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Abstract

The main objective of this study was to obtain knowledge about *Bacillus pumilus* B367 with relevance to food safety and investigate if *B. pumilus* B367 would be inactivated at lower temperatures for shorter time by applying pressure-assisted thermal sterilisation (PATS) than the classical thermal processing for food matrixes. *B. pumilus* is a spore-forming bacterium, and at favourable environmental conditions it can grow in foods and produce toxin.

The experiment started with finding the temperature that inactivated *B. pumilus* spores in nutrient broth in a water bath. Similar experiments in water bath were then carried out with spores in plastic bags and tuna fish, and finally inactivation experiments in high pressure equipment combined with moderate temperature. Growth of potential of surviving *B. pumilus* was also examined with different types and concentration of preservatives used in food using a turbidometer (Bioscreen C). Different concentration of sodium chloride (NaCl), potassium chloride (KCl) and sodium nitrite (NaNO$_2$), as well as different levels of pH were applied to *B. pumilus* to investigate the growth process of vegetative cells and activated and non-activated spores. The impact of sporulation temperature on heat resistance of spores was also studied. Pressure-assisted thermal sterilisation (PATS) was used to investigate the inactivation of spores in tuna fish.

Heat treatment in water bath gave 1.8, 3.4 and 5.6 log-reduction of spores in LB medium at 80, 90 and 95 ^{o}C for 10 minutes, respectively. Further, a 2.8 and 4.4 log-decrease of spores in tuna fish were obtained at 90 and 95 ^{o}C for 10 minutes, respectively. The heat treatment with preservatives (2 % NaCl, 2 % KCl and 250 ppm NaNO$_2$) showed a 2.2-2.5 log-reduction of spores at 90 ^{o}C for 5 minutes, and a 3.6-3.8 log-decrease at 95 ^{o}C for 5 minutes. As the pH of the medium was reduced from pH 6.2 to 4.0, the inactivation of *B. pumilus* spores increased at the given temperature-time combinations of heat treatment. The spores that sporulated at higher temperatures showed higher heat tolerance. In the experiment with PATS, a 3.5 and 3.7 log-reduction of spores in tuna fish were achieved at 600 MPa-55 ^{o}C and 600 MPa-65 ^{o}C, respectively. The growth experiment with different preservatives revealed that mean time to detection (TTD) measured at optical density (OD) of 0.2 absorbance were longer as the concentration of NaCl and KCl increased from 1 % to 4 %, concentration of NaNO$_2$ increased up to 500 ppm and pH decreased from 6.2 to 4.0. Thus, as the

concentrations of NaCl, KCl and $NaNO_2$ were increased and when the pH of the media decreased, the growth of *B. pumilus* was reduced.

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Abbreviations

a_w	Water activity
CFU	Colony forming units
CytK	Cytotoxin type K
D-value	Decimal reduction time
DNA	Deoxyribonucleic acid
Hbl	Hemolysin
HPP	High pressure processing
HHP	High hydrostatic pressure
LB	Lysogeny broth
MilliQ water	Ultrapure water (trademark)
MHz	Megahertz
MPa	Megapascal
Nhe	Nonhemolytic enterotoxin
OD	Optical density
PATS	Pressure-assisted thermal sterilization
ppm	Parts per million
rpm	Rotation per minute
rRNA	Ribosomal ribonucleic acid
tRNA	Transfer ribonucleic acid
TTD	Time to detection
UV	Ultraviolent
z-value	Thermal resistance constant

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1 Introduction

During the last decade, the food industry is in great demand for ready-to-eat (RTE) or so-called mild heat-processed foods. During production and storage of these foods, major challenges arise in relation to spore-forming bacteria that can destroy the quality of the product. Some spores are also pathogenic and therefore pose a risk of foodborne illness. Spores are very resistant and have high heat resistance compare to their vegetative stem cells (Driks, 2002; Setlow & Johnson, 2013; Zhang, Delbrück, Off, Benke, & Mathys, 2020). They can thus survive light heat treatment and start to germinate and grow in the food if suitable conditions are present (Ghosh & Setlow, 2009). It is not desirable to heat treat the food at too high temperatures as this can reduce the nutrient content and the sensory quality (Barbosa-Cánovas, Medina-Meza, Candoğan, & Bermúdez-Aguirre, 2014; Lopes, Mota, Gomes, Delgadillo, & Saraiva, 2018). Species from the genus *Bacillus* and *Clostridium* produce spores as a protective mechanism against external influences, such as heat, chemicals, dry conditions and radiation (Løvdal, Granum, Rosnes, & Løvdal, 2013; Reineke & Mathys, 2019).

It has long been known that *Bacillus cereus* can cause food poisoning, but in recent years it has been documented that other *Bacillus* species also can cause intoxication in food. Although there are few described cases of food poisoning with *Bacillus* species other than *B. cereus*, one should be aware of the possibility that other species can cause problems in light heat-treated foods. One of these species is *B. pumilus*, which is an optional facultative anaerobic spore former and belongs to the *Bacillus subtilis* group (From, Pukall, Schumann, Hormazábal, & Granum, 2005). It is reported that *B. pumilus* grows well at 12 °C and can form toxins at 10 °C, although growth here is limited. Best toxin production was found at 15 °C (From, Hormazabal, & Granum, 2007). This means that the temperature in poorly controlled refrigeration chains can quickly rise to levels stimulating growth and toxin production in *B. pumilus*, causing the bacteria to become a potential threat to the food products. A dose of ≥ 10^5 *B. pumilus* cells per gram is the assumed amount needed to cause illness in humans (Løvdal, 2011). All strains of *B. pumilus* can form highly resistant endospores and germinate under favourable conditions. It is widely distributed in the environment, and it can also be transferred to food with raw materials, ingredients and by poor production hygiene (From, Hormazabal, & Granum, 2007; Montville & Matthews, 2013). In 2005, there was an outbreak in Norway caused by this bacterium where rice was left at room temperature during storage

(From, Hormazabal, & Granum, 2007). According to the Institute of Public Health, such outbreaks are not required to report to the National Infectious Disease Surveillance System (MSIS). Therefore, cases like this might be under reported and the problem could be larger than we see from reported cases. In addition of being a pathogenic bacterium, *B. pumilus* can also impair the quality of foods. It has been isolated from semi-fried bread where, together with *B. subtilis* and *B. licheniformis*, it was the cause of ropiness in the bread (Leuschner, O' Callaghan, & Arendt, 1998; Rodríguez-Lozano, Campagnoli, Jewel, Monadjemi, & Gaze, 2010).

Therefore, inactivation of *B. pumilus* spores, along with other spore-forming bacteria, is a challenge that must be considered in food processing. Preservation is a process where the objective is to achieve appropriate microbial safety, to preserve the nutritional content and quality, and sensory properties in the best possible manner. From ancient times, drying, pickling, cooking, and smoking were widely used to preserve food. In recent years, new preservation methods such as modified atmospheric packaging (MAP), cold plasma, irradiation and others have been applied (Schottroff et al., 2018). Although new methods are being developed, heat treatment, either alone or as a step in a process, is considered very effective and still the most widely used method of preserving food (Wells-Bennik et al., 2016). When the heat treatment is combined by a packaging concept, recontamination is avoided provided that the food is packed before the heat treatment. An example is sous-vide products which are vacuum-packed prior to the heat treatment (Yousef & Balasubramaniam, 2013). Furthermore, it is necessary to achieve better understanding of the heat resistance of spores and which conditions are significant for this resistance. The physiological state of the microorganisms as well as the composition of the heat treatment medium is decisive for the heat resistance (Leguerinel, Spegagne, Couvert, Gaillard, & Mafart, 2005; Reineke & Mathys, 2019).

An increasing demand for food safety and a focus on healthy nutritious food require continuous development and improvement to current preservation methods. Pressure-assisted thermal sterilization, microwave-assisted thermal sterilization, high-pressure carbon dioxide, high-pressure homogenization, pulsed electric field and UV processing are some of the emerging technologies, which could sterilise or pasteurise food products of the future and are able to better retain the natural flavour and nutrients of the foods with long shelf-life (Barbosa-Cánovas et al., 2014; Lopes et al., 2018; Mathys, Reineke, & Jäger, 2019; Somerville

& Balasubramaniam, 2009). However, before new processing methods can be used and products sent to the market, the methods need to be validated for safety and quality. This master thesis is a part of such validation process and it is an integrated part of the project "High pressure processing and microwave technology for healthy and sustainable food " (M-PATS) financed by the Norconserv Foundation.

1.1 Aim of the study

The main objective of this work was to examine the inactivation of *B. pumilus* B367 spores in different types of media, such as LB medium and food matrix by using thermal treatment and high pressure combined with heating. Besides, specific concentrations of preservatives (NaCl, KCl and NaNO$_2$) and pH were applied to *B. pumilus* spores in LB medium to investigate its effect on inactivation of the spores. The inactivation of *B. pumilus* spores that sporulate at different temperatures were also carried out to study the impact on heat resistance of the spores. Furthermore, the growth of *B. pumilus* at selected concentrations of preservatives (NaCl, KCl and NaNO$_2$) and pH has been performed to examine the growth process.

2 Theory

2.1 The genus *Bacillus*

Bacteria are ubiquitous in the environment and make an important contribution in natural cycles where they account for the decomposition of organic matter (Saxena, Murugan, Chakdar, Anuroopa, & Bagyaraj, 2019). Most bacteria are useful and harmless, but some types of bacteria can lead to degradation of the food quality and cause disease in humans and animals. Some of these pathogenic microorganisms are found among the *Bacillus* species (Özdemir & Arslan, 2019). The genus *Bacillus* belongs to the family Bacillaceae, established by Ferdinand Cohn in 1872 (Logan & Halket, 2011).

2.1.1 Properties of *Bacillus* species

Bacillus species exist in diverse environmental conditions, such as soils, dusts, rocks, aquatic environments and the gut of various insects and animals, and are a common soil saprophyte (Hong et al., 2009). They represent a genus of aerobic spore-forming, Gram-positive and rod-shaped bacteria growing singly, in pairs, chains or as long filaments, but some are facultative anaerobic. They include both motile and non-motile species. Their cell length range between 0.9-10 µm and the diameter from 0.4-1.8 µm. Majority of the species are mesophilic with minimum growth temperature 5-20 ^{o}C, maximum 35-55 ^{o}C and optimum about 30 ^{o}C. There are also some thermophilic, psychrophile, acidophilic or alkaliphilic and salt tolerant to halophilic (Løvdal, 2011; Oyarzabal & Kathariou, 2014).

The genus *Bacillus* composes of many and various group of bacteria, and it is known as the most diverse genus in the family Bacillaceae. Although there are challenges in taxonomy, different identification methods are being used, such as chemotaxonomic characteristics, genomic characteristics (16S rRNA gene sequence analysis), traditional biochemical tests, morphological and physiological characteristics (Dinsdale & Jordan, 2011; Løvdal, 2011). Despite difficulties in classification, the genus *Bacillus* is divided into two major groups: *B. cereus* group and *B. subtilis* group. Among the *B. subtilis* group, we find *B. pumilus, B. licheniformis, B. amyloliquefaciens, B. atrophaeus, B. sonorensis, B. subtilis, B. vallismortis and B. mojavensis.* They are mesophilic and neutrophilic bacterial species (Løvdal, 2011). It has been shown that the *B. subtilis* group of species can produce toxins that can be linked to food poisoning and can therefore be a potential bacterial disease (From, Hormazabal, &

Granum, 2007; Løvdal, 2011). The *B. cereus* group consists of *B. cereus, B. anthracis, B. mycoides, B. megaterium, B. weihenstephanensis and B. thuringiensis*. Most are mesophilic and neutrophilic, but some are psychrotolerant. Moreover, *B. cereus* group of species is well known for its pathogenic properties, causing foodborne illness (Løvdal, 2011).

The *Bacillus* species are best known for the formation of endospores in response to harsh growth conditions aerobically. Endospores are metabolically inactive, and it is this dormancy that plays a main role to resist several agents, such as chemicals, very high temperature ranges, desiccation and radiation; as a result, they can be able to survive for extended time. *Bacillus* spore, unlike of its vegetative cells, cannot be inactivated by mild pasteurization or simple hygiene steps. If endospores are not completely destroyed during food preservation, they can affect food shelf life if they germinate to vegetative cells and produce enzymes that may change organoleptic properties, leading to food spoilage (Oyarzabal & Kathariou, 2014).

Bacillus spores are recognized for their resistance to heat. D-value is the time required to inactivate 90 % of the bacteria at a given temperature. The $D_{100°C}$ values for *B. pumilus*, *B. subtilis,* and *B. licheniformis* in milk have been found as 0.83, 1.18, and 2.37 minutes, respectively (Rodríguez-Lozano et al., 2010). However, the D-value of the spores can be reduced by decreasing the pH in the food products. The spores can be found in milk and dairy products, and spores connected with toxin formation can also be found in plants, flours, and bakery ingredients. Spores that resist treatments can grow quickly in food products kept under favourable conditions. *Bacillus* with cold adapted strains can form toxin at refrigerated temperature. Probably 10^6 to 10^9 cells per gram of food are needed for *B. licheniformis* and *B. subtilis* to make toxin, but since symptoms are normally moderate and self-limiting, food-borne disease of *Bacillus spp*. is usually under reported (Smith, Daifas, El-Khoury, & Austin, 2003).

2.1.2 *Bacillus* toxins

Regarding foodborne illness, difference in incubation time and symptoms can be explained by the fact that there are two major types of toxin that cause emetic and diarrhoeal type of symptoms. The diarrhoeal type is caused by enterotoxins (Hbl, Nhe or CytK), produced during vegetative growth in the hosts small intestine. It has an incubation time of 8-16 hours

(irregularly > 24 hours) and characteristic symptoms, such as abdominal pain, watery (bloody) diarrhoea and nausea. The symptoms usually resolve after 12-24 hours, but sometimes can stay for days. Foods commonly implicated are soups, vegetables, meat products, milk, milk products and sauces (From et al., 2005; Løvdal, 2011; Montville & Matthews, 2013). The infective dose is assumed to be in the range 10^5-10^7 (total) (Montville & Matthews, 2013).

The emetic type is associated with the cyclic peptide cereluide, preformed in foods by growing cells and often results in more severe symptoms than the diarrhoeal type. The toxin can tolerate heat, pH, and proteolysis (From et al., 2005; Montville & Matthews, 2013). It has short incubation time of 0.5-5 hours, with subsequent symptoms as vomiting and malaise, nausea, and diarrhoea. The symptoms usually disappear after 12-24 hours, and foods generally implicated are pasta, noodles, fried and cooked rice, and baked goods (Løvdal, 2011; Montville & Matthews, 2013). The concentration needed to form emetic toxin to induce food poisoning is around 10^5-10^8 cells per gram (Montville & Matthews, 2013).

Both toxin types have been proven for *B. cereus* (Granum & Lund, 1997), and the diarrhoeal type has been detected for *B. pumilus.* For both types of food poisoning, the food involved has usually been heat-treated, and surviving spores are the source of the food poisoning. However, since *B. pumilus* can also grow anaerobically, at 37 °C there is the possibility that this bacterium can produce toxin in the intestine and be the cause of disease in humans and animals (From, Hormazabal, Hardy, & Granum, 2007; From et al., 2005; Granum & Lund, 1997).

Bacillus spp. growth and toxin formation in foods can be inhibited by adding preservatives, such as propionic acid, calcium or potassium propionate, calcium acetate, potassium sorbate, and sorbic acid. Lactic acid bacteria could also prevent *Bacillus spp*. growth in bread due to production of lactic acid by fermentation. Some strains of lactic acid bacteria also make nisin, which is known to have an antimicrobial effect against *Bacillus spp*. Psychrotrophic *B. cereus* strains are able to grow at cold storage, so it is not possible to control with temperature alone, products should be cooled rapidly, and stored at 4 °C. Modified atmosphere packaging (MAP) with carbon dioxide (CO_2) alone cannot prevent the growth of *Bacillus spp*. However, hurdle technology has proven effectual in inhibiting this pathogen (Smith et al., 2003).

2.2 *Bacillus pumilus*

The species *B. pumilus* shares the general characteristics of the genus *Bacillus*, specifically with *B. subtilis* group, which are described in section 2.1. In addition, the size of *B. pumilus* is about 0.7 μm in diameter, come into view singly or in pairs. It forms spores with ellipsoidal to cylindrical in shape. The minimum growth temperature is 5 °C while the maximum ranges 40-50 °C. Moreover, it is catalase positive that breaks hydrogen peroxide into water and oxygen. Unlike of the other *B. subtilis* group, it does not produce α-amylase which is able to hydrolyse starch. It is also positive for casein hydrolysis; and has no vacuoles (Dinsdale & Jordan, 2011; Løvdal, 2011).

     B. pumilus associates with food poisoning and infections. Foodborne illness caused by *B. pumilus* has symptoms such as diarrhoea, vomiting, nausea, and dizziness. It has incubation period of acute to 12 hours after consuming contaminated food. The infective dose is about $\geq 10^5$ *B. pumilus* per gram (Løvdal, 2011). Foods commonly implicated are meat products, sandwiches, pasta, tomato juice and fried or re-heated rice (From, Hormazabal, & Granum, 2007; Kelley et al., 2019; Løvdal, 2011).

     Besides being a quality-degrading and disease-causing bacterium in food, *B. pumilus* is also used as a beneficial bacterium in various contexts. For example, the bacterium is known to produce fungal inhibitory metabolites which inhibit the growth of fungi on plants (Kelley et al., 2019; Munimbazi & Bullerman, 1998). *B. pumilus* has also been shown to have antibacterial properties, which inhibit the growth of *Listeria monocytogenes*. This was evidenced by the results of an experiment with antibacterial activity of over surface microflora on cheese (Siafaras, Hatzikamari, Litopoulou-Tzanetaki, & Tzanetakis, 2008).

2.2.1 Vegetative cell growth

B. pumilus cells are termed as vegetative cell when they are metabolically active and have ability to multiply. They reproduce by binary fission in which two identical cells are formed from a single mother cell. Vegetative cell growth depends on several factors, such as water activity, temperature, pH, atmospheric conditions, competing flora and nutrient availability (Montville & Matthews, 2013). In the formation of bacterial growth curve, a bacterial culture undergoes various phases, namely the lag phase, exponential phase, stationary phase and

death phase depending on those growth factors (Figure 1) (Clark & Ruehl, 1919; Książek, 2010).
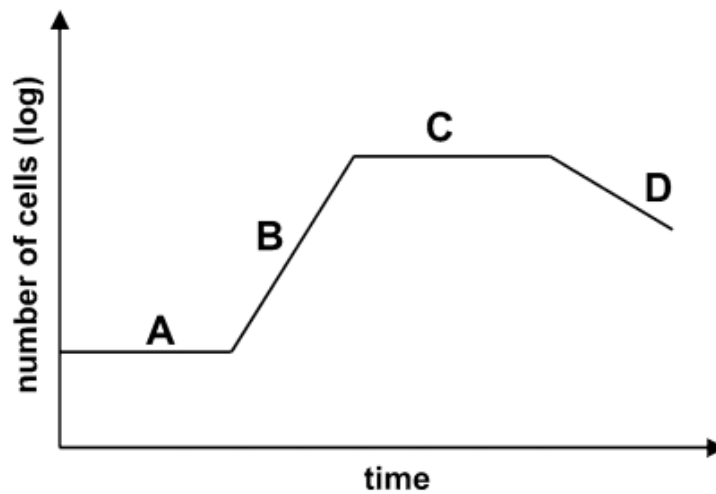

Figure 1: A four-stage pattern of bacteria growth. (A) a lag phase, (B) an exponential phase, (C) a stationary phase and (D) a death phase.

Lag phase is a phase that makes the bacteria ready to begin the exponential growth. It is the less understood growth phase as there is no adequate data that explain physiological and molecular processes of it. However, hypothetical data have showed that the lag phase enables the adaptation necessary for bacterial cells to start utilize new environment by increase their size, store nutrients and the restore of macromolecular injuries as well as formation of cellular elements needed for growth. Moreover, the cells are supposed to be non-replicable in this phase (Książek, 2010; Rolfe et al., 2012).

Exponential phase is the phase where cell division proceeds at a fixed rate. The number of bacterial cells grow exponentially with time provided that the growth factors of the bacteria are favourable for the bacterial cell growth. The bacterial population attain a peak indicating the highest number of cells. At this phase, some changes happen resulting in decreasing of nutrients and gathering of wastes in the environment (Buchanan, Whiting, & Damert, 1997).

