Universidade de Aveiro Departamento de Biologia
2010

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Inactivação por Alta Pressão de Esporos de *Clostridium botulinum* Tipo E em Truta

High Pressure Inactivaction of *Clostridium botulinum* Type E Spores in Trout

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada ramo Microbiologia Clínica e Ambiental, realizada sob a orientação científica do Doutor Rudi F. Vogel, Professor Catedrático do Instituto de Microbiologia Técnica, Universidade Técnica de Munique, Alemanha, e co-orientação da Doutora Sónia Alexandra Leite Velho Mendo Barroso, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro

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palavras-chave

Clostridium botulinum tipo E, Alta Pressão Hidrostática, Truta arco-íris, inactivação, esporos

resumo

Os produtos piscícolas têm um consumo regular em todo o mundo. Mesmo termicamente processado, peixe como a truta arco-íris fumada pode ser contaminada por esporos de *Clostridium botulinum* tipo E podendo levar à sua germinação e possível produção de toxinas. Ingestão de tal produto pode causar a morte em caso extremo. Alta Pressão Hidrostática (APH) tem demonstrado a sua utilidade na pasteurização de alimentos e também a matar estruturas bacterianas de resistência tais como esporos. Neste estudo foram usadas três amostras TMW 2.990, TMW 2.992 e TMW 2.994 de esporos de *C. botulinum* tipo E. Estudos preliminares foram levados a cabo para entender as propriedades de germinação e *outgrowth*. Diferentes meios de cultura foram usados e comparados por meio de fornecer as melhores propriedades para o *outgrowth* dos esporos. Truta fumada foi também inoculada e armazenada a 4 °C, 10 °C and 20 °C para avaliar o *outgrowth* de esporos em peixe ao longo do tempo. Estes estudos conduziram ao cerne deste trabalho: Inoculação de truta fumada e fresca com esporos TMW 2.994 e a sua sujeição a uma gama de valores de pressão entre os 200 e os 800 MPa combinado com temperaturas de 20 a 80 °C com tempo de pressurização de 5 minutos, de forma a alcançar um "produto seguro" equivalente a 6 log de redução de esporos em *outgrowth*. A inactivação máxima obtida foi a 800 MPa a 80 °C com ≥ 5 log de redução para ambos tipos de truta. A pressão trouxe alterações na truta fresca face à sua textura e cor iniciais. Na parte final deste trabalho foi examinado o efeito possível na recuperação dos esporos. Os esporos sujeitos a pressão como factor de stress (tratamento a 600 MPa a 20 °C); a recuperação de esporos não tratados e tratados foi feita por incubação em meio com lisozima. Para ambos os casos, a lizozima não ajudou na recuperação efectiva dos esporos. Este estudo fornece dados úteis relacionados com valores de D e percentagem de germinação dos esporos de *C. botulinum* sujeitos a diferentes condições.

Este estudo permite perceber melhor o comportamento dos esporos de *C. botulinum* tipo E em peixe e em meios de recuperação artificiais, e como podem estes ser inactivados por APH combinada com temperatura num sistema-modelo de alimento como a truta arco-íris fumada e fresca.

keywords

Clostridium botulinum type E, High Hydrostatic Pressure, Rainbow Trout, inactivation, spores

abstract

Fish products are frequently consumed all over the world. Even thermally processed, fish like smoked rainbow trout may be contaminated by *Clostridium botulinum* type E spores which may lead to their germination and possible toxin production. Ingestion of such product may cause dead in an extreme case. High Hydrostatic Pressure (HHP) has been demonstrated to be its useful in food pasteurization and also to kill bacterial structures of resistance such as spores. In this study the three strains *C. botulinum* TMW 2.990, TMW 2.992 and TMW 2.994 were used. Preliminary studies were conducted to understand spore germination and outgrowth properties. Different culture media were used and compared by means of providing the best properties in terms of spore outgrowth. Smoked trout was also inoculated with spores and stored at 4 °C, 10 °C and 20 °C to evaluate the germination and outgrowth of spores in fish product over time. These studies led to the main part of this work: Inoculation of smoked and fresh trout with TMW 2.994 spores and their subjection to a range of pressure levels between 200 and 800 MPa combined with temperatures from 20 to 80 °C with holding times of 5 minutes, in order to obtain a "safe food product" equivalent to a 6 log reduction of outgrowing spores. The maximum inactivation was obtained at 800 MPa at 80 °C with ≥ 5 log reduction for both types of trout. The pressure principally caused the fresh trout to alter its initial texture and color. As last step of this study, the further possible effect on the recovery of spores was exanimated. Spores were subjected to pressure as stress factor (treatment at 600 MPa at 20 °C); the recovery of untreated as well as treated spores was made by incubation on plate counting with lysozyme. For both cases, lysozyme did not help to provide more effective spore recovery. This study also provides valuable data on D values and percentage of germination of *C. botulinum* spores under various conditions.
This study permits to better understand the behaviour of *C. botulinum* type E spores in fish products and artificial recovery media as well as how it can be inactivated by HHP combined with temperature in food systems such as smoked and fresh rainbow trout.

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List of Abbreviations

AMP	adenosine monophosphate
approx.	approximately
ATP	adenosine tryphosphate
a_w	water activity
b.C.	before Christ
BoNT	botulinum neurotoxin
CFU	colony-forming units
cm	centimeter
D	decimal reduction value
DNA	deoxyribonucleic acid
DPA	dipicolinic acid
DRCM	differential reinforced clostridial
e.g.	for example
Eh	oxidation-reduction potential
ELISA	enzyme-linked immunosorbent assay
Etc	etcetera
EU	european union
g	gram
g	gravitational force
h	hour
HACCP	hazards analysis critical control point
HHP	high hydrostatic pressure
HHP/T	high hydrostatic pressure treatments combined with temperature
HP	high pressure
i.e.	that is
kDa	kilodaltons
Kg	kilogram
km	kilometer
L	liter
LHP	low high pressure
log	logarithm base 10
min	minute
mL	milliliter
MAP	modified atmosphere package
mm	millimeter
mm^2	square millimeter
mM	milimolar
MPa	mega pascal
MRI	max rubner-institute
ng	nanogram (10^{-9} kilogram)
PATS	pressure-assisted thermal sterilization
PG	peptidoglycan
PNS	peripheral nervous system
RCM	reinforced clostridial
RNA	ribonucleic acid
s	seconds

SASP-25	synaptossome-associated protein of 25 kDa
t	ton
TMW	technische universität munich – weihenstephan campus
TPY	trypticase-peptone-yeast-extract
TPYC	trypticase-peptone-yeast extract/sugar
TPYG	trypticase-peptone-yeast extract/glucose
TPYC+L	trypticase-peptone-yeast extract/sugar/lysozyme
UK	united kingdom
USA	united states of america
UV	ultraviolet radiation
v	volume
VAMP	vesicle-associated membrane protein
VHP	very high pressure
v/v	volume in volume
µL	microliter (10^{-6} liter)
µm	micrometer (10^{-6} meter)
°C	celsius degrees
%	percentage
γ	gamma

1 Introduction

1.1 Consumer Demands

The perception of consumers has become lately centered on new aspects in food terms. In the last twenty years, consumers have been demanding for freshness, good appearance, good taste and natural flavor; and less acid, salt, sugar and fat of the food products (Rahman 2007). Hereupon, the food industry developed ways to satisfy consumer demands, producing foods free of additives and mildly processed, which have to be safe with regards to pathogenic microorganism. The consumers' interest in non-sensory food properties like nutritional quality, microbiological safety or agrochemical and environmental contaminations represents one of the major reasons that drive food industry towards developing new products using alternative processing techniques (Deliza et al. 2004). New technologies such as irradiation, a combination of mild heat treatment and additives contributing to food safety in line with the 'hurdle concept' or high hydrostatic pressure processing have been used to produce products that satisfy consumers demands. The final decision of a consumer weather to buy a product or choose another is mainly dependent on factors like safety, price, and benefit as well as personal and emotional reasons these novel foods stand for (Cardello et al. 2007). However, the quality and safety, principally related to microbiological considerations of food products are among the most relevant factors influencing consumers choice.

1.2 Commercial Fish and Fish Products

1.2.1 Fishery

In the two last decades, fishery industry severely changed which was mainly driven by the reduction of fish resources and restricted fishery areas. Thereby, 30% of the actual caught fish species are over-exploited, whereas 44% are near to the exploiting limit. This situation leads to a notable enhancement of the aquaculture sector (Bandarra et al. 2005]; FAO 2008). The European Union (EU) represents one of the major world's fishing areas and the biggest market of aquaculture and processed products (European Comission 2010). With a closer look on Portugal and Germany, the average amount of fish consumed in Portugal is

the highest among all EU members (61.1 Kg/head/year), while in Germany the average consumption lies around 12.4 Kg/head/year (European Union 2008).

