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Determination of Spore Heat Resistance of Seven Strains of *Clostridium botulinum* Spores

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This study was carried out in the Department of Food Hygiene and Environmental Health at the Faculty of Veterinary Medicine at the University of Helsinki. The practical part of the study was carried out in the *Clostridium Botulinum* laboratory, in University of Helsinki Viikki campus.

The purpose of this study was to determine the heat resistance of seven different *C. botulinum* strains, belonging to three different groups (I, II and III).

Heat resistance was determined by using a heat test method in which known concentration of spores was heated in a water bath at a certain temperature/time combinations. The concentration of survivor spores was determined by using the MPN-method. The D-value was calculated from the results.

Results were as expected. Heat resistance between groups differs as group I and III spores have higher resistance than spores from group II.

Keywords	*C. botulinum*, botulism, D-value, heat test, MPN-method

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Tämä opinnäytetyö tehtiin Helsingin yliopiston eläinlääketieteellisen tiedekunnan elintarvikehygienian ja ympäristöterveyden osastolla. Käytännön osuus suoritettiin *Clostridium botulinum* -laboratoriossa Helsingin yliopiston kampuksella Viikissä.

Työn tarkoituksena oli selvittää seitsemän eri *C. botulinum* -kannan itiöiden lämmönsietokyky. Kyseiset kannat kuuluivat kolmeen ryhmään, (I, II ja III), jotka eroavat toisistaan muun muassa aineenvaihdunnallisten ja biokemiallisten ominaisuuksien perusteella.

Lämmönsietokyky selvitettiin kuumennuskokeilla, joita toistettiin kullekin ryhmälle kolmessa eri lämpötilassa. Itiöiden määrä kuumennuskokeiden jälkeen laskettiin käyttämällä MPN-menetelmää. Tulosten avulla laskettiin D-arvot ryhmille.

Saadut tulokset olivat odotettujen mukaisia. Itiöiden lämmönsietokyky vaihteli eri ryhmien välillä, ryhmien I ja III itiöillä lämmönsietokyky oli korkeampi kuin ryhmän II itiöillä.

Avainsanat	*C. botulinum,* botulismi, D-arvo, kuumennuskoe, MPN-metodi

Contents

List of Abbreviations

BoNTs	Botulinum neurotoxins. Toxins that are produced by *Clostridium botulinum*
MPN	Most probable number. Method which is used to determine the approximated number of cells in the solution.
°C	°C Celsius
g	Grams
g	Gravitational force
l	Liter
ml	Millilitre
µl	Microliter
min	Minutes

1 Introduction

Clostridium botulinum is an intriguing bacteria which was first identified after a botulism outbreak which occurred in 1895. At first it was named as *Bacillus botulinus* but was later re-named as a *Clostridium* because of its spindle shape [1]. Before identification, symptoms of food-borne botulism were accurately described in the early 1800s by Justinus Kerner who named the disease as sausage poisoning, bad sausages being causative for the symptoms. Latin word for sausages is “botulus” [2].

C. botulinum produces lethal neurotoxins, BoNTs, which are the most potent natural toxin known to science. BoNTs are the cause of botulism, which may lead to death due to respiratory paralysis [3]. Despite of the lethality of the toxin, it has many applications in modern medicine, for example in neurological medicine it is used to treat spasms, dystonia, tremors and voice disorders [4].

C. botulinum produces endospores when conditions are too extreme for the vegetative cell, for example when there is not enough nutrients [5]. These endospores are widely spread in soil and aquatic sediments and can also be present in the intestinal tract of humans and other animals [6]. Endospores survive much higher temperatures than vegetative cells and therefore are the biggest concern when it comes to food spoilage and botulism.

In food industry, heat treatments are used to destroy these spores. The botulinum-cook is a known method used by canning industry to kill these spores by heating the product for an adequate time in an adequate temperature.
The adequate temperature for destroying the spores but not to reducing the quality of the food product is determined using heat tests and calculating the D-value. The D-value tells the time that is required to kill certain type of spores or vegetative cells in certain temperature.

The heat resistance of spore seems to depend on the group of the strain. There are four different groups of *C. botulinum* and three of these groups are known to cause botulism.

In this study, D-values were determined for strains belonging to three different groups of *C. botulinum,* in order to give information about the heat resistance of those groups.

2 Theoretical Framework

2.1 Clostridium botulinum

Clostridium botulinum is an anaerobic, gram positive bacteria which has the ability to form endospores. *C. botulinum* produces botulinum neurotoxins which can cause a severe flaccid paralytic disease leading to death when paralysis reaches the respiratory muscles [7].

Originally bacteria were classified as *C. botulinum* if they were capable of producing botulinum neurotoxin. Today other species of *Clostridium* have been found, for example some strains of *Clostridium baratii* and *Clostridium butyricum,* which can produce some of the botulinum toxins [1].

Toxins of *C. botulinum* are divided into six group according to serological features of toxins produced. Groups are designed from A to G. Toxins from groups A, B, E, and F cause human botulism when toxins from groups D and C causes animal botulism. Group G toxins is not known to cause botulism [7]. Recently a novel toxin type has been described as BoNT H, but it was revealed as being a hybrid between BoNTs A and F [8].

These toxin types are also divided into subtypes, according to the difference in nucleotide sequence of BoNT gene. For example, for group A there are subtypes from A1 to A5 and for group B there is proteolytic B1 to B3, bivalent B and non-proteolytic B4. Bivalent strains produce two different types of toxin, but most *C. botulinum* strains produce only one type of toxin [9].

C. botulinum strains are categorized into four groups, I-IV, according to metabolic and biochemical characteristics of the bacteria [9]. These four groups have been established to correspond also genetically, based on DNA homology and RNA sequencing studies [11].

Group I include neurotoxin types A, B and F, group II includes neurotoxin types B, E, F, group III include neurotoxin types C and D and group IV incudes neurotoxin type G. Each group have its own favourable growing conditions [1].

Strains characterized in group I are straight or slightly curved motile rods. Optimal growth temperature for group I strains is between 30 and 40°C, although some strains grow well in 25°C and a few in 45°C. Minimum growing temperature is 10-12°C. Minimum pH for growing is 4.6 and maximum is 8.5 [11].

Group I strains are proteolytic, meaning that they digest protein into smaller fragments. Since group I strains do not grow in low temperatures, they are not psychrotrophic, thus they do not live in cold environments. Therefore, group I strains are not a big concern among refrigerated products [10, 11]. Group I strains are commonly found in terrestrial sediments. Spores of group I have the highest level of heat resistance among all of the groups. The D-value at 121.1°C of Celsius is 0.05 to 0.32 minutes, meaning that 10% of the spores will survive even after that time at 121.1°C [12].

Group II strains are straight motile rods. Optimum temperature for growing is between 25 °C and 37 °C. Poor or no growth at all occurs above 45°C [12]. Minimum temperature for growing and toxin production is 3.3°C, therefore group II strains are potential cause for food-borne botulism in refrigerated products. Toxin production in temperature as low as 3.3°C requires one to three months of storage periods [11].
Spores of group II are less heat resistant than those of group I. D-value at 82.2°C varies between 0.25 and 73.61 minutes.
Strains from group II are not proteolytic and are often found in aqueous environments [12].

Group III strains are straight, motile rods. Optimum growing temperature is between 30 and 37°C. Most strains also grow well at 45°C. Minimum growing temperature is 15°C, poorly or no growth occurs below 25°C [11].

Group IV strains are straight motile rods. Optimal growth occurs at 25°C and 45°C. Minimum growing temperature is 12°C [11].

2.2 Endospores

In unfavourable conditions, *C. botulinum* is able to sporulate and produce endospores (called spores for simplicity). Spores are highly specialized cells that are produced inside the bacterial cell [5]. Spores can stay dormant for years and are highly resistant toward environmental assaults when compared to bacterial cell, in fact, bacterial spores are the most resistant cell type known [5, 13]. The ability to produce spores allows bacteria to preserve its genetic material in conditions that otherwise would destroy the vegetative cell. For example, spores are able to survive from high temperatures, high UV-radiation, dryness and chemical damages, due to the spore's structure [5].

Endospores have an outer proteinaceous coat which protects it against chemical and enzymatic damages. Below the coat is the cortex, which is thick layer of peptidoglycans. Cortex protects the cell from high temperatures and to do this, the cortex production is important because it involves the dehydration of the spore's core [5].

Below the cortex is germ cell wall which is a thinner layer consisting of peptidoglycan. This layer will become the cell wall of the vegetative cell once the spore germinates. Below the germ cell wall is the inner membrane, which protects the cell from damaging chemicals by acting as a barrier. The core of the spore, which holds the DNA, is very dehydrated. The structure of the endospore is represented in figure 1 [5].