The exponential growth of the bacteria ceases at the stationary phase where the number of bacterial cells remain fixed. This is owing to the availability of nutrients are decreased and formation of toxic substances rises. When it comes to food microbiology applications, if the stationary phase is attained, the food is often spoiled if the bacteria are non-pathogenic or the food may be risk to public health if the bacteria are pathogenic

(Buchanan et al., 1997). Following is the death phase, the period of fall off when the numbers of living bacteria are reducing; and the rate is not constant. Then, the bacteria could be changed into inactive form or survive (Clark & Ruehl, 1919).   .

2.2.2 The spore

The spores of *B. pumilus* are produced during sporulation, which is initiated by decreased amount of nutrients in the environment, so that the bacterium can survive for long times and become metabolically inactive (Schottroff et al., 2018; Setlow, 2006). Spores are formed by the bacterium as a protective mechanism to withstand external influences, for instance absence of nutrients, irradiation, strong chemicals, pressure, high temperature, lytic enzymes and presence of antimicrobials and solvents (Driks, 2002; Kohler, Quirk, Welkos, & Cote, 2018; Setlow & Johnson, 2013). The spore's chemical constitution and structure play main roles in spore resistance. The spore has also dissimilar structure to the growing cell with some unique components to spores (Figure 2) (Driks, 2002; Reineke & Mathys, 2019; Setlow, 2006).
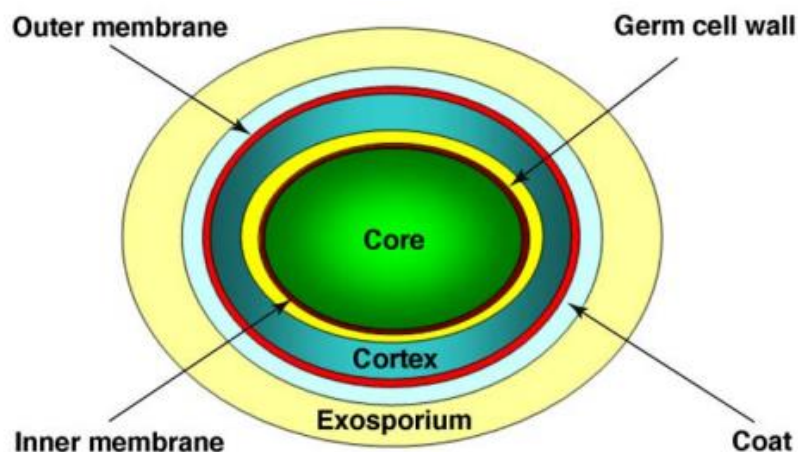

Figure 2: Spore structure of *Bacillus species*. Beginning from the outside and stepping inward the spore layers consist of the exosporium, coats, outer membrane, cortex, germ cell wall, inner membrane and central core (Paredes-Sabja, Setlow, & Sarker, 2011)

Each part of the spore of *Bacillus* species has specific functions, the exosporium is essential in the interaction of the spore with environment and is potentially involved in pathogenicity. The inner membrane is a powerful permeability barrier that plays a key role in spore withstand to several chemicals, while the outer membrane has no clear role in resistance (Kohler et al., 2018; Nicholson, Munakata, Horneck, Melosh, & Setlow, 2000; Setlow, 2006). The spore-coat serves as resistance of spore to some chemicals as well as to

exogenous lytic enzymes that can dissolute the spore cortex; and the germ call wall becomes cell wall after germination (Driks, 2002; Reineke & Mathys, 2019). The cortex contains spore-specific peptidoglycan that is important for making of an inactive spore and for the lowering of the water content of the spore core (Setlow, 2006; Wells-Bennik et al., 2016). The cortex is dissolute during spore germination, and this break down is vital for spore core elongation and outgrowth (Reineke & Mathys, 2019; Setlow, 2003). The last spore layer is the core, which is strongly dehydrated and composes of most spore DNA, enzymes, ribosomes and tRNAs (Driks, 2002; Kohler et al., 2018; Setlow, 2006). A specific type of protein present in the core is small acid-soluble protein (SASP), which protects the DNA. The core also contains dipicolinic acid (DPA) chelated with Ca^{2+} (Wells-Bennik et al., 2016)

Later in the sporulation, the vegetative cells grow, and the spore is released. When considering spores killed by stress treatments, it is important to verify that the treated spores are indeed dead, and not just only lacking the ability of germinating under normal conditions as they could still germinate (Løvdal et al., 2013). Those spores that are not inactivated following heat treatment can return to their vegetative growth cycle in processes including germination and outgrowth (Ghosh & Setlow, 2009; Moir, 2006; Shah, Laaberki, Popham, & Dworkin, 2008). Germinated spores have a lower heat resistance than dormant spores and can be killed at lower temperatures (Setlow, 2003). Induced germination by heat treatment (activation) and subsequent inactivation of germinated spores could be a method to improve spore reduction (Akhtar, Paredes-Sabja, Torres, & Sarker, 2009; Brown, Wiles, & Prentice, 1979; Indest, Buchholz, Faeder, & Setlow, 2009). Double heat treatment (a modified Tyndallization) is such a method consisting of a primary spore heat activation followed by germination and a secondary heat inactivation (Brown et al., 1979). By applying such processes, it can therefore be possible to enhance spore inactivation, particularly for spores that cause food poisoning and spoilage. This will reduce the risk of foodborne illness while maintaining the quality of the product (Løvdal, Hovda, Granum, & Rosnes, 2011). Endospores reflect light and can be seen in bright spheres in a phase contrast microscope. Using this type of microscope, it is easy to distinguish the free spores from the vegetative cells.

2.3 Sporulation

Bacillus species like any other endospore forming bacteria, such as *Clostridium spp.,* perform sporulation, a process where a vegetative cell is transformed into a spore as a reaction to the ceasing of exponential growth of the cell (Lopes et al., 2018; Løvdal et al., 2013). Endospores are the toughest cell types and are obviously the longest surviving. Sporulation involves several complex events. Many of the steps in the sporulation process of *Bacillus* species are genetically controlled and include large number of genes. Sporulation requires a stop in the synthesis of proteins involved in normal vegetative cell metabolism and a start in the synthesis of specific spore proteins. This demands the activation of many different genes, such as *spo* and *ssp* that encode small acid-soluble spore protein, SASP (Errington, 2003; Piggot & Hilbert, 2004).

Sporulation in *Bacillus* species involves several steps that were initially categorized by morphological properties, and then latter by genetic, biochemical, and molecular genetic point of reference. Seven stages (stage 0 - VII) are usually included to describe the total sporulation process. Cells that are still growing are nominated as stage 0; at stage I, two copies of the chromosome form an elongated structure called axial filament. At the stage 0 - II, sporulating cells produce several enzymes, such as α-amylase, proteases, and nucleases, as well as different extracellular antibiotics. During stage II, a division septum is produced at one end of the cell leads to unequal division, with its own chromosome for each. At this stage, the larger compartment (mother cell) engulfs the smaller one (forespore), leading to stage III in that the forespore is within the mother cell, enclosed by layers of two cytoplasmic membrane. At stage IV, peptidoglycan, both cortex and germ cell wall, is set between the two membranes of forespore, then during stage V proteins of spore coat are settled on the exterior of the developing spores. At stage VI, the spore matures and develops full heat tolerance and finally during stage VII the mother cell lyses, setting free the mature spore. The spore is now metabolically inactive and more tolerant to different stress conditions than the growing cells as well as survive for extended time (Figure 3) (Kloosterman et al., 2016; Løvdal, 2011). The ideal time for sporulation in *Bacillus* species at 37 °C is around 7 hours (Errington, 2003; Piggot & Hilbert, 2004).
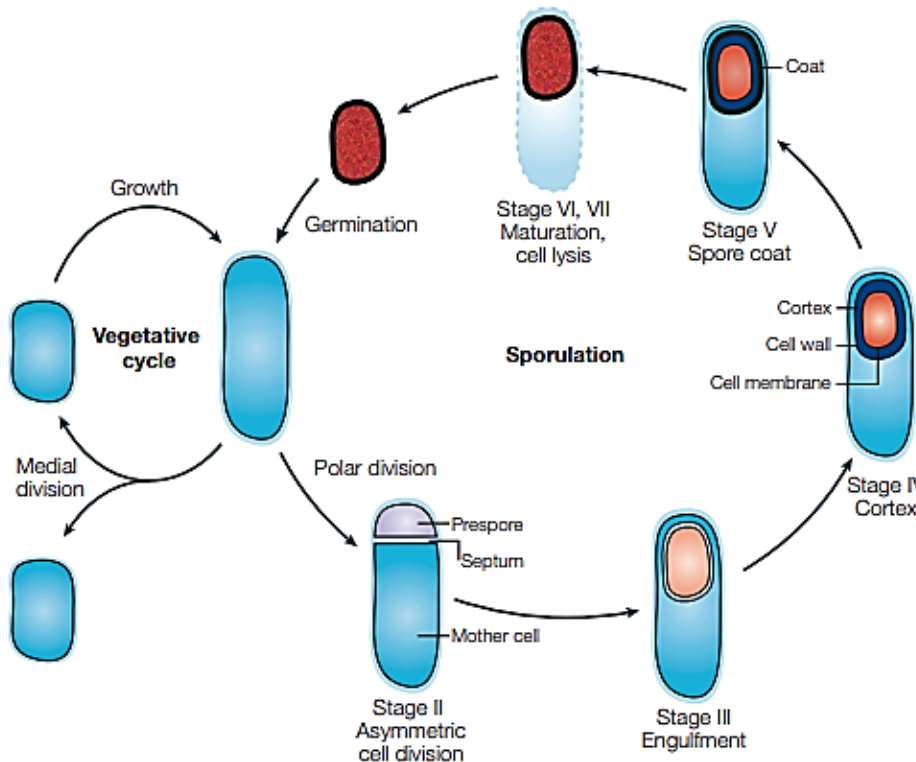
Figure 3: The main stages of the sporulation cycle of *Bacillus subtilis* (Errington, 2003)

2.4 Activation

Activation is a reversible process that enables the spore to germinate when conditions are suitable, otherwise the spore will be deactivated and remain in the resting phase (Collado, Fernández, Rodrigo, Camats, & Lopez, 2003). Exposing the spores to a sublethal heat treatment is the simplest way to activate it. Sublethal heat treatment is often used in laboratory settings and increases the germination rate as well as percentage of germination (Løvdal et al., 2013; Løvdal et al., 2011). Choosing the right temperature and time is the key to activation leading to optimal germination. Temperature and exposure time for optimal activation vary within species and strains (Ghosh & Setlow, 2009; Turnbull, Frawley, & Bull, 2007). Thermophilic strain *Bacillus stearothermophilus*, for instance, achieves activation at temperatures of 110 or 115°C, depending on strain and even batch (Finley & Fields, 1962), and some strains of *B. pumilus, B. cereus, B. weihenstephanensis* and *B. licheniformis* might be activated at moderately elevated temperatures (30-33 °C) (Løvdal et al., 2013). However, for mesophilic bacteria within a range of 60 °C for 90 minutes to 80 °C for 10 minutes is an optimal combination (Samapundo, Heyndrickx, Xhaferi, de Baenst, & Devlieghere, 2014).

For double heat treatments processes, a high germination percentage is desirable so that as many spores as possible can grow up in the first step of moderate heat treatment and be killed in the second heat treatment step. It is believed that a spore suspension should be heat activated before the concentration is determined to obtain a correct calculation. The concentration of a spore suspension will be higher after heat activation compare to a spore suspension without heat activation prior to plating (Turnbull et al., 2007). Other factor that can trigger spore activation is low or high pH, addition of reducing agents like acids, ionizing radiation and various chemicals (Keynan, Evenchik, Halvorson, & Hastings, 1964).

2.5 Germination

Spore germination could lead to deterioration of food products and even serious foodborne illnesses, depending on the kind of spore-forming bacteria. It is essential to take into consideration the germination mechanisms and the factors that activate it (Lopes et al., 2018). The germination of dormant spores of *Bacillus* species is the first important step in altering the spores to vegetative growth. It is divided into three groups based on the inducers: nutrient induced germination, non-nutrient induced germination and muropeptide induced germination. Nutrients that are widely used to induce germination are L-alanine (amino acid) and inosine (nucleotide). Other combinations are also used like a mixture of asparagine, glucose, fructose, and K^+ that has been shown to cause germination. The non-nutrient agents include lysozyme, Ca^{2+}-DPA, cationic surfactants, salts and physical strains (high hydrostatic pressures and abrasion) (Løvdal, 2011; Paredes-Sabja et al., 2011; Setlow, 2003; Zhang et al., 2020). Muropeptides (peptidoglycan fragments) released from growing cells of the same or closely related species can trigger germination via a pathway independent of the germinant receptors (Setlow, Wang, & Li, 2017; Shah et al., 2008).

A spore can remain in the resting phase and withstands external environmental stresses for many years, however, can quickly be revived and restore metabolic activity when conditions are facilitated (Setlow et al., 2017). The three processes that change a spore to vegetative cell are activation, germination and outgrowth, where germination is an irreversible process unlike of activation (Keynan et al., 1964). In nutrient induced germination, when nutrients attach to germinant receptors in the inner membrane of the spore, the release of the spore core's huge depot of dipicolinic acid (DPA) and cations (mostly Ca^{2+}) are induced and replacing of these elements by water. The water initiates the hydrolysis of

peptidoglycan cortex of the spore by one of the two redundant enzymes in *Bacillus spp.*, and finalization of cortex hydrolysis and succeeding germ cell wall enlargement lets full spore core hydration and restarting of spore metabolism and macromolecular formation. The cell will now be in its vegetative form until signals that trigger sporulation may occur (Figure 4) (Lopes et al., 2018; Setlow, 2003; Setlow et al., 2017).
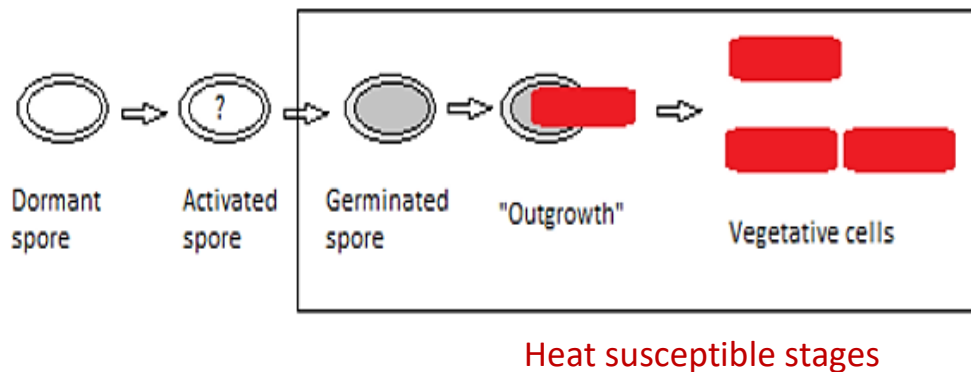

Heat susceptible stages

Figure 4: The alteration of inactive spore to metabolically active vegetative cell. By applying heat-treatment the heat susceptible stages of the *Bacillus* species can be inactivated (Løvdal, 2011).

2.6 Inactivation kinetics

Inactivation kinetics complies with log-linear kinetics that can be represented by D and z-values. The D and z-values are used as parameters for estimating inactivation of bacterial cells or spores. The D-value can be defined as the time required to inactivate 90 % of the bacteria at a certain temperature (T). It is a measure of the rate of inactivation and the heat sensitivity of microorganisms. The temperature change needed to reduce the spores by 90 % or to achieve 1 log-reduction when time is kept constant is known as z-value (Kim et al., 2019; Russell, 1971; U. S. Food and Drug Administration, 2000). It provides information on the relative resistance of microorganism at different temperatures. The D and z-values vary considerably among bacterial species and strains, even within the same group or type. For instance, the D-values at 100 °C for *B. subtilis* strain 4062 and strain 4060 are found to be 3.53 and 4.39 minutes, respectively. The z-values for strain 4073 and strain 4144 are also determined as 5.82 °C and 8.32 °C, respectively *(Berendsen, Zwietering, Kuipers, & Wells-Bennik, 2015)*. Another factor that has impact on the D and z- values is growth stage. It seems that spores are usually less tolerant during exponential or lag phase of growth than during stationary phase. This might be due to the development of stress resistance proteins in stationary phase. The D and z-values could also differ within a species of bacteria of various

extrinsic factors, namely pH, high/low water activity or growth medium (Kim et al., 2019; U. S. Food and Drug Administration, 2000).

In assessing the safety of heat treatment of food, elimination of all pathogenic microorganisms in food seems impossible. However, some general guidelines have been set up. By sterilization, it is meant that there is 12D reduction in bacterial count. 12D is the requirement for heat treatment of canned food (botulinum cook). For conventional pasteurization, the requirement is 6D which means that one has a 6 × D-value at a given temperature (Liato, Labrie, Viel, Benali, & Aïder, 2015; Sevenich, Rauh, & Knorr, 2016).

Pathogens in food product have different tolerance to heat, and those with high heat resistance could grow at the storage conditions. Hence, proteolytic *Clostridium botulinum* type A is the target organism for shelf stable foods as it tolerates most heat. The D-value for this microorganism is 0.21 minutes at 121°C ($D_{121°C}$ = 0.21 min), which is the reference sterilization temperature applied. The demand is to have a 12D lowering of bacteria in the product and 12D will then be 2.52 minutes. The present heat treatment that shelf stable foods acquire is 3 minutes at 121°C, so there is a safety margin for temperature difference in the autoclave. This temperature-time combination corresponds to an integrated process lethality of 3 (F_0 = 3) (Reineke & Mathys, 2019; Sevenich et al., 2016).

To determine the D-value, the different log concentration of spores can be plotted against the heat treatment time or from the linear regression slope of log reduction and time. This curve is known as a survivor curve (Figure 5):

D = (t2 – t1) / (Log N1 - Log N2)   or   D = -1 / slope (Liato et al., 2015; Russell, 1971).
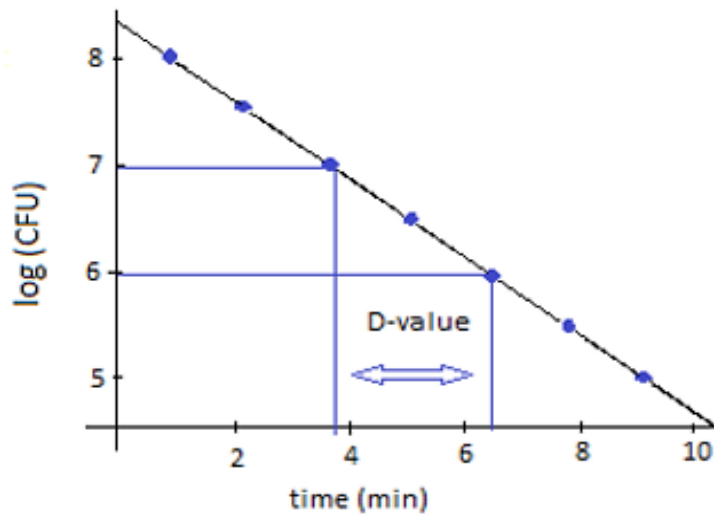
Figure 5: Graphical presentation of inactivation kinetics for the D-values

Whereas, to calculate the z-value the Log D-value is plotted against temperature. This curve known as a heat resistance curve (Figure 6):

$Z = (T2 - T1) / (Log\ D1 - Log\ D2)$ or $Z = -1 / slope$ (Liato et al., 2015; Russell, 1971).
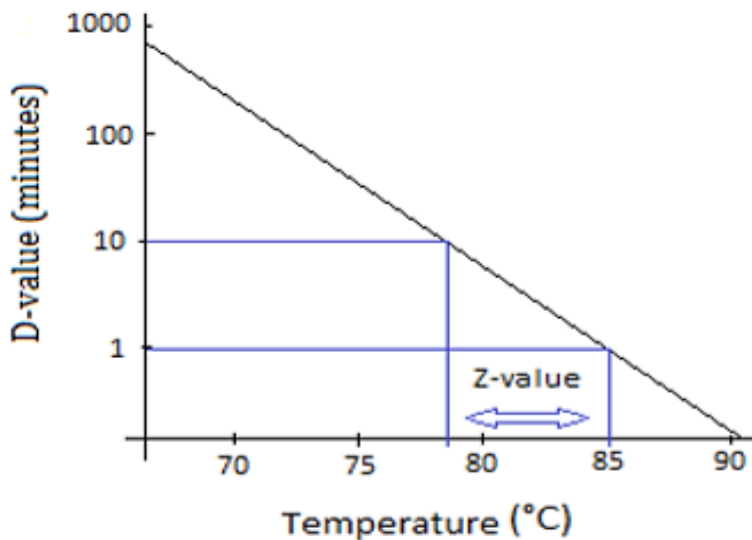
Figure 6: Graphical presentation of inactivation kinetics for the z-values

2.7 Food processing

Food processing is a method where the goal is to achieve appropriate microbial safety and to preserve the nutritional content, quality, and sensory properties in the best possible manner. Shelf stable products are food products that have been sufficiently processed and can safely be stored at room temperature in a sealed container, as canned food. However, food preservation that will have a long shelf life, where the food´s quality is acceptable has been a

challenge to food industries. Major issues arise in relation to spore-forming bacteria that can destroy the quality of the products during processing and storage, as well as cause foodborne diseases (Setlow & Johnson, 2013). Thus, inactivation of these spore-forming bacteria to an acceptable low level in a food is the main objective of food processing. In addition, foods are complex ecosystems consist of intrinsic factors inherent to the food, such as pH, water activity (a_w) and nutrients, and extrinsic factors external to it, for example temperature, gaseous environments and the presence of other bacteria. Both intrinsic and extrinsic factors can be manipulated to preserve the food by designing conditions in the food that limit bacterial growth (Reineke & Mathys, 2019).

