the most studied. The only known surrogate for proteolytic *C. botulinum* is *C. sporogenes*. This is why this organism is of such importance in research and laboratory work (Byun et al., 2011). *C. sporogenes* is a Gram positive, anaerobic, spore forming and motile bacteria. In studies of food sterilization, non-pathogenic *C. sporogenes* spores are often used. They have a high heat resistance similar to *C. botulinum* spores. *C. sporogenes* spores have approximately four times higher thermal resistance at 121 °C than *C. botulinum* spores, but they have a similar z-value of approximately 10 °C. (Ramaswamy et al., 2010)

C. sporogenes is genetically similar to the proteolytic *C. botulinum* (Bassi et al., 2013). Due to HSE (health, safety and environment) regulations many laboratories around the world are not classified to work with *C. botulinum*. *C. sporogenes* have very similar morphology as proteolytic *C. botulinum*, but it does not produce the botulinum neurotoxin, i.e. it is a non-pathogenic alternative to the proteolytic *C. botulinum (Bradbury et al., 2012; Brown et al., 2011)*. Classification of the different strains of *Clostridium* has previously been discussed and many strains are so similar that it is difficult to tell which species it should belong to (Brown et al., 2011). *C. sporogenes* and *C. botulinum* are heterogeneous, and individual members of *C. sporogenes* may be more similar to proteolytic *C. botulinum* than other *C. sporogenes* and vice versa (COLLINS et al., 1994; Dixit et al., 2005; Stringer et al., 2013). The genetic relatedness of the four *C. botulinum* groups suggests that they could have been different species. *C. botulinum* is considered to be a strict anaerobic spore-forming bacterium that produces one or more of the botulinum neurotoxins. The neurotoxin production is the main feature that distinguishes proteolytic *C. botulinum* from *C. sporogenes*. *C. sporogenes* consists of at least two variants (Brown et al., 2011). Spores of *C. sporogenes* are ubiquitous in the environment and *C. sporogenes* are usually involved in food spoilage and contamination (Bassi et al., 2013; Schill et al., 2015).

A recent phylogenetic study, using whole genome sequences (Weigand et al., 2015), indicates that a major cluster of strains within *C. botulinum* group I, can be distinguished from major clusters of *C. sporogenes*, which includes some *C. botulinum* virulent strains, like

Prevot 1662, Prevot 594, Osaka 05 and ATCC 51387 (Janganan et al., 2016). All toxin types of proteolytic *C. botulinum* are very similar (99.7 - 100 % 16S rRNA sequence identity). Together with *C. sporogenes* they form a single phylogenetic unit (Figure 1) (Bassi et al., 2013).

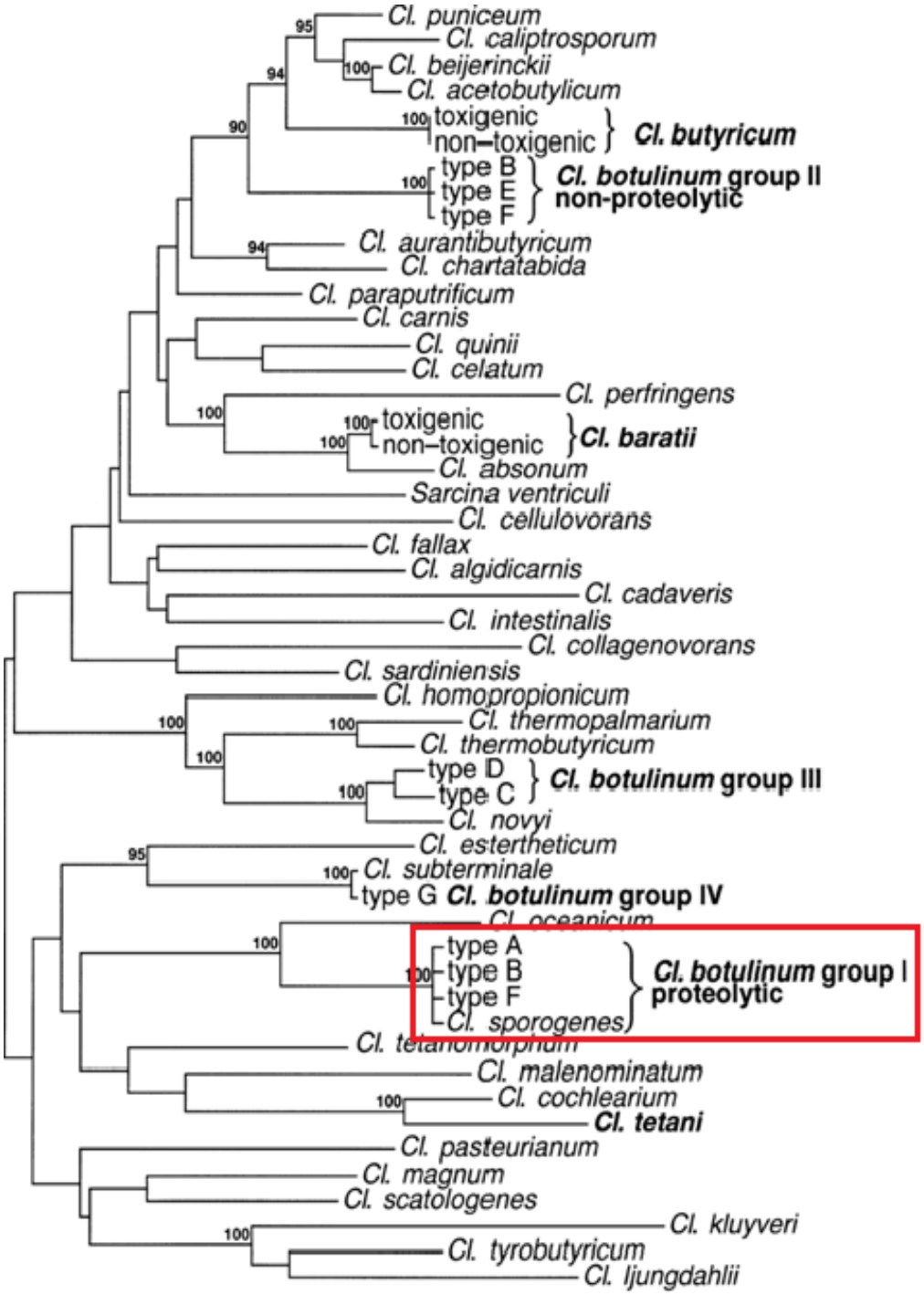

Figure 1: Dendrogram showing the phylogenetic position of the different *Clostridium* strains (Collins and East, 1998). The red rectangle represents the one phylogenetic unit including proteolytic *C. botulinum* and *C. sporogenes*.

2.4 *Clostridium* sporulation

Clostridium species (and other endospore forming bacteria) form highly heat resistant, dormant spores that ensures long time survival under non-optimal environmental conditions or starvation (Brunt et al., 2016; Dürre and Hollergschwandner, 2004; Lindstrom et al., 2006; Løvdal et al., 2013; Rodríguez-Lozano et al., 2010). This dormant state is metabolically inactive and highly resistant. The spores have a multilayered morphology, which can withstand multiple hostile conditions such as presence of oxygen, absence of nutrient, high temperature, pressure, irradiation, lytic enzymes and presence of antimicrobials and solvents. These conditions are lethal to the vegetative cells (Reineke et al., 2013; Talukdar et al., 2015; Yang et al., 2008). The fact that the spores of *C. sporogenes* and *C. botulinum* survive such harsh conditions is the main reason why they present a significant food safety issue (Brunt et al., 2015). Sporulation is a successful survival strategy because of the ability to exit dormancy and continue vegetative growth when favorable conditions re-appear (Løvdal et al., 2013).

All spore-forming bacteria have similar morphological stages during spore formation. A mother cell and a forespore are two forms present in the cell during a sporulation process. Seven stages (stage I-VII) are usually included to describe the total sporulation process. An additional stage 0 can be added as the growth of vegetative cells before sporulation. In the first two stages (I and II), the cell DNA discharges as an axial filament. Then two compartments form, the prespore and the larger mother cell resulted by asymmetric cell division. Initially, a third of the DNA is accumulated in the prespore, but the rest of the DNA is also pumped into the prespore by the action of DNA translocase. At stage III, the mother cell consumes the prespore and is now called a forespore, which has both inner and outer membranes and can float as a protoplast. During stage IV, the primordial germ cell wall and the cortex is synthesized by a peptidoglycan between the inner and outer membranes. The formation of a structure of proteins is formed at stage V. This protein complex is known as the spore-coat and is located on the surface of the forespore. At stage VI, the spore establishes its resistance to heat and UV radiation. During the final stage (VII), the mature spores are released from the mother cells and the mother cells are lysed (Figure 2) (Dürre and Hollergschwandner, 2004; Talukdar et al., 2015).

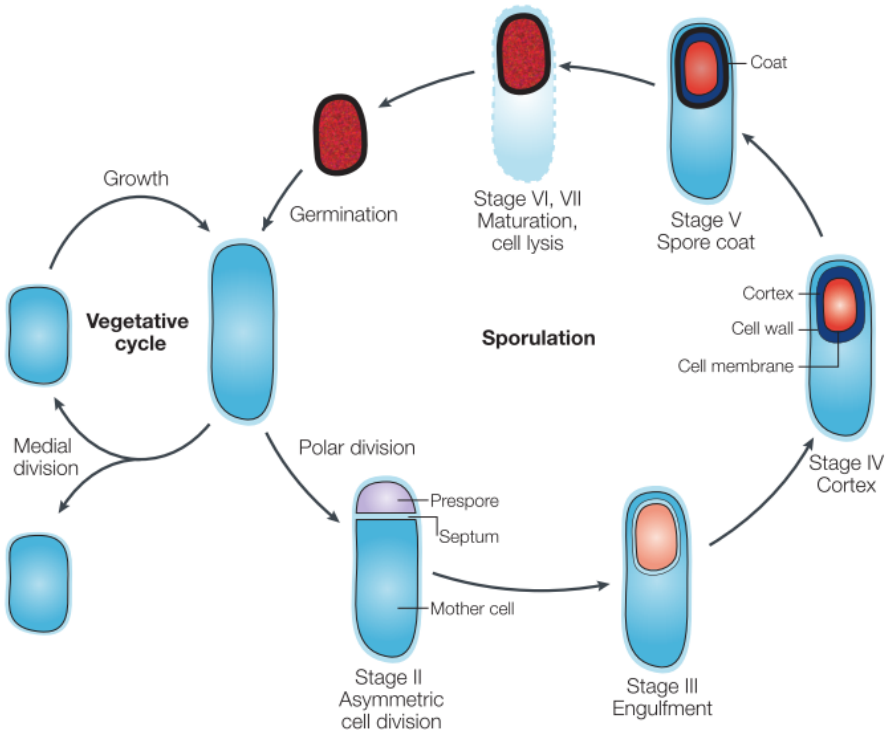

Figure 2: The key stages of the sporulation cycle (Errington, 2003).

Previously it has been assumed that endospore formation are identical in all genera that form spores, but later studies have shown a larger variation in endospore formation than originally believed (Dürre and Hollergschwandner, 2004). Sporulation of *Bacillus* and *Clostridium* is still considered very similar and the majority of sporulation studies are done on different *Bacillus* strains. Therefore most of the information found is describing *Bacillus* spore formation. However most clostridia undergo an additional step before sporulation; the clostridial step, where the cells become swollen, they look phase bright and somewhat cigar shaped (Dürre and Hollergschwandner, 2004).

Janganan *et al.* (2016) reported different features on the surface of *C. sporogenes* spores, e.g. a "beaded fibril" that has not been reported before, "hairy naps" and appendages. The exosporium which encircles the spores is more elongated at one pole and has a 'hairy nap' that emerges uniform (30 ± 5 nm deep) along the perimeter of the exosporium. Some studies carried out with *C. sporogenes* suggest that the exosporium may play a part in germination, outgrowth and attachment. The spore gets most of its protection from the proteinaceous spore coat, but the first part to be in contact with the environment is the exosporium. When the environmental conditions become favorable, activation and germination occurs, cell multiplication starts once again and the dormancy of the spore is

broken (Brunt et al., 2015). Studies done by Bassi *et al*. (2013) indicates that gene transcription entrance into sporulation of *C. sporogenes* may begin at the onset of vegetative cell growth after dormancy and germination, when all other living functions start.

2.5 *Clostridium* germination

When growth conditions become favorable the dormant spore goes through a number of steps before the exponential growth phase. The first of these steps are activation and germination, where the spore goes from being phase bright and dormant to phase dark and germinated when examined with a phase contrast microscope. The activation step leads the dormant spores to germinate when environmental conditions become favorable. Even though some aspects of spore activation are still unclear, it can be achieved by treatments like reducing agents, high or low pH, various chemicals, aging and exposure to sub lethal temperatures called heat activation (Brunt et al., 2015; Løvdal et al., 2013). Germination of *C. sporogenes* spores is probably more time consuming than germination of *Bacillus subtilis* spores, considering that Bassi *et al*. (2013) found over expressed genes in *C. sporogenes* 3 hours after onset of germination while *B. subtilis* starts to germinate after 5 min. One of the first events in the *C. sporogenes* germination process is calcium release as dipicolinic acid, a reduction in the optical density (OD) value and the loss of the spore's luminescence.

Even though *C. sporogenes* is an anaerobic bacterium it is possible to perform germination experiments on *C. sporogenes* spores in aerobe conditions. Plowman and Peck (2001) showed that germination of *C. sporogenes* in aerobe and anaerobe conditions gave very similar results. When performing germination experiments on *C. sporogenes* a mixture of L-lactate and L-alanine as germinators gives the most optimal results (highest percentage of germinated spores) (Brunt et al., 2014; Plowman and Peck, 2001). Amino acids are known to promote *Clostridium* spore germination (Ishimori et al., 2012). The germinators added can activate germination receptors in the spore committing it to go through an irreversible reaction, causing loss of heat resistance and peptidoglycan hydrolysis in the spore cortex (Figure 3) (Brunt et al., 2016; Plowman and Peck, 2001). Thereafter the spore cortex is degraded by cortex lytic enzymes, which allows further core hydration and expansion (Brunt et al., 2016).

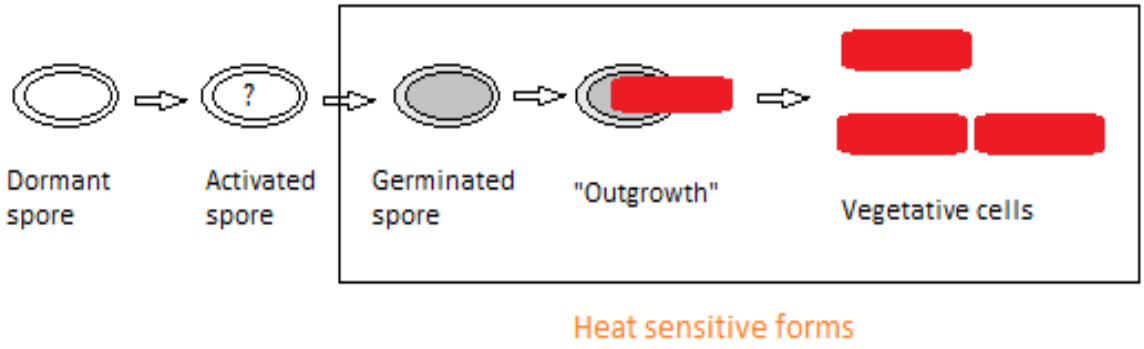

Figure 3: The transformation from dormant spore to vegetative cell. The heat sensitive forms of the bacteria will be inactivated by heat treatment (Løvdal, 2011).