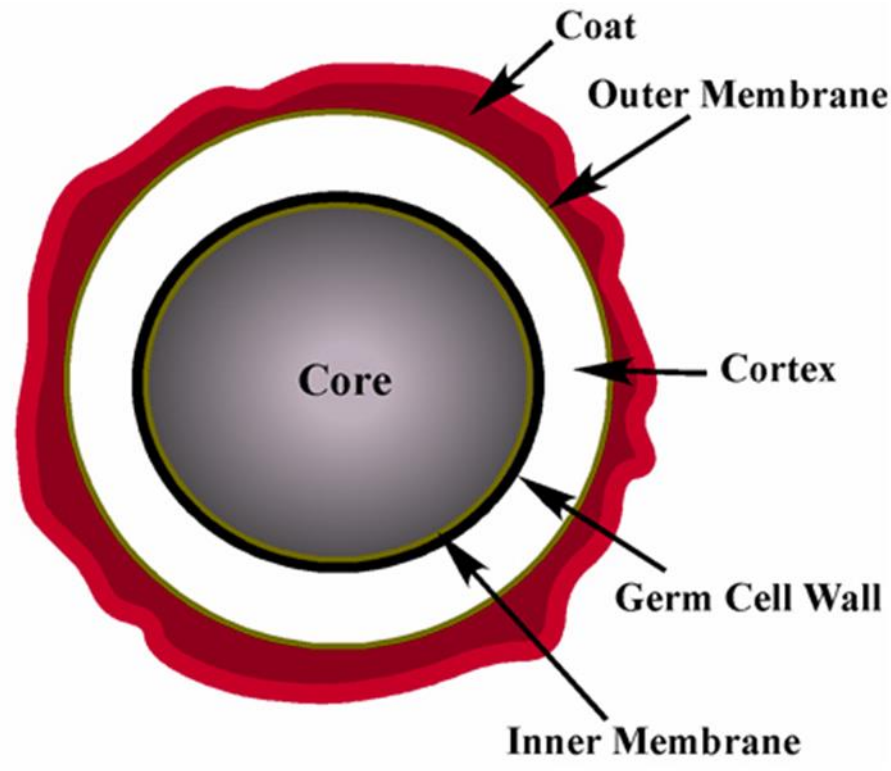

Figure 1. The structure of endospore [5].

Sporulation is an energy demanding process, which cannot be stopped after it, has started, taking several hours to finish.

Sporulation of *Bacillus subtilis* is used as a model version and it is assumed to be similar with sporulation process of *C. botulinum*. This is because the master regulator for sporulation is Spo0A in both micro-organisms, in fact, Spo0A is found in all endospore formers [13]. Spo0A is a DNA-binding protein, which affects the expression of more than 500 genes during the sporulation [14].

Sporulation is divided into eight stages according to the morphological changes of spore and mother-cell. The first stage, labelled as 0, consists of the elongation of the mother-cell. Next stage, I, chromatin filament is formed. In stage II the mother-cell divides assimetrically so that at the end of this-stage there is a mother-cell and a forespore. In stage III, the mother-cell engulfs the forespore. In stage IV, the cortex is formed around

the engulfed forespore. At stage V spore coat and proteins which are needed in the germination process are added to the spore's cortex. Maturation of the spore occurs at the stage VI, and at the stage VII mother-cell lyses and releases the mature spore. This sporulation process is demonstrated in figure 2 [15].

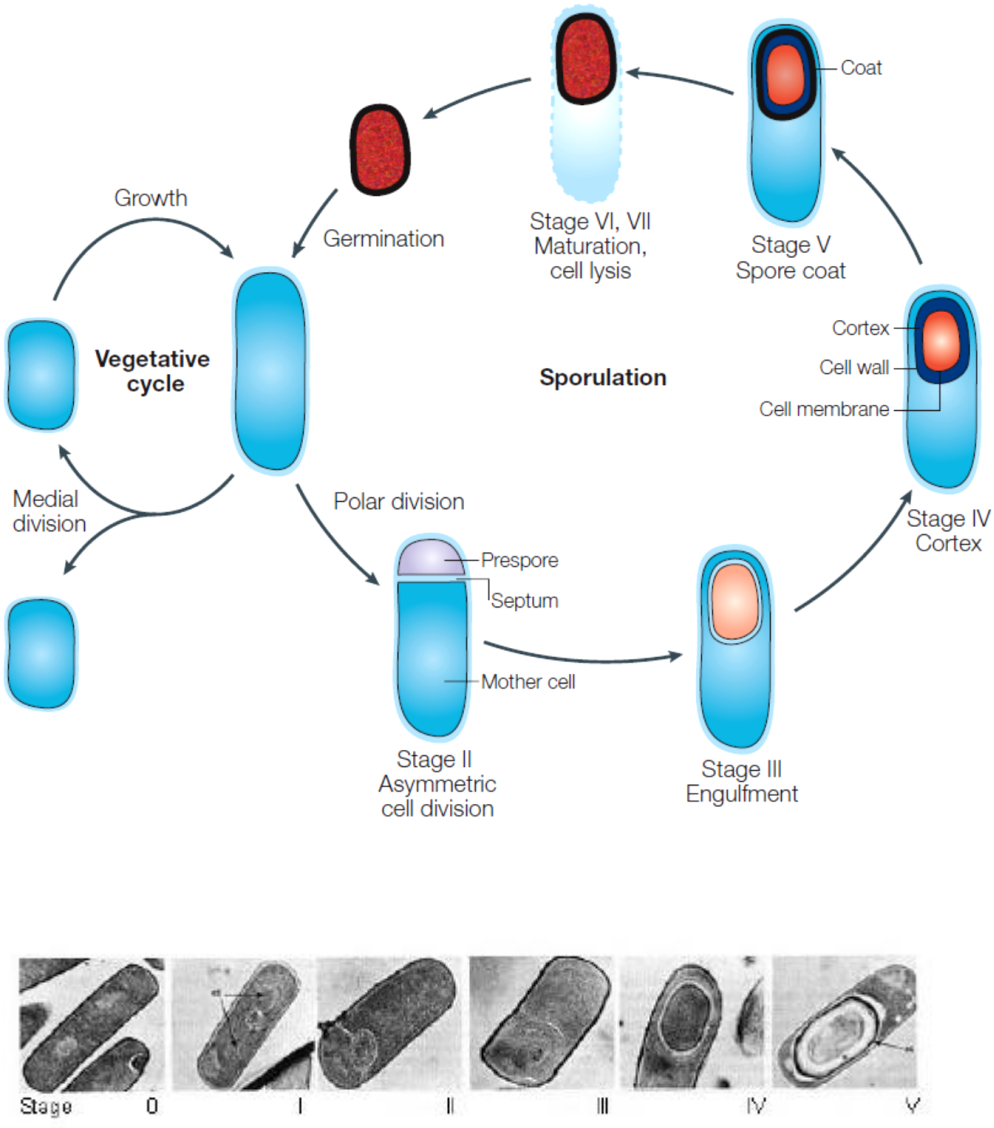

Figure 2. Sporulation process of endospore forming cell. Adapted from [16].

Spores of *C. botulinum* are widely spread in environment. Spores are found in both plant- and animal-based food [12]. Due to high resistance for high temperatures, spores are a higher concern in food industry compared to vegetative cells [11].

The germination of spores is a process in which the spore converts back to vegetative cell. This process needs appropriate environmental conditions [13].

2.3 Botulism

Botulism is life a threatening disease caused by botulinum neurotoxins (BoNTs). BoNTs end up in the body mostly via gastrointestinal tract or mucous membrane, where toxins are absorbed into general circulation system and finally reach the motor nerve endings and inhibit the release of the acetylcholine neurotransmitter. When neurotransmittion is prevented, messages from nerve cell do not reach muscle cells. BoNTs can not pass through the blood-brain barrier and therefore do not affect brain functions [17]. The mechanism of how BoNTs inhibit the release of neurotransmitters depends on the toxin type [1].

Treatment of botulism is mainly supportive. Administration of antitoxin should be prompt, as the antitoxins neutralize only toxins that are not yet bound to nerve terminals [18]. It may take as long as 8 months for surviving patients to recover [11]. The mortality rate is relatively high (20-50%) but it depends on the speed of treatment, the amount of toxin ingested, the type of food, and the toxin type - type A has higher mortality compared to types B and E [11].

The first symptoms of botulism are usually weakness and fatigue. Problems with swallowing and speaking follow. Blurred or double vision is also common [18], followed by symmetrically flaccid paralysis. Fewer is not usually a symptom [19].

Botulism is divided into three main classes in most of the references, these are food-borne botulism, infant botulism (intestinal botulism) and wound botulism. Also cases of inhalational botulism are reported.

2.3.1 Food-borne Botulism

In addition to normal symptoms of botulism, food-borne botulism can also cause nausea, vomiting and constipation [7]. Symptoms can occur 8 hours to 8 days after ingestion of toxin, but symptoms most commonly occur during the first 12-48 hours [11].