1.2.2 Aquaculture

Farming of aquatic organisms including fish, mollusks, crustaceans and aquatic plants could be a general definition of aquaculture. It represents 31% of the total amount of fishery production in the EU. The major producers are France, Spain, Italy and the United Kingdom (UK), while fish processing industry is bigger in Germany. Portugal's aquaculture fish production includes 27 freshwater fish farms. While the significance of aquaculture productions and species produced by the industry varies between EU members, salmon, mussels and trout represent the important species in terms of amount and business volume.

In 2001, rainbow trout was the second most frequently produced fish in aquaculture among all EU members with 226, 549 t. With a closer look on Germany and Portugal, in 2002, rainbow trout was the second most frequently produced fish in aquaculture in Germany (25, 027 t) and the third one in Portugal (1, 249 t) (European Comission 2010).

1.3 Rainbow Trout

Rainbow trout (*Oncorhynchus mykiss*) belongs to the family of *Salmonidae*. It is benthopelagic and anadromous and it is classified as an oily fish (Fishbase [updated 2010]; Moore et al. 2006). It is one of the most widely spread fish species and commonly used as food in at least 45 countries. Rainbow trout food products are retailed fresh, smoked, canned and frozen and can be prepared for eating by steaming, frying, broiling, boiling, microwaving or baking.

1.4 The 'Hurdle Concept' for Fish Products

Many different conservation methods are commonly applied in the conservation of fish products ranging from salting to modified atmosphere packaging (MAP). Moreover, fish is also eaten raw, although such raw products have a very short shelf life because of the rapid growth of psychrotropic Gram-negative bacteria. Even at 0 °C, gutted fish and fish fillets usually spoil within 7-10 days of storage. For each 5 °C increase in storage temperature the shelf life of the product is halved because of the increased growth of the bacteria and their

enzymatic activity (Hauschild and Dodds 1993). In the following paragraphs a summary of some of the most commonly used preservation methods is given.

1.4.1 Fish Additives

1.4.1.1 Salt

The use of sodium chloride (NaCl) for the preservation of the fish is probably the oldest method of preservation and applied for long in many parts of the world. Nowadays the consumer concerns about the amounts of salt present in food is rising. This is mainly driven by getting aware of health risks associated with a nutrition containing high levels of salt, which may lead to problems in controlling food microflora as salt often represents an important 'hurdle' to prevent spoiling bacteria form growing (U.S. FDA [updated 2009c]). However, a minimum salt content of 3.5% is required to prevent *Clostridium botulinum* growth (Bell and Kyriakides 2000).

1.4.1.2 pH

A decrease in the pH value of a fish product can be achieved by fermentation or pickling. The safety of these products relies on the addition of adequate NaCl or fermentation at low temperatures until the proper pH is obtained (Hauschild and Dodds 1993). A low pH value, high salt concentration and low storage temperature in combination may help to yield a maximum of safty in terms of the growth of *C. botulinum* (Bell and Kyriakides 2000). However, depending on the product, for sensoric reasons the pH as well as the salt content cannot be chosen low at will.

1.4.2 Processing Methods

1.4.2.1 Ionizing Radiation

This method permits the inactivation of spoiling microorganisms. With low radiation doses, over 99% of the microorganism population is inactivated and the refrigerated shelf life can be enhanced two or three times. Numerous studies showed that *C. botulinum* would not be a problem in radurized fish products if the products are stored at 5 °C (Hauschild and Dodds 1993).

1.4.2.2 Smoking

The preservation of fish employing smoking varies between different parts of the world. As already mentioned, consumers demand less salt quantities in fish products. On the other hand, relatively high concentrations (minimum 3% (Cann 2001)) are needed for corresponding to the traditional flavor and for microbiogical safety of smoked fish products. Refrigeration of fish products is a crucial factor after the conservative process to inhibit or slow down the growth of possible contaminants During the smoking process, fish products undergo several steps including brining with NaCl, potassium chloride (KCl) and sodium nitrite ($NaNO_2$), smoking using fire-generated or liquid smoke at various temperatures for a varying period of time and, finally, packaging (Hauschild and Dodds 1993). This whole process has the aim to inactivate possible spoiling microorganisms.

1.4.2.2.1 - Hot smoking

The process of hot smoking, additionally to the action of preservatives (NaCl, KCl $NaNO_2$), has the aim to thermally inactivate a large proportion of spoilage microorganisms and dehydrate the surface of the product. Along with the preservative agents added and the reduced initial microbial load, the lower surface water activity protects the product from spoilage. The processing parameters altogether eliminate or modify the type of spoilage microflora that is typical for the raw product. Hot smoking mainly differs from cold smoking based on the temperatures used (60 to 70 °C internal temperature). The resulting product is also significantly different in terms of sensory properties such as different in structure due to heat coagulation of proteins and reduced surface water content (U.S. FDA [updated 2009c]; Hauschild and Dodds 1993).

1.4.2.2.2 - Cold Smoking

Cold smoking represents a preservation process that has a long history of smoked fish products production. This process involves just a moderate heat treatment to the product undergoes only incomplete heat coagulation of proteins. The involved mild heat step does not eliminate any vegetative bacterial cell. Cold smoked fish products that, in many cases,

also contain only a reduced amount of salt are often vacuum packaged (U.S. FDA [updated 2009b]).

1.4.3 Storage conditions

1.4.3.1 Vacuum Packaging

During vacuum packaging most of the air inside of the package containing the food product is removed. The possibly present aerobic bacteria rapidly consume the residual O_2, whereas carbon dioxide (CO_2) is generated. The resulting concentration of CO_2 is not as high as it is the case in modified atmosphere packages, but it is enough to prevent many spoiling organisms from growing and to increase the shelf life of fish products (Bell and Kyriakides 2000).

1.4.3.2 Modified Atmosphere Packaging (MAP)

During MAP the air of a package is removed or replaced by a defined mixture of CO_2, nitrogen (N_2) and possibly a small amount of oxygen (O_2). Each different gas components added has a different role. CO_2 is added to inhibit the growth of spoiling bacteria, molds and yeasts, N_2 is an inert gas and is used primarily to reduce the CO_2 concentration and O_2 can have the purpose of retarding the growth of obligate anaerobic bacteria, meanwhile the possible strictly aerobic existing microflora could not grow very longer due to the CO_2 presence, and their selves are producing more CO_2. Besides of growth conditions where O_2 is absent, under certain circumstances, *C. botulinum* is able to grow and form toxin in 100% O_2 atmosphere (U.S. FDA [updated 2009c]; Hauschild and Dodds 1993)!

1.4.3.3 Refrigeration

Refrigeration is the most important factor to preclude potential food-poisoning problems. Storage of fish products after the processing methods control and delays the eventual growth of spoilage bacteria. Refrigeration temperature below 3.3 °C is required to inhibit the *C. botulinum* growth (Hauschild and Dodds 1993).

1.5 Fish Microflora

The microflora of temperate-water fishes predominantly consists of psychrotrophic or psychrophilic, Gram-negative bacteria belonging to the genera *Pseudomonas, Moraxella, Acinetobacter, Acaligenes, Shewanella and Flavobacterium. Vibrio* spp. are typical from marine waters, whereas *Aeromonas* spp. are characteristic for fresh-water fish (U.S. FDA

[updated 2009b]). *Clostridium botulinum* type E can be frequently found in aquatic environments and particularly fish from temperate and subarctic zones are likely to be contaminated by this organism. Is not surprising that aquaculture fish microflora is different from the one of fresh-caught, seeing that the first is more likely to be contaminated with certain bacterial species existing in aquaculture environment, also varying between aquacultures depending on the location, temperature, pH, etc.

1.6 Clostridium botulinum

1.6.1 Historical Context

The first references mentioning human botulism date from the period of 886 to 912 b.C., when the Emperor Leo VI., byzantine governor banned the consumption of blood sausage for being baneful for health. One of the first recorded outbreaks (defined as incidents involving one or more botulism cases (CSC, 1979) (Hauschild and Dodds 1993), happened in Germany in 1793 with blood sausage as causative food, accounting 13 ill and 5 dead people. Between 1820 and 1822, Justinus Kerner also studied some cases of the deathly sausages. Later, in 1896, Emile van Ermengem, studying an outbreak derived from raw *serrano* ham, isolated a toxin formed by an anaerobic bacillus and called the causative agent *Bacillus botulinus* (botulus, from the Latin word "sausage"). The organism as well as the corresponding illness, botulism ("sausage poisoning"), are called like this because for many years this type of poisoning has been associated with the consumption of sausages, principally in Germany where the consumption of raw ham and sausages was widespread (Jay et al. 2005).

Later on, the anaerobic and spore forming *Bacillus botulinus* was generally reclassified as *Clostridium botulinum* and subdivided according to the physiological characteristics of as well as the serological properties of the toxin formed by a specific subtype. Most of the early cases described involving ham or sausages were likely to be caused by *Clostridium botulinum* type B (Hauschild and Dodds 1993).