2.5.1 Germination measurements

Germination of bacterial spores can be measured by different methods. Phase contrast microscopy and Bioscreen C are two of those methods. The Bioscreen C instrument measures turbidity (optical density) of solutions. It can incubate, shake and measure OD of samples for a long period of time. This method is fast, reliable and reduces the amounts of test materials required for each experiment (Johnston, 1998). Germination experiments using Bioscreen is described in Section 3.7. With a phase contrast microscope it is possible to see the difference between dormant spores and germinated spores. The dormant spores appear phase bright while the germinated spores appear phase dark (or grey) in the phase contrast microscope.

2.6 Inactivation kinetics

The decimal reduction time (D-value) can be described as the time required to inactivate 90 % of the bacteria at a certain temperature (Figure 4). The temperature increase required to achieve a 1-log reduction (inactivate 90 %) of spores at a specific heat treatment time is called the thermal resistance constant z (Figure 5) (Hassan and Ramaswamy, 2011). The D - and z-values are specific for each species of bacteria. The z-value is commonly referred to as 10 °C for *C. botulinum* and *C. sporogenes*, but recently an average z-value of 11.3 °C has been recorded in a meta study (Diao et al., 2014). No significant differences between the z-values of *C. sporogenes* and *C. botulinum* are established (Diao et al., 2014). The $D_{121.1°C}$ value for *C. sporogenes* is estimated to be 1.28 min. The $D_{121.1°C}$ value for *C. botulinum* is estimated to be 0.19 min in liquid medium and neutral pH (Brown et al., 2011; Diao et al., 2014). The D -and z-values can also vary within a species of bacteria with different extrinsic factors such as

growth medium, pH and water activity (Diao et al., 2014). The z-value for *C. sporogenes* 7955 in meat is estimated to be 10.1 °C (Hassan and Ramaswamy, 2011).

The target organism for shelf stable foods is proteolytic *C. botulinum* type A. The D-value for this organism is 0.21 min at 121 °C ($D_{121\,°C}$ = 0.21 min), which is the reference sterilization temperature used. The requirement is to have a 12-D reduction of bacteria in the product and 12-D will then be 2.52 min. The current heat treatment that shelf stabile foods receive is 3 min at 121 °C, which means that there is a safety margin for temperature variation in the autoclave. This time temperature combination corresponds to an integrated process lethality of 3 (F_0 = 3) (Sevenich et al., 2016).

The D-value can be calculated by plotting the difference between log concentration of spores against the heat treatment time or from the linear regression slope of log reduction and time. This curve is called a survivor curve:

D = (t2 – t1) / (Log N1 - Log N2) = -1 / slope

In this thesis the D-value was calculated from the linear regression slope of the survivor curve:

D=-1/slope

The z-value (thermal sensitivity parameter) can be calculated by plotting the Log D-value against temperature. This graph is known as a heat resistance curve:

Z = (t2 – t1) / (Log D1 – Log D2) = -1 / slope (Hassan and Ramaswamy, 2011; Liato et al., 2015)

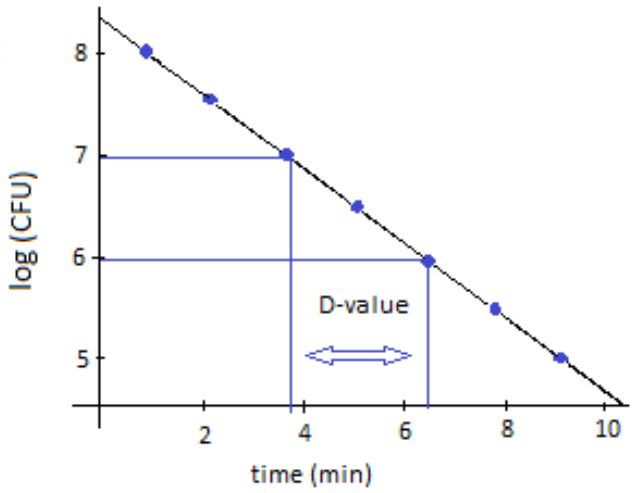

Figure 4: Inactivation kinetics representing D-value. The time required to reduce the bacterial numbers by 1-log unit at a specific temperature is represented.

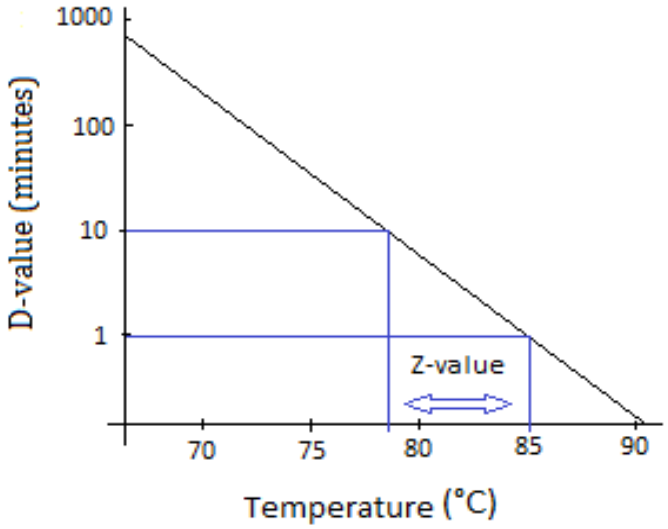

Figure 5: Inactivation kinetics representing z-value. The temperature increase required for a 1-log reduction in the D-value is represented.

2.6.1 Measurement of inactivation kinetics

There are different methods to measure the inactivation kinetics of microorganisms. Some of these methods are the capillary tube method, the spiral method and thermal food processing by autoclave (Stumbo, 1973). These methods can be divided into isothermal and non-isothermal processes. An isothermal process is a change of a system where the temperature remains constant ($\Delta T = 0$). In this thesis the capillary tube method and thermal food processing by autoclave was used.

The capillary tube method is an isothermal temperature measuring method. The thin glass walls of the capillary tubes have a high heat perforation and the narrow inner diameter of the tubes allows rapid heat exchange (Haas et al., 1996).The temperature of the spore-suspension inside the capillary tubes remains equal throughout the heat. Isothermal methods are often used to determine the D-values of microorganisms and to use these values for thermal food processing in the food industry.

Thermal food processing allows inactivation of microorganisms in food products by autoclaving. This is a non-isothermal process because the time required for the food core to reach the same temperature as the surroundings is longer. To ensure "commercial sterility" low acid shelf stabile foods are thermally processed. This requires sufficient heat treatment (above 100 °C) at every point of the container. When products are heat treated in an autoclave the temperature in the autoclave as well as in the food core is closely monitored to make sure that the food core is properly heated (**Feil! Fant ikke referansekilden.**). This results in a total inactivation of vegetative cells and partial or total inactivation of spores (André et al., 2013). The thermal food processing performed in this thesis had lower temperature than the reference sterilization process to monitor the partial inactivation of *C. sporogenes* spores in a meat product.

Low-acid shelf stabile foods are given the 12-D cook or the "botulinum cook". This is based on the kinetic parameters (D- and z-values). The increasing customer demand for high quality foods and a fresher appearance causes a challenge to meet the requirements of safe foods. Hurdle technology was developed to meet these demands. This approach is based on a combination of several hurdles (preservative factors) e.g. high or low temperature, low pH, low water activity (a_w), redox potential and competitive microorganisms at lower individual intensities. This makes the food treatment more gentle but effective without compromising on food safety (Jaeger et al., 2015; Liato et al., 2015; Ros-Chumillas et al., 2015; Sevenich et al., 2016).

3 Materials and methods

3.1 Test organism

The methods were based on previous work performed by Hossain (2015) and Yang (2010). In this thesis *C. sporogenes* variant CIP 104607 was used. The bacteria were stored in mikrobank at -80 °C prior to use. CIP 104607 was purchased from the Pasteur Institute, Paris. An overview of the different methods and experiments performed in this thesis is presented in Figure 6. *C. sporogenes* variant MF 02712 was used in some initial experiments in water while all the other experiments were concentrated on CIP 104607.

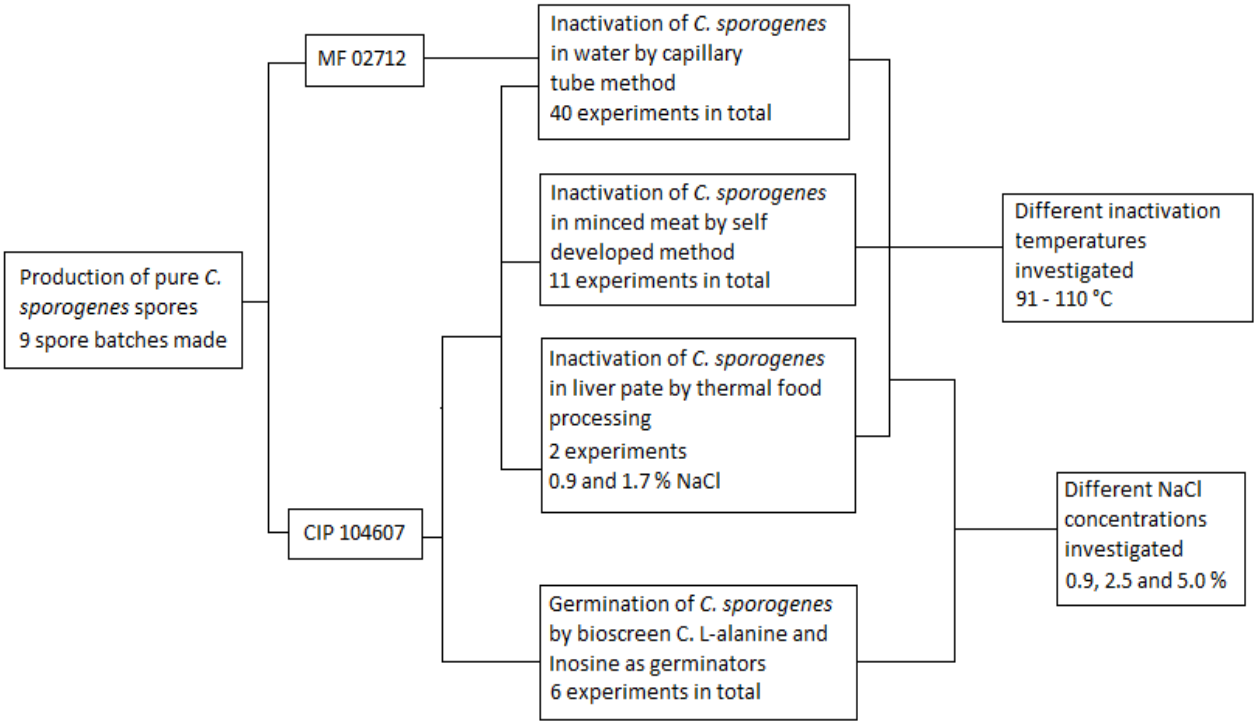

Figure 6: The different methods and experiments performed in this thesis

A total of nine spore batches were produced. Some of the spore batches were produced in different ways to investigate the effect of specific steps in the sporulation process. The two initial batches were made double where one part was the CIP 104607 variant and the other part was the MF 02712 variant. Spore batch two was made double to check the effect of the heat shock treatment prior to sporulation. Spore batch three was harvested and cleaned without adding lysozyme. Spore batches four and five were produced according to Yang (2010) (without any variables). Spore batch six was sporulated on RCA plates. Spore batches

seven to nine were produced in larger quantity (1.5 liters) than the other spore batches in order to obtain enough spores for the food production experiment.

Different *C. sporogenes* variants are deposited in different micro-banks and given new names in the specific banks in different countries, i.e. the CIP 104607 strain is in theory the same strain as the more known PA 3679 strain (Figure 7).

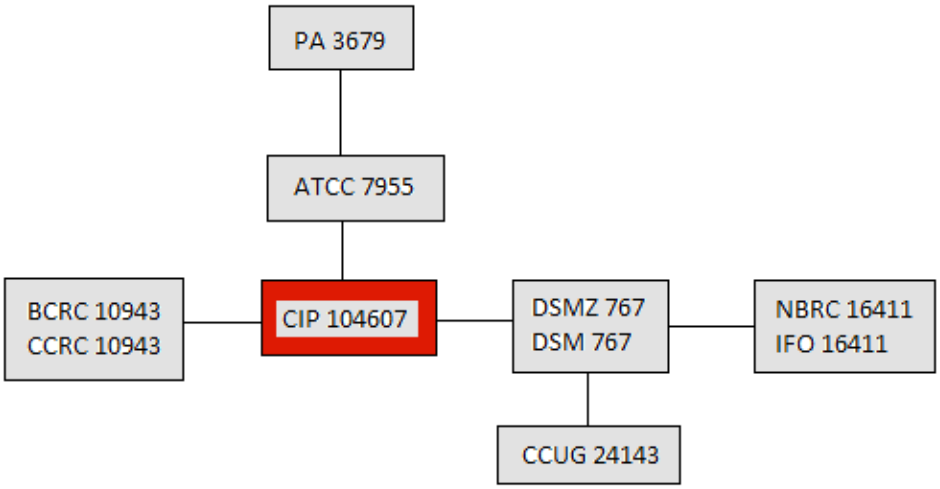

Figure 7: Relation between different *C. sporogenes* variants. CIP 104607 is marked with a red frame.

3.2 Storage

The stock microorganisms were stored frozen in tubes containing beads in glycerol (Figure 8 a**Feil! Fant ikke referansekilden.**) (Mikrobank®, Prolab Diagnostics, Wirral, U.K.). *C. sporogenes* CIP 104607 were grown on Reinforced Clostridial Agar (RCA) (CM0151, Oxoid, Basingstoke, UK) plates at 37 °C for 24 hours. Then 2 - 3 colonies from each dish were collected using a sterile plastic loop and inoculated in separate Mikrobank® tubes. The access fluid was removed and the tubes were stored at -80 °C prior to use.

3.3 Growth conditions and media

When a new sporulation process was started, one bead from the Mikrobank® tube was collected and inoculated in a sterile tube containing Reinforced Clostridial Medium (RCM) (CM0149, Oxoid). The tubes were incubated anaerobically at suboptimal temperature (30 °C) for 24 hours. Then 1 ml from these tubes was added to either 100 or 200 ml serum bottles containing 80 or 160 ml RCM respectively by a sterile syringe (Figure 8 b). These bottles were

also incubated at suboptimal temperatures until there were enough spores to start the sporulation process (approximately 10 %). All incubations were performed in jars containing an anaerobe atmosphere generation system (anaerogen, Thermo Scientific, Oxoid) or in sealed serum bottles. The headspace of the serum bottles were flushed immediately after autoclaving with gas (Aga AS, Oslo, Norway) containing 70 % N_2 and 30 % CO_2 until a redox potential of -110 mV or lower was obtained. To assure sterility in the bottles after autoclaving a 0.2 µm sterile filter was attached to the tube from the gas tank used for flushing. Resazurin was used as an indicator to demonstrate anaerobic conditions (Guerin et al., 2001). Resazurin is dark blue before boiling and pink after boiling. When the redox potential sinks below -110 mV resazurin becomes colorless.

The serum bottles (clear glass, Apodan Nordic, Copenhagen, Denmark) used was either 100 or 200 ml with a narrow neck. The bottles were tightly sealed with a rubber cork (20 mm bromobutyl rubber, Apodan Nordic, Copenhagen, Denmark) after flushing and then an aluminum capsule (model 2035P/7.5, Apodan Nordic, Copenhagen, Denmark) was attached to the bottle, over the rubber cork. The aluminum capsule has a hole in the middle that makes it possible to penetrate the rubber cork with a sterile syringe to add or remove content from the bottles after they had been sealed (Figure 8 b).