Many types of food processing have been utilized and optimized to achieve better quality products and ensure food safety. Typical processing used by processors include dehydration, cooking, freezing, smoking, active packaging, and others. When applying hurdle technology, pH and water activity depressors (salts and sugars), antimicrobials and spices need to be considered (Barbosa-Cánovas et al., 2014; Reineke & Mathys, 2019). Researchers from the food industry are responding to the consumer's desire for microbiologically safe and minimally processed foods by developing various advanced thermal based technologies, such as aseptic processing and ohmic, microwave, and radio-frequency heating, and nonthermal processing methods, such as irradiation and high pressure, pulsed electric field, and UV processing (Balasubramaniam, Martinez-Monteagudo, & Gupta, 2015)

2.7.1 Thermal processing

Thermal processing is used to produce safe and shelf stable foods and to eliminate pathogenic microorganisms. In other words, it reduces the probability of survival and growth of the microorganisms to an acceptable low level in given food products. Heat treatment is the principal and presumably oldest method for ensuring microbial safety of food products, which includes simple methods, such as roasting and cooking, or combinations of thermal and chemical preservation methods, such as smoking. Besides to the formation of a unique flavour profile and the digestibility of cooked food, the application of moderate heat inactivates vegetative cells of bacteria, remarkably increasing the shelf life of heat-treated food (Reineke & Mathys, 2019). During the development of any new thermal processed product it is important to evaluate the combined effects of the total system, such as heat process,

preservatives, packaging and storage conditions so as to ensure that the product is of a good microbiological standard and does not possess any food safety risk (Wells-Bennik et al., 2016). To produce such a high-quality product there is a tendency towards applying fewer preservatives and minimal heat treatment. Different bacteria have inherently different resistance to high temperatures. Vegetative cells are usually the most vulnerable whilst endospores are much more resistant. The type of foodstuff to be heat treated will often have associated microorganisms with a high heat tolerance which it is essential to inactivate to ensure the safety of the food.

Most of the time, shelf-stable foods such as canned food require intense thermal processing to warrant sterility. For example, products with 1-5 years of target time that are stored at room temperature. The products are processed based on specifications, where only temperature and time are manipulated. The aim of this type of treatment is to attain a target sterilization temperature (121 $^\circ$C for a given time) to kill harmful pathogenic spores of *Clostridium botulinum* and other species that cause spoilage in products. Thus, the purpose of sterilization is to make sure that the number of microorganisms in the food products are decreased by 12 log CFU/ml. The time and temperature required to reach this final microbial load is known as 12D. Thus, safety of packaged low-acid food products can be ensured by eliminating pathogenic microorganisms (Barbosa-Cánovas et al., 2014; Liato et al., 2015). In foods susceptible to heating, it is only possible to eliminate vegetative cells and the more sensitive bacterial spores, and some additional factors are needed to ensure preservation even for limited periods of time. This type of treatments is commonly known as pasteurisation. A 6 log CFU/ml reduction of microorganisms is the requirement for pasteurisation at a given temperature, and 6D is the time required to pasteurise the product (Liato et al., 2015).

The application of high temperatures, however, affects adversely to the quality of foods since they cause the degradation of some nutritional compounds such as vitamins, carbohydrates, lipids, proteins, or minerals. The increase of chemical reactions leading to off-flavours, destruction of health-related compounds in foods such as pigments, polyphenols and antioxidant compounds, and taste or colour changes, among others, are particularly important drawbacks of conventional thermal processing technologies (Barbosa-Cánovas et al., 2014; Reineke & Mathys, 2019; Somerville & Balasubramaniam, 2009). Because of these

limitations and the increased perception of consumers towards food products with fresh-like appeal and long shelf life, which are minimally processed with fewer preservatives, food industries today are looking for alternative technologies for processing low-acid, shelf-stable foods (Lopes et al., 2018; Mathys et al., 2019).

2.7.2 High pressure processing

During the last decade there has been a growing interest in novel technologies that applies high pressure processing (HPP) for food preservation. As an emerging nonthermal spore inactivation method, HPP can retain food quality better than the heat treatment in terms of nutritional value, colour and other sensorial attributes (Zhang et al., 2020). Pressure treatment, with or without heat, can result in either pasteurization or sterilization of food products depending upon the intensity of the treatment. Pressure treatment at ambient temperatures has minimal influence on product chemistry and can be used to pasteurize foods. Application of pressure decreases the need of high thermal exposure of the product during processing, thereby protecting a variety of bioactive compounds (Balasubramaniam et al., 2015).

Industrial processes are typically performed in a pressure range of 200-600 MPa, with holding times of up to 10 minutes with typical temperature increase rates of around 3 to 9 $^{\circ}$C per 100 MPa due to the so-called adiabatic heating, that is, a product-dependent temperature increase caused by compression, and thus allowing pasteurization at distinctly lower temperatures compared to thermal treatments. If the product contains a large amount of fat, such as cream or butter, the temperature rise is larger (8-9 $^{\circ}$C/100 MPa). Using pressure allows to decrease the sample´s volume, which results in an increase in sample temperature due to physical compression of the food. (Barbosa-Cánovas et al., 2014; Lopes et al., 2018; Schottroff et al., 2018). The following are typical components of HPP equipment: cylindrical pressure vessel, two end closures (to cover the cylindrical pressure vessel), yoke (for restraining end closures while under pressure), high pressure pump and intensifier (for generating target pressures), process control and instrumentation, and a handling system (for loading and removing the product) (Balasubramaniam et al., 2015).

During HPP, the product is placed into the pressure chamber, and the chamber is closed and sealed. The chamber is linked to a high-pressure pump and a corresponding

pressure intensifier and filled with a pressure transmitting liquid, for example water, and additional water is pumped into the system to increase the pressure. The pressure cycle begins by pumping a pressure-transmitting fluid, at the selected starting temperature, into the chamber and allowing the remaining air to escape through a vent valve. Water is the commonly used pressure-transmitting fluid in commercial-scale pasteurization equipment due to its availability, non-toxicity, and low cost. Once the remaining air has been removed, the vent valve is closed, and more fluid is pumped into the chamber using hydraulic pumps and pressure intensifiers to form the desired pressure. Depending on the power of the hydraulic pump, the ramp rates to the target pressure may range from a few seconds to several minutes. At the end of the pressure cycle, when the pressure has been released from the system, the chamber is opened and the product is unloaded (Evelyn & Silva, 2018; Schottroff et al., 2018; Somerville & Balasubramaniam, 2009).

Inactivation kinetics of HPP treated pathogenic *Clostridium* and *Bacillus* usually show tailing behaviour, that is, a decline in inactivation amounts compared to a linear progression, toward the end of the process, with increasing treatment intensities. The reasons for this tailing could be due to the existence of microbial spore population with different individual pressure tolerances owing to genetic variation, as well as adaption to external stresses. The tails in the survival curves pose a challenge to HPP processors, thus severe processing conditions (higher temperature and/or pressure) might be more effective. It should be ensured that preservation processes requiring HPP are designed in such a way that they are completely inactivate the bacterial target populations or to design treatment concepts for the specific control and avoidance of recovery by applying the hurdle concept. Moreover, the usual HPP pre-packaging can be combined with other hurdles such as acidification and addition of preservatives or inhibitors to slow down the growth of resistant spore-formers which may have survived the HPP process, and thus extend the food shelf life and enhance the food safety. (Lopes et al., 2018; Schottroff et al., 2018; Silva & Evelyn, 2019). Table 1 summarizes some main advantages and drawbacks of high-pressure processing in the food industry.

Table 1: Advantages and drawbacks of HPP in food products (Balasubramaniam et al., 2015)

Description	Advantages	Drawbacks
Hydrostatic pressure	Fast, uniform distribution throughout the product	Semi-continuous performance
Thermal distribution	Decreased thermal exposure Immediate temperature rise and subsequent cooling upon depressurization	The preheating step for pressure-assisted thermal sterilization (PATS) Thermal non-uniformity during PATS
Physical compression	Suited to high moisture-content products	Not suited to foods consisting of dissimilar compressibility materials, such as marshmallows
Product handling	Suited to both liquid and pumpable foods	Throughput limited owing to Semi-continuous operation
Process time	Independent of food shape and size	
Functionality	Novel product formulation opportunity Distinct products through pressure effects such as, protein denaturation, carbohydrate gelatinization, and fat crystallization	
Reaction rate	At some pressure-thermal ranges, pressure accelerates microbial inactivation	Pressure alone cannot inactivate bacterial spores, variable efficiency in enzyme inactivation
Consumer acceptance	Consumer acceptance as a physical process	Higher processing costs and semi-continuous operations are obstacles for commodity product processing

2.7.3 Pressure-assisted thermal sterilization

Pressure-assisted thermal sterilization (PATS) is the innovation which expands the uses of HPP by taking advantage of the self-generated heat while compression is taking place. It is one of the promising technologies to sterilise the food products of the future and provides better nutrient retention and superior taste than the traditional food processing (Schottroff et al., 2018). A PATS system consists of six major steps: (i) vacuum packaging and product loading, (ii) preheating to target temperature, (iii) product equilibrium to initial temperature, (iv) product temperature increase to pressurisation temperature by means of compression

heating, (v) product temperature decrease during decompression, and (vi) product cooling to ambient temperature (Barbosa-Cánovas et al., 2014).

PATS uses a combination of high pressure, moderate temperature, and short time to produce superior quality, shelf-stable food products. The pressures applied in PATS are ranging from 500 to 900 MPa and initial temperatures of 60 to 90 °C for up to 5 minutes to low-acid food products to achieve commercially sterile products. The initial temperature can reach 90 to 130 °C during internal compression heating at pressure of 600 MPa or greater and cool rapidly during decompression at the time of processing due to adiabatic heating. Applying pressure allows to reduce the sample´s volume, which causes an increase in sample temperature owing to physical compression of the food. The rapid temperature increasing during compression and the temperature decrease in the product upon decompression is a unique benefit of the process as compared to traditional retort process. This helps the food products to be sterilized with less thermal exposure (Dhawan et al., 2014; Lopes et al., 2018; Somerville & Balasubramaniam, 2009; Wang, Ismail, & Farid, 2017).

The PATS process of a low acid food product usually begins with removing air from the food, and vacuum packaging the product in a high-barrier, flexible pouch. The selected packaging material should have at least one flexible interface, which will ensure pressure transfer through the package to the food material. Different flexible packaging structures can be used, allowing the technology to be applied to both liquid and solid foods. Thus, rigid metal containers may not survive the pressure treatment (Somerville & Balasubramaniam, 2009). The pre-packaged product is preheated to a chosen temperature prior to the pressure-assisted processing. The pressure chamber is also preheated to reduce heat loss to the environment and ensure uniform treatment. After preheating, the packaged product is placed into the pressure chamber, and the chamber is closed and sealed. After processing, the product is then chilled to ambient temperature to prevent any further thermal degradation (Evelyn & Silva, 2018; Schottroff et al., 2018; Somerville & Balasubramaniam, 2009).

In PATS, both pressure and temperature are applied concurrently. While temperature changes during processing can result in both volume and energy changes, pressure mainly influences the volume of a product being treated. Pressure treatment at ambient temperature is an effectual method for sterilizing vegetative microorganisms via inhibition of protein synthesis, enzyme denaturation and decrease of lipid membrane fluidity. A

combination of high pressure and temperature is needed to inactivate bacterial spores (Schottroff et al., 2018). In 2009, the commercial use of PATS for low-acid foods as well as a pressure-enhanced sterilization process for commercial production of shelf-stable foods were approved by the U.S. Food and Drug Administration (FDA). PATS renders better flavour, texture, colour and aroma retention than the classical retorted products in pouches. The combination of high pressure with heat gives opportunities to develop new food types such as those derived from gel and starch. Animal tissues, fruits and vegetables are also handled to be the bases to produce completely new food products. (Barbosa-Cánovas et al., 2014; Reineke, Mathys, Heinz, & Knorr, 2013).

Table 2: Overview of different HHP, PATP and PATS experiments

Conditions	Target microorganisms	Type of food	Main results	Reference
600 MPa, 70°C, 1 min	*B. pumilus, B. cereus, B. licheniformis, B. subtilis B. circulans, B. coagulans, B. mycoides,* and *B. sphaericus*	Skim milk	Variable spore sensitivity according to the strain. Spore reduction ranged from 0-6 log. *B. sphaericus* was the most resistant and *B. circulans* was the least resistant	(Lopes et al., 2018) (Wimalaratne & Farid, 2008)
600 MPa, 120 °C, 5 min	*B. amyloliquefaciens*	Milk	Inactivates 7.0 log CFU/ml	(Sergio & Marleny, 2015)
400-600 MPa, 0-40 min, 40-60 °C	*B. licheniformis*	Carrot juice	241 to 465 MPa (D value range 23.3 to 31 °C)	(Tola & Ramaswamy, 2014)

2.7.4 Spore inactivation by PATS

PATS processing has been reported to be more effective than heat treatment for bacterial spore inactivation and has the potential to deliver novel chilled or shelf-stable products with improved sensory and nutritional qualities through reduction in overall thermal exposure during processing (Barbosa-Cánovas et al., 2014). The heat resistance of the spore can be reduced due to rapid hydration of the spore core as a result of increasing the treatment temperature (> 60 °C) and pressure (> 500 MPa). It has been established that spore inactivation under PATS proceeds as at least a two-step process. First, spores are induced by

high pressure to rapidly release Ca^{2+}-DPA, and the hydrated spores are subsequently killed by the combination of pressure and heat due to their increased overall stress sensitivity. Consequently, the ability of a spore to retain Ca^{2+}-DPA under high pressure at a certain process temperature is a major factor for the pressure resistance of a single spore. The temperature rise during the treatment is a significant factor to establish the efficiency of inactivation (Reineke et al., 2013).

Although the effectiveness of PATS on the inactivation of microorganisms directly is increased with pressure and temperature, inactivation rates are also dependent on the bacterial species and strains within the same species. *Clostridium botulinum* spores are the target pathogen for PATS treatment (Sevenich et al., 2016; Silva & Evelyn, 2019; Somerville & Balasubramaniam, 2009). In addition, the food composition, pH, and water activity among other factors also affect the efficiency of using PATS for inactivation of microorganisms. For example, high acidic foods (pH < 4.6) with high water activity (a_w > 0.95) favour spore inactivation, while low acidic foods with low water activity need longer treatment time or higher pressure and temperature to attain microbial inactivation. Besides, since some bacterial spores during processing get injured, monitoring of bacterial growth should be examined during storage (Lopes et al., 2018; Somerville & Balasubramaniam, 2009).

The sporulation temperature and the composition of the sporulation media can also influence the resistance of spores to pressure. *Bacillus* species that sporulated at lower temperatures showed higher resistance to high pressure. For example *B. subtilis* spores prepared at 30°C are more pressure-resistant than those sporulated at over 37 or 44 °C, after a treatment at 300 MPa and 55 °C for 30 min (Reineke et al., 2013). Resistance to pressure increased after demineralization of spores and decreased after remineralization of spores with Ca^{2+} or Mg^{2+}, but not with Mn^{2+} or K^+. This may suggest that Ca^{2+} or Mg^{2+} increase the rate of germination under HP (Reineke & Mathys, 2019). However, the highest influence on inactivation in real food is often due to temperature increases caused by the high initial starting temperatures and owing to various adiabatic heats of compression depending on the composition of the food treated. Thus, the same pressure profiles with identical initial starting temperature may be completely different for various food matrixes (Lopes et al., 2018; Reineke et al., 2013).

Furthermore, the product quality and shelf life are usually impacted more by packaging material barrier properties, storage conditions, and time than the PATS treatment itself. The choice of packaging material with adequate barrier properties is critical for preserving PATS product quality and its long-term storage stability. For instance, Nylon packaging material best preserved the colour and β-carotene content, while Polyethylene packaging material changed adversely the colour and β-carotene content in carrot after PATS treatment and following storage. However, no bacterial growth was detected in these two pouches tested after 12 weeks storage at 25 of 37 °C (Ayvaz et al., 2012). Although the PATS process is considered as advantageous due to its shorter time in comparison to thermally treated food products, lower processing temperatures cannot yet be certain to *C. botulinum* inactivation until the right temperature/pressure/time combinations are established (Barbosa-Cánovas et al., 2014).

3 Materials and methods

3.1 Test microorganism

B. pumilus B367 was stored in microbank at -80 °C prior to use. The strain B367 was purchased from the Pasteur Institute, Paris. A total of eight spore batches were produced. Some of the spore batches were produced in different ways to investigate the effect of sporulation temperature on heat tolerance of *B. pumilus* spores. Spore batches four, five and seven were produced at sporulation temperatures of 15, 25 and 42 °C, respectively, while the other spore batches were produced at 37 °C. An overview of the different methods and experiments performed in this thesis is presented in Figure 7.
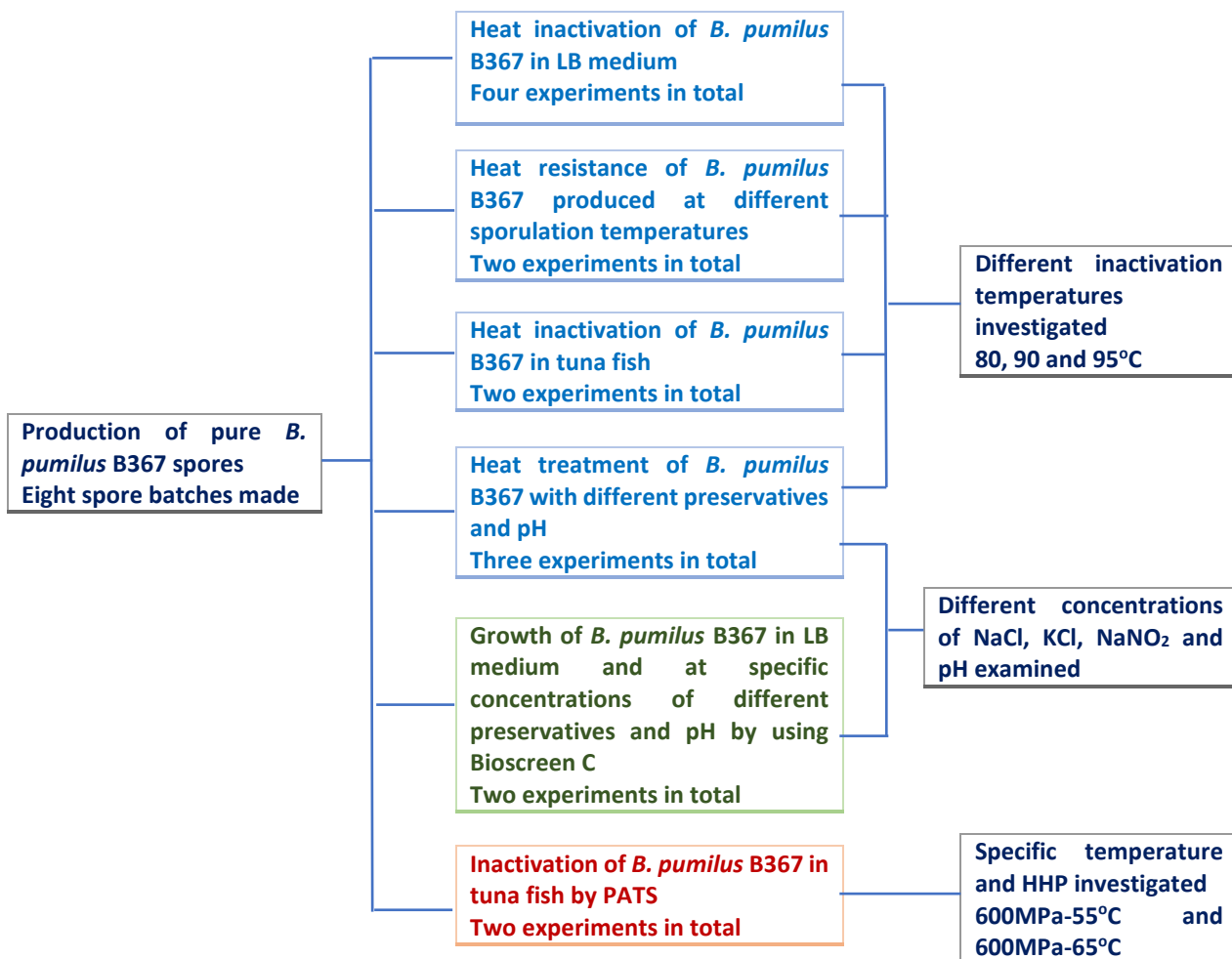

Figure 7: An overview of the experiments conducted in this thesis

3.2 Storage

The stock microorganisms were stored frozen in Microbank® (Prolab Diagnostic, Wirral, U.K.), a vial that contains sterile coloured beads with cryopreservative (glycerol), at the laboratory of Nofima (Figure 8). The *B. pumilus* B367 was grown in an Erlenmeyer tube contained 100 ml LB medium incubated at 37 °C for 24 hours. Then 0.5 ml of the sample from Erlenmeyer tube was pipetted and inoculated into a new Microbank® tubes. The Microbank® was shaken 5-6 times for proper penetration of the bacteria into the pores. The tube was then stored at -80°C until use.
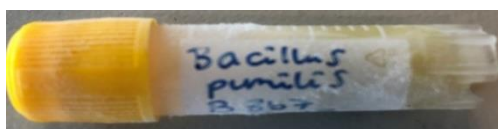

Figure 8: Microbank® (Prolab Diagnostic) with *B. pumilus* B367

3.3 Production of spores

In the laboratory experiments, two different kinds of sporulation techniques were applied. When the sporulation process was begun to perform, a bead from the Microbank® tube was taken and inoculated in a sterile 100 ml Erlenmeyer tube containing 40 ml LB medium. The tube was then incubated at optimal temperature (37 °C) while shaking at 200 rpm for 24 hours on an orbital shaker (Infor HT Multitron, Based, Switzerland). The next day, in the first sporulation technique, a drop from the overnight bacterial cell suspension was added into each of the three 250 ml Erlenmeyer tube containing 37.5 ml of 2× SG medium each. These tubes were incubated at a sporulation temperature of 37 °C, while shaking at 200 rpm for about 3 days until > 90 % of spores were present (Setlow, 2019).