1.6.2 *Clostridium botulinum* Characterization

Clostridium botulinum is a member of the family *Bacillaceae*, while the genus *Clostridium* comprises four relevant pathogenic species producing very potent toxins: *C. tetani*, *C.*

difficile, *C. perfringens* and *C. botulinum*. Within this group of organisms the latter two can be involved in food poisoning (Forsythe 2010).

C. botulinum is typically strictly anaerobic, rod-shaped (0.2-0.7 × 3.4-7.5 μm) with peritrich flagellated (U.S. FDA [updated 2009c]), Gram-positive, catalase negative, spore-forming (terminal or sub-terminal) bacterium which produces a protein with neurotoxic characteristics and is usually arranged as single cells, pairs or chains. This organism can be found widely spread in nature including soil, sediments of oceans and lakes, coastal waters, intestinal tracts of fish and mammals, as well as in the gills and viscera of crabs and other shellfish and seafood (U.S. FDA [updated 2009]).

Based on the properties of the toxin formed, *C. botulinum* can be divided into seven different subtypes (from A to G). Furthermore, based upon serological and physiological characteristics of *C. botulinum*, it can also be separated into four distinct serotype subgroups (from I to IV) (Table 1).

Table 1 – Characteristics of *C. botulinum* subgroups (n.d. – not defined)

Properties	**Group**			
	I	II	III	IV
Neurotoxin type	A, B, F	B, E, F	C (α, β), D	G
Proteolytic	yes	no	no or only midly so	weakly
Saccharolytic	no	yes	no	no
Lipolitic	yes	yes	yes	no
Psychrotrophic	No	yes	yes	no
Associated with botulism in humans	yes	yes	rare	rare or doubtful
Minimal temperature (°C)	10	3.3	n.d.	n.d.
Maximal temperature (°C)	45 to 50	40 to 45	n.d.	n.d.
Optimal temperature (°C)	35 to 40	18 to 25	n.d.	n.d.

(Adapted from Bell and Kyriakides 2000; Hauschild and Dodds 1993)

1.6.3 *C. botulinum* Type E

C. botulinum type E can be commonly found in water and is strongly associated with botulism caused by the consumption of fish products and physiologically belongs to the non-proteolytic group II (Table 1). This subtype of *C. botulinum* is able to grow and produce toxins at temperatures as low as 3.0 to 3.3 °C in a surrounding environment containing up to 5% natrium chloride (NaCl) (U.S. FDA [updated 2009c]). The minimum a_w that allows growth of *C. botulinum* type E lies around 0.97. Depending on the competitive flora present, growth of this organism can be significantly slowed down or even inhibited, especially when organisms with similar biochemical and morphological properties are present (Jay et al. 2005).

1.7 Botulinum Neurotoxins (BoNTs)

1.7.1 Toxin Types

The toxins produced by *C. botulinum* are among of the most potent naturally occurring toxins. Based on serological specificity of the toxins produced, seven types of *C. botulinum* are currently recognized (from A to G) (Bell and Kyriakides 2000). Each of these types produces a different toxin. While types A, B, E and F can cause human botulism and types C and D are related to intoxication of animals, no outbreaks have been reported for type G. Among the animals that are most frequently affected by *C. botulinum* toxin type C and D are wild fowls, poultry, cattle, horses, and some species of fish. (U.S. FDA [updated 2009]).

1.7.2 Synthesis of Botulinum Neurotoxins (BoNTs)

The BoNTs are formed inside of the vegetative cell and liberate by autolysis. Cells that grow in optimal conditions produce toxins, although stationary phase cells are also capable of forming them (Forsythe 2010). Toxin production is inhibited when the conditions of salinity and temperature are lower than optimal (U.S. FDA [updated 2009c]).). Non-proteolytic *C. botulinum* types B, E and F can produce toxins even at refrigeration temperatures (approx. 3 to 4 °C). Toxins of group I do not manifest maximum toxin potential till have been activated by trypsin (U.S. FDA [updated 2009a]).

1.7.3 Action in Human Body

BoNTs can be used for cosmetic applications (as Botox®) to reduce wrinkles on the face and neck as an alternative to cosmetic surgery. On the other hand, BoNTs, when ingested, are highly toxic to humans. They can enter in the human body and be absorbed by crossing the barrier inside of the duodenum and jejunum (Bossi et al. 2004) or the respiratory mucosis going directly into the bloodstream. The BoNTs are not completely inactivated by the stomach proteolytic enzymes, and the BoNTs produced by nonpreoteolytic strains could stay untouched and be activated. So, the toxins reach the small intestine, where they can pass to the bloodstream and achieve the peripheral nervous system (PNS) and their primary target will be the neuromuscular junctions, making them paralyzed by the acethylcolin blocking.

The BoNT is produced as one polipeptidic chain, which is cut by transduction, forming one chain composed by heavy chain 100 kDa and light chain of 50kDa held by a dissulfid bond. Following bacterial death and lysis, the BoNT are cut by bacterial proteases (Forsythe 2010). When the pH of the vesicular membrane drops, the BoNT suffer some conformational change: the heavy chain opens channels into the vesicular membrane for the light chain get into the cytosol and the bond between chains breaks. Light chain is possible incorporated inside the endossome, passing a proteolytic cleavage of the synaptic vesicles, which blocks the release of neurotransmitors. Inside the nerve terminal the light chain acts in distinct ways, reproducing different durations for neuromuscular paralysis. The toxins have different proteins receptors as target (vesicle-associated membrane protein (VAMP), syntaxin and synaptossome-associated protein of 25 kDa (SNAP-25)); what also affects the different blockade time (Davletov et al. 2005).

The most powerful is the toxin from the *C. botulinum* type A (BoNT/A), is just need 1 ng/Kg of body weight to be lethal (Forsythe 2010), and it is also the one that the paralysis remains for longer time, contrary to BoNT/E that has the shortest time, maybe due to its dissociated and damaged target (SNAP-25) being replaced by a new one in a short time, allowing a quick restoration of the synaptic transmission.

There exist some antitoxins, but at most they serve to neutralize the unbound of the chains, being unable to reverse the binding of any toxins that are already spreading their effect.

1.7.4 Weapon Threat

BoNTs' aerosols could be used as a biological weapon. It has been estimated that a point-source aerosol release of BoNT could damage or kill 10% of the population within about 0.5 km downwind (Bossi et al. 2004).

According to Portuguese law, (ordinance No. 1120/95 of 15th September 1995, and article No. 5 of the decree-law No. 436/91 of 8th November 1991), where list of biological agents, pathogenic animals and vegetables and equipments for biological proliferation is published, and; *C. botulinum* (B6) as well as the botulinum toxins (T1) are considered as a biological hazard that could threat national interests (Portuguese Republic Diary 1995).

According to German law (BGBI. I 1990, 2515-2519; Appendix (Paragraph 1, part 1)) *C. botulinum* as well as the botulinum toxins are considered as biological weapons use is strictly limited to, civilian purposes i.e. scientific, medical or industrial research in the fields of pure and applied science (German Federal Ministry of Justice 1990).

1.8 Botulism

1.8.1 Causes

The sequence of events that leads to foodborne botulism is the contamination of food or, in a rare case, aerosols of *C. botulinum*. First, the local where the food come from is somehow with a high level of *C. botulinum* cells or spores, it is probable that the food could be contaminated, but also during harvesting, processing, or storing. Secondly, the cells or spores must survive to the food processing treatment, in another way, the post-processing contamination has to occur (60% of food poisoning happened at home (Wilcock et al. 2005). For this happen, the environment where the spores are has to be favorable for them to germinate and outgrowth, and for the toxin production of the vegetative cell. Finally, the food must be consumed without cooking to destroy the heat-labile toxin (Hauschild and Dodds 1993).

1.8.2 Botulism Types

These are the most common types of botulism that show similar symptoms of the illness:

1.8.2.1 Foodborne Botulism

Foodborne intoxication caused by consumption of foods contaminated with toxins of *C. botulinum*. Worldwide is this type of botulism that causes major incidence that any other

one. As a first step leading to foodborne botulism there is the contamination of food either the environment (e.g. soil or sediment) where the food originates from contains a high level of *C. botulinum* cells or spores which leads to a natural contamination of the food or the contamination occurs during harvesting, processing, or storing food raw materials. Furthermore, the cells or spores must survive to food processing except for there is a post-processing contamination (60% of food poisoning happened at home (Wilcock et al. 2005). If this happens, food matrix and composition as well as environmental parameters like temperature or the presence of oxygen also have to be favorable for germination and outgrowth of spores and toxin production of vegetative cells. Finally, the food has to be consumed without any further cooking which would destroy the heat-labile toxin (Hauschild and Dodds 1993).

1.8.2.2 Intestinal Botulism

Caused by colonization of the gastrointestinal tract in adults by *C. botulinum* with an *in vivo* production of the toxin. That can be caused for any medicine and/or antibiotic that alters the biota of the intestinal tract allowing the colonization.

1.8.2.3 Infant (Intestinal)Botulism

Affects infants under 12 months age, since older children are not susceptible as they have a more stable intestinal biota. This botulism type is caused by the ingestion of *C. botulinum* spores that colonize the intestinal tract and the toxin is synthesized. Sources linked with this case are soil, water, dust, syrups and honey, where the present toxins are A and B.