Otherwise sensitive BoNTs survive the acidic conditions of gastrointestinal tract via the non-toxic neurotoxin associated proteins which form a complex with BoNTs. This complex allows BoNTs to pass through epithelial membrane of intestines into the circulation system [20].

Canning and fermentation often creates anaerobic and low-salt conditions that are favourable to *C. botulinum* germination and toxin production. Commercial canned foods are nowadays sterilized with appropriate heat treatment so that outbreaks of botulism caused commercial foods are rare. Homemade fermentation and canning processes are the main reasons for food-borne botulism [1].

2.3.2 Infant Botulism

The difference between infant botulism (or intestinal botulism in adults) and food-borne botulism is the way the body is exposed to toxins. In food-borne botulism exposition is usually on one occasion, whereas botulism in infants appears with continuous exposure to toxins caused by the ingestion of spores, which germinate and colonize the intestines. This causes the symptoms to develop slower and more chronic [21].

Infant botulism occurs usually in babies from one week to one year due to the immature protective bacterial flora in the intestine and the lack of the bile acid which inhibits *Clostridia*. Infant botulism is mainly connected with honey, but spore contaminated dust is also suggested to be a potential source [18].

Usually infant botulism is connected to group I strains since the normal body temperature is favorable for them [7].

2.3.3 Wound Botulism

Wound botulism is a rare form of botulism. Nowadays it is mostly contracted among intravenous drug abusers. Botulism occurs when a deep wound or abscess is contaminated with *C. botulinum* spores, and its anaerobic conditions which allows spores to germinate and grow [7].

2.4 MPN-method

The Most Probable Number method (MPN) is a quantitative method used to determine the concentration of microorganisms in a sample. It is widely used method in food industry and microbiology industry [21].

The method is based on the presumptions that the micro-organisms in the sample are randomly distributed and sample is homogenous with the micro-organisms. Also, it is assumed that microorganisms are unattached in the sample and do not repel each other [12].

In MPN-method, aliquots of the culture are inoculated into liquid medium appropriate for the growth of the microorganisms of interests. These aliquots are then incubated in appropriate time and temperature, and after that the aliquots either have growth (positive result) or not (negative result). Therefore, the dilutions of the sample should be such that the final few dilutions give negative results. According to the number of positive and negative aliquots, the number of microorganisms in the original sample is calculated according to probability estimation calculations. Since the mathematics needed for the calculations are quite heavy, already developed MPN tables are often used [12, 21].

For how the sample is inoculated in the medium, there are two formats. Multiple dilution test and single dilution test [12].

In multiple dilution test, usually three or five replicate dilutions are prepared and from these dilutions, decimal dilutions are made using serial dilution [12].
In single dilution test all of the inoculated aliquots are the same dilution and therefore contain equal amount of the sample. Single dilution test is commonly used with samples

that have low concentration of microorganisms and therefore there is no reason to dilute the sample. There are usually more aliquots than with multiple dilution test, commonly five or ten [12].

2.5 Heat Tests

The main principle of heat tests is to heat microorganism in various temperatures/time combinations. The concentration of survived microorganisms is found to decrease logarithmically.

Heat tests are usually used in food industry to determine the effective temperature and time combination to kill microorganisms that cause food spoilage. Tests are needed to find the adequate time and temperature to sterilize food, but also retain food's organoleptic and nutritive properties [23].

Botulinum-cook, or 12D-process, is a process used by canning industry to reduce the bacteria by 12 log cycles. This is done by heating the product at 121.1 °C for 3 minutes. Time and temperature of this process are based on the D-value of toxin type A and B spores of *C. botulinum* because these spores are the most heat resistant [24].

2.5.1 D-value

Heat test are usually used to determine the D-value of certain bacteria or spore

Spores from strains producing toxin types A and B are more heat resistant than in example, type E. Types A and B are characterized by D25 values of the order of 0.10 to 0.20 minutes [23].

The D-value, the decimal reduction time, is the time, at a certain temperature, which is needed to reduce survival of microorganisms by 90 percent, or in other words, by 1-log. The D-value usually remains constant, thus the time that is needed for example, to reduce the bacteria from 10^7 to 10^6 is same than the time that is needed to reduce the bacteria from 10^5 to 10^4.

D-value depends on the microorganism and the media they are inoculated in. The death of the bacteria is defined so that bacteria is considered dead when it is not able to reproduce [23]. Death of cells is found to be logarithmic. Since the decomposition of micro-organism is generally logarithmic the D-value is possible to be calculated mathematically.

The time required to kill 90% of the cells is the time required for the curve to traverse one log cycle, if time is represented as D (decimal reduction time), the slope of the survivor curve can be expressed as:

$$\frac{logC_0 - logC}{D} = \frac{1}{D} \quad (1)$$

In which C_0 = number of cells in initial sample and C = number of cells in final sample.

As reciprocal of D is the slope of the survivor curve, the D-value can be determined straight from the survival curve where the time is on x-axis and the logarithmic values from survival cells are on the y-axis.

Z-value is used when calculating the relative resistance of micro-organism to the different temperatures. The following equation is used:

$$\frac{\log D_2 - \log D_1}{z} = \frac{1}{z} \quad (2)$$

In which the D_2 and D_1 are the strain's D-values in different temperatures. Z-value can also be calculated from a curve where D-values are on y-axis and temperatures on x-axis. Z-value is then the reciprocal number of slope.

In practice, z-value tells how much time is required to change the D-value by a factor of ten.

3 Experimental Part

C. botulinum strains used for heat tests were group I strains: ATCC 3502, ATCC 19397 and ATCC 19397 *ΔbotA*, group II strains Beluga and Beluga *ΔbotE* and group III strains BKT015925 and Stockholm.

Waterbath used in heatings at temperatures 70 – 95°C was a VWR® 18 litre shaking water bath (shaking was not used). For 100°C heatings a boiling bath was used.

The compositions of mediums used for laboratory works are presented in appendix.

3.1 Spore Stocks – Preparation and Determination of Concentration

Spore stocks used for heat tests were prepared from the sterile spore stocks. Each strain was inoculated in appropriate media and purified with Gastrografin (Bayer©), as described below.

Strains were inoculated in 5 ml of media for overnight growth from clean sterile spores stocks.
One millilitre of each overnight culture was inoculated into 50 ml of media. Group I strains were inoculated into TPGY media, group II strains into TPGY media with meat agar and group III into TPGY-cystein-strach media. Inoculations were carried out in an anaerobic chamber, group I and III in a 37 °C chamber and group II in a 30 °C chamber. Cultures were let to grow until sporulation was completed, which was assured by microscopy, also confirming that contamination did not occur.

Grown cultures were collected into 50 ml falcon tubes, after confirming via phase contrast microscopy that sporulation had started. Tubes were spinned down for 10 min at 10 000 *g*. Supernatant was removed and pellet was resuspended in cold 0.1% Triton-X.

Tubes were incubated at 4 °C for 2 days. After incubation, spores were pelleted for 10 min at 10 000 *g*. Pellets were resuspended with sterile ddH_2O and again spinned down for 10 min at 10 000 *g*.

Supernatant was removed, and the pellet was carefully resuspended in 1 ml of 20% Gastrografin and layered over 20 ml of 50% Gastrografin, already placed in a centrifuge tube. Tubes were centrifuged at 4 °C max speed for 30 min. Supernatant was removed carefully in a way that did not disturb the pellet and without allowing any visible cell debris to touch the pellet.

Pellet was resuspended in sterile ddH_2O and spinned down at 6 000 *g*. Supernatant was discarded and washed 5 times with cold 0.1 % Triton-X.
Spore stocks were stored at 4 °C.

Concentration of spore stocks was determined using MPN-method. Work was carried out in an anaerobic chamber. 270 µl of appropriate media for each strain was pipetted with multichannel pipet into each well of 96-well microplate. 30 µl of each spore stock was pipetted into 3 wells on the first row. After first row was done, with a multichannel pipet 30 µl of diluted spore stock from the wells on the first row was pipetted into wells on the second row. These decimal dilutions were continued until the final row, so that final dilution was 10^8.

Plates were sealed with sealing tapes and were incubated anaerobically for 48 hours. Group I and group III plates were incubated at 37 °C and group II plates were incubated at 30 °C. After incubation, concentration of spore stocks was calculated with MPN calculator. Calculated concentrations are shown on table 1.

Table 1. Concentrations of used spore stocks, the number in parenthesis is number of the stock.

Strain	spores/ml
Beluga (1)	$5*10^8$
Beluga Δ*botE* (1)	$8*10^8$
ATCC 3502 (2)	$8*10^8$
ATCC 19397 (2)	$3.1*10^8$
ATCC 19397 (3)	$2.5*10^7$
ATCC 19397 Δ*botA* (3)	$8*10^8$
ATCC 19397 Δ*botA* (2)	$5*10^8$
BKT015925 (1)	$8*10^5$
Stockholm (1)	$8*10^6$

3.2 Heat Tests

For each group, three different temperatures were used in the heat tests. Temperatures and time of heating for each group are stated in table 2.