The 2× Schaeffer's-glucose (SG) medium is a modification of Schaeffer's medium. Schaeffer's medium is sporulation agar consisting glucose that can be used as a solid medium or liquid medium with the absence of agar for indication of sporulation by exhaustion of growth substrates. The 2× SG medium is richer and gives higher cell densities than Schaeffer`s medium (Nicholson & Setlow, 1990).

When the sporulation medium contained > 90 % spores, the spores were then harvested and cleaned. In the first step, spore suspension was pooled in smaller centrifugation tubes (30 ml) and centrifuged (Avanti J-26XP, USA) at 10,000 × g and 4 °C for 10 minutes. After discarding the supernatants, the spore pellet was resuspended in ¼ volume of autoclaved MilliQ water and centrifuged again at 10,000 × g and 4 °C for 10 minutes three times on day one. Afterwards, the spores were resuspended with 1/5 the original culture volume of autoclaved MilliQ water and incubated at 4 °C for a few days. The spore suspension was then centrifuged at 20,000 × g for 20 minutes at 4 °C and the supernatant was discarded. 1/5 the original culture volume of autoclaved MilliQ water was then added to the spores, incubated at 4 °C for 24 hours (Nicholson & Setlow, 1990; Setlow, 2019). The next 2 days, the spore suspension was centrifuged, resuspended, and incubated with the same procedures as the day before until < 1 % vegetative cells remained.

The percentage of vegetative cells remaining was monitored by counting with the phase contrast microscope (LEICA 1000, Wetzler, Germany) at PH3 100× magnifications after each centrifugation. 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 9).
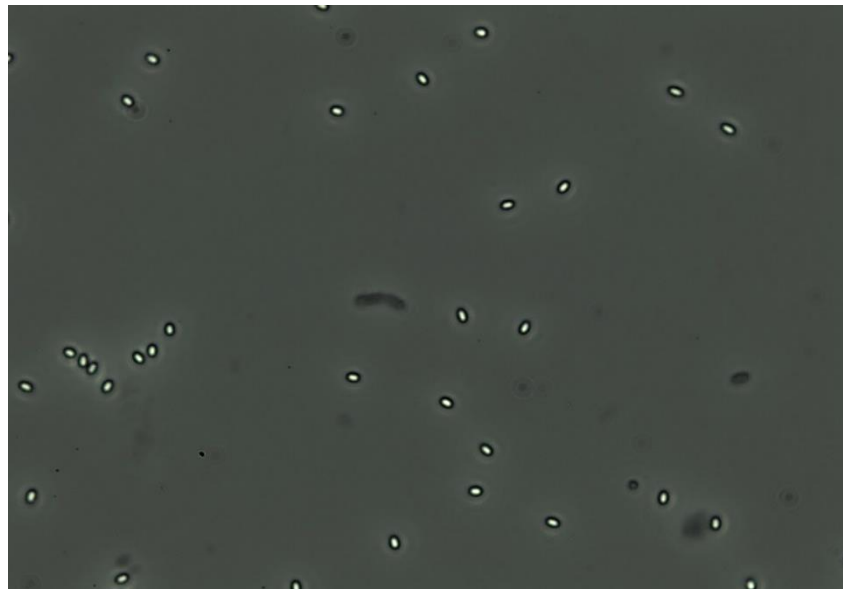

Figure 9: *B. pumilus* B367 spores under PH3 100× magnification phase contrast microscope. Dormant spores are phase bright (white), while the vegetative cells are phase dark (grey).

The concentration of spores was calculated using both standard plate-count method and EddyJet. Each spore batch was diluted to a final volume of 22.5-27 ml. The final concentration of B367 spores was 1.3×10^8 - 1.8×10^9 spores/ml in a 22.5 ml spore-suspension and stored at refrigerated temperature (4 °C) until further experiments (Table 3).

In the second technique of sporulation, the aim was to produce spores at different sporulation temperature (15, 25, 37 and 42 °C). Thus, 0.1 ml from the overnight culture was plated and spread on the 2× SG medium plates using L-Rod, then incubated at 15, 25, 37 and 42 °C. After 5 days, harvesting of spores were started for those spores that were sporulated at 25, 37 and 42 °C. However, harvesting of spores incubated at 15 °C were started after 7 days, this was due to it took two extra days to obtain > 90 % spores. The process of harvesting and cleaning remained the same as the first technique of sporulation (Table 3).

Table 3: Different conditions used for production of spore batches in 2x SG liquid medium.

Batch	Sporulation temperature (°C)	Incubation time (days)	Concentration (spores/ml)	Date washed	Sporulation medium
1	37	3	8.3×10^8	15.09.19	45 ml in 300 ml bottle
2	37	3	1.8×10^9	17.10.19	37.5 ml in 250 ml bottle
3	37	3	8.4×10^8	10.12.19	37.5 ml in 250 ml bottle
4	15	7	1.3×10^8	19.01.20	medium plates
5	25	5	2.8×10^8	17.01.20	medium plates
6	37	5	1.1×10^9	17.01.20	medium plates
7	42	5	3.0×10^8	17.01.20	medium plates
8	37	3	1.2×10^9	11.03.20	37.5 ml in 250 ml bottle

3.3.1 Calculation of spore concentration

To determine the concentration of *B. pumilus* B367 spores, the spores were diluted using sterile MilliQ water in Eppendorf tubes (10-fold serial dilutions). The samples were plated and spread using L-rod on the LB agar media plates. Likewise, the samples were plated using EddyJet. The plates were incubated at 37 °C for 24 hours. The next day, the number of colonies were counted and the concentrations (CFU/ml) calculated.

3.4 Inactivation of *B. pumilus* spores in water bath

In water bath experiments using hot water, it is important that the temperature in the water bath is correct and stays stable for the results to be reliable. A water bath with insufficient heat distribution will result in measurement errors. The water bath used to measure inactivation in this task was Hetotherm (DBT, Heto Lab Equipment, Denmark). In addition to the digital displayed temperature on the water bath, an external thermometer was also used to monitor the temperature of the water (Figure 10).

The spores were diluted with 10-fold serial dilutions with LB medium in Eppendorf tubes, then 500 µl of the dilution was pipetted into 2 ml screw cap tubes (Nunc tubes) and stored in the ice slurry prior to inactivation to avoid germination. Three parallels of Nunc tubes for each heat treatment were prepared. At each inactivation, three parallels of Nunc tubes were placed on a floating element and transferred to a closed water bath. The spores were heat treated at 80, 90 and 95 °C for 10 minutes.

The tubes were then taken out of the water bath and put on ice-water immediately for rapid cooling. Then 10-fold serial dilutions were made using sterile MilliQ water, and the samples were plated and spread using L-rod on the LB agar media plates. The plates were incubated at 37 °C for 24 hours. The untreated samples were used as a control for each experiment to obtain the initial spore counts. After incubation, the number of colonies were counted and the concentration (CFU/ml) calculated. The logarithm of survivors (log CFU/ml) was used as the spore reduction after different treatments (Liang et al., 2019). The experiment was performed twice at two different days with the same spore batch.
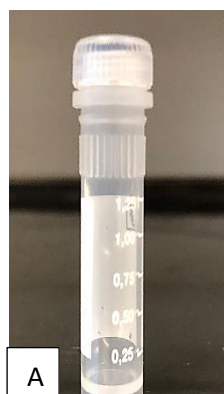
Figure 10: Equipment used in the water bath experiments. a) Screw cap tube (Nunc tube), b) Temperature detector, and c) A floating element holds the nunc tubes in water bath for heat treatment.

3.5 Growth of *B. pumilus* vegetative cells

Bioscreen C (OY Growth Curves Ab Ltd, Helsinki, Finland) was used to measure optical density (OD) of the vegetative cells in real time to study the growth process. Bioscreen C is a computer-controlled incubator and reader equipped with eight filters from 450 to 600 nm including a wideband filter, and it has a sensitive light source measuring turbidity (Figure 11). This technique is more sensitive than a normal spectrophotometer. The incubator system in Bioscreen C can incubate, shake, and calculate optical density of samples for a long period of span. This technique is reliable (maintain temperature with a 0.1 °C accuracy), rapid and decreases the numbers of test materials needed for each experiment (Johnston, 1998). Honeycomb microtiter plates are sterile multi well plates packed individually in boxes of 10 × 10 wells. This disposable microtiter plates (Greiner bio-one (GBO), Germany) that are used in Bioscreen C are sterile and can measure 200 samples wells (two microtiter plates) per test.

Figure 11: Equipment used for the growth experiment. a) Bioscreen C used to read the growth process and b) Honeycomb microtiter plate of 10 × 10 wells that is used in the Bioscreen C.

The overnight bacterial cell suspension was prepared with initial number of 10^9 cells/ml. To run the Bioscreen C, 10^9 cells/ml were diluted with LB medium to obtain 10^4 cells/ml. Then the 10^4 bacterial cell/ml suspension was further diluted with both LB medium alone and 2 % w/v NaCl added LB medium (10-fold serial dilutions). Afterwards, 100 µl from each serial diluted sample and the control (LB medium and 2 % w/v added NaCl media without cells) were inoculated in two replicates in the wells of mircotiter plate (Table 4). The microtiter plate with samples added was then mounted in Bioscreen C, and absorbance read at 600 nm (A_{600}) at regular time intervals. The plate was shaken for 10 seconds before each measurement. The measurements were performed every 20 minutes for a period of 24 hours. The experiment was conducted at 37 °C.

Table 4: A Bioscreen C microtiter plate setup

Well number	Samples
1 - 10	100 µl cell suspension with LB medium (10^{-1} to 10^{-10} dilutions)
11 - 20	100 µl cell suspension with LB medium (10^{-1} to 10^{-10} dilutions)
21 - 30	100 µl cell suspension with 2 % w/v NaCl + LB medium (10^{-1} to 10^{-10} dilutions)
31 - 40	100 µl cell suspension with 2 % w/v NaCl + LB medium (10^{-1} to 10^{-10} dilutions)
41 - 42	100 µl of LB medium (negative control)
43 - 44	100 µl of 2 % w/v NaCl + LB medium (negative control)

3.6 Growth of *B. pumilus* at selected levels of different preservatives and pH

The initial concentration of spore suspension (stored in MilliQ water at 4 °C) from batch 1 was 10^8 CFU/ml, then further diluted to 10^4 CFU/ml with LB medium to run the Bioscreen C. Similarly, the initial 10^9 overnight bacterial cells were diluted to 10^4 cells. Afterwards, 5 ml from the diluted spores were activated by applying 80 °C for 10 minutes in the water bath. All the samples were kept in ice water prior to inactivation to avoid germination.

From the stock solutions, 2, 4 and 8 % w/v (NaCl and KCl) solutions, and 125, 250 and 1000 ppm $NaNO_2$ solutions of LB medium were prepared. In addition, pH 3, 4 and 5 solutions were prepared. Then, 50 µl from each concentration of NaCl, KCl, $NaNO_2$ and pH were loaded to Bioscreen C microtiter plate in three parallels. 50 µl from each of non-activated spores, activated spores and overnight bacterial cells were added to each preservatives and pH in the three parallels, forming 100 µl sample in each well of microtiter plate. As a result, the concentrations of preservatives were decreased from 2, 4 and 8 % to 1, 2 and 4 % w/v (NaCl and KCl), respectively; and from 125, 250 and 1000 ppm to 62.5, 125 and 500 ppm $NaNO_2$, respectively. The sample with pH 3, 4 and 5 were increased to pH 4, 5 and 6.2, respectively when measured using pH-meter (Table 5). The Bioscreen C was then run for 7 days at 25 °C with reading A_{600} at each 30 minutes. The plate was shaken for 10 seconds before each reading. Moreover, the initial concentrations of the diluted non-activated spores, diluted activated spores and diluted overnight bacterial cells were further diluted, incubated, and later calculated.

Table 5: A Bioscreen C mircotiter plate setup

	01	02	03	04	05	06	07	08	09	10
1	4 % NaCl	4 % KCl	500 ppm $NaNO_2$	pH 4	4 % NaCl	4 % KCl	500 ppm $NaNO_2$	pH 4	pH 4	4 % NaCl
2	4 % NaCl	4 % KCl	500 ppm $NaNO_2$	pH 4	4 % NaCl	4 % KCl	500 ppm $NaNO_2$	pH 4	pH 4	4 % NaCl
3	4 % NaCl	4 % KCl	500 ppm $NaNO_2$	pH 4	4 % NaCl	4 % KCl	500 ppm $NaNO_2$	pH 4	pH 4	4 % NaCl
4	2 % NaCl	2 % KCl	125 ppm $NaNO_2$	pH 5	2 % NaCl	2 % KCl	125 ppm $NaNO_2$	pH 5	pH 5	LB
5	2 % NaCl	2 % KCl	125 ppm $NaNO_2$	pH 5	2 % NaCl	2 % KCl	125 ppm $NaNO_2$	pH 5	pH 5	LB
6	2 % NaCl	2 % KCl	125 ppm $NaNO_2$	pH 5	2 % NaCl	2 % KCl	125 ppm $NaNO_2$	pH 5	pH 5	LB
7	1 % NaCl	1 % KCl	62.5 ppm $NaNO_2$	pH 6.2	1 % NaCl	1 % KCl	62.5 ppm $NaNO_2$	pH 6.2	pH 6.2	LB
8	1 % NaCl	1 % KCl	62.5 ppm $NaNO_2$	pH 6.2	1 % NaCl	1 % KCl	62.5 ppm $NaNO_2$	pH 6.2	pH 6.2	LB
9	1 % NaCl	1 % KCl	62.5 ppm $NaNO_2$	pH 6.2	1 % NaCl	1 % KCl	62.5 ppm $NaNO_2$	pH 6.2	pH 6.2	LB
10	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB

Keys:

	Type of spores/bacterial cells	Treatment
	Non-activated spores	Spores diluted with LB medium to 10^4 CFU/ml
	Activated spores	Spores diluted with LB medium to 10^4 CFU/ml and heated at 80 $^{\circ}$C for 10 minutes
	Overnight bacterial cells	At 37 $^{\circ}$C, 200 rpm for 24 hours and diluted with LB medium to 10^4 cells
	LB (negative control, no bacteria)	Only LB medium

3.7 Heat treatment of *B. pumilus* spores in LB media added different preservatives and pH

The water bath used to measure inactivation in this experiment was Hetotherm (DBT, Heto Lab Equipment, Denmark) (Figure 12). In addition to the digital displayed temperature on the water bath, an external thermometer was used to monitor the temperature of the water.

The initial concentration of the spores was 10^9 CFU/ml. 4 % w/v NaCl, 4 % w/v KCl, and 500 ppm $NaNO_2$ solutions of LB medium were prepared from the stock solutions. In addition, three different concentrations of pH (pH 3, 4 and 5) of LB medium were made. Then 250 µl from the spore suspension was added into each of 250 µl of 4 % w/v NaCl, 4 % w/v KCl, and 500 ppm $NaNO_2$ media. Similarly, 250 µl from the spore suspension was also added into each of 250 µl of pH 3, 4 and 5 media by using a sterile syringe, each sample then forming 500 µl in small glass tubes. As a result, the concentrations of preservatives were decreased from 4 % to 2 % w/v (NaCl and KCl) and from 500 to 250 ppm $NaNO_2$. The samples with pH 3, 4 and 5 were increased to pH 4, 5 and 6.2, respectively (Table 6).

Table 6: Scheduled program for heat treatment in a water bath

Samples (initial concentration)	Samples (final concentration)	Temperature (ºC)	Time (min) (heating process 1)	Time (min) (heating process 2)
4 % NaCl	2 % NaCl	90	5	20
4 % KCl	2 % KCl	90	5	20
500 ppm $NaNO_2$	250 ppm $NaNO_2$	90	5	20
pH 3	pH 4	90	5	20
pH 4	pH 5	90	5	20
pH 5	pH 6.2	90	5	20
4 % NaCl	2 % NaCl	95	5	
4 % KCl	2 % KCl	95	5	
500 ppm $NaNO_2$	250 ppm $NaNO_2$	95	5	
pH 3	pH 4	95	5	
pH 4	pH 5	95	5	
pH 5	pH 6.2	95	5	

A rubber stopper was used to close the glass tubes (Figure 12). The tubes then kept in ice slurry until heat-treatment to avoid germination. For each inactivation, three parallels from each preservatives and pH media were prepared in glass tubes; and then transferred to a closed water bath. The spores were heat treated at 90 °C for 5 and 20 minutes, and at 95 °C for 5 minutes. After inactivation, the glass tubes were placed on ice slurry immediately for rapid cooling; and 10-fold serial dilutions were made using sterile MilliQ water. Then, the samples were plated by using EddyJet spiral plater on LB agar medium. The plates were then incubated at 37 °C for 24 hours. After the incubation, the number of colonies were counted and the concentration (CFU/ml) calculated.

Figure 12: Equipment used in the water bath experiments. a) A metal rack holds the glass tubes in water bath for heat treatment, b) A glass tube with a rubber stopper and **c)** Sterile syringe that used to inoculate the samples into the glass tubes.

3.8 Heat resistance of *B. pumilus* spores produced at different sporulation temperatures

Sporulation temperature is the temperature at which the bacterial vegetative cells are grown to sporulate. Optimum sporulation temperature for most mesophilic bacteria is around 37 °C. In this heat resistance experiment, the spores were produced at various temperature (15, 25, 37 and 42 °C), which is described in section 3.3. The aim was to examine the effect of sporulation temperature during heat treatment. 1.0 ml of LB medium was added to each of 1.0 ml spore suspension that was sporulated at different temperatures (15, 25, 37 and 42 °C) in Eppendorf tube, making 2 ml in total volume for each suspension. Then, 0.5 ml of the spores in LB medium were transferred into plastic bags in three parallels from each suspension sporulated at various temperatures using sterile syringes. The plastic bags were then sealed using a Dyno packaging machine (462/463 VGA half automatic vacuum/gas, Dynopack A/S) and immersed in ice water before the heat treatment to avoid germination. The heat resistance experiment was carried out at 90 and 95 °C for 10 minutes for spores made from all sporulation temperatures.