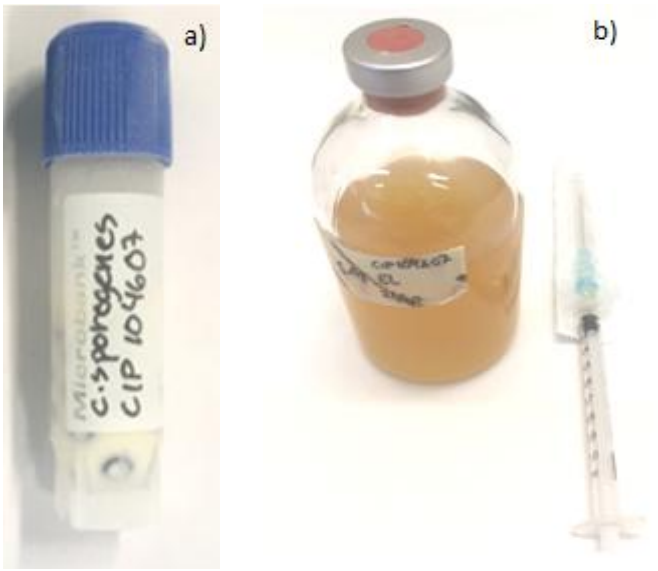

Figure 8: a) Mikrobank® containing *C. sporogenes* variant CIP 104607. b) Serum bottle containing *C. sporogenes*. Sterile syringes were used to inoculate the sporulation medium and also to take samples out of the bottle.

If not otherwise is stated, all media and chemicals used were prepared according to the label on the bottles. After each inactivation experiment the spores were 10 fold serial diluted in

0.1 % Peptone water and 100 µl of the diluted samples were spread on RCA plates and incubated anaerobically at 37 °C for 2 days.

3.4 Production of spores

Sporulation medium (SM) containing 3 % Tryptone Soya Broth (CM0129, Oxoid), 1 % Peptone Water (CM0009, Oxoid) and 1 % Ammonium Sulfate ($(NH_4)_2SO_4$) (A991417 808, Merck, Darmstadt, Germany) was used for the production of *C. sporogenes* spores (pH = 6.8 – 7.2) (Ponce and Yang, 2009). Resazurin (50 mM) (199303-5G, Sigma Aldrich, St. Louis USA) was added as an indicator to make sure the redox potential was below -110 mV in all the serum bottles containing SM. The bottles were flushed when the content was as warm as possible, immediately after autoclaving. The 200 ml serum bottles (containing 160 ml SM) were flushed (with N_2/CO_2 gas) for 6 - 7 min in order to achieve a redox potential below -110 mV (Figure 9 a-c).

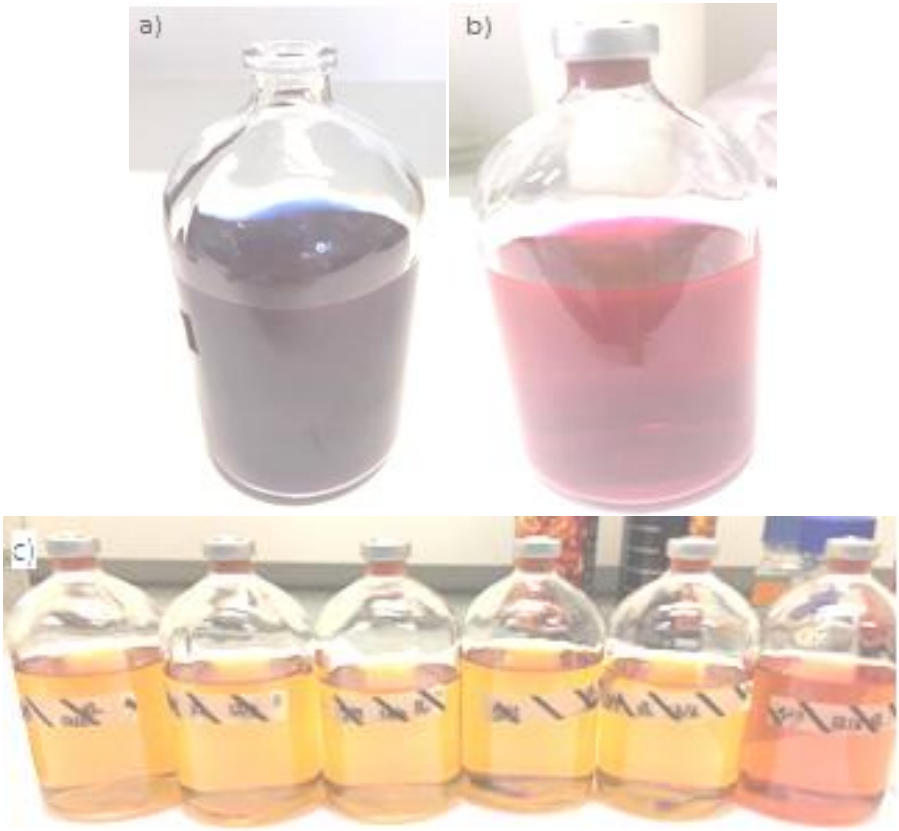

Figure 9: a) Serum bottle with resazurin in sporulation medium before autoclaving. b) Sealed serum bottle with resazurin and sporulation medium immediately after flushing and sealing. c) Serum bottles with resazurin 30 min. after sealing. They become more and more colorless indicating a redox potential of -110 mV or lower.

Then 1 ml of a 10 % inoculum from RCM was transferred to the serum bottles containing SM with a headspace of 70 % N_2 and 30 % CO_2. The SM inoculums were then heat shocked at 80 °C for 30 min and incubated at suboptimal temperature (30 °C) while shaking at 155 rpm on an orbital shaker (Infors HT Multitron, Basel, Switzerland) (Ponce and Yang, 2009). The culture was incubated for seven to ten days until > 90 % spores were present. The sporulation was monitored by phase contrast microscopy (LEICA 1000, Wetzler, Germany) at 400 magnifications each day. The dormant spores, which are desirable, appeared phase bright while the vegetative cells and the germinated spores appeared phase dark in the phase contrast microscope (Figure 10).

Figure 10: Spores of *C. sporogenes* at 400 magnifications. Dormant spores are phase bright (white) and the vegetative cells are phase dark (grey).

When the sporulation medium contained > 90 % spores the spores were harvested and cleaned. The first step was by centrifugation (Heraeus Multifuge X3 FR Centrifuge, Thermo Scientific, Osterode, Germany) at 15,300 x g for 10 min at 4 °C in 250 ml centrifugation tubes. After the supernatant was discarded the spore pellet was resuspended in autoclaved Phosphate Buffered Saline (PBS) (P4417-100TAB, Sigma Aldrich, St. Louis, USA) (pH = 7.0 - 7.2) and pooled in smaller centrifugation tubes (30 ml). Then they were centrifuged again at 19,630 x g for 10 min at 4 °C. The supernatant was discarded and the spore pellets were resuspended in PBS containing 500 µg/ml lysozyme (L6876-1G, Sigma Aldrich, Canada). Lysozyme helps to digest the vegetative cells making it easier to separate the spores from the cell debris. After addition of lysozyme, the spore-suspension was sonicated with an ultra

sonicator (Cole Parmer, Vernon Hills, United States) at 60 Hz for 8 min to release the spores from the vegetative cells, and then incubated at 37 °C for 2 hours for additional digestion of vegetative cell debris.

After the 2 hour incubation the spore-suspension was centrifuged at 1,860 x g for 20 min at 4 °C, the supernatant was discarded and the pellet was washed with water (autoclaved milliQ water). This washing was repeated 6 - 8 times until < 1 % vegetative cells remained. The percentage of vegetative cells remaining was monitored by counting with the phase contrast microscope after each centrifugation (Ponce and Yang, 2009). The pure spore-suspension (40 ml) was stored at refrigerated temperature (4 °C) until further experiments.

The concentration of spores was calculated using a Thoma cell counting chamber (Celeromics Technologies, Cambridge, UK). All spore batches were diluted to a final volume of 40 ml. The final concentration of CIP 104607 spores was 4 x 10^8 – 2 x 10^9 spores/ml in a 40 ml spore-suspension.

3.4.2 Calculation of spore concentration using Thoma cell counting chamber

The frame of the Thoma cell counting chamber contains a large central square (1 mm). This central square consists of 16 medium squares (0.25 mm) divided into 25 small squares (0.05 mm). When a sample is put under the cover slip, it reaches a height of 0.1 mm. The volume of the large square will be: 1 x 1 x 0.1 = 0.1 mm^3 = 10^{-4} ml. Four or five medium size squares (containing 25 small squares each) were counted with the phase contrast microscope (with 400 magnifications). The average of these were calculated and multiplied by 16 (because one large square contains 16 medium squares). If N cells are counted in one of the large squares, the concentration will be: N x 10^4 cells/ml. Prior to counting the sample was diluted and must also be multiplied by the dilution factor (f): N x 10^4 x f cells/ml. E.g. the sample were diluted hundred times and an average of 27 spores were counted in one medium square; N = 27 x 16 = 432 spores. 432 x 10^4 x 10^2 = 4.32x10^8 spores/ml.

3.5 Inactivation of *C. sporogenes* in water

Capillary tubes (inside diameter 1.7 mm) (200 µl color code red, Blaubrand® intraMARK, Wertheim Germany) were filled with 100 µl spore-suspension in water with the help of a sterile syringe (70mm) and sealed in both ends with a gas burner (Figure 11). The tubes were inverted to confirm that it was a closed system. (When the liquid inside did not move it was

closed.) All the tubes were immersed in ice water before the heat treatment to avoid germination.

Figure 11: Capillary tubes 200 µl.

The capillary tubes were stacked in a special rack with a magnetic rail to keep the racks in place in the water/oil bath and to make it easy to remove during the heat treatment. Each rack can hold three tubes and it also has an adjustable wire that can be tightened if necessary (Figure 12). The heat treatment was carried out in water bath when the temperature was ≤ 99 °C and in silicone oil bath when the temperature was ≥ 100 °C. The time intervals were 1 - 7 min, but adjustments were made depending on the expected inactivation rate. Longer treatment times were used at slow inactivation. The tubes were removed at different time intervals in triplicates. After removal, the tubes were rapidly cooled by immersing in ice-water.

The time required for the spore-suspension in the capillary tubes to reach the same temperature as in the water bath is approximately 3 sec. The spore-suspension inside the tube is evenly distributed in the tube with no air bubbles in between.

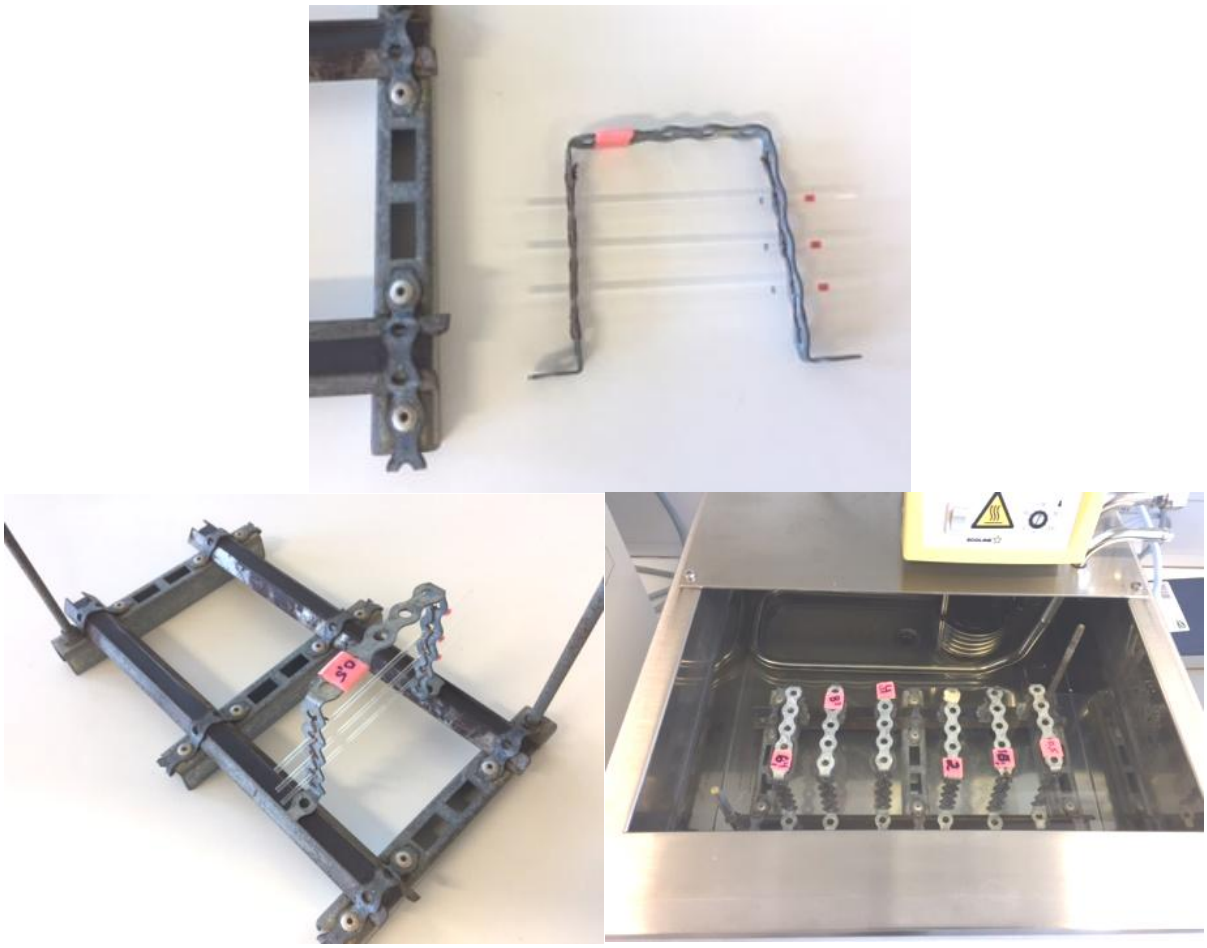

Figure 12: Rack on magnetic rails used to hold capillary tubes in water and oil bath. Each rack can hold three capillary tubes. The rack allows easy removal from the water bath.

After the heat treatment the tubes were surface sterilized by rinsing in 70 % ethanol and broken in one end. Sterile syringes were used to transfer the content from the tubes into an empty eppendorf tube. Then 50 µl of each sample of the spore-suspension were further used for dilutions.

After incubation all bacterial numbers were counted and the colony forming units per ml (CFU/ml) was determined (Figure 13). All experiments were performed with three parallel tubes. The number of culturable spores after specific times and temperature intervals were used to calculate D -and z-values.

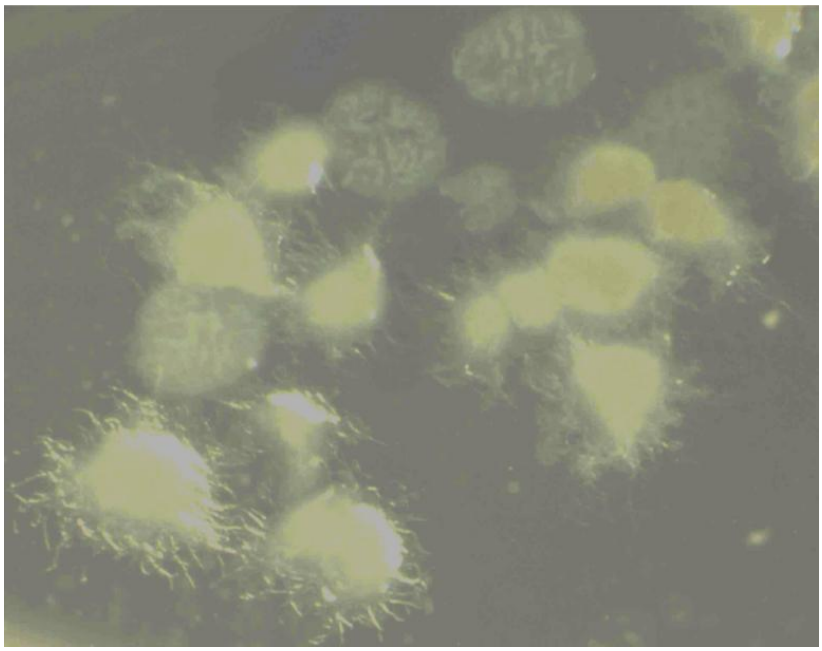

Figure 13: Colonies of *C. sporogenes* on TSA plates.