1.8.2.4 Wound Botulism

Wound botulism represents the rarest type of botulism and occurs when *C. botulinum* enters a wound and produces its toxin. Subsequently, the toxin is spread inside of the body via the bloodstream and causes the common symptoms. However, BoNTs do not penetrate intact skin. (U.S. FDA [updated 2009]).

1.8.2.5 Inhalation Botulism

This type of botulism does not occur naturally or coincidentally but could rather result from accidental or intended inhalation of the toxin released as aerosol. This route of intoxication is poorly documented in humans. For instance, an accident involving the exposure of three humans to botulinum toxins in aerosol form occurred (1962) in a German laboratory (Bossi et al. 2004).

1.8.3 Symptoms

Botulism may be misdiagnosed because symptoms resemble other illnesses. The impact of the illness can vary from individual to individual and depends on the amount of toxin ingested, toxin type, occurrence and time point of vomit, individual weight, sex, age and health condition. Onset of symptoms usually occurs 12 to 72 h after ingestion of the food containing the toxin, although there are reported cases where the time of onset was 4 to 8 h or even longer than 72h. Toxins induce similar effects no matter if ingested or inhaled, although following aerosol exposure the time of onset may be faster i.e. possibly less than 1 h. A transmission from on person to another has never been described (Jay et al. 2005; U.S. FDA [updated 2009a]).

The symptoms described include nausea, vomiting, fatigue, dizziness, headache, dry skin, mouth and throat, constipation, symmetrical muscular paralysis that begins from the head, lassitude, weakness and vertigo, dyspnea (shortness of breath), muscle weakness, abdominal distention, diploplia (double vision), ptosis (drooping of upper eyelid), blurred vision, enlarged or sluggishly reactive pupils, photophobia (sensitivity to light), facial weakness, dysphonia (disorder of the voice), dysphagia (difficulty in swallowing), dysarthria (poor articulation), postural hypotention (sudden fall of blood pressure), cardiovascular, gastrointestinal and urinary dysfunction (Bossi et al. 2004), respiratory failure and possibly death. Besides all of these symptoms, there is no loss of sensation and the patient is generally well oriented. The endurance of the illness could be from 1 to 10 days or even more,depending on the resistance of the host and other factors as decribed above. The mortality rates vary between 30 to 65 % with lower values for Europe than the USA in general (Jay et al. 2005).

Botulism clinical diagnosis is most effectively conducted and confirmed by identifying BoNTs in the blood, serum, feces, patient's vomit or the food that was consumed (U.S. FDA [updated 2009a]).

1.8.4 Foods Associated with Botulism

Almost any food that does not have a very acidic pH (above pH 4.6) can support growth and toxin production of *C. botulinum* (Bell and Kyriakides 2000). BoNTs may be found in a big variety of foods like canned corn, peppers, green beans, soups, beets, asparagus, mushrooms, ripe olives, spinach, chicken and chicken livers and liver pate, luncheon meats, ham, sausages, stuffed eggplant, fruits, vegetables in oil or brine, cheese, honey,

lobster, general seafood, tuna fish, smoked and salted fish, etc. (U.S. FDA [updated 2009 Set]). The majority of botulism cases originate from inadequately processed home-prepared food. Home-prepared foods, particularly vegetables in oil or brine that are improperly treated (e.g. suffer inadequate thermal treatment) to destroy *C. botulinum* spores, continue to be a great concern. Outbreaks caused by commercially produced products, do occur very frequently. However, contaminated food can be possibly spread worldwide due to the various channels of distribution representing a even greater risk potential.

Despite of the synergistic effect between clostridia and some lactic acid bacteria, lactobacilli act in an antagonistic way, in terms of growth and toxin production by *C. botulinum*; evidences of this fact is the presence of toxins in milk, seeing that Lactate is inhibitory for *C. botulinum* (U.S. FDA [updated 2009c]). Even though the contamination level of the milk is usually very low; botulism outbreaks have been associated with the consumption of Liederkranz cheese, Brie cheese, and a commercial cheese containing onions. Fruits, meat and vegetable are occasionally contaminated by *C. botulinum*. More frequently, honey appears to be contaminated with spores that are not able to germinate inside of the product but can potentially affect children under 12 months of age or or immonocompromised adults as described above. Botulism outbreaks have also been reported after the consumption of thermally processed fishery products prepared commercially and at home. However, commercially processed products are far less related to fish botulism outbreaks. In contrast to this, homemade fishery products caused the majority of cases associated with fish products. Most of the fish outbreaks probably occured due to the higher percentage of water-phase NaCl (Hauschild and Dodds 1993), but the incorrect distribution, manipulation and storage can also have influence on fish contamination. Commercial products associated with *C. botulinum* contamination are salted and dried fish as well as fish egg products, which are frequently vacuum packaged and may cause type E botulism, whereas, hot smoked fish products are more frequently affected than cold smoked products. Hytiä et al. (1998) found that 3% of 64 samples of cold-smoked rainbow trout were positive in terms of contamination of *C. botulinum* type E, with numbers between 40 and 290 spores/Kg (U.S. FDA [updated 2009c]). Moreover, 7% of vacuum-packaged hot-smoked rainbow trout and white-fish products marketed in

Finland contained *C. botulinum* spores, indicating that spores often survive during the heat treatments commonly employed in industrial hot smoking (Lindström et al. 2003).

In another study, the number of spores present in various foods has been determinate and the results show low values in meat < 10 spores/Kg, often < 1/Kg and in raw milk < 1 spore/L. Higher number can be found in vegetables <1 – 2100 spores/Kg or in fish 1 – 2400 spores/Kg (Bell and Kyriakides 2000).

Most of the contaminated food maintains has normal appearance and taste which is especially valid, principally for the group II *C. botulinum* bacteria, since no proteolysis occurs (Hauschild and Dodds 1993). However, a food may contain viable *C. botulinum* and still be not capable of causing botulism. If the organism does not grow, no toxins are produced (U.S. FDA [updated 2009a]). If spores of *C. botulinum* are present and there are no other microorganisms may compete, and the storage temperature is high enough and no other preservatives as well as oxygen are present to cannot avoid the spore germination this food may certainly be dangerous for the consumer. Besides the presence of O_2 the oxidation-reduction potential (Eh) is an important factor to inhibit spore germination although it has to be considered that it may vary during the shelf life of a product. It has also to be noticed that a single food can have different micro-environments in which the presence/absence of O_2 may vary. For example, *C. botulinum* may occur just a few millimeters below the surface of a meat or fish product (Bell and Kyriakides 2000).

1.8.5 Outbreaks in Europe: Focus on Portuguese and German Cases

European countries are not safe from botulism, on the decade 80/90 botulism were disseminated almost in all Europe being the most affected Poland, Italy, France, Spain and Germany. Poland had more botulism cases involving meat contaminated with type B. If the rest of the countries were botulism outbreaks happened by meat consumption, Italy and Spain beside of the type related being B, the food involved was vegetables and in Scandinavia countries fish was the most problematic food caused by type E *C. botulinum* (Hauschild and Dodds 1993).

The data on Portuguese outbreaks from 1970 to 1989 were reported from Hospital de São João in Porto (north of Portugal), where botulism appears to be more common than the

central and south areas. The cases have increased on the recent years, caused by *C. botulinum* type B (group II). Portuguese incidents were 91% associated with meat, discriminated as smoked hams, sausages and bacon, all home prepared. In Lisbon were reported four incidents with a total of 15 cases, between 1972 and 1980 (Hauschild and Dodds 1993).

Recent data continue to show more cases on the northern part of Portugal in relation of the rest of the country. Most of the cases were with men individuals, with no correlation with a specific time of the year (Portuguese General-Direction of Health 2007; Saraiva 2009). More recently, in 2006, two outbreaks were related with Serrano ham (Jorge 2008).

During 6-years periods, a total of 96 outbreaks and 206 cases were reported in East and West Germany. On a population basis, the number of cases in the two regions was similar, but the relative number of outbreaks was somewhat higher in the East part. The estimated involvement of type B (group II) in German outbreaks has over 90% (Hauschild and Dodds 1993). In contrast to the type, the responsible food was in nearly all the outbreaks, primarily as meats, and discriminated as smoked hams. An outbreak of four cases (associated with toxin type E) was notified in Germany in January 1998 and caused by consumption of smoked and vacuum-sealed salmon and trout from a professional smokery (Therre 1999). On recent years (2002-2008) a total of 82 patients suffered from botulism, some of wound botulism, but mainly food spoilage in meat, particularly in sausages (German Annual Book of Epidemiological Infections 2008).