The plastic bags were attached to a metal rack at multiple areas to prevent them from floating around. Afterwards, the bags 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 the bags were kept separate from each other in the water bath to obtain an even heat distribution in all bags (Figure 13). The temperature in the water bath as well as between the bags were measured using temperature detector at time intervals during the experiments as the distribution of heat might not be uniform in the water bath. After inactivation, the rack with the three parallels were immediately immersed in ice water. The plastic bags were cut at one end, and the samples were transferred into a 1.5 ml Eppendorf tube by sterile syringe. Then 10-fold serial dilutions were made using sterile MilliQ water. The samples were then plated by EddyJet spiral plater. The plates were incubated at 37 °C for 24 hours. After the incubation, the number of colonies were counted and the concentration (CFU/ml) calculated.
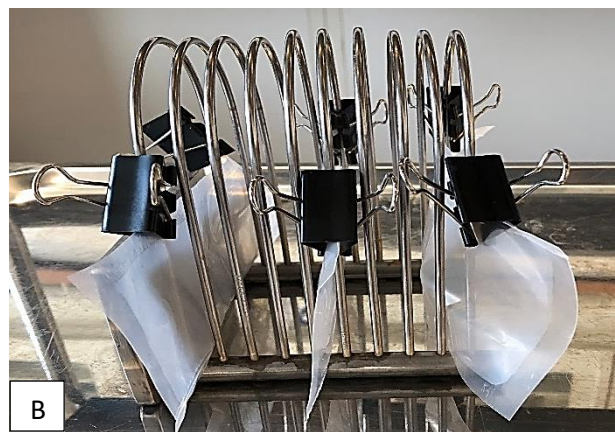
Figure 13: Equipment used in the water bath experiments. a) The rack used to hold the plastic bags in water bath for heat treatment and b) Each rack can hold three bags. The rack allows easy removal from the water bath.

3.9 Inactivation of *B. pumilus* spores in tuna fish by heat treatment

Tuna fish was used as a food matrix for inactivation experiment. The fish used contained 29 % water and 1 % salt. A 340 gram of tuna fish was first mixed with 77 ml of sterile MilliQ water in a laboratory blender, then 150 grams of the tuna fish were added to 1.5 ml of the spore suspension. The initial concentration of the spore suspension was 10^9 CFU/ml. The concentration of the spores was then reduced to 10^7 CFU/ml after mixing with the tuna fish. Afterwards, 6 grams of the tuna fish solution with spores were transferred to each 18 plastic bags. The plastic bags were sealed in all sides with a Dyno packaging machine, and the tuna fish was rolled flat to get an even heat distribution in the whole product. Six parallels were

prepared for both temperature-time combinations (90 °C and 95 °C for 10 minutes). In addition, six parallels were made for the untreated sample.

The bags were attached to a metal rack preventing them from floating around. Then they were immersed in the water bath with three parallels at a time. The metal rack without the samples was preheated in the water bath to avoid a large temperature drop at the beginning of the heat treatment. All bags were kept separate from each other in the water bath to achieve an even heat distribution in all bags (Figure 14). Further, the temperature in the water bath as well as between the bags were measured using temperature detector at time intervals during the experiments as the distribution of heat might not be uniform in the water bath. The rack with each of the three parallels were then removed after treatment and immersed in ice water.

Out of the 18 bags, nine bags were kept chilled at 4 °C to be analysed later. The rest nine bags (three parallels from each time-temperature combination and the untreated ones) were analysed at day zero, and homogenized with peptone water, that is, 4 grams of the sample mixed with 36 grams of 1 % peptone water (1:10) in a stomacher bag using the stomacher machine for 2 minutes. Then, 10 ml of the homogenized sample were transferred into a 15 ml sterilized tube, and this was the 10^{-1} dilution. After that, 10-fold serial dilutions were made using sterile MilliQ water. The samples were then plated by using EddyJet spiral plater. The plates were incubated at 37 °C for 24 hours. After the incubation, the number of colonies were counted and the concentration (CFU/ml) calculated.
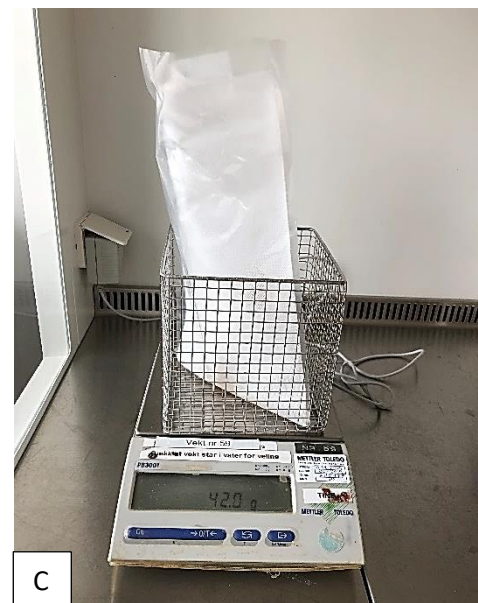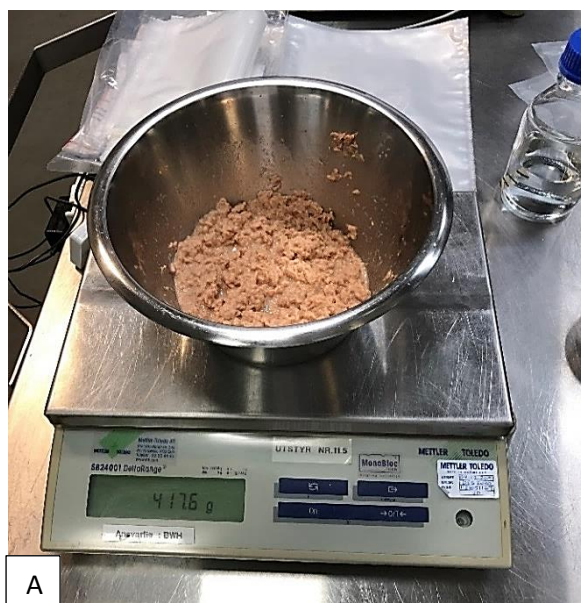
Figure 14: a) The tuna fish was diluted with sterile MilliQ water in a laboratory blender prior to processing, b) The rack with plastic bags attached for heat treatment, three parallels at a time, and c) The tuna fish samples were weighed and homogenized. Samples of 4 grams were collected in a stomacher bag and diluted (1:10) with 1 % peptone water.

3.10 Inactivation of *B. pumilus* spores in tuna fish by PATS

As in the previous experiment, tuna fish was used as a food matrix in this experiment. The PATS technology was applied to measure the inactivation of the spores. 80 ml of sterile MilliQ water was mixed with 340 grams of tuna fish in a laboratory blender to soften the food, then 150 grams of the tuna fish were added to 1.5 ml of spore suspension. The concentration of the spore suspension was then decreased to 10^7 CFU/ml after mixed with the tuna fish, the initial concentration of the spore was 10^9 CFU/ml. 6 grams of the tuna fish solution with spores were then transferred into each 18 plastic bags. The bags were sealed in all sides with a Dyno packaging machine, and the fish was rolled flat to get a uniform heat distribution in the whole product. Three parallels were prepared for both temperatures (55 and 65 ^{o}C) set with pressure of 600 MPa. Besides, three parallels were made for the untreated sample. The experiment was carried out twice at two different days with two different spore batches. Experiment number 1 was performed using spore batch 6 and experiment number 2 was performed using spore batch 8.

In the first experiment (spore batch 6), six parallels were preheated to initial temperature of 55 and 65 ^{o}C for 10 minutes in water bath prior to HHP treatment (Liang et al., 2019). Meanwhile, the HPP unit was initially heated to 56.6 ^{o}C and 65.2 ^{o}C, then the bags were loaded into the pressure chamber (Figure 15) and the temperature was reduced to 55.6 and 64 ^{o}C, respectively prior to the experiment. PATS was performed at 600 MPa for 2 minutes, and the temperature then increased to 73.7 and 83.7 ^{o}C at the maximum and

decreased to 68.2 and 76 ℃, respectively at the end of the holding period of 2 minutes (Table 7).

In the second experiment (spore batch 8), six parallels were preheated to initial temperatures of 55 and 65 ℃ for 10 minutes in water bath prior to PATS treatment. Meanwhile, the HPP unit was initially heated to 55.3 and 64.7 ℃, then the bags were loaded into the pressure chamber (Figure 15) and the temperature was decreased to 55.4 and 64.3℃, respectively prior to the experiment. HPP was carried out at 600 MPa for 2 minutes, and the temperature was risen to 73.4 and 83.6 ℃ at maximum and lowered to 68.4 and 77 ℃, respectively at the end of the holding time of 2 minutes (Table 7). The increase of in-process temperature was owing to the internal adiabatic heating (Lopes et al., 2018). The bags were then removed after treatment and immersed in ice water.

Table 7: Processing at 600 MPa for 2 minutes

Spore	Start temperature (℃)	Maximum temperature (℃)	Final temperature (℃)
Batch 6	56.6	73.7	68.2
Batch 6	65.2	83.7	76.0
Batch 8	55.3	73.4	68.4
Batch 8	64.7	83.6	77.0

After processing, nine of the bags were kept chilled at 4 ℃ to be analysed later. The rest nine bags (three parallels from each pressure-temperature combination and the untreated ones) were analysed at day zero, and homogenized with 1 % peptone water, that is, 4 grams of the sample was mixed with 36 grams of 1 % peptone water (1:10) in a stomacher bag using the stomacher machine for 2 minutes. Then, 10 ml of the homogenized sample were transferred into a 15 ml sterilized tube, and this was the 10^{-1} dilution. After that, 10-fold serial dilutions were made using sterile MilliQ water. The samples were then plated by using EddyJet spiral plater. The plates were incubated at 37 ℃ for 24 hours. After the incubation, the number of colonies were counted and the concentration (CFU/ml) calculated.

Figure 15: A) The plastic bag was filled with 6 grams of tuna fish solution, sealed with the Dyno packaging machine and rolled flat. B) The plastic bags inside the pressure chamber of the PATS for processing at pressure of 600MPa combined with 55 ºC and 65 ºC for 2 minutes, three parallels at a time.

4 Results and discussion

The *B. pumilus* B367 strain was not previously used in the laboratory at Nofima and experience with growth and heat resistant was not available. Therefore, the experiments started with finding the temperature for inactivation of spores in nutrient broth in a water bath. Similar experiments in water bath were then carried out with spores in tuna fish, and finally inactivation experiments in high pressure combined with moderate temperatures. Growth of potential of surviving *B. pumilus* was also examined with different types and concentrations of preservatives used in food products.

4.1 Inactivation of *B. pumilus* spores in water bath

B. pumilus B367 spores were heat treated at different temperatures (80, 90 and 95 ^{o}C) for 10 minutes as described in section 3.4. The temperature measurements carried out in this experiment showed that the actual temperature in the water bath was 0.2-0.6 ^{o}C lower than the set temperature of the water bath. This means that when the water bath was set to 95°C the actual temperature was 94.4-94.8 ^{o}C. This is the case for all experiments conducted with the water bath. The reported temperature in the figures for each experiment is the set temperature for the water bath. The whole experiment was repeated twice with the same spore batch to document the stability of the process.

A 1.8 log and 3.4 log-reduction were achieved at 80 and 90 ^{o}C, respectively, in experiment 1. In experiment 2, a 1.7 log and 3.0 log-reduction were obtained at 80 and 90 ^{o}C, respectively. Highest inactivation was achieved at 95 ^{o}C with a 5.6 and 5.8 log-reduction in experiment 1 and 2, respectively (Figure 16). With increasing of temperature, the rate of inactivation was increased, and the D-value decreased in both experiments. However, inactivation of spores and the D-values can vary among various spore batches tested at the same temperature. Different spore batches can have different heat resistance depending on many factors.
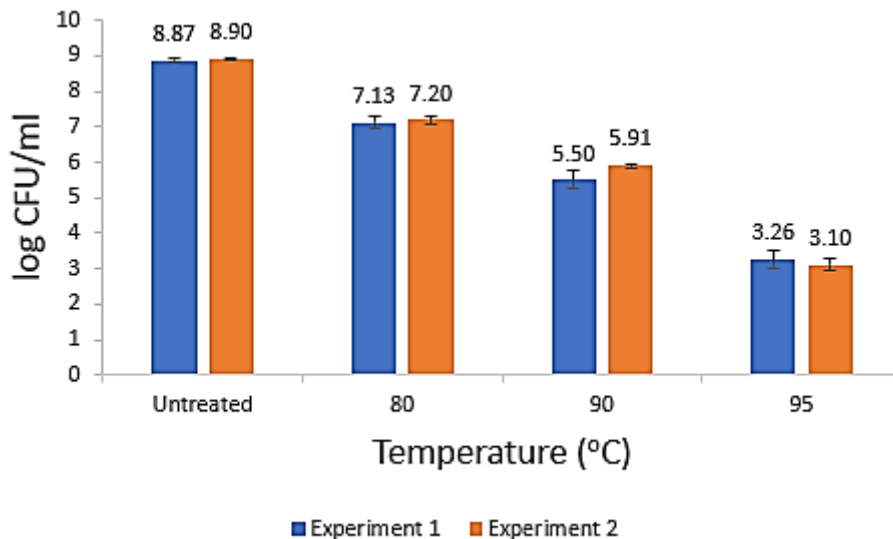
Figure 16: Inactivation of *B. pumilus* B367 spores after heat treatment for 10 minutes at 80, 90 and 95°C. The data is mean values of three parallels and the standard deviations are shown. The spores used were from batch 1 for both experiment 1 and 2.

Sporulation medium is a medium on which the vegetative cells of bacteria are plated to sporulate. Different sporulation media have varying effect on the percentage of sporulation. Majority of the sporulation media are consisted of different nutrition levels that could have an influence on the heat resistance of the spores. For instance, manganese has a role for developing heat and high pressure resistant spore while calcium contributes in heat resistant spore production (Lenz & Vogel, 2014). The medium that is used for heat treatment has also an important role on the inactivation kinetics. In the laboratory context, a buffer or distilled water is often used. The water activity (a_w) of the medium is of great importance for the heat resistance of a spore. Spores have a higher heat resistance in media with low water activity, such as grease and oil-containing media (Coroller, Leguérinel, & Mafart, 2001; Gaillard, Leguérinel, & Mafart, 1998).

Experiment number 1 and 2 showed very similar results for each temperature indicating that the results were reproducible for the same spore batch used. During the experiment, care was taken to confirm the screw cap tubes (Nunc tubes) being submerged enough that the spores in the tubes were inside the hot water during heating and were quickly transferred from the water bath to ice slurry. Besides, attention was paid to avoid uneven heating and post contamination.

4.2 Growth of *B. pumilus* vegetative cells

The growth experiments were conducted using the Bioscreen C programmed to measure absorbance at 600 nm (A_{600}). The initial number of bacteria were 10^9 cells/ml in the spore batch. To run the Bioscreen C, the cells were diluted with LB medium to obtain 10^4 cells/ml. This bacterial suspension was then further diluted with both LB and 2 % w/v NaCl added LB medium (10-fold serial dilutions). The growth experiment was done as described in section 3.5. Growth was measured at 37 °C for 24 hours, and the experiment was performed with two replicates.

Maximum turbidity is defined as the maximum measured turbidity. It can be an expression for the maximum number of cells in a stationary phase after optimal growth. A lower maximum turbidity can be a result of lower numbers or changed cell structure due to the physio chemical growth conditions, for example that the cells are smaller, or more translucent to light. Therefore, a lower maximum turbidity indicates that growth is inhibited by some growth factors. The time to detection (TTD) is determined from a point where there is a rapid increase in optical density (OD) and can be related to the maximum turbidity (number of cells present) (Johnston, 1998). It was taken to be the time to an increase of 0.2 OD units at 600 nm in all growth experiments. The results showed that high maximum turbidity levels gave faster change in ODs, which resulted in shorter TTD values. The highest maximum turbidity and shortest TTD of *B. pumilus* vegetative cells were observed in the well of lowest diluted samples (10^{-1} dilution) with both LB and 2 % w/v NaCl added LB media (Table 8). On the other hand, the lowest maximum turbidity and longest TTD were detected in the well of highest diluted samples (10^{-10} dilution) with both LB and 2 % w/v NaCl added LB media (Table 8).

Table 8: The TTD (in hours) for *B. pumilus* B367 vegetative cells measured for different diluted samples (10^{-1} - 10^{-10}) in LB and 2 % w/v NaCl added LB media at 37 °C. The data is collected from Bioscreen C.

Dilution series	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
TTD (hours) LB	1	2	3	4	6	7	7	8	8	9
TTD (hours) 2 % w/v NaCl	1	2	3	5	6	7	8	11	12	12

In figure 17 the TTD times for the dilution series in LB and NaCl are plotted and a linear trend line is given. The R^2 values are high indicating a good fit to a straight line. The TTD values are very similar for LB and NaCl from 10^{-1} - 10^{-10}, but the deviation increases with higher dilutions. This shows that 2% NaCl is inhibitory for the cells compared to growth in LB medium.
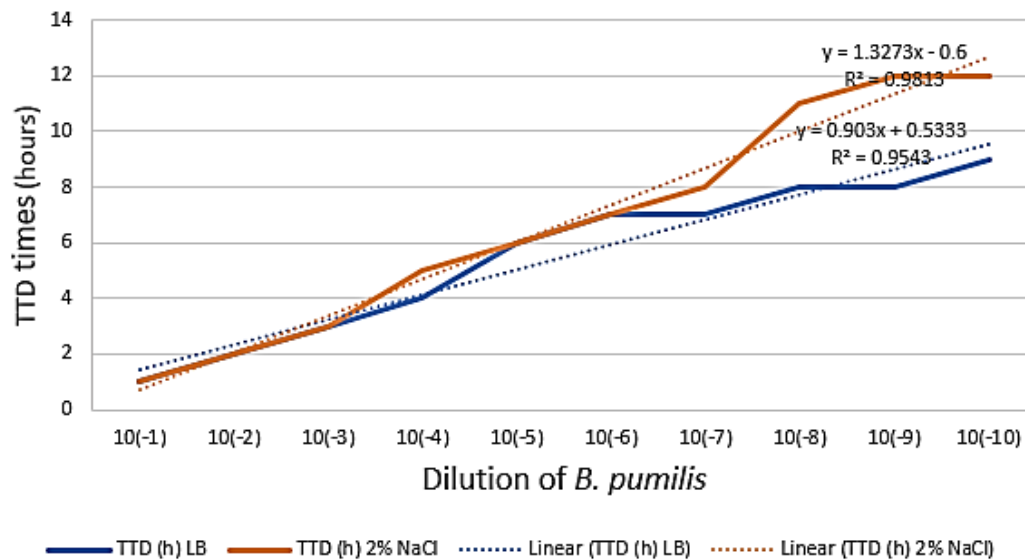

Figure 17: TTD values in LB and 2% NaCl added LB medium from table 8 is presented against dilution of *B. pumilus*. The equation for the trend line is given with the R^2 value.