Table 2. Times and temperatures used in the heat test.

Group I		**Group II**		**Group III**	
Temperature (C)	**Time (min)**	**Temperature (C)**	**Time (min)**	**Temperature (C)**	**Time (min)**
90	20, 40, 60	**70**	20, 40, 60	**80**	20, 40, 60
95	10, 20, 30	**72.5**	10, 20, 30	**85**	10, 20, 30
100	1, 2, 5	**75**	10, 15, 20	**90**	10, 15, 20

Measurements in each temperature were carried out two or three times.

Heat tests were started with group II strains Beluga and Beluga Δ*botE*. At first dilutions were made so that concentration for Beluga was $1.0*10^6$ /ml and concentration of Beluga *ΔbotE* was $1.6*10^6$ /ml.

Spore stocks were diluted with sterile ddH_2O, using 12 ml of water and 24 µl of spore stock. However, when later calculating results, it appeared that concentration was too low, and spores did not grow enough.

Initial concentrations were then optimized, diluted concentrations for each strain are shown in table 3.

Table 3. Spore concentration of these study strains.

Strain	Diluted concentration /ml
Beluga (1)	$3.0 * 10^6$
Beluga Δ*botE* (1)	$4.8 *10^6$
ATCC 3502 (2)	$4.8 *10^6$
ATCC 19397 (2)	$1.86 *10^6$
ATCC 19397 (3)	$1.5 *10^5$
ATCC 19397 Δ*botA* (3)	$4.8 *10^6$
ATCC 19397 Δ*botA* (2)	$3.0 *10^6$
BKT015925 (1)	$4.8 *10^3$
Stockholm (1)	$4.8 *10^4$

Concentrations should be as so that concentration of survival spores in an initial sample is 10^6.

For each temperature, there were three timepoints and for each timepoint three replicate samples were prepared. Also three replicates of initial sample, a sample that was not heated, were made.

First the dilution was prepared into 15 ml falcon tube. 12 ml of sterile ddH_2O plus 72 µl of vortexed spore stock were used. From this dilution, one millilitre was pipetted into short

glass tube. Thus, if there were three time points, there were in total 12 tubes. Tubes were then incubated in a water bath, except for initial tubes which were left to room temperature.

After each time point, those three replicate samples were taken out from the water bath and immediately transferred to an anaerobic chamber to prevent the death of vegetative cells via oxygen. If the time points were close to each other, plating was started at the end of the timing, and if there were enough time between time points plating was done after each time point. The number of surviving organisms was determined by MPN-method.

Samples were then incubated 48 hours in an anaerobic chamber. After incubation time, plates were read using a MPN calculator. Plates were read so that the last wells where growth occurred were marked and then the combination of three replicates was read so that usually the final row had no growth. There is an example in a figure 3.

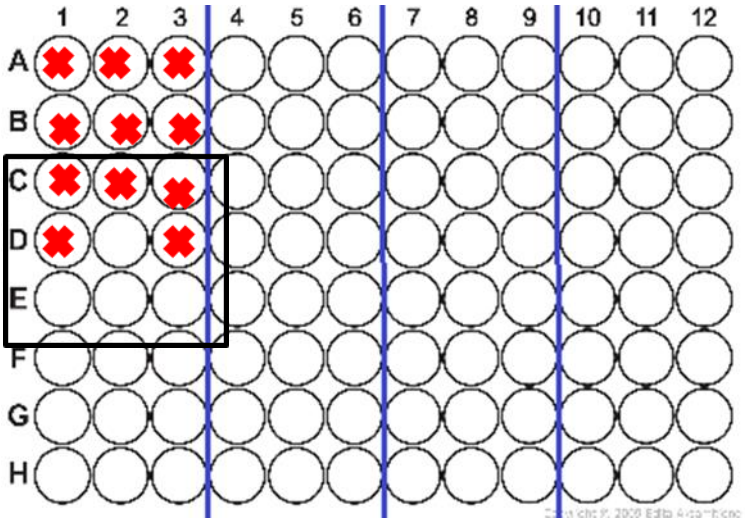

Figure 3. An example of how to read the MPN plate.

In a figure 3, marked wells would be read so that it has combination of three, two and zero. The dilution of middle row would be four. This means that the second row from the combination of three is the fourth row in where the growth occurs.

Using the Botulinum group's MPN calculator the most probable number would be read next to this combination and the number would be added in the equation, the dilution of

middle row and the amount of diluted solution pipetted into the microwell (30 µl) would also be given to calculator. Calculator would then give the answer for number of spores per millilitre.

Number of spores of each sample were added into Excel spreadsheet program and D-values were calculated, as described below.

4 Results

Results were calculated with Excel.

First, the average of the spores per millilitre from the three replicates for each temperature/time combination was calculated. Ten base logarithms were taken from the average. This is called post-log in the graphs. The average of the spores per millilitre from the initial sample replicates was calculated and ten base logarithms was taken from it, this is called pre-log in the graphs. Then the difference between pre-log and post-log was calculated. This pre-log minus post-log was put on the y-axis of the graphs, while the time is on the x-axis. The intercept of the linear regression with the X-axis was forced on zero. D-value was then calculated with the equation:

$$\mathrm{D} = \frac{-1}{\mathrm{a}} \qquad (3)$$

In which *a* = slope of the survival curve.

The number of survivor spores from heat tests are shown in appendix 1, 2 and 3.

From those experiments that were repeated three times, it was possible to calculate the D-value. and the z-value from group II strains. All of the calculated D-values are shown on table 4.

Table 4. Calculated D-values for groups. D-values are usually presented as D(t), in which the t is the time in minute.

Group I	**ATCC 3502**	**ATCC 19397**	**ATCC 19397 Δ*botA***
D(90)	-	-	7.3
D(100)	1.2	0.8	0.8
Group II	**Beluga**	**Beluga *ΔbotE***	
D(70)	113	113	
D(72.5)	25.5	30.7	
D(75)	6.3	5.4	
Group III	**BKT015925**	**Stockholm**	
D(85)	16.6	8.5	

From the table 4, it can be seen that group I and group II strains have higher heat resistance than group II strains.

4.1 Group I

At 90 °C, heat test was carried out for ATCC 19397 *ΔbotA*, the survival curve is shown in figure 4.

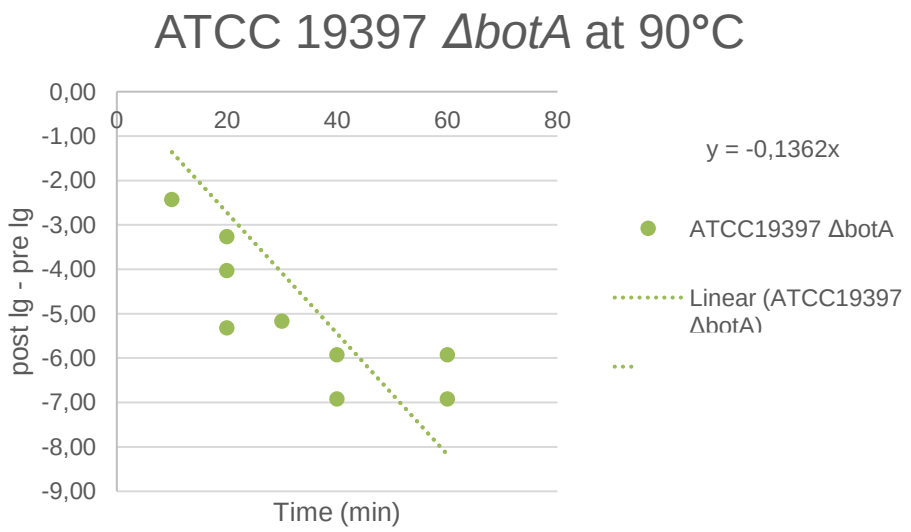

Figure 4. The survival curve for ATCC *ΔbotA* at 95 °C.

The D-value was calculated for ATCC 19397 Δ*botA:*

$$D = \frac{-1}{-0{,}136} = 7.34 \qquad (4)$$

This means that when heating temperature is 90°C, heating time acquired to kill 90% of the spores is 7.34 minutes.

D-values for the ATCC 3502 and ATCC 19397 in 90°C were not calculated. For both strains, heat test were carried out only two times.

The spore stocks of ATCC 19397 and ATCC 19397 Δ*botA* were changed from number 2 to number 3 between 95°C and 100°C, due to the inadequate amount of stock. Changing the stock might have affected a little the results; in 95°C heat test there did not occurred growth after heating, whereas when heated in 100°C there were growth after one-minute heating.

Heat tests were carried out twice in 95°C, so the D-value was not calculated. Also, for ATCC 19397 and ATCC 19397 Δ*botA* there did not occur any living spores after ten minutes at 95°C.
D-values for all of the strains in group I were calculated in 100°C. Survival curves are shown in figure 5.