1.8.6 Medical Care

Laboratory diagnosis relies on the isolation and identification of the neurotoxin from the biological body samples or the contaminated food. The laboratory tests remain the mouse assay (injection of the serum collected to a mice) and enzyme-linked immunosorbent assay (ELISA) (It recognizes protein antigenic sites and in general is somewhat less sensitive than the mouse bioassay) (Ferreira et al. 2004). Trivalent (A, B and E) equine antitoxins must be giving to the patients as soon as possible after clinical diagnosis by intravenous infusion. Heptavalent (A-G) antitoxins are available in certain countries. The use of antibiotics is not recommended since they would increase the amount of toxin available for absorption. Muscle functions just return 3 to 6 months after the botulism case when neuromuscular junctions regenerate (excluding BoNT/E) (Bossi et al. 2004).

1.9 *Clostridium botulinum* Spores

1.9.1 General Properties

As well as other bacteria, *C. botulinum* has created structures to resist to different environmental stresses, known as spores.

Spores are dormant, non-reproductive structures produced by Gram-positive bacteria that could be found in soil or water. They could appear positioned central, lateral, sub-terminal and terminal, the last two positions are found in *C. botulinum* vegetative cells (Prescott et al. 2005). Dormant spores lack common high-energy compounds such as adenosine tryphosphate (ATP), although the "low energy" forms has adenosine monophosphate (AMP) are present. Dormant spore resistance is much greater than the germinated one or even of the growing cell for the same strain. They are extremely hard to kill, since they have resistance to wet heat, desiccation, dry heat, freezing and thawing, ultraviolet radiation (UV), γ-radiation, and many toxic chemicals including acids, bases, aldehydes, oxidizing agents, alkylating agents, and aliphatic and aromatic alcohols (Black et al. 2007; Doona et al. 2007).

1.9.2 Structure

The spore has a number of layers that are not present on the vegetative cells, see on Figure 1. Starting from outside, when present, the exosporium is the outmost layer on the spore and its thickness could vary between the species. The exosporium has carbohydrates, proteins and specific macromolecules, only presents in spores (Black et al. 2007). Exosporium itself does not play any role on the resistance or in the germination of the spore, its function is principally to cover the coat, which is itself may be composed by several layers.

Diverse proteins compose the coat. The coat layer acts like a barrier to some chemicals, perhaps inactivating their toxicity before they enter deeply into the spore. Coat also protects the spore from lytic (lysozyme-like) enzyme attacks, by blocking the access to peptidoglycan (PG) principal target for these enzymes. Spores with a defective coat still resistant to (high) pressure and also not sensitive to many toxin molecules. It can contain enzymes and could be involved in spore germination (Hauschild and Dodds 1993).

Beneath the spore coat is located the outer membrane, this structure is a functional membrane in developing spores, but is not well-clarified if is complete on dormant spores; it can be removed by minimal external effects and has no effect on spore resistance or on germination by high pressure (Black et al. 2007; Doona et al. 2007).

The next layer is the cortex, composed mostly of PG, fairly similar to the vegetative cells one. The cortex seems to be quite related with the spore dormancy and it's maintaining, most likely for keeping the central core region with a low water level. The cortex PG is degraded soon during the spore germination, this cortex hydrolysis is essential for spore to "emerge to life".

The following layer beneath the cortex is the germ cell wall, also constituted by PG but this is similar to that one present in the growing cells. The germ cell wall is not disseminated during spore germination and became the cell wall of the outgrowing spore (Black et al. 2007). The cortex is degraded in the first minutes of spore germination, and degradation of the cortex is very important to permit the spore to germinate, initiate its metabolism and grow (Doona et al. 2007).

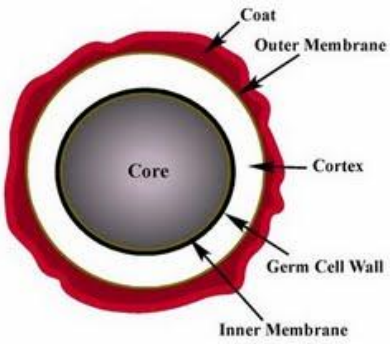

Figure 1 - Simplified structure of bacterial spore (adapted from Angert [updated 2007])

Positioned immediately after appears the inner membrane. In dormant spores is a complete membrane that has low permeability to small molecules. Despite its unusual properties, the phospholipid and fatty acid composition of this membrane is not markedly different for the plasma membrane existing on the growing cell. Molecules of the inner membrane appear to be largely immobile. The proteins that recognize the nutrients that led to spore germination are located here (Doona et al. 2007). This membrane also seems to be compressed during spore dormancy, but when the germination happens and the cortex is hydrolyzed, the thickness of the inner membrane increases 2 to 3-folds and the production

of ATP starts. The inner membrane appears to be extremely important in restricting access of DNA-damaging chemicals (Black et al. 2007).

The core wall lies beneath the cortex and surrounds the core of the spore. The core contains most of the enzymes, ribosomes and deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) of the spore, but is metabolically inactive (Prescott et al. 2005). The water content here is low, contrary to growing cells that have about 80 % of the water (wet weight), for spores these values are about 25 to 55%, depending on the species. The low water content is the major contributor for the resistance to moist heat, and also a big reason for spore dormancy, as normal free and mobile proteins are stocked in the core. Differently to the core, most of the other spore layers do not have the percentage of water content similar to the growing cell. The spore core has pH 1.0 to 1.5 units; meanwhile a growing cell has pH 7.5 to 8.0. The low core pH is triggered in sporulation and plays an important role on modulating the activity of several key enzymes in the developing spore at this time (Black et al. 2007). The spore cortex is in some fashion involved to bringing out the low water content of the core. With the reducing of the water content is also implicated the pyridine-2,6-dicarboxylic acid (dipicolinic acid or DPA). DPA is made late in sporulation and accumulates 20% of the core dry weight. DPA and the ions of metal calcium (Ca^{2+}) together (1:1) protect the spore DNA from a variety of damaging agents and act as a spore germinant. There is also another compost unique to the spores that has a small role in protecting the spore DNA called α/β-type small acid-soluble proteins (SASP), they are expressed in developing spore late in sporulation and also mediate the spore heat resistance (Doona et al. 2007). These proteins are small (65 to 75 amino acids), comprise about 5 to 20% of the total spore protein composition (Hauschild and Dodds 1993) and are located just in the spore core. The binding of SASP to the complex Ca-DPA changes the DNA structure and also protects it for several external factors as dry heat, oxidizing agents and ultraviolet radiation on spore DNA.

1.9.3 Sporulation

When a bacterium detects environmental conditions are becoming unfavorable, it may starts the sporulation process, which will takes about 8 h. First early stages (Figure 2) begin with asymmetric cell division having two daughter cells with the same morphologic and genetics characteristics, one will multiply and the other enters in the sporulation process (Hendrickx and Knorr 2002). Inside of a vegetative cell, proteins are remodeled in forming rings in each sporangium pole. Then, one of the two rings is converted to a division septum and the other is dissembled. Next, DNA condenses and aligns itself in the center of the cell, being the vegetative cells referred now by mother cell. A symmetric division takes place and the DNA is transferred and located by division septum pumps to a small chamber of the sporangium. So when this process is complete, there are two cells side by side each having a complete chromosome (Losick [updated 2009 Set]). In next stage, the mother cell membrane invagines to form the developing forespore, where already exists an intact membrane. The mother cell continues to grow and migrates around the forespore by fully engulfment and pitch of as a free cell within a cell. The three membranes enclose the forespore compartment, which will later develop to the central protoplast of the spore (Hendrickx and Knorr 2002). Now, the process of sporulation is well underway and the inner cell will mature into a spore. This conversion of inner cell into a spore involves some principle morphogenetic processes as the remodeling of the cromossome to the forespore in a donut-like structure, and also the PG is laid down between the two membranes of the developing spore to form a thick layer around the forespore called cortex and also the germ cell wall. The chromosomes of the forespore are remodeled and has a donut-like appearance (core), while a thick protein shell is formed outside the cortex (coat), some spores can create an external an additional layer called

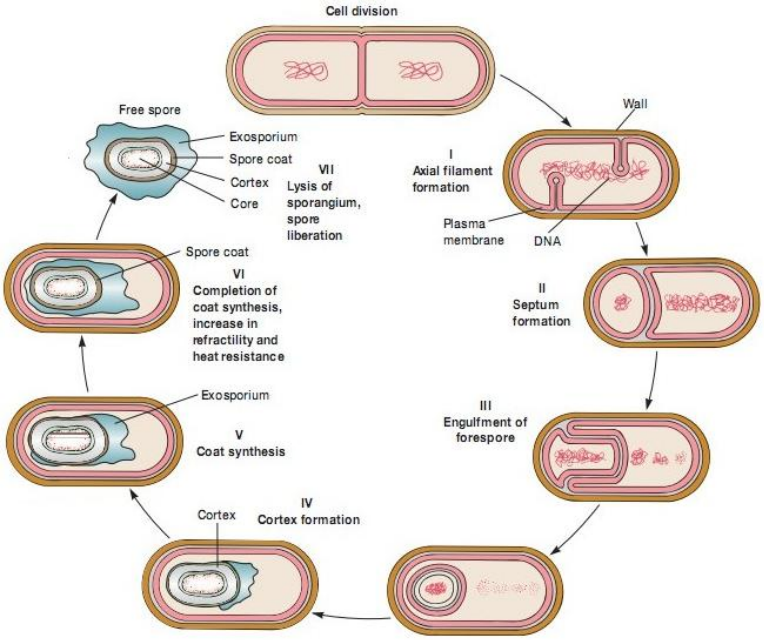

Figure 2 – Sporulation process (adapted from Prescott et al. 2005)

exosporium. The DPA is formed inside of the developing spore and Ca^{2+} enters from outside, meanwhile potassium (K^{+}) is release from the spore and water is removed at the same time that pH drops (approx. 1 unit) (MCGraw-Hill Higher Education [updated 2005]; Hendrickx and Knorr 2002). The spore is mature and is released from the mother cell after its lysis. The spore remains inert (dormant) for many years.