4.3 Growth of *B. pumilus* at selected levels of different preservatives and pH

4.3.1 Growth of *B. pumilus* non-activated spores

In all growth experiments, Bioscreen C was used with various combinations of preservatives and pH. 50 µl from the non-activated spores were inoculated in each 50 µl of NaCl (1, 2 and 4 % w/v), KCl (1, 2 and 4 % w/v), NaNO$_2$ (62.5, 125 and 500 ppm), and pH (4, 5 and 6.2) media, as explained in section 3.6. Growth was measured at 25 °C for 7 days. The mean of the three sets of data was determined and the resulting growth curves were plotted. The result showed that the highest maximum turbidity and shortest TTD of *B. pumilus* non-activated spores were found when incubated with LB medium, and lowest maximum turbidity and longest TTD were seen with 4 % w/v NaCl medium. The non-activated spores with 1 % w/v NaCl medium had also relatively higher maximum turbidity and shorter TTD than 2 % w/v NaCl medium (Figure 18 and Table 9).
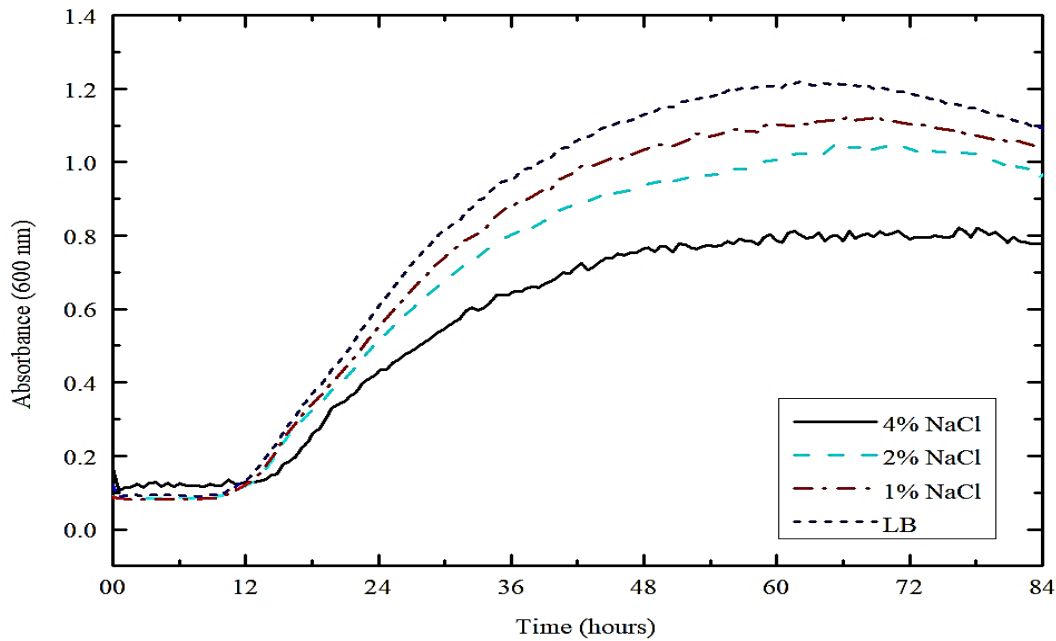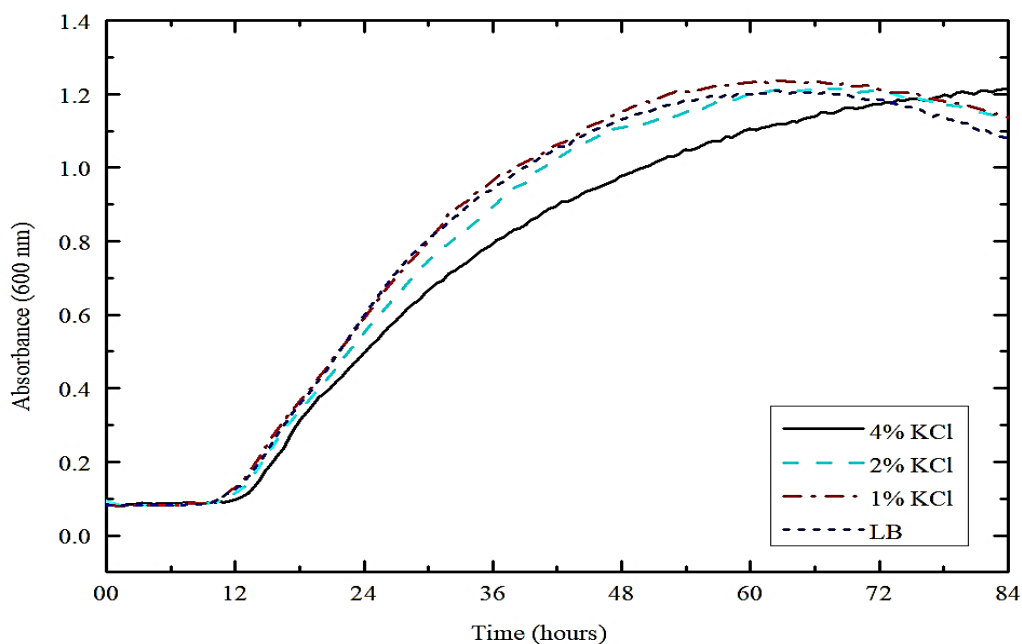
Figure 18: Growth of *B. pumilus* B367 non-activated spores with LB and different NaCl concentrations (1, 2 and 4 % w/v NaCl) of LB media at 25 °C. The data is mean values of three parallels collected from Bioscreen C, and the spores used were from batch 1.

For *B. pumilus* non-activated spores inoculated with KCl medium, no differences in maximum turbidity were observed between 1 % and 2 % w/v KCl, as well as LB medium. However, the maximum turbidity of non-activated spores with 4 % w/v KCl medium was lower compared to 1 % and 2 % w/v KCl as well as LB media up to 72 hours (Figure 19). The TTD was shorter with LB medium and longer with 4 % KCl medium (Table 9).

Figure 19: Growth of *B. pumilus* B367 non-activated spores with LB and different KCl concentrations (1, 2 and 4 % w/v KCl) of LB media at 25 °C. The data is mean values of three parallels obtained from Bioscreen C, and the spores used were from batch 1.

Non-activated spores of *B. pumilus* inoculated with 500 ppm $NaNO_2$ medium had lower growth rate (based on the shape of the curve) and lower maximum turbidity than the 62.5 and 125 ppm $NaNO_2$, as well as LB medium before 60 hours. However, no variations in maximum turbidity were seen among the samples after 60 hours (Figure 20). The TTD was shorter with LB medium and longer with 4 % $NaNO_2$ (Table 9).
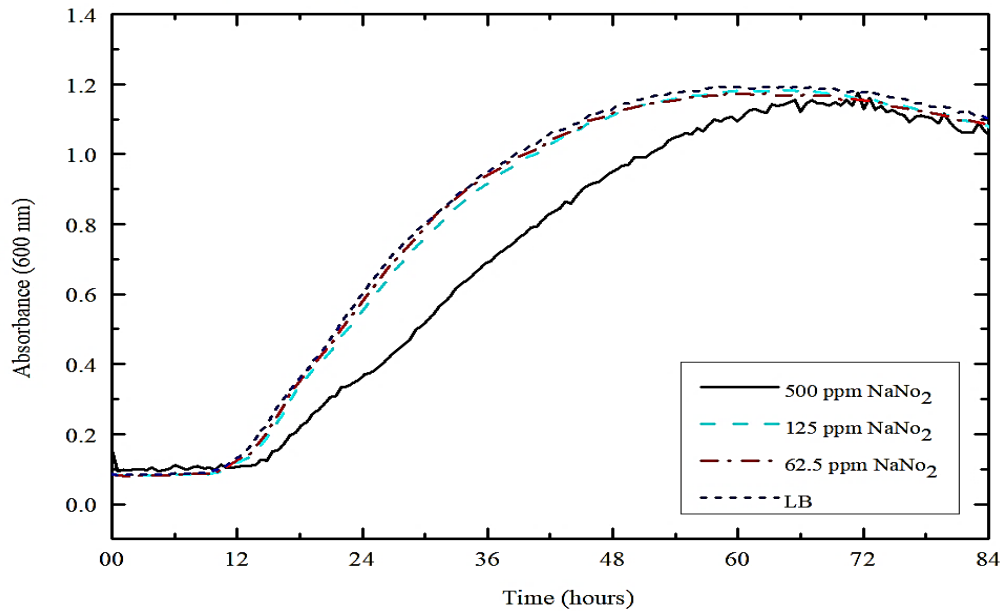

Figure 20: Growth of *B. pumilus* B367 non-activated spores with LB and different $NaNO_2$ concentrations (62.5, 125 and 500 ppm $NaNO_2$) of LB media at 25 °C. The data is mean values of three parallels collected from Bioscreen C, and the spores used were from batch 1.

Growth at different pH levels showed that the highest maximum turbidity of *B. pumilus* non-activated spores were observed in the media with pH 6.2 and LB medium (Figure 21). The TTD was shorter with LB medium and longer with pH 4 medium (Table 9); the pH of LB medium was 6.9.
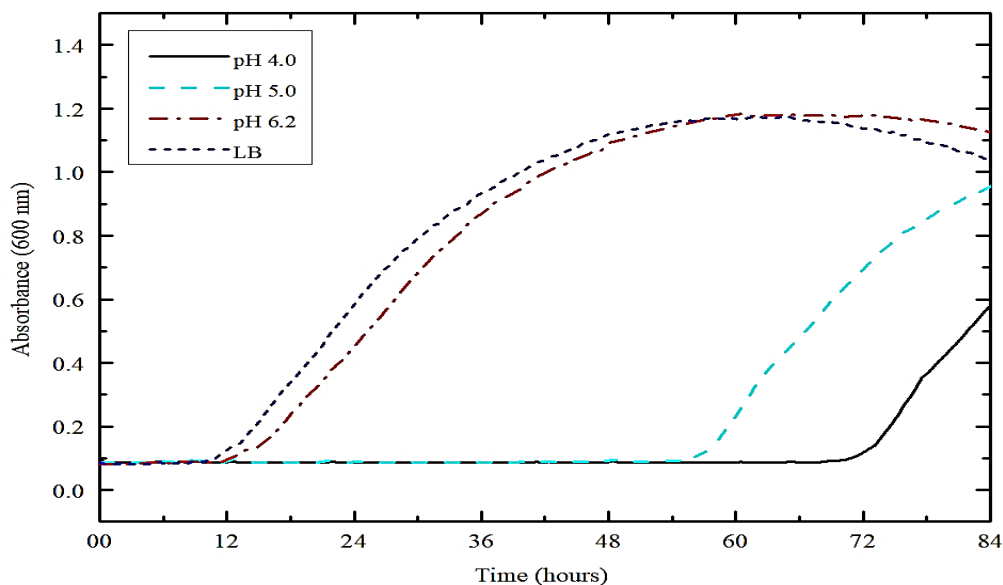
Figure 21: Growth of *B. pumilus* B367 non-activated spores with LB and different pH concentrations (pH 4, 5, and 6.2) of LB media at 25 $^{\circ}$C. The data is mean values of three parallels obtained from Bioscreen C, and the spores used were from batch 1.

From this experiment, it can be observed that with increasing concentrations of the different preservatives (NaCl, KCl and NaNO$_2$) and decreasing of the pH of the media, the maximum turbidity (the number of cells present) decrease. Further, the TTD measured from the experiments were longer with higher concentrations of NaCl, KCl and NaNO$_2$ samples and with lower pH samples (Table 9). The decrease in O.D$_{600\ nm}$ value implies that there is inhibition of spore germination and growth (Cortezzo, Setlow, & Setlow, 2004). Therefore, germination and growth of *B. pumilus* non-activated spores can be inhibited as the concentrations of NaCl, KCl and NaNO$_2$ increase, and when the pH values decrease. In combination with lower temperatures, close to minimum growth temperature, a total growth inhibition may be achieved in the food product.

Table 9: The mean TTD (hours) for *B. pumilus* B367 non-activated spores from batch 1 at various concentrations of NaCl (1, 2 and 4 % w/v), KCl (1, 2 and 4 % w/v), NaNO$_2$ (62.5, 125 and 500 ppm) and different pH values (4, 5 and 6.2), as well as LB medium at 25 $^{\circ}$C . The data is obtained from Bioscreen C.

NaCl %	Mean TTD (hours)	KCl %	Mean TTD (hours)	NaNO$_2$ ppm	Mean TTD (hours)	pH	Mean TTD (hours)	LB	Mean TTD (hours)
1	16	1	16	62.5	15	6.2	16	6.9	15
2	17	2	16	125	16	5.0	69		
4	19	4	17	500	17	4.0	75		

4.3.2 Growth of *B. pumilus* activated spores

Activated spores by applying 80 $^{\circ}$C for 10 minutes in the water bath were used to conduct the growth experiment. The procedures were the same as the procedures used for the non-activated spore growth testing, and growth was measured at 25 $^{\circ}$C for 7 days as explained in section 3.6. The mean of the three sets of data was determined and the resulting growth curves were plotted. The result revealed that highest maximum turbidity and shortest TTD of *B. pumilus* activated spores were seen when incubated with LB medium, and lowest maximum turbidity and longest TTD were found with 4 % w/v NaCl medium. The activated spores with

1 % w/v NaCl medium had also relatively higher maximum turbidity and shorter TTD than with 2 % w/v NaCl medium (Figure 22 and Table 10).
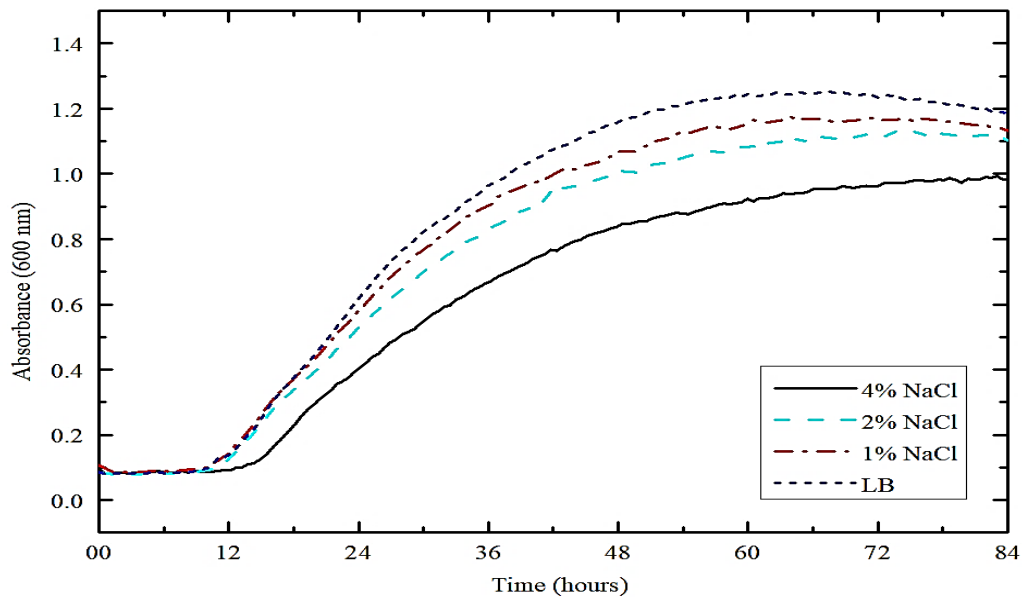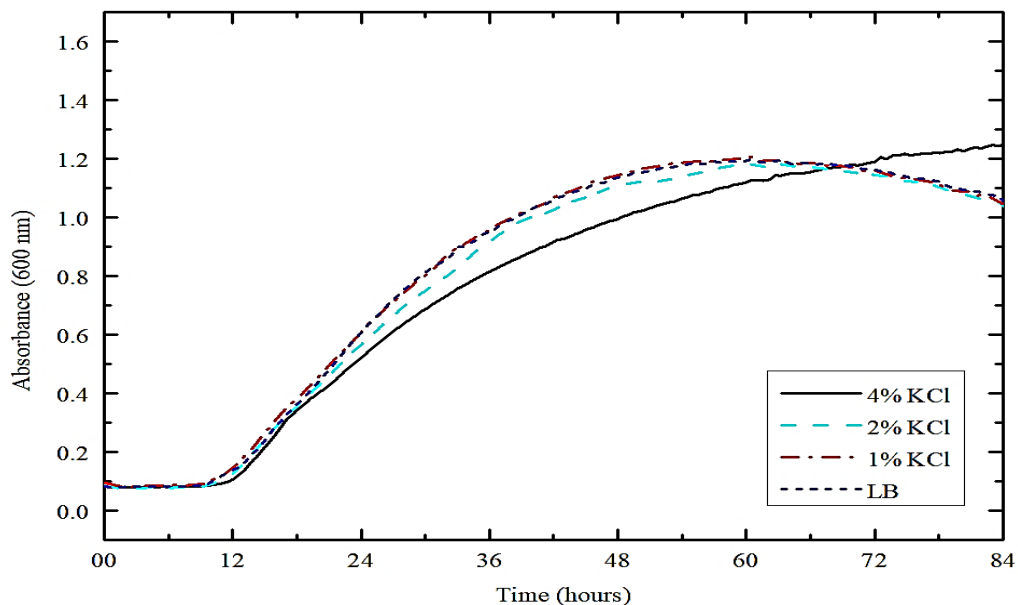

Figure 22: Growth of *B. pumilus* B367 activated spores with LB and different NaCl concentrations (1, 2 and 4 % NaCl) of LB media at 25 °C. The data is mean values of three parallels collected from Bioscreen C, and the spores used were from batch 1.

For activated spores of *B. pumilus* inoculated with KCl medium, no variations in maximum turbidity were detected with 1 % and 2 % w/v KCl, as well as LB media. However, the maximum turbidity of activated spores with 4 % w/v KCl medium was lower compared to 1 % and 2 % w/v KCl, as well as LB media up to 65 hours (Figure 23). TTD was shorter with LB medium and longer with 4 % KCl medium (Table 10).

B. pumilus activated spores inoculated with LB medium and 62.5 ppm $NaNO_2$ medium had similar maximum turbidity, which was higher than the 125 ppm and 500 ppm $NaNO_2$ media at the stationary phase (Figure 24). The TTD was shorter with LB medium and longer with 4 % $NaNO_2$ (Table 10).
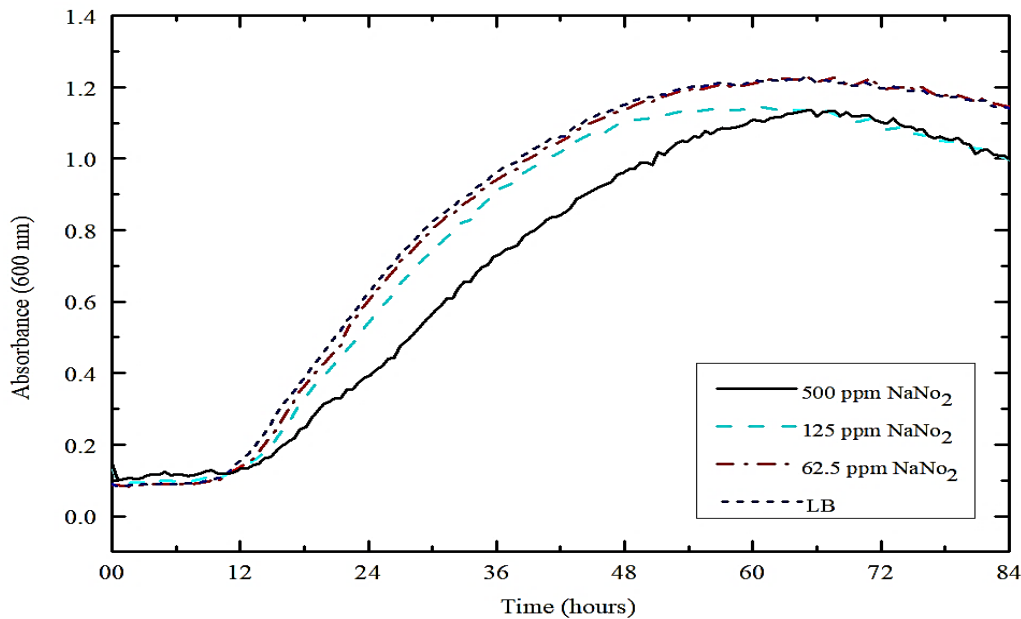

Figure 24: Growth of *B. pumilus* B367 activated spores with LB and different $NaNO_2$ concentrations (62.5, 125 and 500 ppm NaNO2) of LB media at 25 °C. The data is mean values of three parallels obtained from Bioscreen C, and the spores used were from batch 1.

The growth of the *B. pumilus* activated spore with pH 6.2 and LB media had highest maximum turbidity (Figure 25). In addition, the TTD was shorter in LB medium and longer with pH 4 medium (Table 10).
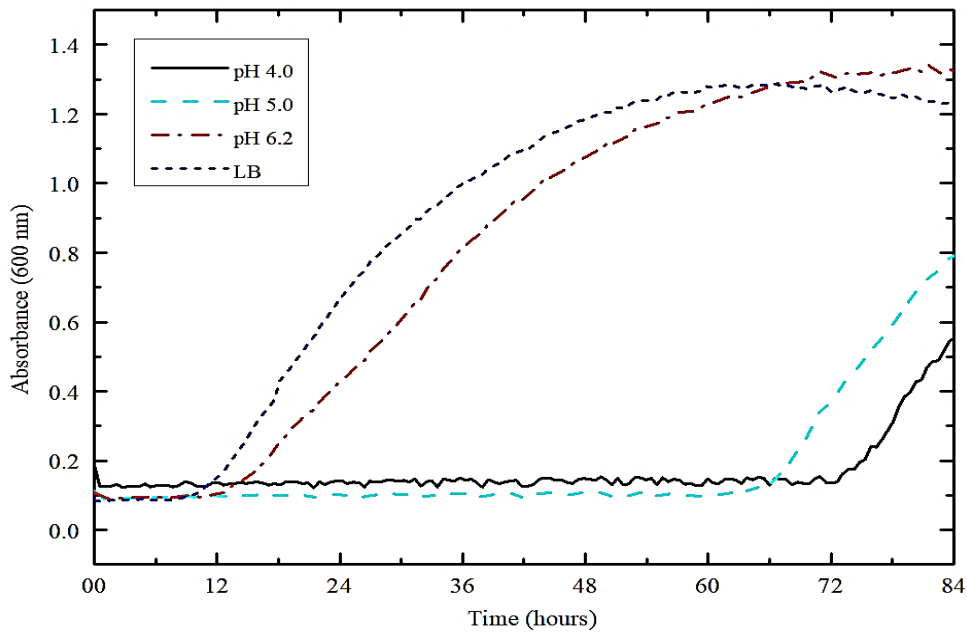
Figure 25: Growth of *B. pumilus* B367 activated spores with LB and different pH concentrations (pH 4, 5, and 6.2) of LB media at 25 °C. The data is mean values of three parallels collected from Bioscreen C, and the spores used were from batch 1.