3.6 Inactivation of *C. sporogenes* in water with different NaCl concentrations

Three different NaCl concentration were tested; 0.9, 2.5 and 5.0 % NaCl. For each experiment 3 ml of spore-suspension were prepared with a specific percentage of NaCl (Table 3). These experiments were performed at 93 °C. They were incubated at 37 °C for 2 days. All other aspects of the experiments with NaCl were carried out the same way as in Section 3.5.

Table 3: The amount of NaCl added to achieve the specific NaCl concentrations in the spore-suspension.

Volume of spore-suspension (ml)	NaCl (%)	NaCl (mg)
3	0.9	27
3	2.5	75
3	5.0	150

3.7 Germination of *C. sporogenes*

Bioscreen C (OY Growth Curves Ab Ltd, Helsinki, Finland) was used to measure the OD of the spores in real time to study the germination process. The measurements were carried out every 10 min for a selected period (6 - 20 hours). The experiments were performed at 37 °C under aerobic conditions. The effect of only germinators and spores, the effect of

germinators in combination with different NaCl concentrations and the effect of a heat activation step (prior to the onset of the germination experiment) were tested. When different NaCl concentrations were investigated, the spores were prepared with NaCl according to Table 3. When heat activation prior to the germination experiment were investigated, the spores were heated at 60 °C for 15 min (Plowman and Peck, 2001). The germinators used were 1 M L-alanine (A7469-25G, Sigma, Aldrich, St. Louis USA) in Tris-NaCl buffer and 50 mM Inosine (I4125-10G, Sigma, Aldrich, China) in Tris-NaCl buffer.

Initially the spores were centrifuged at 6,000 x g for 10 min at 4 °C to wash away any potential germinators that could have occurred during storage. The supernatant was discarded and the spores were resuspended in 200 µl water. Then they were centrifuged again at the same conditions. The supernatant was discarded and the spores were resuspended in 1 - 1.5 ml Tris-NaCl buffer (50 mM Tris-HCl (T3253-500G, Sigma, Aldrich, St. Louis USA) and 10 mM NaCl) to get an initial OD of 0.7 - 1.0.

The Bioscreen C instrument uses 10x10-well Honeycomb micro plates with a lid that minimizes evaporation and the incubation chamber can hold a certain temperature throughout the experiment. The capacity is 200 samples (2 honeycomb plates) at a time enabling replication to be easily performed. The honeycomb plates were loaded first with 100 µl spores (with and without different NaCl concentrations), with 100 µl water to the negative controls and then 100 µl germinators to the others (Table 4). The Bioscreen C instrument was preheated to 37 °C prior to the experiment. Then the honeycomb plate containing all the samples was loaded and the measurements were initiated.

The germination was also monitored using a phase contrast microscope after the germination experiment and the phase dark (germinated) spores were counted using a Thoma cell counting chamber.

Table 4: An example of a Bioscreen setup.

Well nr.	sample
1-3	100 µl spores in Tris NaCl, 100 µl water (negative control)
4-6	100 µl spores in Tris NaCl, 100 µl 1M L-alanine
7-9	100 µl spores in Tris NaCl, 100 µl 50mM Inosine
11-13	100 µl spores in Tris NaCl, 0.9 % NaCl, 100 µl water
14-16	100 µl spores in Tris NaCl, 0.9 % NaCl, 100 µl 1 M L- alanine
17-19	100 µl spores in Tris NaCl, 0.9 % NaCl, 100 µl 50 mM Inosine
21-23	100 µl spores in Tris NaCl, 2.5 % NaCl, 100 µl water
24-26	100 µl spores in Tris NaCl, 2.5 % NaCl, 100 µl 1 M L- alanine
27-29	100 µl spores in Tris NaCl, 2.5 % NaCl, 100 µl 50 mM Inosine
31-33	100 µl spores in Tris NaCl, 5 % NaCl, 100 µl water
34-36	100 µl spores in Tris NaCl, 5 % NaCl, 100 µl 1 M L- alanine
37-39	100 µl spores in Tris NaCl, 5 % NaCl, 100 µl 50 mM Inosine

3.8 Inactivation of *C. sporogenes* in minced meat (laboratory scale) with different NaCl concentration

Minced meat was used in a laboratory scale food experiment. The minced meat used did not contain any added NaCl or water, but according to the label it contained 0.1 % NaCl ("karbonadedeig" with 5 % fat from Gilde). The minced meat was frozen in 100 g packages five days before the expiration date. Before each experiment one package of minced meat was thawed overnight in the refrigerator.

The following day the minced meat was mixed with 20 ml water in a laboratory blender. The spores and NaCl were added to the water before it was mixed with the minced meat. The amount of spores added to the minced meat solution was 10^7 spores/g. Then NaCl was added to the correct concentration (Table 5).

Table 5: The amount of NaCl added to achieve the specific NaCl concentrations in the meat solution.

Minced meat mixture (g)	NaCl added (%)	NaCl (g)
120	0.1	0.00
120	0.9	1.08
120	2.5	3.00
120	5.0	6.00

Then 5 grams of the minced meat solution with spores and NaCl were transferred to plastic bags and sealed. The plastic bags were sealed in multiple places to make a 5 x 6 cm pocket for the meat in the middle and empty pockets on the sides for easier attachment to the rack

(Figure 14). The meat was rolled flat to get an even heat distribution in the whole product and faster heating of the core of the meat product. The thickness of the bags was approximately 2 - 3 times greater than the diameter of the capillary tubes. Five parallels for each time interval were made (0, 2, 4 and 6 min).

Figure 14: Bag containing 5 grams of minced meat solution rolled flat. There are empty pockets on the sides of the bag for easy attachment to the rack.

The bags were attached to a metal rack five at a time to prevent them from floating around. Then they were immersed in the water bath. The metal rack without the samples was preheated in the water bath to avoid a large temperature drop in the beginning of the heat treatment. All bags were kept separate from each other in the water bath in order to obtain an even heat distribution in all bags (Figure 15). The experiments were first performed at 93 °C, but due to low inactivation the temperature was adjusted to 97 °C for the rest of the experiments. The rack with the five parallels were removed after a specific time and immersed in ice water. Then ten-fold serial dilutions were made.

The temperature in the water bath as well as in the minced meat bags were logged during one of the experiments. Five temperature loggers were placed at specific locations in the water bath in order to control actual temperature in three dimensions. The actual temperature inside two of the bags with minced meat (without *C. sporogenes* spores) was also measured by thin temperature probes (Testo 176 T4 data logger, Lenzkirch, Germany). Five bags with minced meat were attached to the metal rack. The bags that were prepared with inside temperature loggers were the two bags in the middle of the rack (Figure 16). One of the temperature loggers for the water was attached to the side of the rack to get the

actual temperature of the water around the rack when it was immersed and removed from the water bath. The temperature measurements are presented in Section 4.5.

Figure 15: Rack with bags attached. Five parallels at a time.

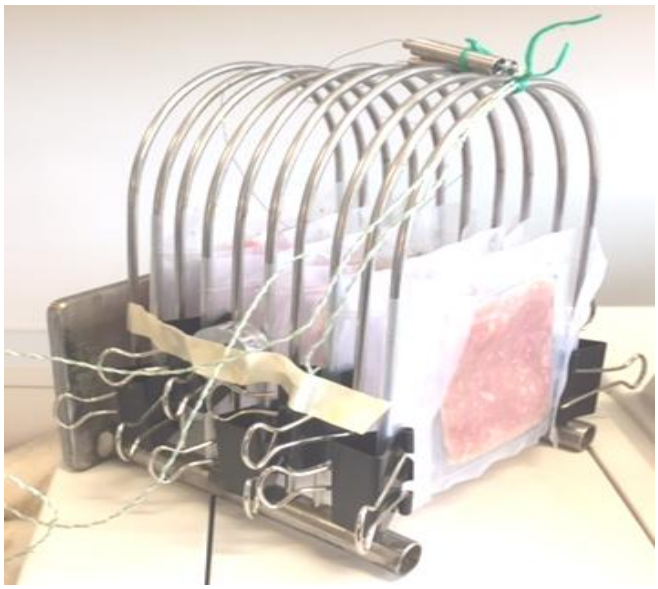

Figure 16: Rack with minced meat bags and temperature probes attached. The probes that go into the minced meat bags are attached at the top of the rack and a temperature logger for the water is attached to the side of the rack.

3.9 Inactivation of *C. sporogenes* in liver pate (semi scale) added different NaCl concentrations

Liver pate with two different NaCl concentrations was used as a food matrix. The NaCl concentrations tested were 0.9 and 1.7 %. In addition it contained 60 ppm of nitrite. The

liver pate was stored frozen and thawed overnight. It was preheated for 2 hours at 45 °C before the experiment. The liver pate was inoculated with *C. sporogenes* (CIP 104607), 10^7 spores/g. Then trays (PE-HD, 120 ml, DYNO 561, Dynopack A/S, Kristiansand, Norway) were filled with 100 g of liver pate and sealed with a Dyno packaging machine (Figure 17) (462/463 VGA half automatic vacuum/gas, Dynopack A/S).

Three of the trays for each temperature were fitted with a temperature probe (E-Val Flex, Ellab A/S, Hilleroed, Denmark) in the center for exact temperature measurements. These trays contained liver pate without spores from the 1.7 % NaCl mixture. The samples were then autoclaved (Shaka 900, Steriflow, Paris, France) at specific programs at 93 and 97 °C (Figure 18). The autoclave programs are presented in Section 4.6. Three parallels for each condition were made. The majority of the samples were analyzed at day zero, but some samples were stored at 4 °C to be analyzed later. Untreated samples for both NaCl concentrations of liver pate were also collected (Table 6).

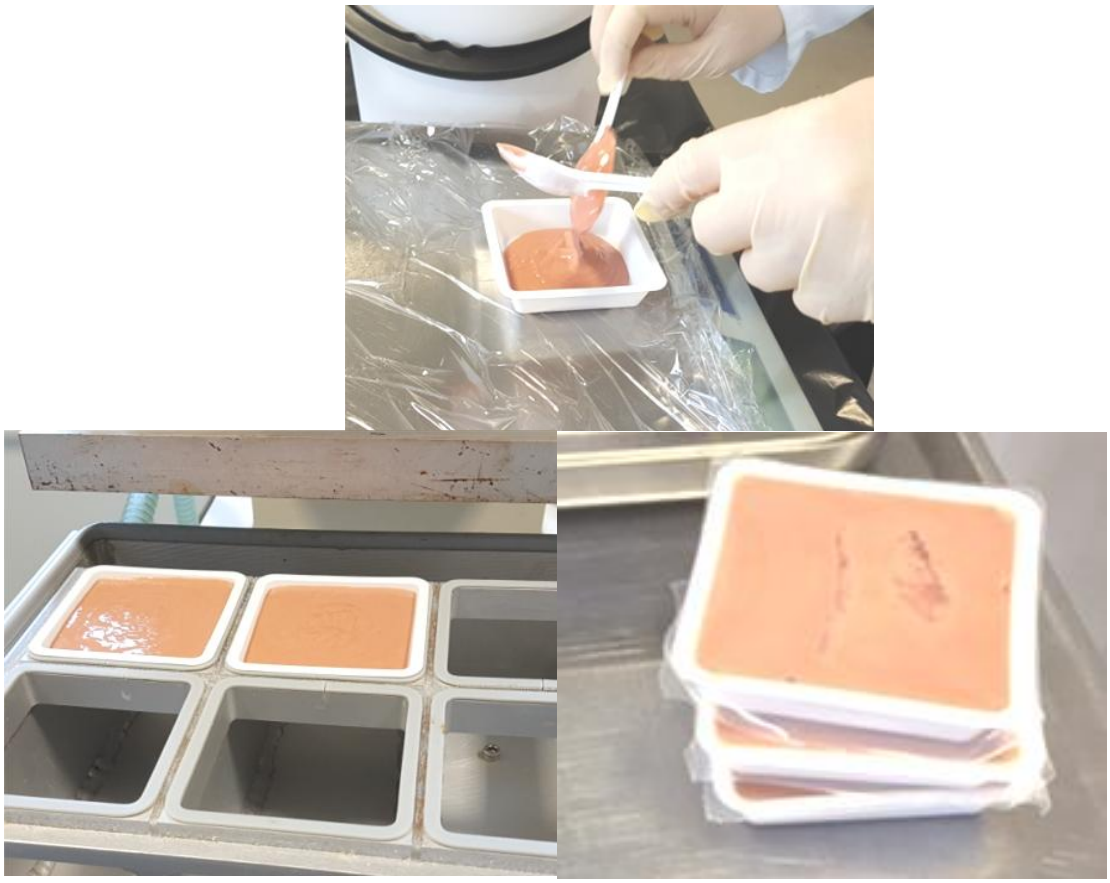

Figure 17: Trays were filled with 100 grams of liver pate and sealed with the Dyno packaging machine.

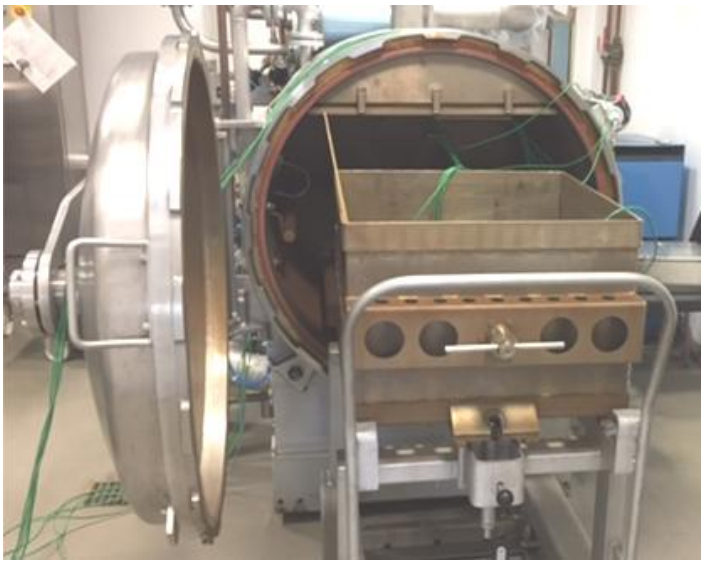

Figure 18: Steriflow autoclave used for thermal processing of the trays containing liver pate.

After the heat treatment 25 grams of each sample was measured and diluted 1:10 with 0.1 % Peptone water in a stomacher bag (Figure 19). Then the bags were homogenized in the stomacher machine at normal speed for 120 sec. The homogenate from the bags was transferred to 15 ml tubes and this was the 10^{-1} dilution. Then tenfold-dilutions were performed and the samples were spread on RCA plates. The background flora in the liver pate was also enumerated. Some of the liver pate without spores were diluted and spread on Plate Count Agar (PCA) (84608.0500, VWR, Hardy Diagnostics, Santa Maria, USA) plates. These plates were made in duplicates, one set were incubated aerobically and the other set anaerobically at 37 °C for 2 days. The RCA plates were incubated anaerobically at 37 °C for 2 days. The log-reduction representing the two heat treatments was then calculated. The experiment was performed twice at two different days with two different spore batches. Experiment number 1 was performed using spore batch 8 and experiment number 2 was performed using spore batch 9.

Table 6: Number of samples made for the different heat treatments of liver pate with different NaCl concentrations.