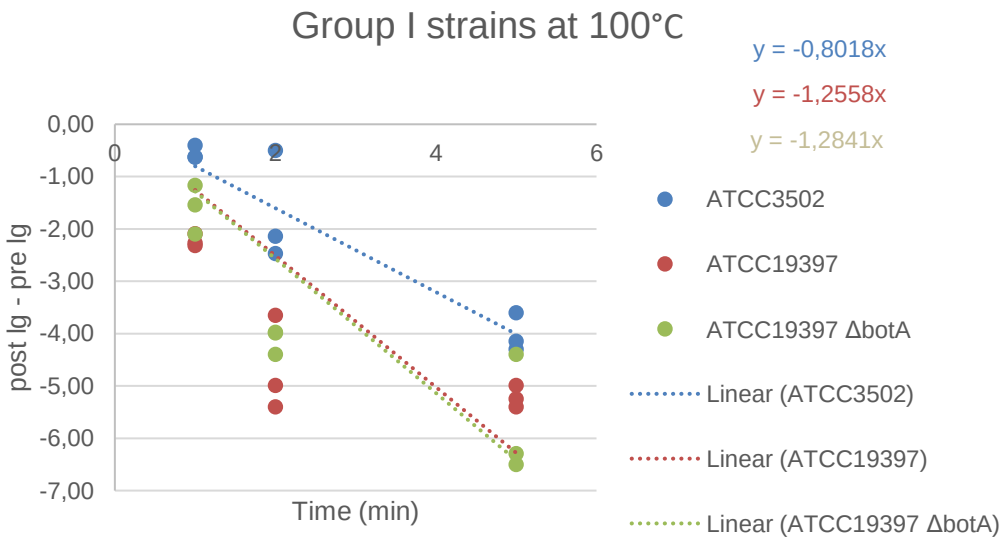

Figure 5. Survival curves for group I strains at 100°C.

D-value for ATCC 3502 in 100°C is 1.24. D-value for ATCC 19397 in 100°C is 0.79 and D-value for ATCC 19397 Δ*botA* in 100°C is 0.77.

4.2 Group II

For group II, heat tests were performed three times in each temperature. Graphs for survival curves from each heat treatment is shown in figures 6, 7 and 8.

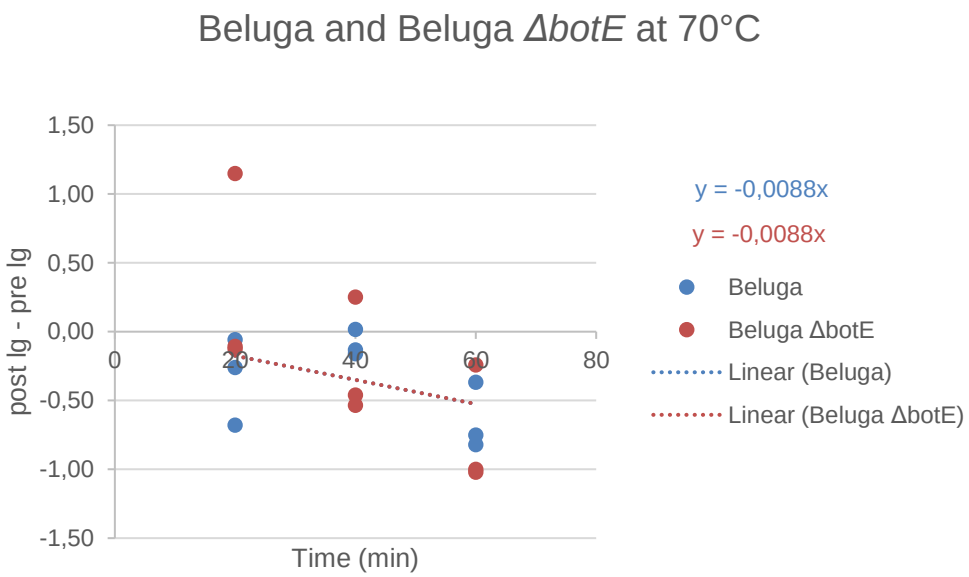

Figure 6. Survival curves for Beluga and Beluga Δ*botE* at 70°C.

D-value for Beluga at 70°C is 113.6. D-value for Beluga Δ*botE* at 70°C is also 113.6.

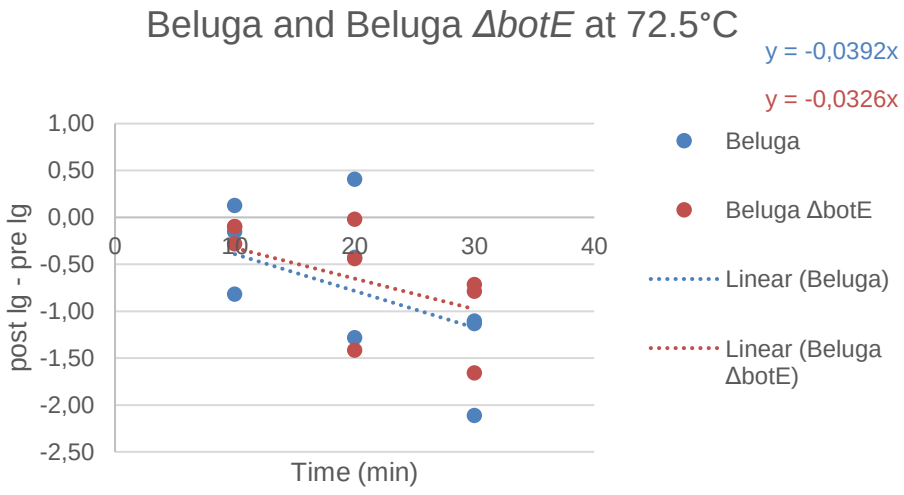

Figure 7. Survival curves for Beluga and Beluga Δ*botE* at 72.5°C.

D-value for Beluga at 72.5°C is 25.5 and for Beluga Δ*botE* D-value is 30.6

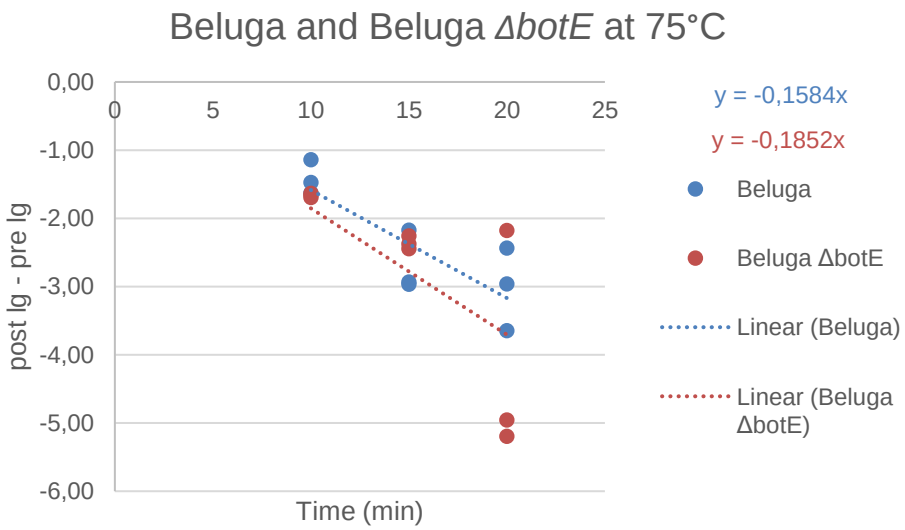

Figure 8. Survival curves for Beluga and Beluga Δ*botE* at 75°C.

D-value for Beluga at 75°C is 6.3 and for Beluga Δ*botE* D-value is 5.4.

The z-value for Beluga was calculated by plotting the ten base logarithms of D-values against temperatures used, and then taking the negative reciprocal number from the slope. The graph is shown in figure 9.

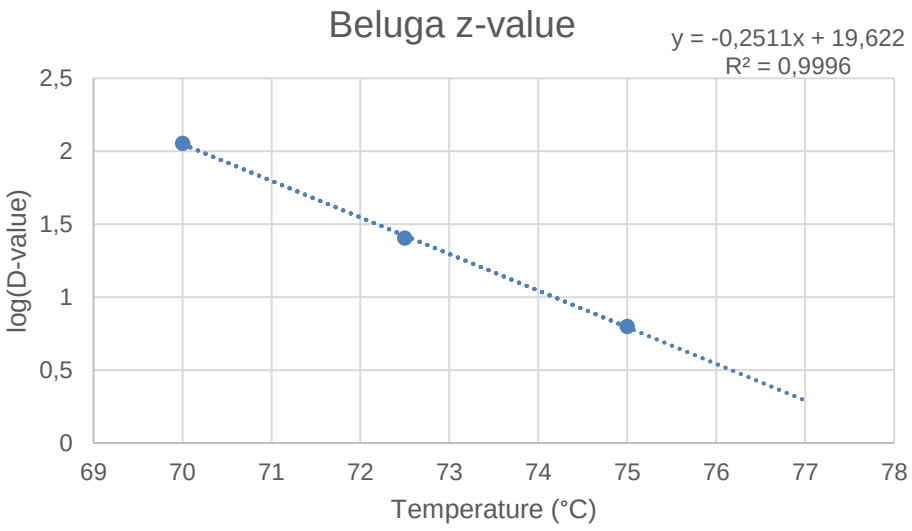

Figure 9. Z-value for Beluga.

z-value was calculated using the slope

$$z = \frac{-1}{-0.2511} = 3.98 \quad (5)$$

This means that if temperature is increased 3.98°C, the thermal reduction time would be reduced by one log cycle.