From this experiment, it can be concluded that with increasing concentrations of the different preservatives (NaCl, KCl and $NaNO_2$) and decreasing of the pH in the samples, the maximum turbidity (the number of cells present) decrease. Further, the TTD measured from the experiments were longer with higher concentrations of NaCl, KCl and $NaNO_2$ samples and with lower pH samples (Table 10). Thus, germination and growth of *B. pumilus* activated spores can be inhibited when the concentrations of NaCl, KCl and $NaNO_2$ increase, and as the pH values is reduced.

Table 10: The mean TTD (hours) for *B. pumilus* B367 activated spores from batch 1 at various concentrations of NaCl (1, 2 and 4 % w/v), KCl (1, 2 and 4 % w/v), $NaNO_2$ (62.5, 125 and 500 ppm) and different pH values (4, 5 and 6.2), as well as LB medium at 25 °C . The data is obtained from Bioscreen C.

NaCl %	Mean TTD (hours)	KCl %	Mean TTD (hours)	NaNO₂ ppm	Mean TTD (hours)	pH	Mean TTD (hours)	LB	Mean TTD (hours)
1	15	1	15	62.5	15	6.2	16	6.9	14
2	16	2	17	125	15	5.0	60		
4	18	4	18	500	17	4.0	74		

The results of the TTD values in table 9 (non-activated spores) and table 10 (activated spores) are very similar and indicate that heat activation of spores does not have a great

influence on germination and growth of *B. pumilus* spores. Surviving spores from a food heat treatment will therefore behave similarly as newly produced spores from the environment. Furthermore, both non-activated and heat-activated spores show that with the increasing of NaCl, KCl, $NaNO_2$ and decreasing of pH values lead to increased TTD values. Highest growth inhibition is achieved at pH 4 medium of non-activated spores where the TTD value is around 75 hours.

4.3.3 Growth of *B. pumilus* vegetative cells

The overnight bacterial cells that were incubated at 37 °C for 24 hours at 200 rpm were used to run the Bioscreen C with the selected pH media (pH 4, 5 and 6.2) and 4 % w/v NaCl medium, as explained in section 3.6. The result revealed that higher maximum turbidity of *B. pumilus* vegetative cells were observed when incubated with pH 6.2 and LB media compared to pH 4 and pH 5 samples (Appendix 4). TTD was shortest with LB medium and longest with pH 4 sample (Table 11).

The bacterial cells inoculated with 4 % w/v NaCl medium had lower maximum turbidity than the cells with LB medium. The LB medium without bacterial addition (blank) showed no increase in maximum turbidity (Appendix 5). TTD was longer for *B. pumilus* cells with 4 % w/v NaCl added LB medium than LB medium (Table 11). A longer TTD values indicate that there are growth inhibitors and growth will be slower (Johnston, 1998). Therefore, the growth of *B. pumilus* vegetative cells can be inhibited as the pH value of the samples decrease and the concentration of NaCl media increase.

Table 11: The mean TTD (hours) for *B. pumilus* B367 vegetative cells from the overnight bacterial cells at various pH values (pH 4, 5 and 6.2), 4 % w/v NaCl medium and LB medium (pH 6.9) at 25 °C. The data is collected from Bioscreen C.

Media	pH 4	pH 5	pH 6.2	LB	4 % w/v NaCl
Mean TTD (hours)	84	24	21	18	23

4.4 Heat treatment of *B. pumilus* spores in LB media added different preservatives and pH

The strain B367 spores were inoculated in 2 % NaCl, 2% KCl, 250 ppm $NaNO_2$ and pH (4, 5, and 6.2) media in small glass tubes with a rubber stopper. The experiment was performed at 90 °C for 5 and 20 minutes, as well as at 95 °C for 5 minutes, which is described in section 3.7.

When the spores were treated at 90 °C for 5 minutes, a 1.5 log-reduction in LB medium, 2.2 log-decrease at both 2 % NaCl and 2 % KCl, and 2.5 log-inactivation at 250 ppm $NaNO_2$ media were obtained. The heat treatment of spores at different pH values (4, 5, and 6.2) showed that highest inactivation was achieved at lowest pH (pH 4) with 4 log-reduction (Figure 26).

As the inactivation time increased from 5 to 20 minutes at the same temperature (90°C), the rate of inactivation of spores increased. A 3.0 log-reduction of the spores in LB medium, 3.5 log-decrease at both 2 % NaCl and 2% KCl, and 3.7 log-reduction at 250 ppm $NaNO_2$ media were obtained. Moreover, at lower pH higher reduction of spores were attained, which were 4.8, 4.2 and 3.4 log-decrease at pH 4, 5 and 6.2, respectively (Figure 26).
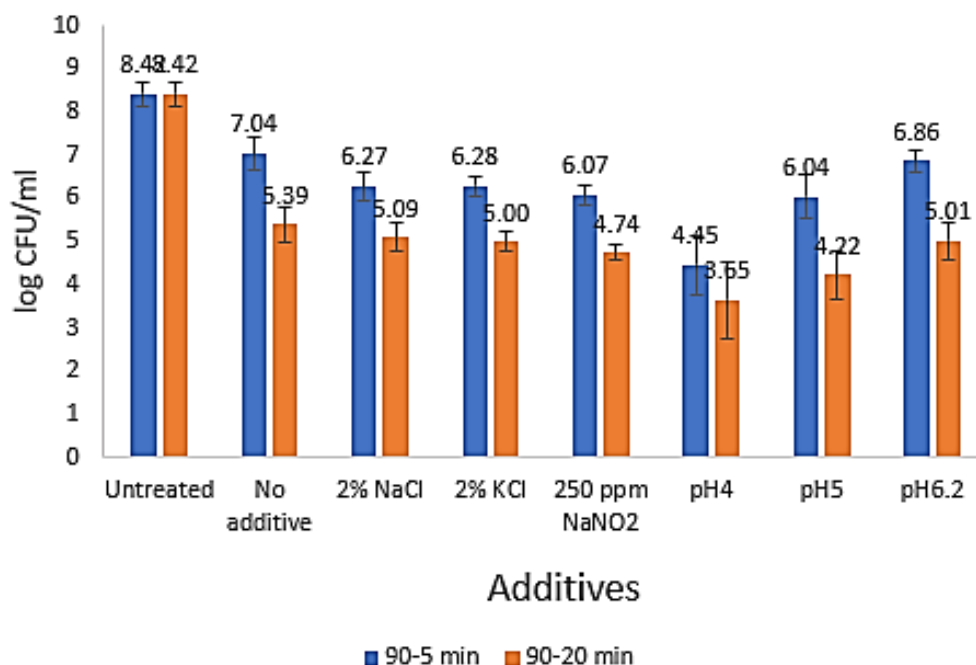

Figure 26: Inactivation of *B. pumilus* B367 spores with different additives after heat treatment at 90°C for both 5 and 20 minutes. The data are mean values of three parallels and the standard deviations are shown. The spores used were from batch 2.

When the treatment temperature was increased from 90 to 95 °C for 5 minutes, higher inactivation of spores was observed. A 2.6 log-decrease of spores was found with no additives and 3.7, 3.7 and 3.8 log-reduction at 2 % NaCl, 2 % KCl and 250 ppm NaNO$_2$ media, respectively. Other research has revealed that addition of salt in the medium results in a lower heat resistance of spores (Coroller et al., 2001). Moreover, highest inactivation at lowest pH (pH 4) was obtained, which was 4.4 log-reduction of spores (Figure 27).
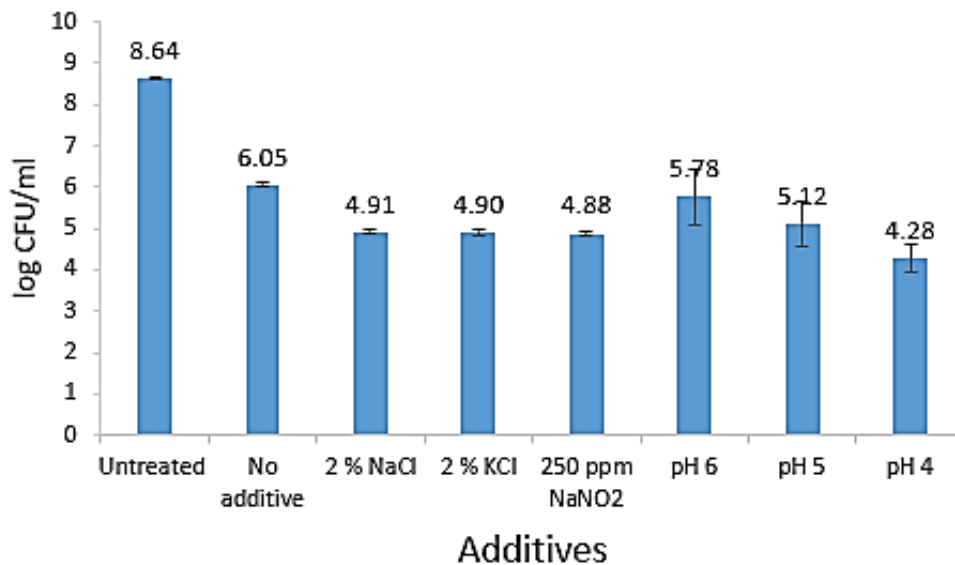

Figure 27: Inactivation of *B. pumilus* B367 spores with different additives after heat treatment at 95°C for 5 minutes. The data are mean values of three parallels and the standard deviations are shown. The spores used were from batch 3.

Low pH during inactivation of the spores leads to lower resistance of spores. The effect pH has on the heat resistance is often utilized in the food context. By lowering the pH of a product, the heat treatment can be reduced (Couvert, Leguerinel, & Mafart, 1999; Gaillard et al., 1998; Iciek, Papiewska, & Molska, 2006). Others have previously reported that at a heat treatment of 90 °C for 10 minutes, as the pH decreased from pH 7 to pH 5, a 1 -2 log-reduction of *B. pumilus* spores were detected (Betts & Everis, 2014). In another study, a decrease in the thermal resistance of *B. pumilus* spores in soup (cream of tomato) was showed as the temperature increased from 93 to 107 °C and when the pH was decreased from 6.4 to 3.6 (Rodríguez-Lozano et al., 2010).

Overall, the results reveal that addition of 2 % NaCl, 2 % KCl and 250 ppm NaNO$_2$ in LB medium had an additional effect on the heat inactivation of spores, and highest inactivation of spores were achieved in medium adjusted to pH 4 and heat treated at 90 and 95 °C.

However, to do more experiments with higher concentrations of these preservatives and more repetitions are advisable to obtain more accurate knowledge on the effects of additives and the inactivation of *B. pumilus* spores.

4.5 Heat resistance of *B. pumilus* spores produced at different sporulation temperatures

Spores were produced at various sporulation temperatures (15, 25, 37 and 42 °C) as described in section 3.3. The heat treatments in water bath were carried out at 90 and 95 °C for 10 minutes as explained in section 3.8.

Three parallels were used for each spore-suspension that were sporulated at the different temperatures. In the first experiment, the spores were treated at 90 °C for 10 minutes. The spores that sporulated at 15 and 25 °C were the most sensitive with a 4.9 and 4.6 log-reduction, respectively. However, those spores that sporulated at 37 and 42 °C revealed higher heat tolerance with 3 and 2.2 log-reduction, respectively. The results showed that spores which sporulated at lowest temperature appeared to have lowest heat resistance (Figure 28).
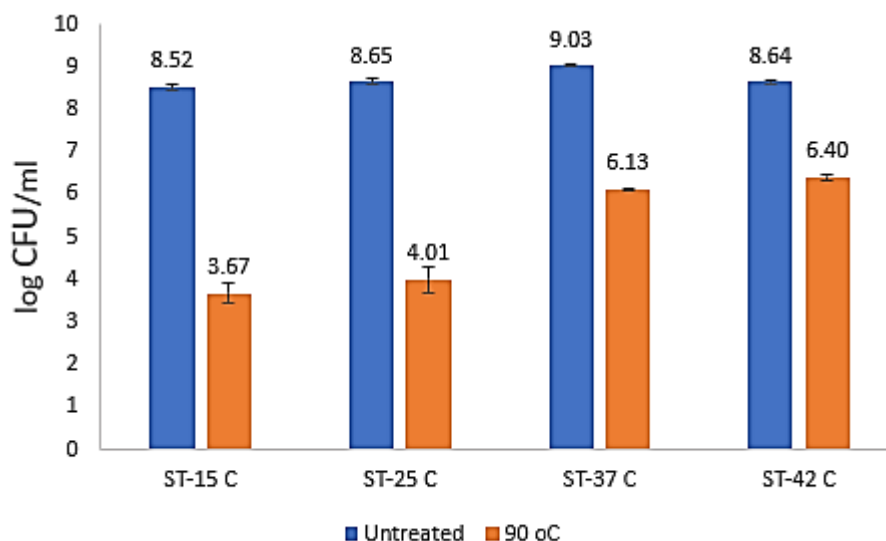

Figure 28: Inactivation of *B. pumilus* B367 spores that sporulated at different temperatures after heat treatment at 90 °C for 10 minutes. The data is mean values of three parallels and the standard deviations are shown. The spores used were from batches 4-7.

Others have previously reported that spores have a higher heat resistance when sporulated at higher temperatures (Gounina-Allouane, Broussolle, & Carlin, 2008), for instance, the sporulation temperature of *B. cereus* spores at 20°C will reduce the survival of the spores by

an average of 1.4 log CFU/g more than at 37ºC (Stanford, Reuter, Gilroyed, & McAllister, 2015). The extent to which the sporulation temperatures affect the heat resistance is often dependent on the bacterial species. It has also been observed that the relationship between the logarithm of the D-value at a given temperature and the sporulation temperature is directly proportional (Leguérinel, Couvert, & Mafart, 2007; Wells-Bennik et al., 2016). Most of studies for the effects of sporulation temperatures on the heat resistance of spores have been done for *B. cereus* and other *Bacillus* species, but due to genetic and phenetic similarities between *Bacillus* strains, it is likely to assume that *B. pumilus* has similar properties.

In the second experiment, the spores were treated at 95 ºC for 10 minutes. A 6.0, 5.8, 5.8 and 5.1 log-reductions were achieved for the spores that sporulated at 15, 25, 37 and 42ºC, respectively (Figure 29).
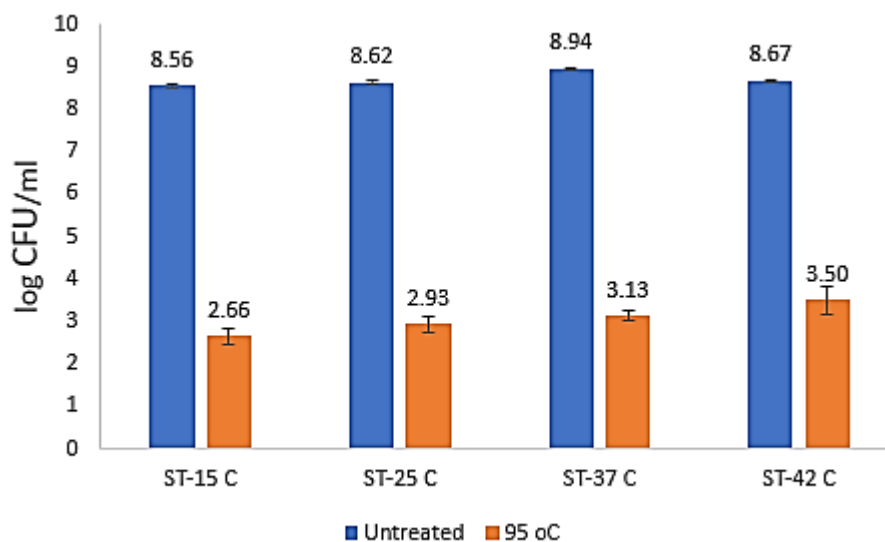

Figure 29: Inactivation of *B. pumilus* B367 spores that sporulated at different temperatures after heat treatment at 95 ºC for 10 minutes. The data is mean values of three parallels and the standard deviations are shown. The spores used were from batches 4-7.

As the temperature was increased to 95 ºC in the second experiment, the inactivation was higher compared to the first experiment at 90 ºC for each spore batch formed at various sporulation temperatures. At 95ºC the inactivation had still a trend with highest inactivation at the lowest spore production temperatures, but the difference in inactivation between spores produced at 15 ºC and 37 ºC was less than 1 log.

4.6 Inactivation of *B. pumilus* spores in tuna fish by heat treatment

Canned tuna fish added spores were heat treated at two different temperatures, 90 and 95℃ for 10 minutes, as described in section 3.9. Three replicates were used to measure inactivation of spores at both temperature-time combinations.

The spores in tuna fish that were treated at 90 ℃ for 10 minutes gave a 2.8 log-reduction. Further, in the treatment of spores in tuna fish that were processed at 95 ℃ for 10 minutes, a 4.4 log-reduction was obtained. For comparison, spores in the LB medium that were treated at 90 ℃ and 95 ℃ for 10 minutes showed a 2.9 and 5.9 log-reduction, respectively (Figure 30).
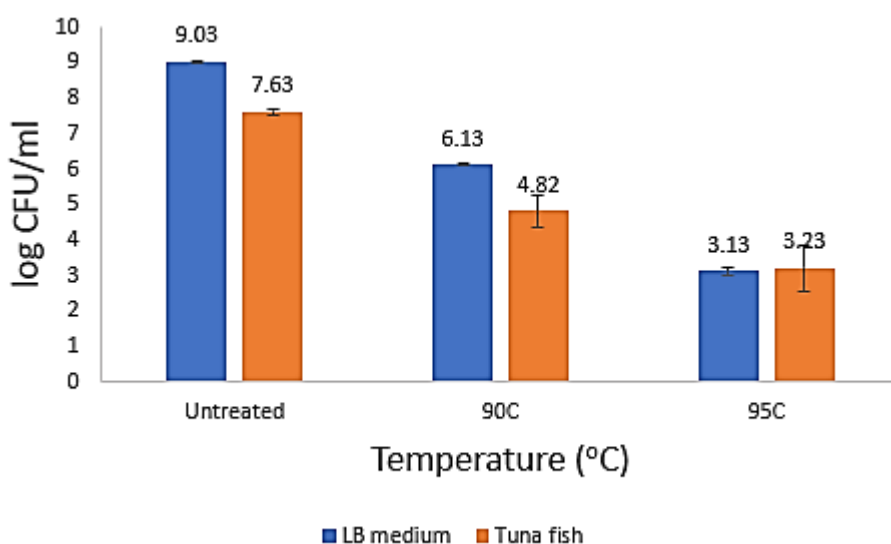

Figure 30: Inactivation of *B. pumilus* B367 spores in LB medium and tuna fish after heat treatment at 90 and 95 ℃ for 10 minutes. The data is mean values of three parallels and the standard deviations are shown. The spores used were from batch 6.

The results indicate that tuna fish had a protective effect on the spores, the spores have a higher heat tolerance in tuna fish than was found in the LB medium. This could be due to a higher fat content and a reduction in water activity (a_w). Ready meals have thicker consistency, lower water activity and contain salt (Rodríguez-Lozano et al., 2010; Sevenich et al., 2016). Thus, a higher heat tolerance for *B. pumilus* in food products can be expected compared to values for heat resistance measured in liquid neutral medium. The medium that is used for heat treatment has an important role on the inactivation kinetics. The water activity (a_w) of the medium is of great importance for the heat resistance of a spore. Spores have a higher heat resistance in media with low water activity (Coroller et al., 2001). This means that to inactivate the spores in food matrixes, either a high concentration of

preservatives or a hard heat treatment is needed. To maintain the nutritional values and sensory qualities of pasteurised ready meals, development of new and milder processes is needed. High pressure processing in combination with heat is an example of such a new process that can be beneficial for the food industry. The heat treatment temperature is reduced while food safety is still maintained (Lopes et al., 2018).