1.9.4 Reactivation

When the dormant spore has favorable environmental conditions it will be reactived. This reactivation involves activation, germination and outgrowth in a process named as sporogenesis. Even if a spore is located plentiful nutrient medium, if may fail to germinate unless it has been activated (Prescott et al. 2005). In a reversible process, heating the dormant spore may trigger its activation and prepare the spore for germination. The next stage, germination, refers to the step that the metabolic activity is started and thus, the hibernation state is finished. Common nutrients germinants are L-amino acids, D-sugars and purine nucleosides. These nutrients bind to germinant receptors that are located in the inner membrane of the spore. Nutrient-germinant receptor-binding leads to the release of some ions as K^{+}, H^{+} and Ca-DPA. Germination is commonly characterized by rupture or absorption of the spore coat, increase of metabolic activity, loss of environmental stress resistance, and loss of refractility and swelling of the spore. This Stage I of germination, where spores have lost some resistance properties, due to the increase of water content in the core, however, these stage I-germinated spores remain metabolically dormant and enzymes in the core still not active and proteins not diffused. Outgrowth is the last stage (stage II), and in contrast to germination, that requires no exogenous or endogenous nutrients or even any energy metabolism, outgrowth eventually requires exogenous nutrients; however, energy reserves stored in the dormant spore, in particular the amino acids derived from the SASP degradation, can support some metabolism early in outgrowth. Ca-DPA appears to trigger spore germination, leading to the cortex hydrolysis by lytic enzymes as lysozyme *[1] (Black et al. 2007). On this step the water raises on the core to that of a growing cell, this allows resumption of protein mobility on the core, lipid mobility in the inner membrane, and initiation of SASP degradation and metabolism followed by macromolecular synthesis. With the enzymatic events and complete the DNA replication the outgrowing spore is a fully functional vegetative bacterial cell (Doona et al. 2007).

*[1] – lysozyme is an enzyme abundant in egg white and human tears that catalyzes the hydrolytic cleavage of polysaccharides in the protective cell walls of some bacteria. Because it can lyse, or degrade, bacteria cell walls, serves as a bacterial agent (Lehninger et al. 2008). In terms of spores, is known that lysozyme facilitates the germination of heat-damaged spores of nonproteolytic *C. botulinum* thereby increasing measured spore heat resistance (Lindström et al. 2003).

1.10 Preventions

The best way to avoid *C. botulinum* contamination in foods and the resulting botulism is to make some control on the manufacturing, processing, storage and manipulation of the food. Measures to prevents *C. botulinum* include reduction of the microbial contamination level, acidification, reduction of moisture level, and whenever possible, destruction of all *C. botulinum* spores present in food. (U.S. FDA [updated 2009a]).

The industry is not the most responsible for *C. botulinum* food contamination, but the implementation of Hazards Analysis Critical Control Point (HAACCP) and Good Manufacturing Practices (GMP) had contributed to numbers reduction (Hauschild and Dodds 1993). Refrigeration that is strictly required for proper foods, will not ever prevent growth and toxin formation of nonproteolytic strains unless the temperature is kept under 3 °C (U.S. FDA [updated 2009a]). The main problem is indeed the manipulation and home processed food. Improvements to traditional food preservation methods should be one of the most effective means of preventing (Hauschild and Dodds 1993).

Besides the preservatives added to food (salt, smoke, modified atmosphere packaged, etc.), thermal and physical technologies could be used to forearm the contamination by *C. botulinum.*

1.10.1 pH

All types and strains of *C. botulinum* can grow and produce toxin about pH 5.2 (all other conditions being optimal) (Bell and Kyriakides 2000). So, an alternative approach to control germination of spores in food is to produce high acid products, such as fruit juices, jams and jellies, salsa, avocado dips and similar products, insofar as the spores are enable to germinate in low pH medium (Kalchayanand et al. 2004a). The spore demineralization appears to be the main reason for the inhibition of the germination at low pH, under ambient condition and also at high-pressure one (Doona et al. 2007).

1.10.2 Temperature

In certain foods (canned, pasteurized, smoked, boiled, etc. food), temperature is being solving the possible contamination by *C. botulinum* vegetative cells and their toxins since they are heat sensible (Bell and Kyriakides 2000), *e.g.* toxins can be destroyed if heated at 80 °C for 10 min or longer (U.S. FDA [updated 2009 Set]), so the best preventive measure is the heating of a suspect food in boil temperature for such time.

As said in epigraph, (dormant) spores are quite resistant to several agents; one of them is (high) temperature. If the vegetative cells and toxins are not so difficult to eliminate, spores are more complicated to inactivate or destroy (Reddy et al. 2006). Burning or autoclaving can eradicate spores. They are able to survive to boiling at 100 °C for hours, although longer the hours fewer they will survive. At temperatures below 90 °C, *C. botulinum* was inactivated by less than 2 log (Doona et al. 2007), while a "cook" of 121 °C for 3 min is generally considered to provide a 12 log reduction in *C. botulinum* spores (Bell and Kyriakides 2000). An indirect way to destroy them is to reactivate them to germinate to vegetative cell, and new-formed cells can be straightforwardly killed. This indirect method is called tyndallization, which was used mostly before the use of modern autoclaves.

Setlow and Setlow (1998) said that might be a possibility that the inactivation of bacterial spores by moist-heat is "associated with protein denaturation and enzyme inactivation". Earlier, Belliveau et al. (1992), using a scanning calorimetry showed that "a crucial protein is the rate-limiting primary target in the heat-killing of dormant bacterial spores".

But seeing that traditional thermal processing methods can solve the problem of killing the diverse *C. botulinum* forms, some of the food can suffer some undesired modifications on texture, flavor, color and nutrient value (Gao 2010)

1.10.3 High Pressure (HP)

HP can be used as a commercial sterilization process, and as the other, HP as the scope of producing quality and safe food free of any microbial pathogens. If the yeast, mold and vegetative cells are not so hard to inactivate by HP even at room temperature, spores are the most difficult challenge for the industry. In the next chapter, is going to be explained more detailed about this technology and its effect on the structures of resistance.

1.11 High Pressure Processing (HHP)

1.11.1 State of Art

This technique had been used largely from many years in cosmetic, chemical, ceramic, carbon allotropy, steel/alloy, composite materials and plastic industries but also in military research improving the effectiveness of guns and weapons (Rastogi et al. 2007; Doona et al. 2007).

On 19th century, resulting from studies in microorganisms of the deep-sea, this technique was introduced to food preservation. The early studies of High Hydrostatic Pressure (HHP) started with Bert Hite (1889) in West Virginia (USA) (University Agricultural Experimental Station), who performed the investigation of HHP in milk, which was exposed to 689 MPa for 10 min at room temperature, this treatment showed a 5 to 6 log reduction in microflora and he reported that the milk "kept sweet for longer", saying that HHP treatment enhanced the milk's shelf life (Reddy et al. 2007). Later, Hite *et al*. (1914) continued studies on meat, fruits and vegetables that were exposed to different pressure levels, different pH and temperatures. They found that microorganisms associated with sweet, ripe fruit were more sensitive to pressure than those related with vegetables. Their hypothetical answer was that on fruit and fruit juices, present yeast and molds are easy to destroy under HHP and the other possible, is that contaminants could not grow on such acidic environment. The vegetable are a concerning problem due to the spore forming bacteria that survive to pressure and support low-acid environment Doona et al. 2007; Reddy et al. 2007).

Other scientists also studied the effects of HHP on food. Cruess (1924) also proposed that HHP could be used to the fruit juices preservation, where the low pH inhibited the growth of spore forming bacteria. Bridgeman (1914) reported the coagulation of egg albumen by HHP in a different way from the thermal processing. Payens and Heremans (1969) described the effects of pressure on β-casein milk molecules, and Macfarlane (1973) reported that, under certain conditions, the HHP could be used to tenderize meat (Doona et al. 2007).