Treatment of meat product	0.9 % NaCl	1.7 % NaCl	Samples for temperature measurements
Control without spores	3		
Untreated with spores	3	3	
93 °C	3 (+ 3 for storage)	3 (+ 3 for storage)	3 (1.7 % NaCl without spores)
97 °C	3 (+ 6 for storage)	3 (+ 6 for storage)	3 (1.7 % NaCl without spores)

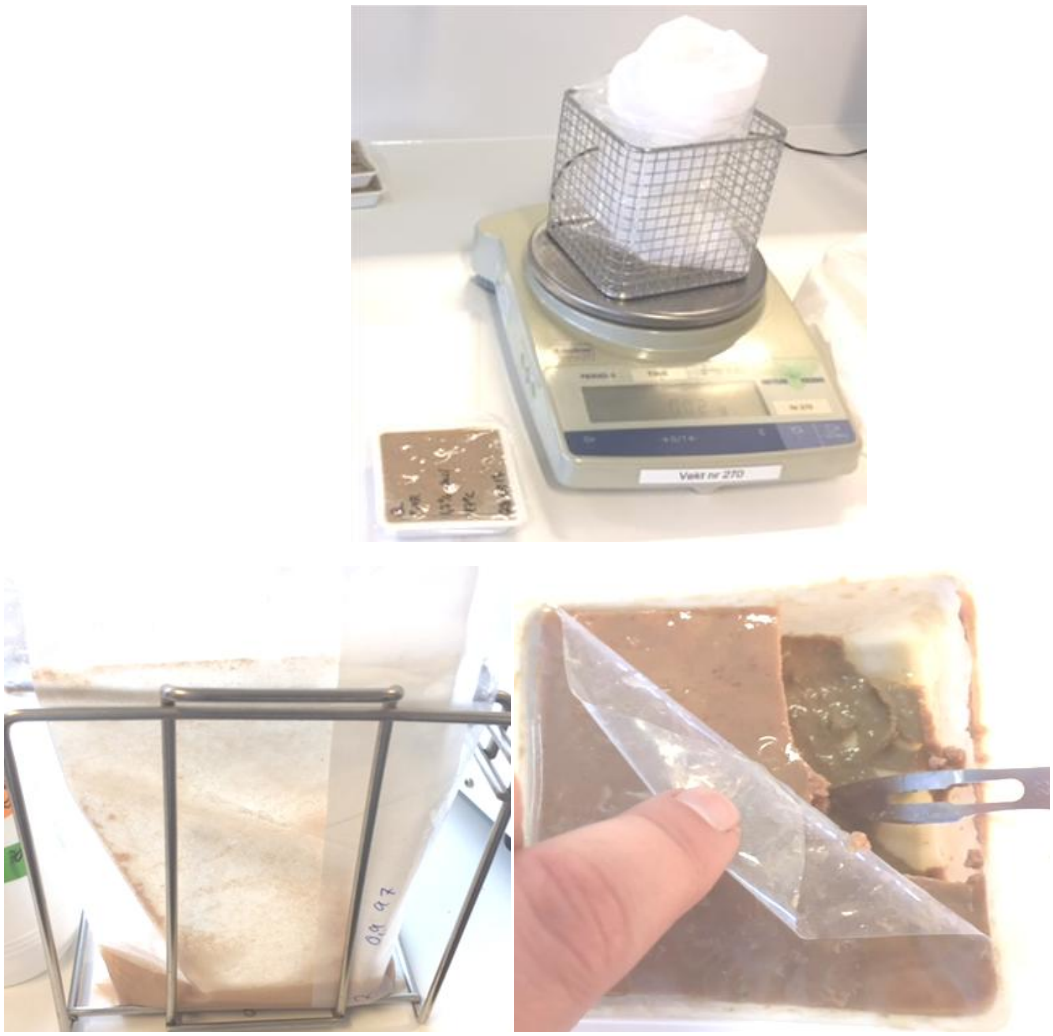

Figure 19: The liver pate samples were weighed and homogenized. Samples of 25 grams were collected in a stomacher bag and diluted 1:10 with 0.1 % Peptone water.

4 Results and discussion

4.1 Sporulation and harvesting of spores

The sporulation and especially harvesting and cleaning procedures of the spores are important and the procedure may influence the properties of the spores, related to heat tolerance, activation and germination. It is therefore included as a separate section in the results. Both sporulation and harvesting contain a number of steps that can potentially have an effect on the heat tolerance of the spores. Some of these steps are the heat shock step to initiate sporulation, the time required for optimal sporulation, the redox potential in the bottles used for sporulation, sporulation in liquid or on solid medium, addition of lysozyme in the harvesting and cleaning process, how many times the spore-suspensions are cleaned and how much of the debris that is removed between each washing and centrifugation.

In the beginning, two *C. sporogenes* variants (CIP 104607 and MF 02712) were investigated to find the best variant for the planned experiments. To do experiments with both variants was too laborious. Based on the preliminary experiments it was therefore decided to concentrate all experiments on the CIP 104607 variant.

4.1.1. The heat shock treatment

A heat shock treatment is performed to initiate the sporulation process and inactivate the vegetative cells. The spore production method was performed according to Ponce and Yang (Ponce and Yang, 2009; Yang, 2010; Yang et al., 2008). They have performed systematic experimental work on spore production and found a method to achieve the highest amount of pure dormant spores from the production, harvesting and cleaning steps. The initiation of sporulation performed by a heat shock treatment was carried out at 80 °C for 30 min (Ponce and Yang, 2009). This step was used to obtain a high percentage of sporulation. Spore batch number two were separated into two parts: one part got the heat shock treatment at 80 °C for 30 min and the other one did not get any heat treatment before incubation in sporulation medium. After eight days of incubation, approximately 50 % were determined as phase bright spores in the phase contrast microscope in the non-heat treated portion and > 90 % phase bright spores in the heat shock treated portion. Based on this result, all following spore batches were given a heat shock to initiate sporulation.

4.1.2. Redox potential, sporulation medium and storage temperature used

To achieve strict anaerobic conditions the redox potential has to be -300 mV (DSMZ). Any exact redox potential for optimal growth and sporulation of *C. sporogenes* was not found in the literature, but high percentage of sporulation was achieved when the redox potential in the serum bottles was below -110 mV. The redox potential was monitored by adding resazurin to the sporulation medium which turns colorless at -110 mV.

Sporulation in sporulation medium was used for all spore batches, except number six in which sporulation on RCA plates was performed. The percentage of sporulation on solid medium was comparable with sporulation on liquid medium after equal incubation time and temperature, but it was difficult to dissolve the spore pellet during the spore cleaning and therefore difficult to dispose of the cell debris. The desired purity of < 1 % vegetative cells was not achieved for the spores that were sporulated on solid medium. This spore batch had a purity of approximately 95 %, i.e. there were 5 % vegetative cells in the spore-suspension. Therefore, the sporulation was performed in serum bottles containing liquid medium for the following spore batches.

The storage temperature may influence the heat resistance of the spores. The pure spore-suspensions were stored in the refrigerator at 4 °C for up to four weeks. Mah *et al.* (2009) reported that when the spores are stored frozen they maintain the heat resistance, but when stored in the refrigerator (4 °C) or room temperature (25 °C) the heat resistance decreases somewhat throughout the storage period. This could indicate that the storage temperature of 4 °C may have an influence on the heat resistance of the spores throughout the storage period, but this was not experimentally tested.

4.1.3. Harvesting and cleaning of the *C. sporogenes* spores

The effect on sporulation of adding lysozyme to the spores in the cleaning process was investigated. Lysozyme is added to separate the vegetative cells from the spores and digest the cell debris. Batch number three was harvested and cleaned without addition of lysozyme and without the following incubation for two hours. The other steps of the cleaning procedure were performed according to the method described in Section 3.4. The number of cleaning repetitions required to remove the cell debris from the spore batch without added lysozyme were the same as for the other spore batches. The number of spores was similar,

but there were approximately 5 % more mother cells attached to the phase bright spores in the batch without lysozyme added. It was therefore decided to continue with the lysozyme step for the following spore batches.

Table 7: Different conditions used for the production of spore batches. The final volume for storage of all spore batches was adjusted to 40 ml.

Batch	Incubation time	Strain	Concentration (spores/ml)	Date washed	Variables *
1	8 days	CIP 104607/ MF 02712	$3.63*10^8$/ $3.68*10^8$	08.09.16	+ L/HS, SM in 100 ml bottles, ≥ 95 % ph.bs
2	10 days	CIP 104607/ MF 02712	$3.47*10^8$/ $4.16*10^8$	13.10.16	+L, +/- HS, SM in 100 ml bottles, ≥ 95 % ph.bs
3	10 days	CIP 104607	$6.77*10^8$	01.11.16	-L, +HS, 1 liter SM in 100 ml bottles, ≥ 95 % ph.bs,
4	9 days	CIP 104607	$4.39*10^8$	24.11.16	+ L/HS, 1 liter SM in 100 ml bottles, ≥ 98 % ph.bs
5	10 days	CIP 104607	$8.00*10^8$	13.12.16	+ L/HS, 1 liter SM in 100 ml bottles, ≥ 99 % ph.bs
6	9 days	CIP 104607	$1.42*10^9$	18.01.17	+ L, sporulated on RCA plates, > 95 % ph.bs
7	8 days	CIP 104607	$1.90*10^9$	21.02.17	+ L/HS/resazurin, 1.5 liter SM in 200 ml bottles, > 99 % ph.bs
8	7 days	CIP 104607	$3.16*10^9$	02.03.17	+ L/HS/resazurin, 1.5 liter SM in 200 ml bottles, > 99 % ph.bs
9	8 days	CIP 104607	$1.1*10^9$	31.03.17	+ L/HS/resazurin, 1.5 liter SM in 200 ml bottles, > 99 % ph.bs

*+ = with, - = without, L =lysozyme, HS = heat shock, SM = sporulation medium, ph.bs = phase bright spores, RCA = reinforced clostridium agar

The spore harvesting and cleaning process includes a series of steps that requires experience to achieve the desired pure spore-suspension containing > 99 % phase bright spores without any cell debris that can affect the inactivation of the spores (Hossain, 2015; Yang, 2010). The top layer of the spore pellet was removed after each centrifugation in order to remove the cell debris from the tube and to achieve a pure spore-suspension. The spore-suspension was cleaned 6 - 8 times to achieve the desired purity. An overview of the sporulation and

harvesting process of the different spore batches at the start and in the end of the thesis is presented in Table 7.

4.2 Inactivation of *C. sporogenes* in water

The inactivation experiments were performed at a temperature range from 91 - 110 °C. Temperatures from 91 - 97 °C showed slower inactivation rates with lower standard deviations than 99 - 110 °C. Experiments performed in the latter temperature range showed high inactivation, high standard deviations and low R^2 values. A selection of the inactivation curves from 91 - 97 °C is presented in Figure 21. The temperature range was selected because of results in the previous work of Hossain (2015). His work was performed with other strains of *C. sporogenes* (PA 3679, CCUG 24143 and CCUG 7489) with temperatures ranging from 80 - 90 °C. These strains had a lower heat tolerance than expected and no survivors were found at 100 °C. New *C. sporogenes* strains were purchased for this project in the expectance of a higher heat tolerance than the strains Hossain used. Therefore the selected temperature for the first inactivation experiments was 99 - 110 °C. When these results showed a fast inactivation rate and high standard deviations, the temperature was reduced to 91 - 97 °C (Table 8).

With increasing temperature, the D-value is expected to decrease. When the average of the D-values from the same temperatures is calculated it is possible to see a decrease in D-value for increasing temperature. However, D-values are varying between the different spore batches tested at the same temperature. With reference to previous described factors that may influence the heat resistance of spores, this may have caused the varying D-values. Spore batch 5 is more heat resistant at 91 °C than the other spore batches. In the calculation of z-values spore batch 5 is not included in the data set. The z-value of *C. sporogenes* CIP 104607 was then 9.86 °C (Figure 20). This is close to the z-values found by others (Diao et al., 2014).

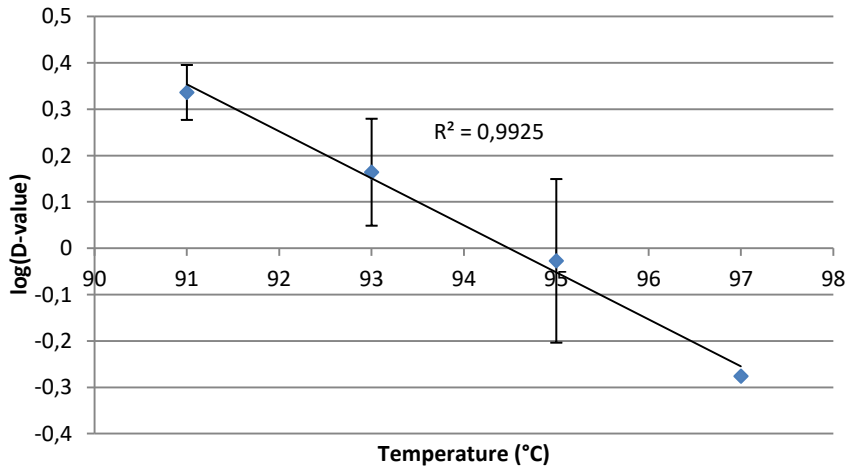

Figure 20: z-value of *C. sporogenes* CIP 104607.

Table 8: D-values and temperatures for the different inactivation experiments using *C. sporogenes* CIP 104607 in water.

Temperature(°C)	Batch no.	D-value	R^2	Average D-value*
91	4	1.78	0.98	2.17
91	4	2.34	0.87	
91	4	2.15	0.97	
91	4	2.41	0.94	
91	5	12.56	0.60	
91	5	16.60	0.60	
93	3	1.16	0.97	2.02
93	4	1.27	0.94	
93	4	1.32	0.94	
93	5	2.19	0.95	
93	6	2.10	0.90	
93	6	4.28	0.80	
95	2	0.66	0.95	0.94
95	3	0.75	0.88	
95	4	1.41	0.95	
95	5	1.44	0.99	
97	3	0.53	0.93	0.53

*Spore batch 5 is excluded from the average D-value calculations.

The initial inactivation experiments gave inactivation curves with a rapid decrease in bacterial numbers (in the beginning of the inactivation curve) from 0 to 1 min (data not shown). This may be a result of a high percentage of spores (and injured cells) that were less

heat tolerant and died almost immediately after exposed to the high temperatures. Therefore the initial data point was excluded and only the log-linear line was used for D-value calculations. For the last experiments performed this rapid decrease in bacterial numbers in the beginning of the inactivation curve was not present. Deviations from log linear curves are known and may be associated with heterogeneity of resistance within a population or with changes in resistance during heating. An initial shoulder may denote clumping of cells or demonstrate that inactivation occurs via a "multihit" process on target sites. Tailing indicates the presence of small numbers of large clumps or of heterogeneity of resistance in the population. A sigmoidal curve results from a combination of factors.