The same calculations were done to Beluga Δ*botE*, graph is shown in figure 10.

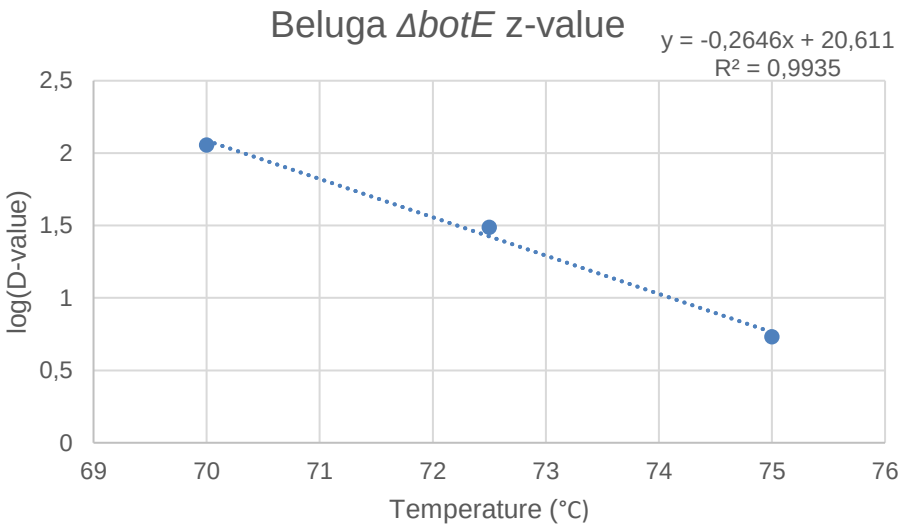

Figure 10. Beluga Δ*botE* z-value

Calculated z-value for beluga ΔbotE was 3.77°C.

4.3 Group III

For group III, D-values were calculated at 85°C. The survival curves of strains in that temperature is shown in figure 11.

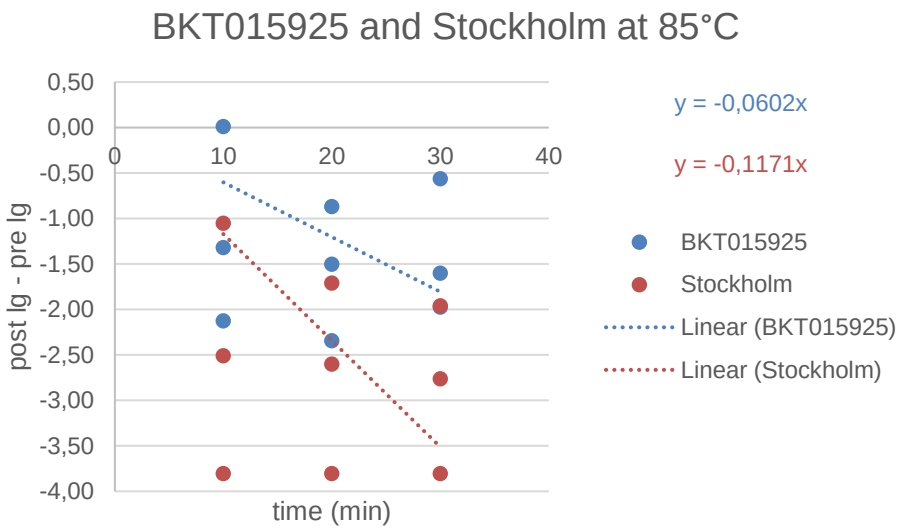

Figure 11. Survival curve for BKT15925 and Stockholm at 85°C

D-value for BKT015925 at 85°C is 16.6 and for Stockholm D-value is 8.5.

5 Discussion and Conclusions

The aim of this study was to find out the heat resistance of spores of *C. botulinum* from three different groups. This was done by carrying out heat treatments and calculating D-values.

Although it was not possible to determine the D-value for all of the strains in every temperature, results still give the trend of the heat resistance of spores of those three groups.

The difference in strains' heat resistance was as expected. When results between groups are compared it is shown that group I and III have higher heat resistance than group II. This was presumed to be the case according to the living environments of bacteria, group II bacteria mostly isolated from aquatic sediments and therefore colder environments, whereas group I and group II bacteria are more likely to live in territorial environments.

The results of the heat tests are being used in a project of the research group. The name of the project is; Understanding the Clostridium spore, a prerequisite.

References

1 Bell, C. & Kyriakides, A. Clostridium botulinum, A practical approach to the organism and its control in foods. Blackwell Science Ltd. Editorial offices. (2000)

2 Erbguth, F. Historical notes on botulism, Clostridium botulinum, botulinum toxin, and the idea of the therapeutic use of the toxin. Movement Disorders 19:s8, pp: s2-s6. (2004)

3 World Health Organization. [Internet] Botulism (2018). Read 25.10.2018. Available from: http://www.who.int/news-room/fact-sheets/detail/botulism

4 Ting P. & Freiman A. The story of Clostridium botulinum: from food poisoning to Botox. Clinical Medicine 4:3, pp: 258-261. (2004)

5 Cornell Department of Microbiology in the College of Agriculture and Life Sciences (CALS). [Internet] Bacterial Endospores. Read 15.10.2018. Available from: https://micro.cornell.edu/research/epulopiscium/bacterial-endospores/,

6 Hatheway, C. Toxigenic Clostridia. Clinical Microbiology Reviews 3:1, pp: 66-98. (1990)

7 Lindström, M. & Korkeala, H. Laboratory diagnostic of botulism. Clinical Microbiology Reviews. 19:2, pp: 298–314. (2006)

8 Maslanka S.; Lúquez C.; Dykes J.; Tepp W.; Pier C.; Pellett S.; Raphael B.; Kalb S.; Barr J.; Rao A. & Johnson E. Novel Botulinum Neurotoxin, Previously Reported as Serotype H, Has a Hybrid-Like Structure With Regions of Similarity to the Structures of Serotypes A and F and Is Neutralized With Serotype A Antitoxin. The Journal of Infectious Diseases, 213:3, pp: 379–385. (2015)

9 Fillo. S.; Giordiani, F.; Anniballi, F.; Gorgé, O.; Ramisse, V.; Vergnaud, G.; Riehm, J.; Scholz, H.; Splettstoesser, W.; Kieboom, J.; Olsen, J.; Fenicia, L. & Lista, F. Clostridium botulinum Group I Strain Genotyping by 15-Locus Multilocus Variable-Number Tandem-Repeat Analysis. Clinical Microbiology Reviews, 49:12, pp: 4252–4263. (2011)

10 Smith, T.; Hill, K. & Raphael, B. Historical and current perspectives on Clostridium botulinum diversity. Research in Microbiology 166:4, pp: 290-302. (2014)

11 Adams, M. R. & Moss, M. O. Food Microbiology (3rd edition). The Royal Society of Chemistry. (2008)

12 da Silva, N.; Taniwaki, M.; Junqueira, V.; Silveira, N.; do Nascimento, M. & Gomes, R. Microbiological Examination Methods of Food and Water, a Laboratory Manual. CRC Press Taylor & Francis Group. (2013)

13 Dürre, P., Physiology and Sporulation in *Clostridium.* (2012). Microbiology Spectrum. 2:4 (2014)

14 Molle, V.; Fujita, M.; Jensen, S..; Eichenberger, P.; González-Pastor, J..; Liu, J. S & Losick, R. The Spo0A regulon of Bacillus subtilis. Molecular Microbiology, 50:5, 1683–1701. (2003)

15 Dahlsten, E. Genetic mechanism of stress response and sporulation in *Clostridium botulinum*. The Faculty of Veterinary Medicine, University of Helsinki. (2013)

16 Errington, J. Regulation of endospore formation in *Bacillus subtilis*. Nature Reviews Microbiology, 1, 117-126. (2003)