The mid-80's decade marked the resurgence of the HHP as a commercial interesting treatment as an alternative to thermal processing of foods. The first country to repechage the HHP technique was Japan. The use of this technology comes about so quickly that it took just three years for two Japanese companies to launch products, as Meidi-ya who

brought a line of jams, jellies, and sauces packaged and processed without heat application. This has encouraged others Japanese and American companies to introduce HHP products on the market. In Europe, the first program dedicated to pressurized food, started in Poland (1993) with fruit products by Institute of High Pressure Physics (IHHP) also known as Unipress (Rahman 2007). Other products arise as fruit juices, rice cakes, and raw squid in Japan, fruit juices, especially apple and orange juice in Portugal by Frubaça and France by Pernod Ricard ; sliced cooked ham in Spain by Espuña and guacamole by Avomex and oysters by Motivate in the USA (Rastogi et al. 2007; Doona et al. 2007).
The recent approaches on HHP treatments are related with enzymes, protein and microbial systems, developing on fruit product pasteurization, ready-to-eat food and studies on meat, dairy products, fish, etc. new food taste or appearance combinations (Simpson and Gilmour 1997; Rahman 2007).

In Germany, beside of the existing of several institutes on the research of HHP, there is no company selling pressurized products on the German market. In Portugal, Frubaça is selling pressurized (at room temperature) fruit juices to Portuguese and Spanish markets in a production of 600, 000 L (Gomes [updated 2004]).

European Union legislation on products treated with HHP in food industry is based on the EC Regulation 258/97, which entered into force on 15th May 1997. It brought, before any sale, permission marketing in the EU for new foods and new ingredients. Products treated with HHP are considered as such. This approach is mandatory for all member states (European Parliament and Council of the European Union 1997).

1.11.2 Principles

In HHP systems could be treated liquid or solid food subjected to pressures between 50 and 1000 MPa, being treated by continuous or semi-continuous process (Considine et al. 2008). Besides of being called non-thermal process, HHP can be use combined temperature since under-zero (< 0°C) till about 100 °C (Masschalck et al. 2001; Fellows 2000), but for industrial treatments is mostly used at room temperature. HHP can also be used combined with processing methods such as γ-radiation, alternating current, ultrasound, CO_2, antibiotics, etc. (Rastogi et al. 2007; Doona et al. 2007).

This technology underlies two principles. First is that transmittance of pressure is enabled rapidly and uniformly in all directions throughout the food, independently of the size or shape of the respective food, this allows solid food to retain their original geometric after treatment, which is could be a problem in thermal processing for large or bulky food products (Smelt 1998). This fundament is called isostatic principle. Another HHP basis is the *Le Chatelier* principle, this state that every reaction, conformational change or phase transition that are followed by a decrease in volume would be enhanced by high pressure, meanwhile reactions that involve increase in volume would be inhibited by such pressure (Rahman 2007).

A HHP system consists in a HP vessel and its closure, pressure-generation system, temperature-control device, and material handling system. Once the samples are loaded into the pressure-transmitting fluid and the vessel on the top is closed. Air is removed from the vessel by of a low-pressure fast-fill-and-drain pump that pumps the pressure medium (usually water, High Hydrostatic Pressure), that in combination with a daeration valve, generates HP (Rahman 2007). The pressure is exercised by compression time (pressure raises till the desired pressure level), holding time (pressure is held for the set time) and decompression (pressure release). During the compression is possible that the temperature in the vessel enhances due to, what is called, adiabatic heating. This term relates to a moderate temperature increase (approx. 5 to 15 °C *per* 100 MPa) of the liquid component of the pressurized food (Considine et al. 2008). When the food contains a significant amount of fat the temperature rise is greater (approx. 8 °C *per* 100 MPa). When the decompression happens, foods cool down to their original temperature if no heat is lost to (gained from) the walls of the pressure vessel during the hoding time (Rastogi et al. 2007).
In the HHP system, the HP could be generated direct or indirectly compression, a scheme of this is presented on Figure 3. The direct compression is generated by pressurizing a medium with a small diameter end of a piston. The large diameter end of a piston is driven by a low-pressure pump. This method allows very fast compression, but has the restriction of cannot be used in a small laboratory or pilot plant system. Indirect compression uses a HP intensifier to pump a pressure medium from a reservoir into a closed HP vessel until the desired pressure level is reached. This is the most common used method.

The most recognized companies on the construction of HHP equipment are Avure technologies (Kent, Washington, USA) and NC Hyperbaric (Burgos, Spain).

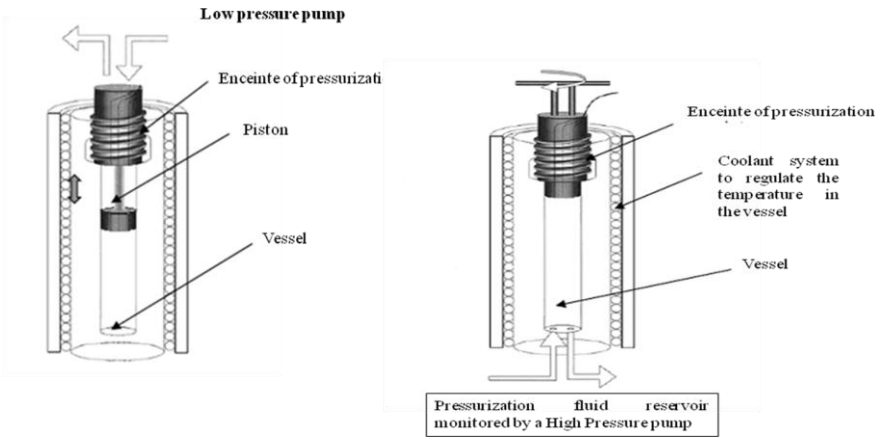

Figure 3 - Generation of compression in a High Hydrostatic Pressure equipment: direct (left side) and indirect (right side) (Adapted from Rahman)

The HHP technology has many advantages in relation to others techniques that can be summarized as follows:

1. It inactivates (pathogens) microorganisms at room temperature or lower temperatures;
2. It can reach all the object points at the same time by its isostatic pressure;
3. It can be used to create ingredients with novel functional properties (e.g. gelification);
4. It could provide the maintenance of the food quality parameters (nutrients, flavor, sensorial preservation and vitamins);
5. It is a clean technology with reduce loss of energy; and
6. It permits the combination of other processes (e.g. temperature).

(Rastogi et al. 2007; Ross et al. 2003; Ramaswamy [updated 2004]; Gao 2010)

Likely to other techniques, this one also has some limitations resumed as follows:

1. It is necessary to reach a right pressure (sometimes unknown) to inactivate microorganisms;
2. It can alter polysaccharides and proteins structures, causing, in some cases, adulterations in appearance, texture and functionality of some food products;

3. It is not a cheap technology and the prices raises for equipment that can reach higher pressures; and
4. It has to reach very high-pressure levels to inactivate spores, in other way; it needs the combined use of temperature.

(Ross et tal. 2003; Jung [updated 2009]; İbanoğlu and Karataş 2001)

1.11.3 Effect of HHP on Food Quality

HHP has the potential to produce high-quality products keeping the original appearance and most of the organoleptic characteristics, microbiological safe products with extended shelf life. But, the products are not intact after a pressure treatment, several properties can became altered (depending on the food type), this could be seen as a limitation, although industry is taking advantage of this properties changing to create new products that are satisfactory for consumers. For them, even in some cases would be not easy to understand the meaning of "pressurized food", but in a study conducted by Cardello et al. (2007) about the interests of the consumers in innovative and emerging technologies, HHP had the most positive utility value for the most respondent groups, while, for comparative purposes, irradiation and genetic modification had extremely low interest among the respondents. (Cardello et al. 2006)

1.11.3.1 Functional Properties

The functional properties of biological molecules are usually dependent on conformational changes. HP affects only non-covalent bonds (hydrogen, ionic and hydrophobic bonds). Hydrogen bonding, which stabilizes protein structures, is influenced by pressure but to a lesser extent than ionic or hydrophobic interactions. Hydrogen bond formation results in the shortening of interatomic distances with the volume decrease, enhanced by HP. Application of HP contributes also to phase changes in lipids and proteins, whence the gelification which is a consequence of protein and polysaccharides denaturation. Small molecules such amino acids, vitamins and flavor compounds remain unaffected by HHP, while larger molecules as proteins, enzymes, polysaccharides and nucleic acid may be altered. HHP also reduces the rate of browning Maillard reaction (Rahman 2007; Rastogi et al. 2007).

1.11.3.2 Sensory Properties

Food quality parameters such as flavor, color, texture, appearance, etc. can be barely modified (eventually for better) relatively to the original product after HHP treatment in certain products. The disposition of the water molecules in turn of the amino acids promotes the enhancement of bright and transparency of certain foods. These causes are depend on the food structure and composition, but these alterations have always a less effect that the ones caused by thermal treatments. In the other hand, nutritional value and food pigments are not affected by HHP (Rahman 2007; Rastogi et al. 2007).

In animal products HHP induces changes in muscle enzymes, meat proteolysis and myofribrille proteins, as a consequence the structure and texture of meat alter. Further, HHP influences the tenderization and gelation process, color of the product, and the extent of lipid oxidation. Combining pressure with temperature does tenderarize the meat, but the final products have a cooked appearance, and cannot be sold as a fresh product. The change in meat color cause by HHP happens at 300 MPa or even below levels (Rastogi et al. 2007).