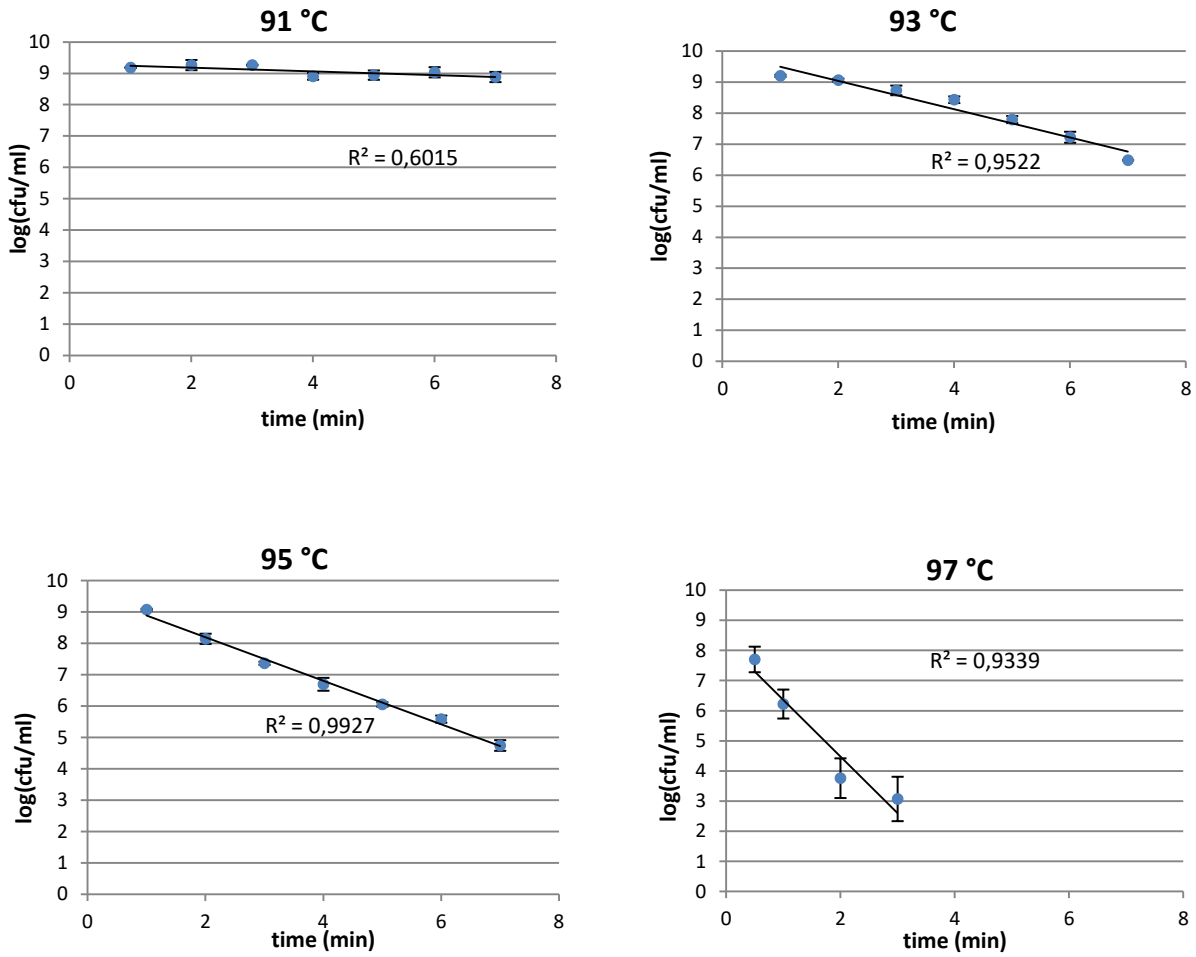

Figure 21: Inactivation of *C. sporogenes* (CIP 104607) spores at 91-97 °C. All data points are a mean of three parallels and the standard deviations are shown.

4.3 Inactivation of *C. sporogenes* in water with different NaCl concentrations

NaCl concentrations in the range of 0.9 and 2.5 % are relevant concentrations used in different food products like canned ham. A NaCl concentration of 5.0 % was previously used in preserved foods, but today this concentration is regarded as high for most foods. All capillary tube experiments with NaCl were performed at 93 °C.

The D-values for the experiments with NaCl present were higher than for the experiments without NaCl at the same temperature. The average D-value for the experiments without NaCl was 3.19 min. The highest D-value was found when 0.9 % NaCl was added with 17.34 min, and then the D-value for 2.5 % was 16.11 min and for 5.0 % it was 10.15 min. The standard deviations were low and the R^2 values were over 0.7 (Table 9 and Figure 22).

Table 9: Different NaCl concentrations and corresponding D-values for the inactivation experiments in water. The experiments were performed at 93 °C with spore batch number 6.

[NaCl] %	D-value	R^2	Average D-value
0	4.28	0.80	3.19
0	2.10	0.90	
0.9	17.82	0.71	17.34
0.9	16.86	0.85	
2.5	18.51	0.76	16.11
2.5	13.71	0.70	
5	10.67	0.89	10.15
5	9.62	0.82	

The D-values representing 0.9, 2.5 and 5.0 % NaCl showed a decrease in D-value when the NaCl concentration increases. The highest inactivation was achieved without added NaCl. The results indicate that the NaCl concentration has a different effect on inactivation of spores than on germination and growth of spores. The NaCl could possibly have a protective effect on the spores resulting in more spores surviving the heat treatment. To do more experiments with higher NaCl concentration and more repetitions is advisable to obtain more accurate knowledge on the effects of added NaCl and the inactivation of *C. sporogenes* spores.

Many food producers reduce their salt content due to health reasons, and products with NaCl content < 1 % is often targeted. The inactivation results with *C. sporogenes* show that

this strain was more heat resistant at low salt concentrations, i.e. at the same heating conditions more spores may survive when salt concentration is reduced. In addition, salt is known to have an inhibitory effect on bacterial growth. This means that surviving spores may have lower inhibition and could possible grow to hazardous levels. In order to achieve safe heat treated foods, manufacturers therefore should both re-evaluate their processing conditions, and evaluate if extra hurdles are needed for safety, in relation to the shelf life used.

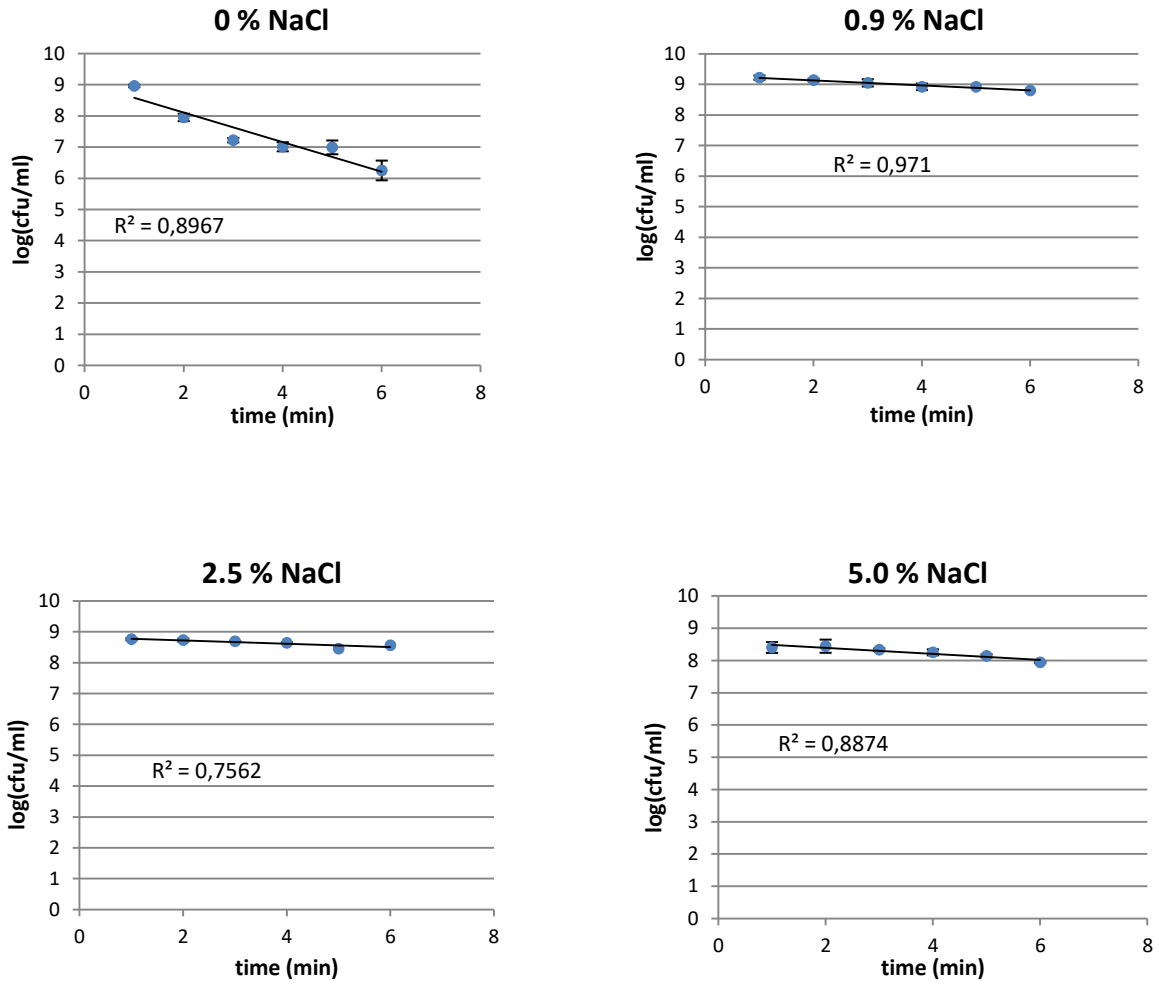

Figure 22: Inactivation of *C. sporogenes* (CIP 104607) spores in water at 93 °C with NaCl concentrations from 0 - 5 %. All data points are a mean of three parallels and the standard deviations are shown.

4.4 Germination of *C. sporogenes*

Germination experiments using optical density measurements with Bioscreen C showed that the percentage of germinated spores after each experiment varied from 15 - 25 %, when L-alanine and Inosine were used as germinators and the spores were dissolved in water. According to the Bioscreen results the spores also germinated to certain extend without any germinators present (10 - 20 % germination). The percentage of germinated spores was in addition documented using a phase contrast microscope. A change from phase bright to phase dark was defined as germination. In all samples without NaCl there were only 1 - 5 % germinated spores. L-alanine and Inosine gave very similar results regarding the number of germinated spores. The time required for the germination process with only germinators added was 6 - 8 hours.

Both heat activation (60 °C for 15 min) (Plowman and Peck, 2001) and no heat activation prior to the germination experiment were tested in order to examine if the heat activation step was necessary for sufficient germination. Others have previously reported that they use heat activation prior to germination experiments to make the spores germinate more easily (Løvdal et al., 2011; Plowman and Peck, 2001). In our experiments, there were no difference between heat activated and not heat activated spores (Figure 23). In the germination experiment with different NaCl concentrations only heat activated spores were used.

When a combination of NaCl and L-alanine was used the percentage of germinated spores was approximately 70 % measured with Bioscreen (Figure 24). When the percentage of germinated spores was enumerated using the phase contrast microscope there were hardly any phase bright dormant spores left (1 - 2 %), i.e. 98 - 99 % of the spores were germinated. The time required for the germination process with a combination of NaCl and L-alanine was 10 - 12 hours. When L-alanine or Inosine were added as germinators, no germination occurred. When L-alanine is combined with 2.5 and 5.0 % NaCl the spores were completely germinated.

Ponce and Yang (2009) reported that spores of *C. sporogenes* did not germinate when only L-alanine was added. When counted in the phase contrast microscope the *C. sporogenes* spores in our experiment did not germinate either when L-alanine or Inosine were added.

The Bioscreen results for this experiment showed a decrease in percentage of absorbance (% A 600) of 15 - 25 %, but that decrease can be caused by other components in the wells.

C. sporogenes is an anaerobic bacterium, requiring anaerobic environment for growth and sporulation. The germination experiments of *C. sporogenes* were, however, performed under aerobic conditions. This was based on results from Plowman and Peck (2001) who reported minimal differences between aerobic and anaerobic germination of *C. sporogenes* using the Bioscreen C instrument. Our results confirmed that it was possible to perform germination experiments on *C. sporogenes* under aerobic conditions.

Two methods, phase contrast microscope and Bioscreen, were used to document the germination present. The results from the two methods show the same trend, based on factors as added salt, preheating, and added germinators. The difference found in exact percent number, may be due to the complex mechanisms that are taking place in the germination process of a spore. The germination process proceeds over several steps with activation, germination and end up with outgrowth (figure 3). This may be expressed and interpret slightly different in the two methods.

The results show that NaCl is important for the germination process to take place. L-alanine or Inosine are amino acids that are present in many protein rich foods. They can increase the germination of *C. sporogenes*, and the germination is more rapid and at a higher percent at higher NaCl concentration (Figure 24). In this experiment, the low NaCl concentration of 0.9 % only slightly increased the germination percent compared to the control.

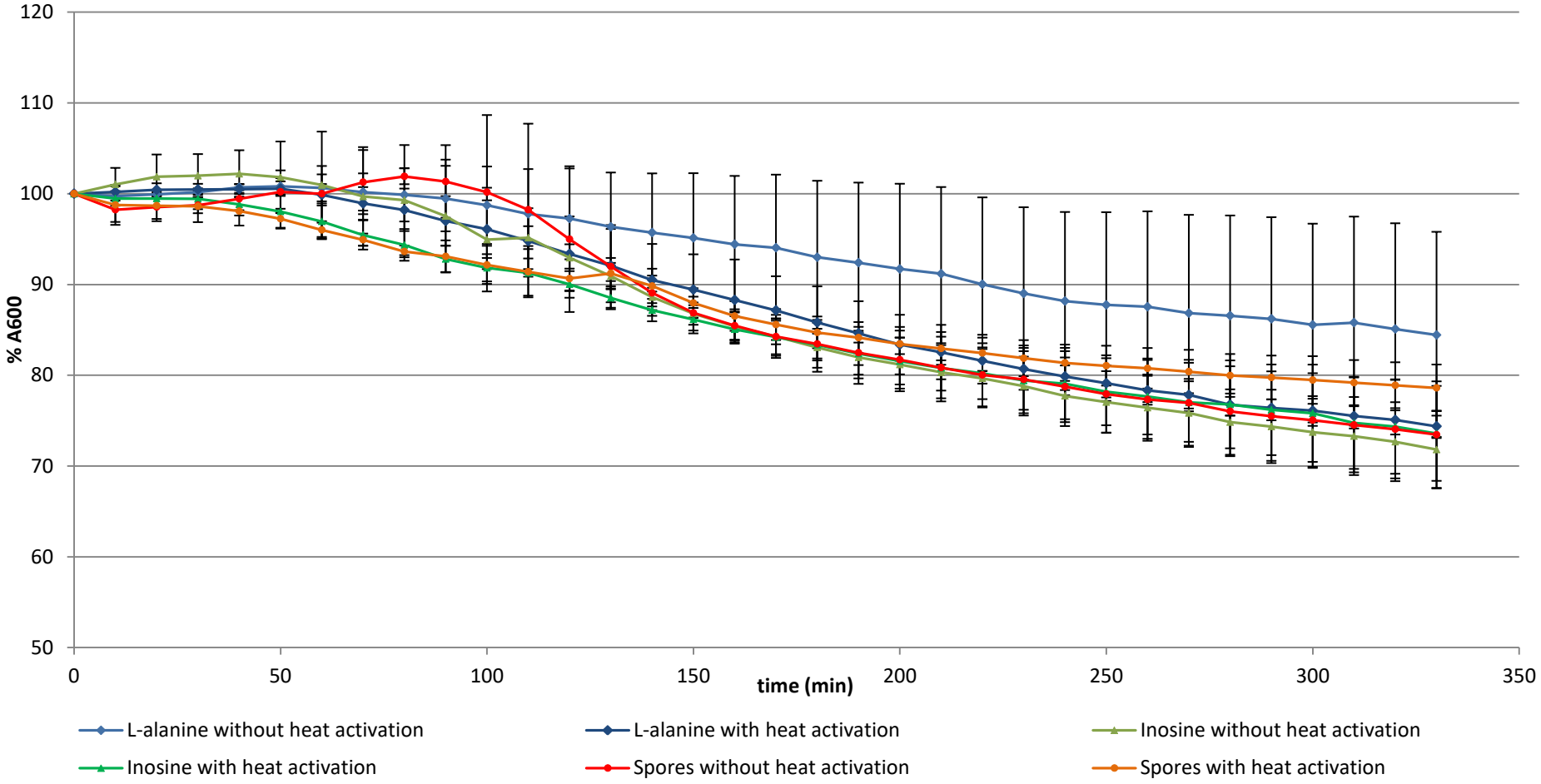

Figure 23: Aerobic germination of *C. sporogenes* spores by Bioscreen C. All data are a mean of three parallels and the standard deviations are shown.