17 Cagan E.; Peker, E.; Dogan, M.; Caksen, H. Infant Botulism. The Eurasian Journal of medicine. 42. pp:92-94. (2010)

18 Evira, elintarviketurvallisuusvirasto. [Internet] *Clostridium botulinum* ja botulismin ehkäisy. Read: 28.10.2018. Available from: https://www.evira.fi/elintarvikkeet/tietoa-elintarvikkeista/elintarvikevaarat/ruokamyrkytykset/ruokamyrkytyksia-aiheuttavia-bakteereja/clostridium-botulinum/

19 Aston, S. & Beeching, N. “Botulism”. *Hunter's Tropical Medicine and Emerging Infectious Disease, 9th Edition,* edited by Magill, A. et al. Elsevier Inc. pp. 511-513. (2012)

20 Gu, S. & Rongsheng, J. “Assembly and Function of the Botulinum Neurotoxin Progenitor Complex”. *Botulinum Neurotoxins*, edited by Rummel, A. & Binz, T. Springer, 364. pp. 21-39. (2013)

21 Fujinaga, Y.; Sugawara, Y. & Matsumura, T. ”Uptake of Botulinum Neurotoxin in the Intestine”. *Botulinum Neurotoxins*, edited by Rummel, A. & Binz, T. Springe. 364. pp. 45-56. (2013)

22 Oblinger, J. & Koburger, J. Understanding and Teaching the Most Probable Number Method. Milk, Food, Technol. 38:9, pp. 540-545. (1975)

23 Stumbo, C. R. Thermobacteriology in Food processing. Academic Press. (1965)

24 Ministry for primary Industries, New Zealand. [Internet] *Clostridium botulinum.* Read: 1.11.2018. Available from: https://www.mpi.govt.nz/dmsdocument/11042/send

Number of spores of group I after heat treatments

strain	heating temperature	time	1	2	3	
ATCC3502	90	0	1433333	800000	8000000	1st
ATCC3502	90	0	800000	3100000	800000	2nd
ATCC3502	90	0				3rd
ATCC3502	90	20	143333,3	80000	31000	1st
ATCC3502	90	20	143333,3	143333,3	8000	2nd
ATCC3502	90	20				3rd
ATCC3502	90	40	8000	31000	3100	1st
ATCC3502	90	40	143333,3	31000	14333,33	2nd
ATCC3502	90	40				3rd
ATCC3502	90	60	1433,333	1433,333	3100	1st
ATCC3502	90	60	8000	3100	31000	2nd
ATCC3502	90	60				3rd
ATCC19397	90	0	14333,33	14333,33	50000	1st
ATCC19397	90	0	31000	50000	14333,33	2nd
ATCC19397	90	0				3rd
ATCC19397	90	10	120	303,3333	700	1st
ATCC19397	90	20	120	0	0	2nd
ATCC19397	90	10				3rd
ATCC19397	90	20	766,6667	303,3333	766,6667	1st
ATCC19397	90	40	120	0	0	2nd
ATCC19397	90	20				3rd
ATCC19397	90	30	120	0	120	1st
ATCC19397	90	60	0	0	0	2nd
ATCC19397	90	30				3rd
ATCC19397ΔbotA	90	0	2500000	14333333	8000000	1st
ATCC19397ΔbotA	90	0	310000	1433333	800000	2nd
ATCC19397ΔbotA	90	0	50000	80000	310000	3rd
ATCC19397ΔbotA	90	20	0	0	120	1st
ATCC19397ΔbotA	90	20	120	0	120	2nd
ATCC19397ΔbotA	90	10	766,6667	120	766,6667	3rd
ATCC19397ΔbotA	90	40	0	0	0	1st
ATCC19397ΔbotA	90	40	0	0	0	2nd
ATCC19397ΔbotA	90	20	120	120	0	3rd
ATCC19397ΔbotA	90	60	0	0	0	1st
ATCC19397ΔbotA	90	60	0	0	0	2nd
ATCC19397ΔbotA	90	30	0	0	0	3rd

strain	heating temperature	time	1	2	3	
ATCC3502	95	0	1433333	4000000	1433333	1st
ATCC3502	95	0	1433333	3100000	1433333	2nd
ATCC3502	95	0				3rd
ATCC3502	95	10	14333,33	8000	3100	1st
ATCC3502	95	10	1433,333	3100	3100	2nd
ATCC3502	95	10				3rd
ATCC3502	95	20	120	100	0	1st
ATCC3502	95	20	303,3333	0	766,6667	2nd
ATCC3502	95	20				3rd
ATCC3502	95	30	120	0	120	1st
ATCC3502	95	30	303,3333	303,3333	120	2nd
ATCC3502	95	30				3rd
ATCC19397	95	0	1433333	1433333	1433333	1st
ATCC19397	95	0	31000	8000	8000	2nd
ATCC19397	95	0				3rd
ATCC19397	95	10	0	0	0	1st
ATCC19397	95	10	0	0	0	2nd
ATCC19397	95	10				3rd
ATCC19397	95	20	0	0	0	1st
ATCC19397	95	20	0	0	0	2nd
ATCC19397	95	20				3rd
ATCC19397	95	30	0	0	0	1st
ATCC19397	95	30	0	0	0	2nd
ATCC19397	95	30				3rd
ATCC19397ΔbotA	95	0	3100000	2500000	1433333	1st
ATCC19397ΔbotA	95	0	1433333	1433333	143333,3	2nd
ATCC19397ΔbotA	95	0				3rd
ATCC19397ΔbotA	95	10	0	0	0	1st
ATCC19397ΔbotA	95	10	0	0	0	2nd
ATCC19397ΔbotA	95	10				3rd
ATCC19397ΔbotA	95	20	0	0	0	1st
ATCC19397ΔbotA	95	20	0	0	0	2nd
ATCC19397ΔbotA	95	20				3rd
ATCC19397ΔbotA	95	30	0	0	0	1st
ATCC19397ΔbotA	95	30	0	0	0	2nd
ATCC19397ΔbotA	95	30				3rd

strain	heating temperature	time	1	2	3	
ATCC3502	100	0	1433333	1433333	3100000	1st
ATCC3502	100	0	8000000	1433333	1433333	2nd
ATCC3502	100	0	8000000	800000	3100000	3rd
ATCC3502	100	1	310000	310000	800000	1st
ATCC3502	100	1	310000	1433333	800000	2nd
ATCC3502	100	1	800000	3100000	800000	3rd
ATCC3502	100	2	14333,33	14333,33	14333,33	1st
ATCC3502	100	2	8000	14333,33	14333,33	2nd
ATCC3502	100	2	310000	310000	3100000	3rd
ATCC3502	100	5	120	303,3333	0	1st
ATCC3502	100	5	303,3333	120	120	2nd
ATCC3502	100	5	766,6667	766,6667	1433,333	3rd
ATCC19397	100	0	310000	143333,3	310000	1st
ATCC19397	100	0	80000	143333,3	70000	2nd
ATCC19397	100	0	143333,3	143333,3	250000	3rd
ATCC19397	100	1	1433,333	766,6667	1433,333	1st
ATCC19397	100	1	500	303,3333	766,6667	2nd
ATCC19397	100	1	1433,333	1433,333	1433,333	3rd
ATCC19397	100	2	0	0	0	1st
ATCC19397	100	2	0	0	0	2nd
ATCC19397	100	2	0	120	0	3rd
ATCC19397	100	5	0	0	0	1st
ATCC19397	100	5	0	0	0	2nd
ATCC19397	100	5	0	0	0	
ATCC19397ΔbotA	100	0	1433333	1433333	3100000	1st
ATCC19397ΔbotA	100	0	800000	800000	1433333	2nd
ATCC19397ΔbotA	100	0	3100000	5000000	1433333	3rd
ATCC19397ΔbotA	100	1	310000	80000	14333,33	1st
ATCC19397ΔbotA	100	1	31000	25000	31000	2nd
ATCC19397ΔbotA	100	1	31000	14333,33	31000	3rd
ATCC19397ΔbotA	100	2	303,3333	303,3333	0	1st
ATCC19397ΔbotA	100	2	120	0	0	2nd
ATCC19397ΔbotA	100	2	766,6667	120	120	3rd
ATCC19397ΔbotA	100	5	0	0	0	1st
ATCC19397ΔbotA	100	5	0	0	120	2nd
ATCC19397ΔbotA	100	5	0	0	0	3rd