4.7 Inactivation of *B. pumilus* spores in tuna fish by PATS

The inactivation experiment was done by applying PATS to study the survival of *B. pumilus* B367 spores in tuna fish. Canned tuna fish added spores was processed at a pressure of 600 MPa combined with temperatures of 55 °C and 65 °C, for 2 minutes, as described in section 3.10. Six parallels were used to measure inactivation of spores at both pressure-temperature combinations. The whole experiment was repeated twice with a different spore batch to document the stability of the process.

In the first experiment, the spores were treated at 600 MPa with a set temperature of 55 and 65 °C of the HPP unit of PATS, but due to adiabatic heating the temperature increased to 73.7 and 83.7 °C at maximum and decreased to 68.2 and 76 °C, respectively, at the end of the holding period of 2 minutes. A 3.5 and 3.7 log-reduction of spores were achieved when the spores were treated at 600 MPa with a start temperature of 55 and 65 °C, respectively (Figure 31). In the second experiment, the spores were treated at 600 MPa with a set temperature of 55 and 65 °C but due to the adiabatic heating the temperature increased to 73.4 and 83.6 °C at maximum and decreased to 68.4 and 77 °C, respectively, at the end of the holding period of 2 minutes. A 3.2 and 3.6 log-reduction of spores were obtained when the spores were treated at 600 MPa with a set temperature of 55 and 65 °C, respectively (Figure 31). Experiment number 1 and 2 showed very similar results for each treatment indicating that the results were reproducible for the two different spore batches used.
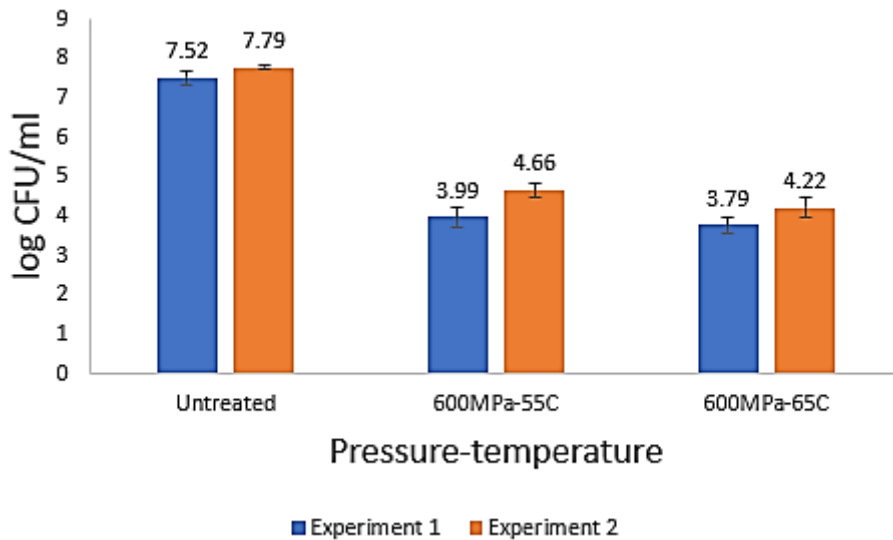
Figure 31: Inactivation of *B. pumilus* B367 spores in tuna fish after processing with PATS at 600 MPa with both 55 and 65 °C for 2 minutes. The data is mean values of three parallels and the standard deviations are shown. The spores used were from batch 6 in experiment 1 and batch 8 in experiment 2.

Some of the PATS treated tuna fish samples were stored chilled for a shelf life study. The concentration of *B. pumilus* in the tuna fish had not changed after 6 weeks of storage at 4 °C. There were low variations in both experiments from day 0 to 6 weeks. The highest difference in log-reduction during storage was 0.4. These results indicate that the *B. pumilus* spore concentrations have not changed during storage at 4 °C for 6 weeks (Figure 32). The minimum temperature for growth of *B. pumilus* is 5°C (Løvdal, 2011), which means that the *B. pumilus* do not grow, and the numbers are not expected to increase at 4 °C.
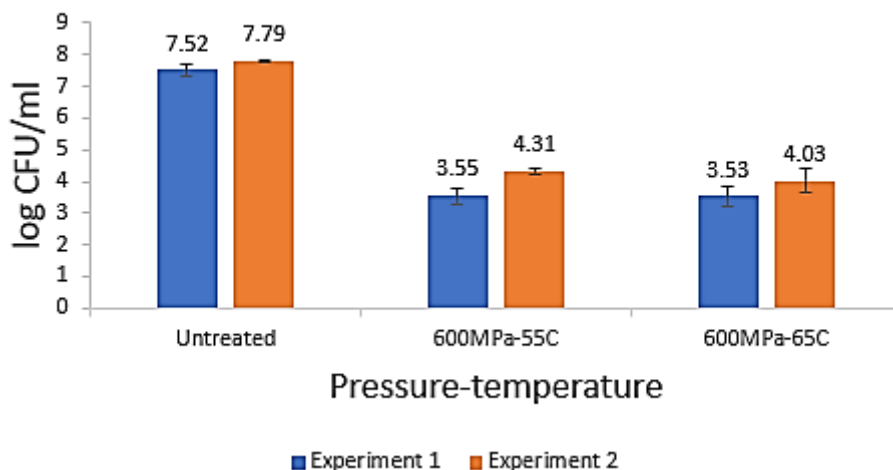

Figure 32: Inactivation of *B. pumilus* B367 spores in tuna fish after processing with PATS at 600 MPa with both 55 and 65 °C for 2 minutes and stored for 6 weeks at 4 °C. The data is mean values of three parallels and the standard deviations are shown. The spores used were from batch 6 in experiment 1 and batch 8 in experiment 2.

Overall, similar reduction was achieved by using a lower temperature for shorter time in PATS while a higher temperature for longer time in conventional atmospheric pressure heat treatment (Figure 30 and 31). This means processing parameters and thereby the stress factors for the spores were different. This shows that it may be beneficial to process the food products by applying the PATS system, as the nutritional quality and sensory properties of the food could be maintained while food safety might still be assured compared to that of heat treatment (Dhawan et al., 2014; Somerville & Balasubramaniam, 2009). Others have previously studied PATS application with 600 MPa at 72 $^{\circ}$C for 1 minute and *B. pumilus* spores suspended in skimmed milk. These results showed a 4.2 and 1.8 log-reduction for spores which was sporulated on soil extract agar and nutrient agar, respectively (Lopes et al., 2018; Scurrah, Robertson, Craven, Pearce, & Szabo, 2006). In another study for *B. licheniformis* spores in carrot juice, spore reduction of about 5 log units were found after treatment of 600 MPa at 60 $^{\circ}$C for 3 minutes at pH 4.5 and complete inactivation by extending the treatment for 5 minutes. The pH variation affected the inactivation level, with lower pH promoting inactivation (Tola & Ramaswamy, 2014). However, inactivation rates in PATS are dependent on the bacterial species and strains within the same species. The food type also affects the efficiency of using high pressure for inactivation of microorganisms. For example, high acidic foods (pH < 4.6) with high water activity (a_w > 0.95) favour spore inactivation, while low acidic foods with low water activity need longer treatment time or higher pressure and temperature to attain microbial inactivation (Lopes et al., 2018; Somerville & Balasubramaniam, 2009).

5 Conclusion

Heat treatment of *B. pumilus* spores in LB medium and tuna fish was applied, and 5.9 and 4.4 log-reduction of spores were achieved in LB medium and tuna fish, respectively at 95 °C for 10 minutes. The result showed that the heat resistance of *B. pumilus* spores increased in the food matrixes compared to the LB medium. The tuna fish appeared to have a protective effect on the *B. pumilus* spores.

The growth experiment of *B. pumilus* with different preservatives showed that the maximum turbidity (number of cells present) was decreased as the concentrations of different preservatives (NaCl and KCl) increased from 1 % to 4 % or from 62.5 to 500 ppm $NaNO_2$ and as the pH was decreased from pH 6.2 to pH 4. Further, the TTD was longer with higher concentrations of preservatives and lower pH samples.

Specific concentrations of preservatives (2 % NaCl, 2 % KCl and 250ppm $NaNO_2$) and pH (4, 5, and 6.2) were applied to the *B. pumilus* spores. When the pH increased from pH 4 to pH 6.2, the heat tolerance of *B. pumilus* spores were increased, and as the concentrations of NaCl, KCl and $NaNO_2$ increased to 2 % (NaCl and KCl) or 250 ppm $NaNO_2$, the heat resistance of *B. pumilus* spores were decreased at some specific temperature-time combinations of heat treatment.

Heat resistance of *B. pumilus* spores that were sporulated at different temperatures was examined. A 4.9, 4.6, 3.0 and 2.4 log-reductions of spores were achieved for spores that sporulated at 15, 25, 37 and 42 °C, respectively at 90 °C for 10 minutes heat treatment. Likewise, a 6.0, 5.8, 5.8 and 5.1 log-reductions were obtained for spores that sporulated at 15, 25, 37 and 42 °C, respectively at 95 °C for 10 minutes heat treatment. The results indicate that spores that sporulated at higher temperatures had higher heat resistance than those that were sporulated at lower temperatures.

PATS processing of tuna fish added *B. pumilus* spores showed that the log-reduction of spores after applying 600 MPa-55 °C and 600 MPa-65 °C for 2 minutes were 3.5 and 3.7 log CFU/ml, respectively. This revealed that similar logarithmic reduction was achieved by using lower temperatures for shorter time in PATS, while higher temperatures for longer time in conventional thermal processing. It could therefore be beneficial to process the food products

by applying the PATS system, as the nutritional quality and sensory properties of the food would be maintained while food safety could still be assured compared to that of classical thermal treatment.

5.1 Future work

There are several aspects in this project that require further study both in conventional thermal treatment and PATS process:

- Expand the testing with different concentration of NaCl, KCl and $NaNO_2$ or applying other types of preservatives to examine the effects on food matrixes during thermal processing.
- Study the application of PATS process with different concentration of preservatives and pH on food matrixes.
- Examine higher inactivation temperatures or pressures for longer times.
- Investigate the inhibition of different preservatives and pH on surviving spores at temperature close to minimum growth temperature, for example 5-10 $^\circ$C which may be found in chill chains.

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Appendices

Appendix 1

All media used in this thesis were autoclaved at 121°C for 15 minutes and stored at 4°C. Below are an overview of ingredients, recipes and preparation:

2× Schaeffer´s-glucose (SG) medium

16 g difco nutrient broth, 2 g KCl, 0.5 g $MgSO_4.7H_2O$ and 17 g agar in 1 litre of MilliQ water. Then the pH was adjusted to 7.0, autoclaved, and added the following sterile component solutions to 1 litre of the cooled (55°C) medium: 1 ml 1M Ca $(NO_3)_2$, 1 ml 0.1M $MnCl_2.4H_2O$, 1 ml 1mM $FeSO_4$ and 2 ml 50 % (w/v) glucose.

LB medium

10 g NaCl, 10 g Tryptone and 5 g yeast extract in 1 litre of MilliQ water, and then autoclaved. The pH of the medium was 7.0 ± 0.2.

LB Agar

10 g NaCl, 10 g Tryptone, 5 g yeast extract and 15 g agar in 1 litre of MilliQ water, and then autoclaved. The pH of the medium was 7.0 ± 0.2.

1 % Peptone water

8.5 g NaCl and 1 g bactopeptone in 1 litre of MilliQ water, and then autoclaved. The pH of the medium was 7.2 ± 0.2.
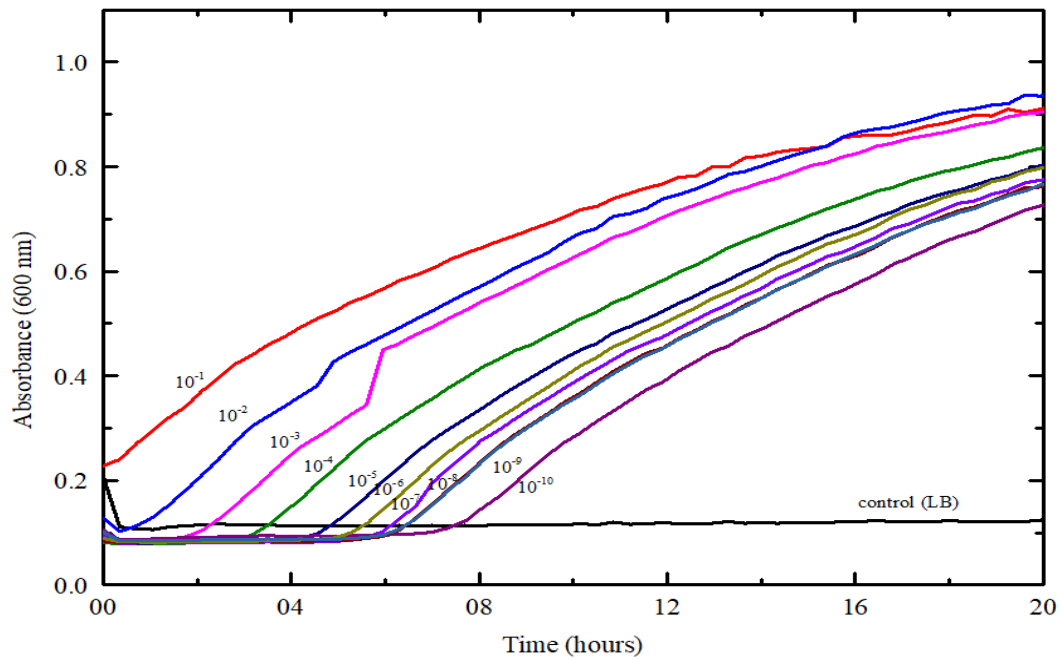
Figure: Growth of *B. pumilus* B367 vegetative cells measured with different diluted samples of LB media at 37°C for 24 hours. The control (blank) was measured without bacterial addition. The data is mean values of two replicates collected from Bioscreen C, and the spores used were from batch 1.
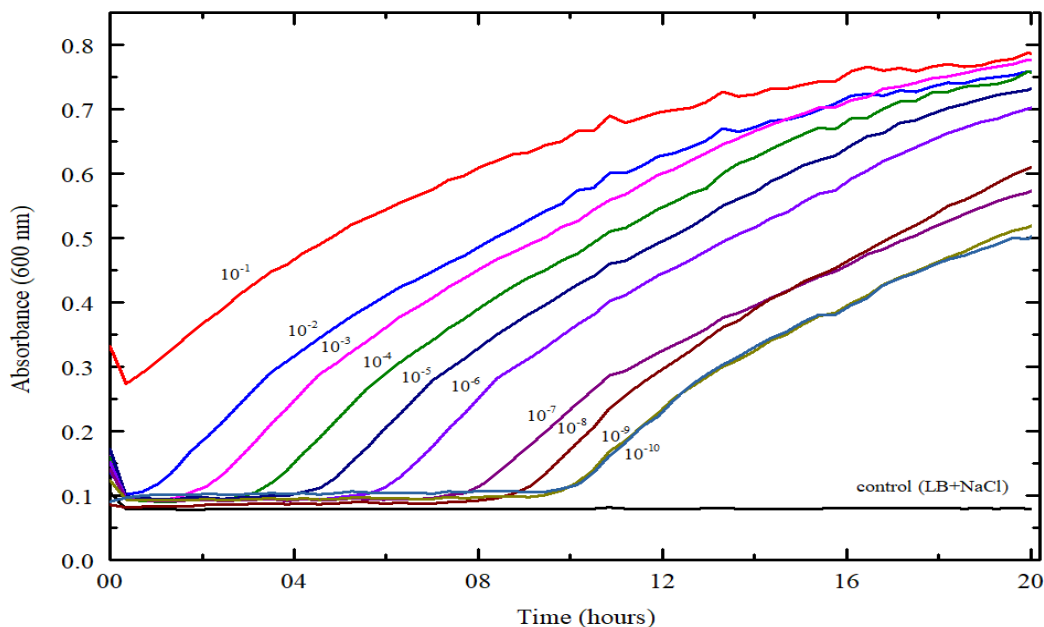
Appendix 3

Figure: Growth of *B. pumilus* B367 vegetative cells measured with different diluted samples of 2 % w/v NaCl added LB media at 37°C within 24 hours. The control (blank) was measured without bacterial addition. The data is mean values of two replicates obtained from Bioscreen C, and the spores used were from batch 1.
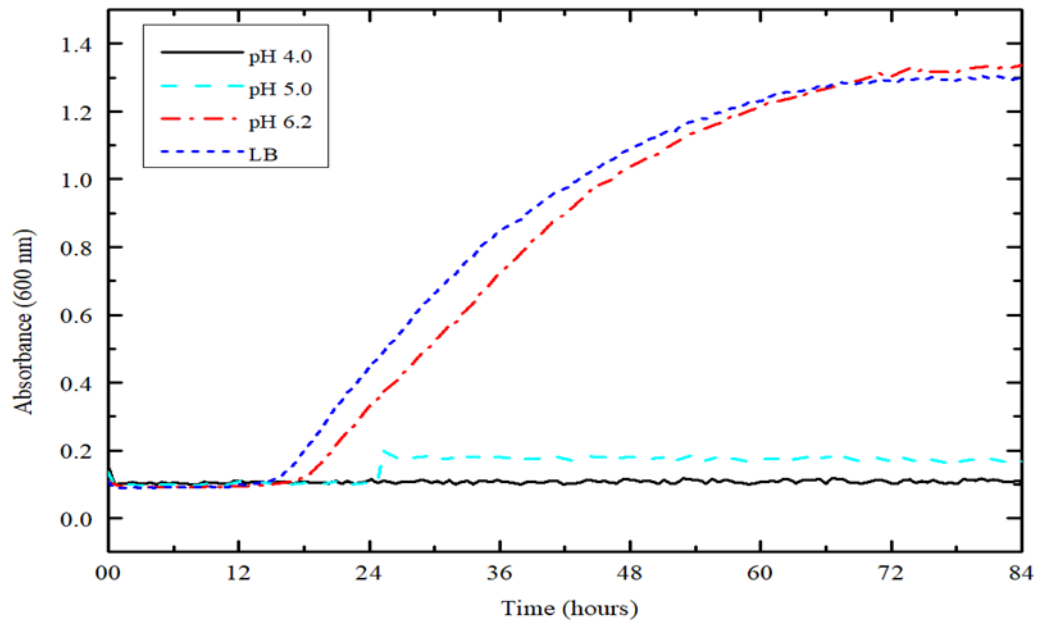
Figure: Growth of *B. pumilus* B367 vegetative cells with LB and different concentrations of pH (pH 4, 5, and 6.2) of LB media at 25°C for 7 days. The data is mean values of three replicates obtained from Bioscreen C.
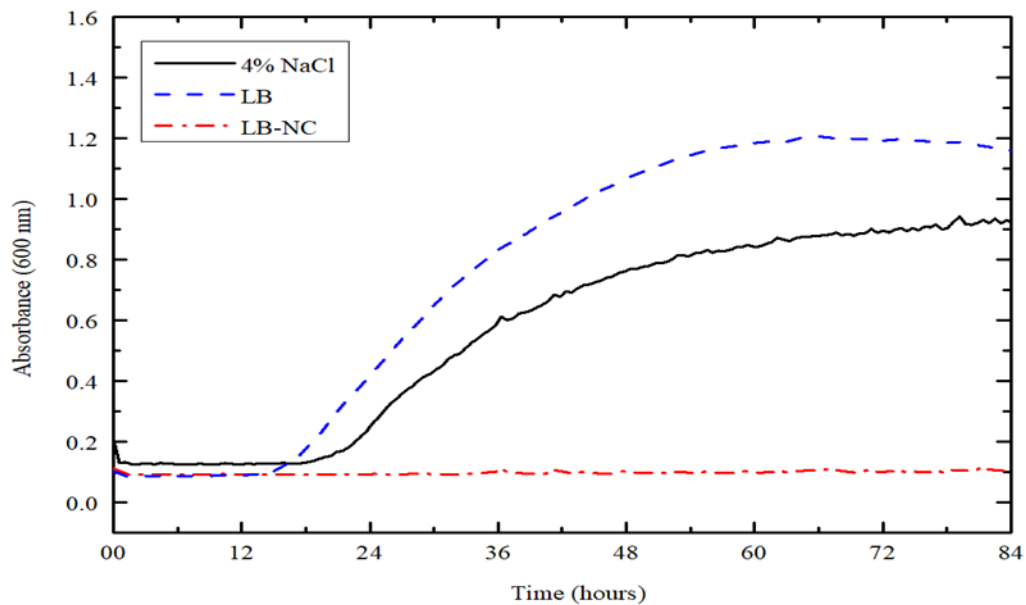
Appendix 5

Figure: Growth of *B. pumilus* B367 vegetative cells with LB and 4 % w/v NaCl added LB media, as well as the negative control (LB-NC) at 25°C for 7 days. The data is mean values of three replicates collected from Bioscreen C.