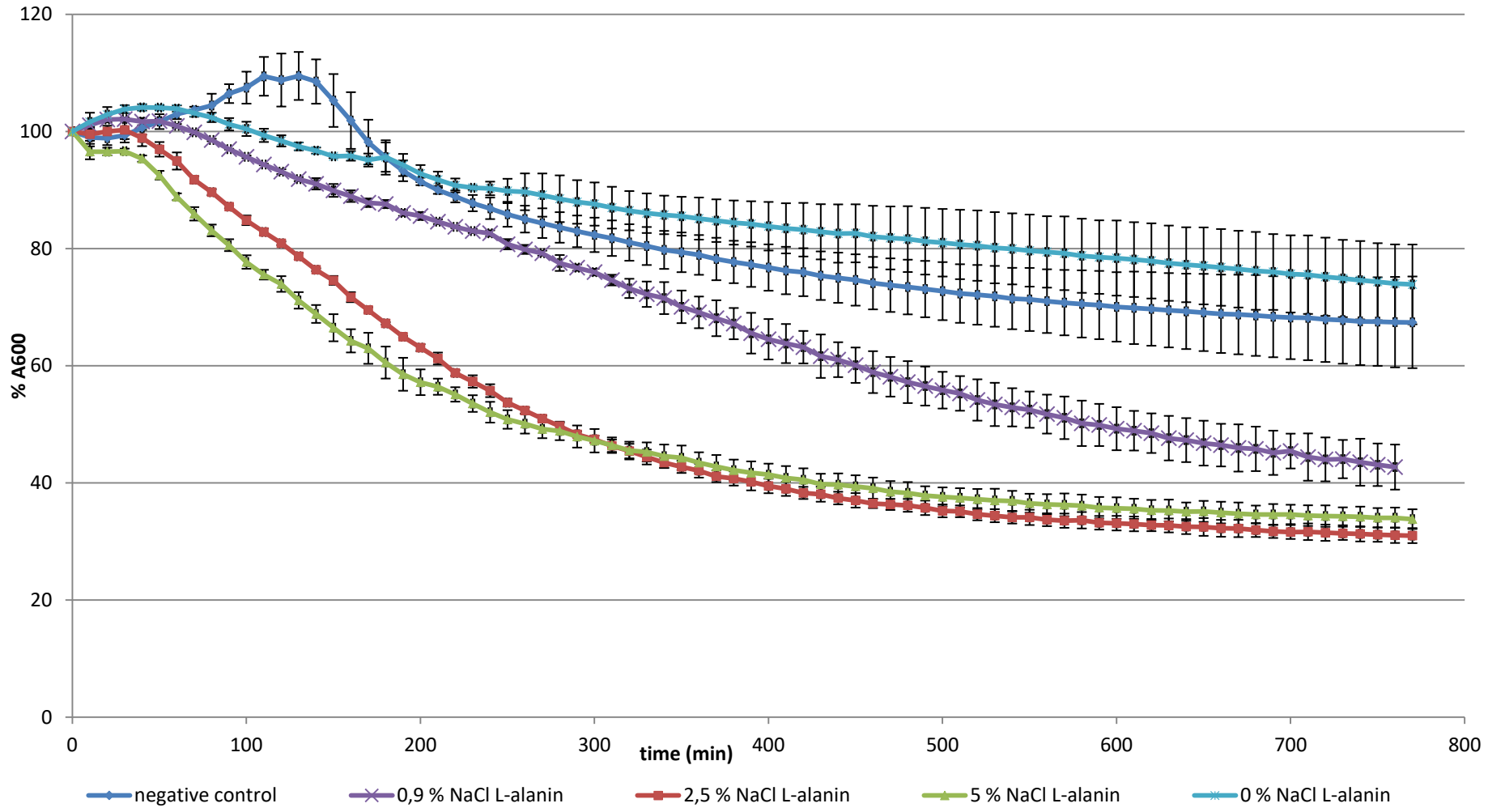

Figure 24: Aerobic germination of *C. sporogenes* spores by Bioscreen C with different NaCl concentrations. All data points are a mean of three parallels and the standard deviations are shown.

4.5 Inactivation of *C. sporogenes* in minced meat (laboratory scale) with different NaCl concentrations

The temperature measurements performed in the minced meat experiment revealed that the actual temperature of the water in the water bath is 0.3 - 0.5 °C lower than the set temperature of the water bath. This means that when the water bath was set to 97 °C the actual temperature were 96.5 - 96.7 °C etc. This is the case for all experiments performed with minced meat. The reported temperature in the tables for each experiment is the set temperature for the water bath.

The practical aspects of temperature measurement before and after the heat treatment were similar to the inactivation experiments performed. Both the minced meat bags and the water showed very stable temperature of 96.2 °C in the bags and 96.7 °C in the water during the heat treatment (Figure 25 and Figure 26).

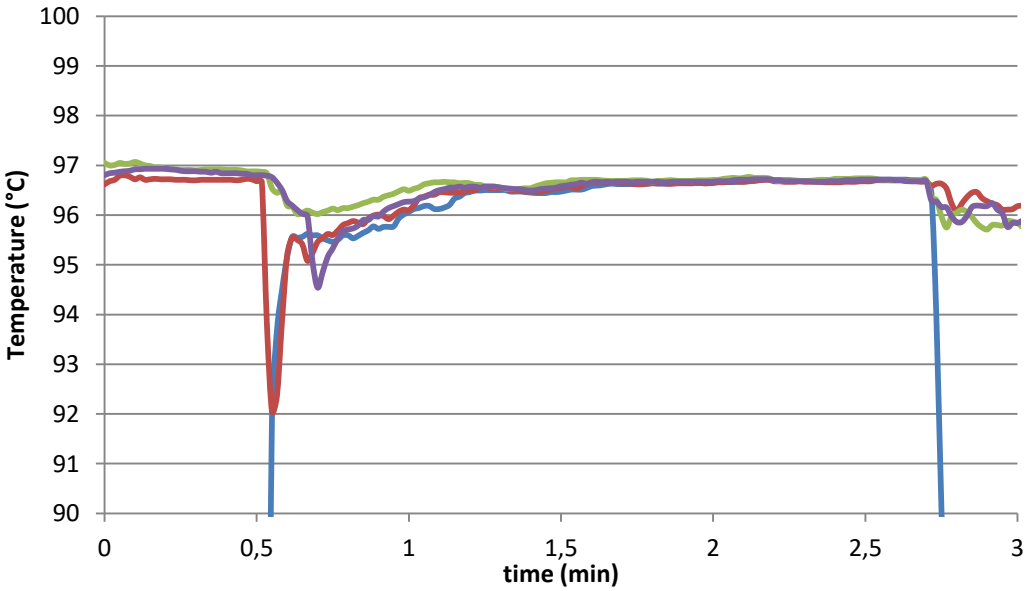

Figure 25: Temperature in water bath. The water bath was set to 97 °C. The temperature in the water bath remained stable at 96.7 °C throughout the measurement. Four probes were used and they were placed at different locations in the water bath.

Probe 1 (blue line) in Figure 25 represents the sensor for water temperature, attached to the rack and this was immersed and removed from the water simultaneously as the rack. Probe 2 (red line) was placed in the middle of the water bath close to the rack. Probe 3 and 4 (green and purple lines) were placed in the front corners of the water bath. The temperature in the water remained stable throughout the experiment.

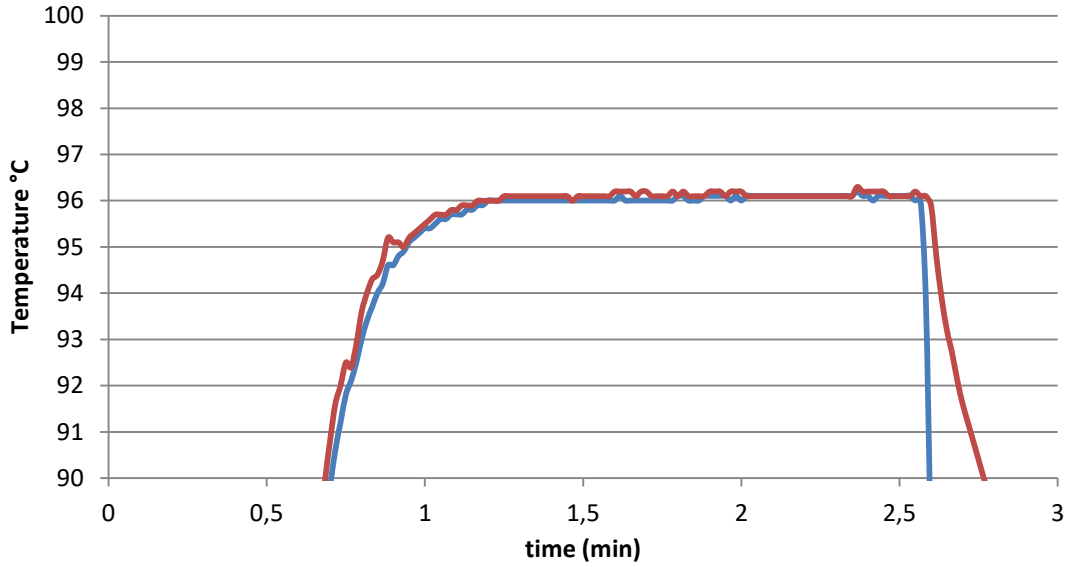

Figure 26: Temperature inside the bags containing minced meat. The temperature inside the minced meat bags remained stable at 96.2 °C throughout the experiment. Two probes were used, one in each of the two bags in the middle of the rack.

The inactivation results from the bags show that the minced meat had a protective effect on the spores, i.e. the spores have a higher heat tolerance in minced meat than was found in capillary tubes with water (Table 10). Therefore, 97 °C was chosen as the suitable inactivation temperature for these experiments. The experiments were performed in water bath at laboratory scale. The time to reach the required core temperature, the come-up time, was 42 sec in the bags. This is longer time than in the capillary tubes, but shorter than it would be in a large package, e.g. a can, heated in an autoclave. An autoclave is common equipment for heat treating food products and the time to reach the intended core temperature can be as long as an hour.

The capillary tube method is an isothermal method while thermal processing for most foods is a non-isothermal method. The heating of a food product is more time consuming than heating a liquid in a thin capillary tube and the heat distribution and temperature of the food product is not equal throughout the heat treatment. Also the inactivation of *C. sporogenes* in minced meat is a non-isothermal process, but a short come-up time was optimized. The minced meat bags were rolled flat (3 -4 mm) to achieve the best possible heat distribution in the product and to achieve an effective heat penetration to the specific core temperature.

NaCl concentrations with increasing NaCl concentrations from 0, 0.9, 2.5 and to 5.0 % gave no obvious trend in increasing or decreasing D-values. At 97 °C the values are varying with

the lowest D-value of 3.39 min with 0 % NaCl to the highest D-value of 13.79 min with 2.5 % NaCl. However, the average D-values shows increasing D-values when the NaCl increases from 0 % to 2.5 %, but this decreases again with 5 % NaCl (Table 10 and Figure 27).

Table 10: The inactivation experiments performed in minced meat with different NaCl concentrations and corresponding D-values. The experiments were performed with spore batch number 7.

Temp** (°C)	[NaCl] %	D-value	R^2	Average D-value
93	0	11.11*	0.78	
93	0.9	10.52	0.84	
97	0	5.33	0.91	4.36
97	0	3.39	0.95	
97	0.9	6.67	0.98	6.33
97	0.9	5.98	0.92	
97	2.5	13.79	0.91	10.51
97	2.5	7.23	0.93	
97	5	10.00	0.89	7.99
97	5	5.98	0.99	

* Initial sample was not heated at 80 °C for 10 min as the other experiments were.
** setting temperature of the water bath. (Core temperature in the minced meat reached 96.2 °C when the setting temperature of the water bath was 97 °C).

The rack used to fix the bags in the water bath for optimal heating exposure was preheated in the water bath at 97 °C before the bags were attached and the temperature measurements started. The temperature probes were fragile and the time to fix them to the rack may have decreased the rack temperature. Therefore the temperature increased at a slower rate when immersed in the water bath than if the rack had still been warm.

The time required to reach the desired core temperature of 97 °C in the minced meat bags were longer than in the capillary tubes. According to the temperature measurements, it took approximately 22 sec to reach 94 °C and 42 sec to reach 96.2 °C. The highest obtained temperature inside the bags with minced meat was 96.2 °C at a water bath temperature set to 97 °C. The temperature in the water itself reached 96.7 °C and both the temperature in the water and in the minced meat bags remained stable throughout the experiment.

The main conclusion from the minced meat experiment is that the heat resistance is higher for *C. sporogenes* spores in a minced meat matrix compared to water. The D-values varies and a clear connection to NaCl concentration is not seen. The variations may be related to the change from isothermal heating to a more complex non-isothermal food system. Variation in the minced meat composition, e.g. fat content, may also have an influence.

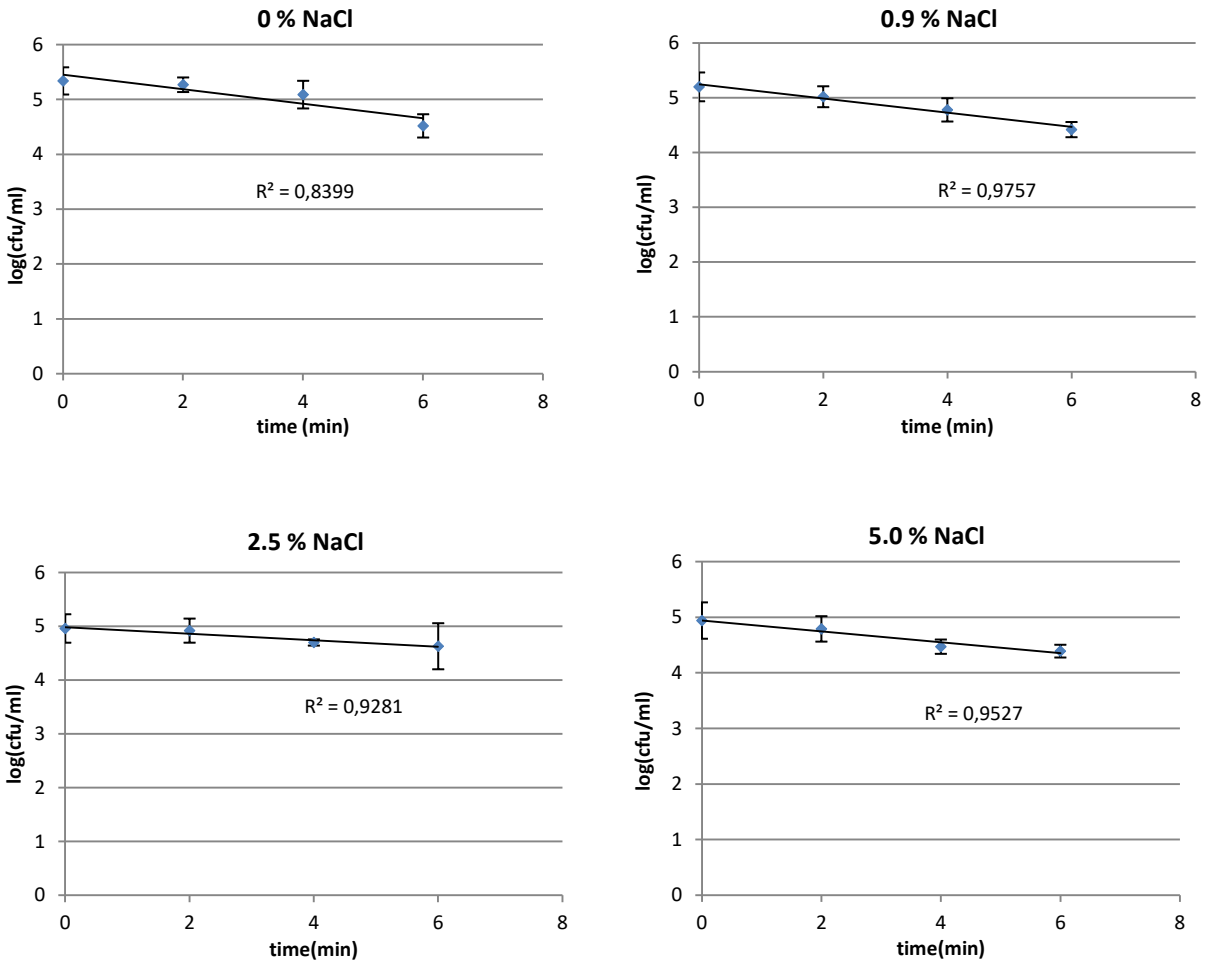

Figure 27: Inactivation of *C. sporogenes* with different NaCl concentrations in minced meat at laboratory scale. All data points are a mean of five parallels and the standard deviations are shown.