Number of spores of group II after heat treatments

strain	heating temperature	time	1	2	3	
Beluga	70	0	50000	14333,33	31000	1st
Beluga	70	0	1433333	2500000	1433333	2nd
Beluga	70	0	1433333	1433333	500000	3rd
Beluga	70	20	14333,33	2500	3100	1st
Beluga	70	20	800000	800000	3100000	2nd
Beluga	70	20	800000	250000	800000	3rd
Beluga	70	40	14333,33	80000	5000	1st
Beluga	70	40	310000	3100000	310000	2nd
Beluga	70	40	1433333	250000	800000	3rd
Beluga	70	60	8000	5000	1433,3	1st
Beluga	70	60	800000	800000	700000	2nd
Beluga	70	60	143333,3	310000	143333,3	3rd
Beluga ΔbotE	70	0	14333,33	8000	31000	1st
Beluga ΔbotE	70	0	310000	310000	310000	2nd
Beluga ΔbotE	70	0	1433333	310000	1433333	3rd
Beluga ΔbotE	70	20	14333,33	2500	25000	1st
Beluga ΔbotE	70	20	5000000	5000000	3100000	2nd
Beluga ΔbotE	70	20	143333,3	800000	1433333	3rd
Beluga ΔbotE	70	40	8000	2500	8000	1st
Beluga ΔbotE	70	40	1433333	143333,3	80000	2nd
Beluga ΔbotE	70	40	310000	310000	310000	3rd
Beluga ΔbotE	70	60	1433,33	800	3100	1st
Beluga ΔbotE	70	60	310000	143333,3	80000	2nd
Beluga ΔbotE	70	60	143333,3	80000	80000	3rd

strain	heating temperature	time	1	2	3	
Beluga	72,5	0	800000	310000	310000	1st
Beluga	72,5	0	800000	1433333	310000	2nd
Beluga	72,5	0	500000	3100000	310000	3rd
Beluga	72,5	10	310000	800000	800000	1st
Beluga	72,5	10	500000	500000	800000	2nd
Beluga	72,5	10	143333,3	143333,3	310000	3rd

Beluga	72,5	20	310000	80000	143333,3	1st
Beluga	72,5	20	1433333	80000	5000000	2nd
Beluga	72,5	20	31000	31000	143333,3	3rd
Beluga	72,5	30	31000	31000	50000	1st
Beluga	72,5	30	143333,3	14333,33	31000	2nd
Beluga	72,5	30	14333,33	8000	8000	3rd
Beluga ΔbotE	72,5	0	70000	80000	310000	1st
Beluga ΔbotE	72,5	0	80000	310000	310000	2nd
Beluga ΔbotE	72,5	0	310000	143333,3	500000	3rd
Beluga ΔbotE	72,5	10	143333,3	80000	143333,3	1st
Beluga ΔbotE	72,5	10	143333,3	143333,3	80000	2nd
Beluga ΔbotE	72,5	10	310000	310000	143333,3	3rd
Beluga ΔbotE	72,5	20	50000	80000	310000	1st
Beluga ΔbotE	72,5	20	31000	143333,3	80000	2nd
Beluga ΔbotE	72,5	20	14333,33	14333,33	8000	3rd
Beluga ΔbotE	72,5	30	50000	8000	31000	1st
Beluga ΔbotE	72,5	30	3100	31000	80000	2nd
Beluga ΔbotE	72,5	30	5000	8000	8000	3rd

strain	heating temperature	time	1	2	3	
Beluga	75	0	143333,3	80000	310000	1st
Beluga	75	0	50000	80000	80000	2nd
Beluga	75	0	800000	310000	310000	3rd
Beluga	75	10	8000	1433,333	3100	1st
Beluga	75	10	3100	8000	4000	2nd
Beluga	75	10	8000	14333,33	25000	3rd
Beluga	75	15	120	3100	366,6667	1st
Beluga	75	15	243,3333	0	0	2nd
Beluga	75	15	766,6667	766,6667	0	3rd
Beluga	75	20	0	0	120	1st
Beluga	75	20	766,6667	0	0	2nd
Beluga	75	20	1433,333	120	0	3rd
Beluga ΔbotE	75	0	80000	40000	143333,3	1st
Beluga ΔbotE	75	0	80000	143333,3	50000	2nd
Beluga ΔbotE	75	0	80000	80000	310000	3rd

Beluga ΔbotE	75	10	3100	766,6667	1433,333	1st
Beluga ΔbotE	75	10	1433,333	3100	1433,333	2nd
Beluga ΔbotE	75	10	1433,333	1433,333	8000	3rd
Beluga ΔbotE	75	15	303,3333	500	303,3333	1st
Beluga ΔbotE	75	15	766,6667	500	243,3333	2nd
Beluga ΔbotE	75	15	120	120	1433,333	3rd
Beluga ΔbotE	75	20	0	1433,333	303,3333	1st
Beluga ΔbotE	75	20	0	0	0	2nd
Beluga ΔbotE	75	20	0	0	0	3rd

Number of spores of group III after heat treatments

strain	heating temperature	time	1	2	3	
BKT015925	80	0	310000	310000	143333,3	1st
BKT015925	80	0				2nd
BKT015925	80	0	800000	3100000	8000000	3rd
BKT015925	80	20	80000	310000	50000	1st
BKT015925	80	20				2nd
BKT015925	80	20	1433,333	1433,333	2500000	3rd
BKT015925	80	40	310000	80000	143333,3	1st
BKT015925	80	40				2nd
BKT015925	80	40	8000	50000	14333,33	3rd
BKT015925	80	60	310000	31000	143333,3	1st
BKT015925	80	60				2nd
BKT015925	80	60	14333,33	31000	8000	3rd
Stockholm	80	0	2500	3100	8000	1st
Stockholm	80	0				2nd
Stockholm	80	0	8000	8000	8000	3rd
Stockholm	80	20	1433,333	3100	766,6667	1st
Stockholm	80	20				2nd
Stockholm	80	20	500	120		3rd
Stockholm	80	40	1433,333	1433,333	1433,333	1st
Stockholm	80	40				2nd
Stockholm	80	40	303,3333	303,3333	120	3rd
Stockholm	80	60	120	0	0	1st
Stockholm	80	60				2nd
Stockholm	80	60	120	0	0	3rd

strain	heating temperature	time	1	2	3	
BKT015925	85	0	310000	310000	310000	1st
BKT015925	85	0	3100000	1433333	3100000	2nd
BKT015925	85	0	143333,3	800000	80000	3rd
BKT015925	85	10	310000	500000	143333,3	1st
BKT015925	85	10	80000	143333,3	143333,3	2nd
BKT015925	85	10	3100	3100	1433,333	3rd
BKT015925	85	20	14333,33	31000	80000	1st
BKT015925	85	20	80000	80000	80000	2nd
BKT015925	85	20	766,6667	766,6667	3100	3rd
BKT015925	85	30	31000	143333,3	80000	1st
BKT015925	85	30	80000	31000	80000	2nd
BKT015925	85	30	1433,333	1433,333	8000	3rd
Stockholm	85	0	25000	25000	31000	1st
Stockholm	85	0	143333,3	143333,3	1433333	2nd
Stockholm	85	0	8000	8000	3100	3rd
Stockholm	85	10	1433,333	766,6667	5000	1st
Stockholm	85	10	3100	766,6667	1433,333	2nd
Stockholm	85	10	0	0	0	3rd
Stockholm	85	20	303,3333	500	766,6667	1st
Stockholm	85	20	1433,333	1433,333	1433,333	2nd
Stockholm	85	20	0	0	0	3rd
Stockholm	85	30	466,6667	120	303,3333	1st
Stockholm	85	30	766,6667	766,6667	1433,333	2nd
Stockholm	85	30	0	0	0	3rd

strain	heating temperature	time	1	2	3	
BKT015925	90	0	3100000	1433333	3100000	1st
BKT015925	90	0	2500000	7000000	800000	2nd
BKT015925	90	0				3rd
BKT015925	90	10	31000	8000	31000	1st
BKT015925	90	10	31000	14333,33	143333,3	2nd
BKT015925	90	10				3rd
BKT015925	90	15	14333,33	14333,33	8000	1st
BKT015925	90	15	50000	8000	8000	2nd
BKT015925	90	15				3rd
BKT015925	90	20	14333,33	8000	14333,33	1st
BKT015925	90	20	14333,33	14333,33	8000	2nd
BKT015925	90	20				3rd
Stockholm	90	0	80000	31000	80000	1st
Stockholm	90	0	80000	80000	143333,3	2nd
Stockholm	90	0				3rd
Stockholm	90	10	0	0	0	1st
Stockholm	90	10	120	0	120	2nd
Stockholm	90	10				3rd
Stockholm	90	15	0	0	0	1st
Stockholm	90	15	0	0	120	2nd
Stockholm	90	15				3rd
Stockholm	90	20	0	0	0	1st
Stockholm	90	20	0	0	0	2nd
Stockholm	90	20				3rd

Compositions of used mediums

TPGY medium consist of tryptone (50 g/l), peptone (5.0 g/l), yeast extract (20 g/l), glucose (4.0 g/l) and natriumtiocyllate (1.0 g/l).

TPGY-cysteine-starch medium consists of tryptone (50 g/l), peptone (5.0 g/l), yeast extract (20 g/l), starch (1.0 g/l), D-glucose (4.0 g/l), L-cystein (1.0 g/l) and natriumtiocyllate (1.0 g/l).

Meat-agar consists of cooked meat (100 g/l), glucose (1 g/l) and agar (15 g/l).