The reference D-value of *C. sporogenes* spores is 1 minute at 121 °C (Diao et al., 2014; Ramaswamy et al., 2010). This is four to five times more than for proteolytic *C. botulinum*. The D-value of *C. sporogenes* in sterile water was approximately 2 min at 93 °C corresponding to 0.02 min at 113 °C when a z-value of 10 °C is used. The D-value of *C. sporogenes* in minced meat without added NaCl was approximately 4 min at 97 °C corresponding to 0.04 min at 117 °C when a z-value of 10 °C is used. These results show that the D-values of *C. sporogenes* CIP 104607 recorded in this thesis are lower than the average D-value reported by others.

4.6 Inactivation of *C. sporogenes* in liver pate (semi scale) with different NaCl concentrations

In this experiment *C. sporogenes* spores were examined in a semi scale experiment simulating a real food production. Spores were added to liver pate in commercial sealed plastic trays and autoclaved in a Steriflow autoclave. Liver pate with 0.9 and 1.7 % NaCl was used as a food matrix. The liver pate was autoclaved at two different temperatures, 93 and 97 °C, based on results from the laboratory experiments. The specific autoclave program that represents the heat treatment given to the liver pate samples at 93 °C is presented in Table 11. Temperature probes were placed in the core of 3 trays and the non-isothermal profile is given in Figure 28. The F_0 (integrated process lethality) value of experiment 1 at 93 °C was calculated to 14.8. The F_0 value of experiment 2 at 93 °C was 15.2.

Table 11: Autoclave program for heat treatment of liver pate at 93 °C.

				Experiment 1	Experiment 2
	Temp (°C)	Pressure (bar)	Program length (min)	Real time (min:sec)	Real time (min:sec)
Heat	93	0.7	5	05:06	05:04
Heat	93	0.9	10	10:00	10:00
Heat	93	0.9	25	25:00	25:38
Cooling	30	0.3	5	06:15	06:23
Cooling	20	0.2	10	10:00	10:00

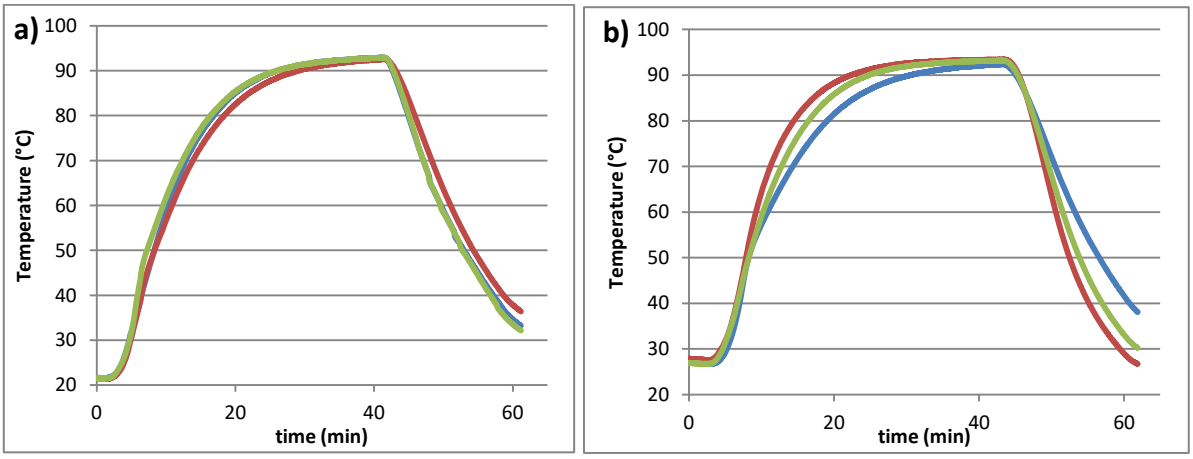

Figure 28: Temperature in liver pate at 93 °C autoclave program. Three temperature probes were used. a) Experiment number 1 performed with spore batch 8. b) Experiment number 2 performed with spore batch 9.

The whole experiment was repeated with a different spore batch in order to document the stability of the process. The specific autoclave program that represents the heat treatment

given to the liver pate samples at 97 °C is presented in Table 12 and Figure 29. The F_0 value of experiment 1 at 97 °C was 18.0. The F_0 value of experiment 2 at 97 °C was 18.3.

Table 12: Autoclave program for heat treatment of liver pate at 97 °C.

				Experiment 1	Experiment 2
	Temp (°C)	Pressure (bar)	Program length (min)	Real time (min:sec)	Real time (min:sec)
Heat	97	0.7	5	05:08	05:08
Heat	97	0.9	10	10:00	10:02
Heat	97	0.9	15	16:40	14:04
Cooling	30	0.3	5	06:33	06:44
Cooling	20	0.2	10	10:00	10:00

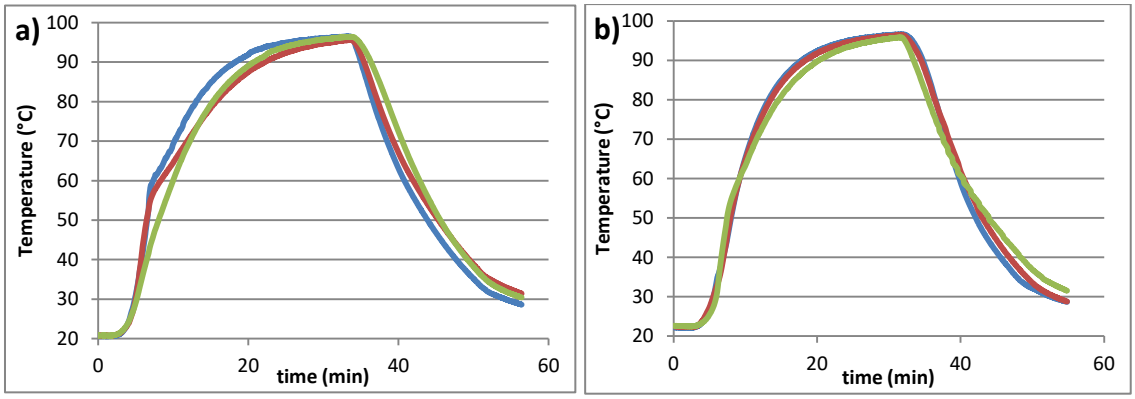

Figure 29: Temperature in liver pate at 97 °C autoclave program. Three temperature probes were used. a) Experiment number 1 performed with spore batch 8. b) Experiment number 2 performed with spore batch 9.

Three parallels were used for each variant in the experiment and 60 ppm Nitrite was used as a commercial addition to the liver pate. In the first experiment a 1.1 log-reduction was achieved for the 0.9 % NaCl mixture and a 1.2 log-reduction was achieved for the 1.7 % NaCl mixture at the 93 °C autoclave program. A 2.7 log-reduction was achieved for the 0.9 % NaCl mixture and a 2.3 log-reduction was achieved for the 1.7 % NaCl mixture at the 97 °C autoclave program. In the second experiment a 1.6 log-reduction was achieved for the 0.9 % NaCl mixture and a 1.6 log-reduction was achieved for the 1.7 % NaCl mixture at the 93 °C autoclave program. A 2.3 log-reduction was achieved for the 0.9 % NaCl mixture and a 2.3 log-reduction was achieved for the 1.7 % NaCl mixture at the 97 °C autoclave program (Table 13 and Figure 30).

Some of the heat treated liver pate trays were stores chilled for a storage study. The concentration of *C. sporogenes* in the liver pate had not changed after 1 month of storage at

4 °C. There were low variations in both heat treatment experiments and for both NaCl concentrations, from day 0 to 27/30 (these data are only presented in the table not the graph). The highest difference in log-reduction during storage was 0.23. These results indicate that the *C. sporogenes* spore concentration have not changed during storage at 4 °C for one month. The minimum temperature for growth of *C. sporogenes* is 10 °C which means that the *C. sporogenes* do not grow, and the numbers are not expected to increase at 4 °C.

Table 13: Log-reduction of *C. sporogenes* in liver pate.

Treatment	Experiment 1 0.9 % NaCl	Experiment 1 1.7 % NaCl	Experiment 2 0.9 % NaCl	Experiment 2 1.7 % NaCl
Directly after heating (day 0)				
93 °C	1.11	1.19	1.65	1.61
97 °C	2.68	2.29	2.35	2.27
Stored at 4 °C for 27 days				
93 °C	1.37	1.35	1.63	1.47
97 °C	2.45	2.18	2.40	2.32

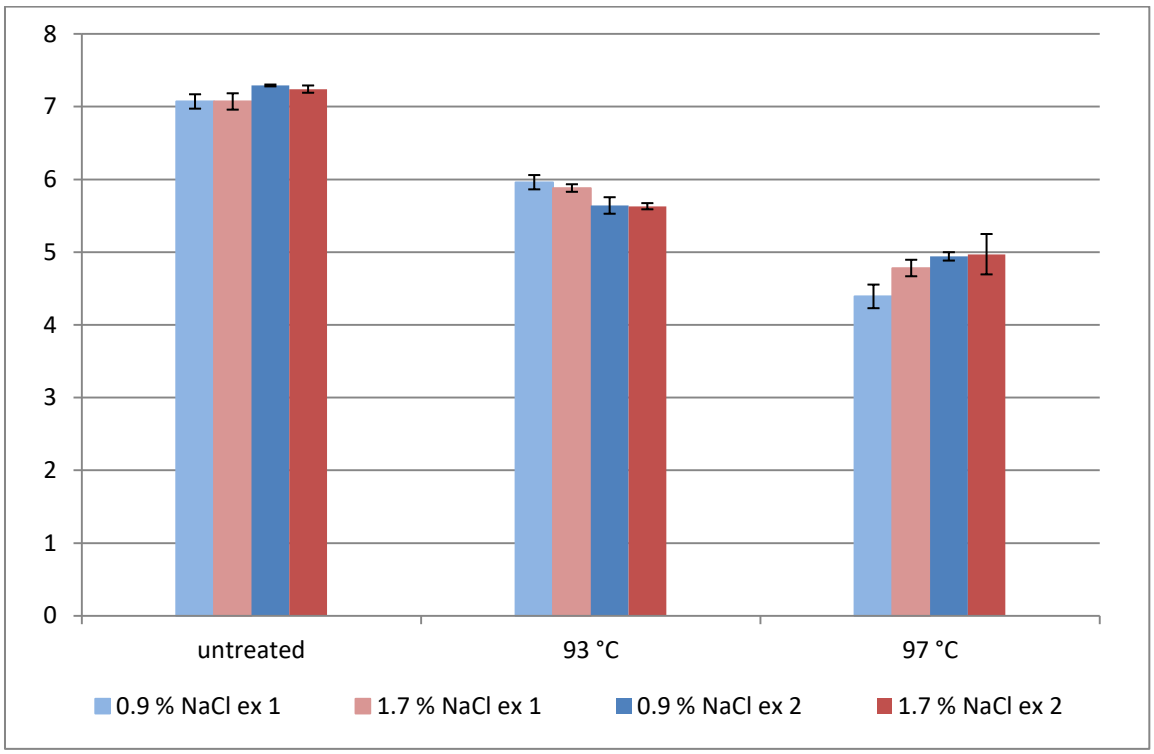

Figure 30: Log-reduction of *C. sporogenes* (CIP 104607) in liver pate. The blue represents 0.9 % NaCl while the red represents 1.7 % NaCl. Experiment number 1 is represented by the lightest colors and experiment 2 is represented by the darkest colors.

Experiment number 1 and 2 showed very similar results for each temperature indicating that the results were reproducible for the two different spore batches used. Both experiment number 1 and experiment number 2 indicated that the different NaCl concentrations in the liver pate did not affect the inactivation of *C. sporogenes*. No differences in the inactivation of *C. sporogenes* in liver pate containing 0.9 and 1.7 % NaCl was found. The two different autoclave programs at two different temperatures however showed a higher inactivation and log-reduction of *C. sporogenes* at 97 °C compared to at 93 °C. The log-reduction at 93 °C was between 1.1 and 1.6 and the log-reduction at 97 °C was between 2.2 and 2.7 for both experiments and NaCl concentrations.

These values are still lower than the log-reductions reported by others (Brown et al., 2011), but the number of surviving *C. sporogenes* at 97 °C in liver pate was greater than the number of surviving spores in the minced meat.

5 Conclusion

All inactivation experiments performed in this thesis show a lower heat tolerance of *C. sporogenes* spores compared to D-values in other published studies (Brown et al., 2011). The heat tolerance of *C. sporogenes* spores increased in the food matrixes compared to in water. The minced meat and the liver pate seemed to have a protective effect on the *C. sporogenes* spores. Several aspects of the sporulation process and the harvesting and cleaning may influence the heat tolerance of the *C. sporogenes* spores.

Specific NaCl concentrations (0.9, 2.5 and 5.0 %) were applied to the CIP 104607 spores in water and in minced meat. The D-values in water increased by approximately a fivefold from 0 to 0.9 % NaCl, but slowly decreased from 0.9 to 2.5 and 5.0 % NaCl. In the minced meat the D-values varied for the different NaCl concentrations applied. These results indicate that NaCl do not affect the inactivation of *C. sporogenes* spores, but more repetitions should be carried out.

The *C. sporogenes* spores were completely germinated by combining L-alanine as germinator and NaCl concentrations of 2.5 and 5.0 %. When L-alanine or Inosine were used alone to germinate the *C. sporogenes* spores, a low percentage (1 - 5 %) of germinated spores were observed. The germination experiments also indicated that it is possible to germinate *C. sporogenes* spores under aerobic conditions.

Thermal processing of liver pate added *C. sporogenes* spores were performed. The log-reduction of *C. sporogenes* after the 93 °C autoclave program was between 1.1 and 1.6 log(CFU/g). The log-reduction of *C. sporogenes* after the 97 °C autoclave program was between 2.2 and 2.7 log(CFU/g). In liver pate added 0.9 and 1.7 % NaCl, there were no difference in the inactivation of *C. sporogenes* spores. After one month of storage of liver pate trays at 4 °C no difference in the spore concentration was observed.

The laboratory experiments and the semi scale experiment have shown that factors like NaCl concentrations, germinators, and food matrixes changes the D-values. In order to achieve safe heat treated foods, after changes in the food composition, manufacturers should both re-evaluate their processing conditions, and evaluate if extra hurdles are needed for safety, in relation to the shelf life used.

5.1 Future work

There are several aspects of this project that require further investigation. Both further work with the experiments performed in this thesis, but also expand with other additives and food matrixes:

- Expand the testing of different NaCl concentrations to investigate if the trend fits other concentrations and temperatures and perhaps other food matrixes.
- Investigate longer inactivation times for several temperatures.
- Study other germinators and components that can influence the germination of *C. sporogenes* spores.
- Investigate the effect of sodium nitrite on inactivation of *C. sporogenes* spores in different food products.

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