1.11.4 Effect of HHP on Food Safety

Although the use of HHP for functional and sensory properties in many products, the main concern is still regarded to food safety with the ability of HHP to inactivate vegetative cells and, in a more difficult manner, inactivate spores.

1.11.4.1 Microorganisms

The relative easiness to inactivate vegetative cells has made them a target for, with HHP, turn food microbiological safe. The more concerning ones are the pathogens that could provoke illness or at extreme cases, death. Pressures between 400 and 600 MPa at room temperature can inactivate vegetative cells in 6 log cycles (Rahman 2007; Kalchayanand et al. 2004a). The vegetative cells response to HHP treatment is strongly dependent on the pressure and holding time applied, type of pressure treatment (i.e. cyclic or continuous), growth phase, culture age, environmental factors like media composition (e.g. ionic strength and type of ions, sugars, a_w, pH), temperature and bacterial species and strain (e.g. shape and Gram type) (San Martin et al. 2002). Gram-positive bacteria are more pressure resistant than Gram-negative ones; cells in the stationary phase of growth are generally

more pressure resistant than those in the exponential phase (Considine et al. 2008). Time-temperature combinations of 90 C for 10 min, 85 °C for 36 to 52 min, and 80 °C for 129 to 270 min have been suggested to reduce the number of spores of nonproteolytic *C. botulinum* by a factor of 10^6 (Lindström et al. 2003).

The HHP gives, to vegetative cells, changes on the morphology, on membrane and cell wall, biochemical reaction, functional integrity, key-enzymes, protein biosynthesis, nucleic acids and on the genetical mechanisms of microorganisms. (Kalchayanand et al. 2004b)

1.11.4.1.1 Suspending Medium Significance

As referred above, the type of substrate and composition of the food can have a decisive effect on the response of microorganisms to HHP. Complex media and some foods containing numerous ingredients have recently been shown to exert a baroprotective effect on microorganisms: carbohydrates, proteins, lipids and other food constituents can offer this protective effect. Besides of the media composition, also a_w protect cells against HHP, but microorganisms that are injured by HHP are usually more sensitive to a_w, in another hand, low pH suspending medium can render pathogens to be sensitive to HHP effects (Considine et al. 2008).

1.11.4.1.2 Pressure-assisted Thermal Sterilization (PATS)

The use of temperature during a HHP treatment can enhance the reduction of existing microorganisms and subsequent growth. Increased inactivation is observed at temperatures above or below room temperature, but at refrigeration temperatures the inactivation could also be improved (Considine et al. 2008). Temperatures in the range of 45 to 50 °C appear to increase the rate of pathogen and spoilage microorganisms' inactivation (Rastogi et al. 2007). The combination between a (possible) non-thermal technology, HHP, with conventional thermal treatment can has a synergical effect, this result in a greater microorganism inactivation, and permit that each one *per se* could use lower intensities. Using a sequential treatment has also shown valuable results. With a treatment of 827 MPa at 75 °C, *C. botulinum* type A were not reduced > 3 log (Margosch et al. 2006).

1.11.4.2 Spores

Bacterial spores are highly resistant when compared to the pressure treatments used for vegetative cells. Might be needed pressure levels around 1200 MPa to inactive them. This has mostly do with the spore structure and thickness. Spores form *Bacillus* and *Clostridium* are the more concerning, being the last one more pressure resistant (Rastogi et al. 2007).

1.11.4.2.1 Application of PATS in Spore Inactivation

In most cases, pressure and heat act synergically to deliver lethality. For spores is needed an initial temperature over 60 °C to have a destruction of 5 to 6 log. Processing times as short as about 1 to 5 min, the effect of pressure on the lethal action of the combined pressure-temperature treatment is marginal unless the pressure exceeds 1400 MPa. A 5 log reduction in spores was achieved at 404 MPa at 70 °C, whereas only 0.5 log reduction could be achieved at 25 °C at the same pressure (Black et al. 2007).

For *C. botulinum* TMW 2.357, high-pressure thermal process using pressures above 1000 MPa at 90 °C resulted in a greater inactivation than those at ambient pressure and the same inactivation. However, treatments of 600 MPa resulted in a slower spore inactivation than at room temperature, which indicates the effective pressure effect (Bull et al. 2009). A combination of moderate heating (40 to 50 °C) and pressure level of 827 MPa can be used to inactivate spores of *C. botulinum* type E; the use of pressure alone may not inactivate the spores (Reddy et al. 2007).

1.11.4.2.2 Spores Resistance to PATS

Spore population can be heterogeneous in terms of HP resistance. Margosh et al. (2004) measured the resistance of spores of seven strains (five proteolytic and the other two nonproteolytic) of *C. Botulinum* and other species from the genus *Bacillus* in mashed carrots for a series of combined pressure (600 to 800 MPa) and temperature (80 to 116 °C) treatments. Log reduction of spores after treatments with 600 MPa at 80 °C for 1 s ranged from > 5.5 log units in nonproteolytic strains and no reduction was observed on the proteolytic ones. Type A spores are more to HHP than type E spores (Reddy et al. 2006) The heat resistance of spores does not correlate with the pressure their pressure resistance (Doona et al. 2007).

Such drastic treatments need to inactivate the spores can adversely affect the acceptance quality of many foods and thus will have limited commercial applications (Kalchayanand et al. 2004a).

1.11.4.2.3 Suspending Medium Relevance

As mentioned before, the medium counts significantly to the baroprotection of the microorganisms to pressure. The same can be said for spores. Proteolytic type A BS-A and 62A spore were inactivated by 2 and 3 log and 2.7 log, respectively, in a crabmeat blend (pH 7.2 to 7.4) after 15 min (Bull et al. 2009). Wuytack and Michiels (2001) suggested that pressure treatments of spores at low pH promote the exchange of minerals from the spore core with protons and decrease the heat resistance of the spores. Margosch et al. (2004) determined the effect of pH on the pressure-induced inactivation of *C. botulinum* in a pressure-stable Tris-buffer. In this case, the inactivation kinetics was not affected by a pH shift from 6.0 to 5.15. Decreasing the pH to 4.0 accelerated the reduction of viable spore counts and the release of DPA from the spores (Doona et al. 2007).

1.11.5 Pressure-induced Germination

HP can trigger spore germination, and this appears to be the most effective way to kill spores. Pressure levels that trigger spore germination range between 50 to 300 MPa (Low High Processure, LHP) that activate the nutrient receptors of the spore and 300 to 800 MPa (Very High Pressure, VHP), which causes DPA release, trigging to germination events.

1.11.5.1 LHP Pressure-induced Germination

This proceeds via activation of any of the nutrient germinant receptors. Spores with elevated germinant receptors level germinate more rapidly with LHP. Spore germination triggered by LHP acts by the same pathway as nutrient germination, including Ca-DPA release followed by cortex hydrolysis. After cortex lysis, the LHP germinated spores go through, at least, the early outgrowth stages, including SASP degradation and ATP synthesis. These spores are more sensitive after germination, so is probable that higher temperatures (70 to 80 °C) do not initiate complete germination efficiently as happen at lower temperatures (20 to 45 °C). Release of Ca-DPA is essential for triggering later

germination events, since DPA-lessand demineralized spores do not germinate well if at all with LHP. A large number of compounds have been shown to inhibit spore germination with nutrients, which some such as D-alanine blocking the function of individual germinant receptors. Another parameter that could affect the spores' rate germination by LHP is the precise sporulation conditions (Doona et al. 2007; Black et al. 2007).

1.11.5.2 VHP Pressure-induced Germination

VHP does not trigger germination by nutrient receptors inactivation; the action mode opening channels to Ca-DPA release (Margosch et al. 2004). Cannot be expected that DPA-less spores germinate even in VHP. An increase of DPA release occurs with temperatures up to at least 60 °C. VHP spores pass to germination or outgrowth (or both slowly) and these spores degrade SASP and synthesize ATP slowly. Spores of *Clostridium* were inactivated by 3 to 4 log cycles at 400 MPa or higher for 10 to 30 min at 70 to 110 °C. These spores are, in general, not induce to germinate in 5 min even submitted to high pressure and temperature (Kalchayanand et al. 2004a; Doona et al. 2007).

Induced germination reduces markedly with the decreasing sporulation temperature, salt concentration or poor sporulation medium (without germinant nutrients). Also low pH has shown to enhance or have little effect on inactivation increasing, however higher levels of inactivation can be achieved when pressure-induced spores after treatment are exposed to acidic environment, due to the pos-pressure treatment sensitivity to lethal or bacteriostatic effects of acid.

There also exist a small percentage of dormant spores in spore population that are germinated slowly by HP relatively to the rest of the population (Black et al. 2007).

Fish products are very important nowadays, for their consumption, economics, employment and health enhancement. Is imperative that the consumed fish correspond to a safe product, free of spoilage bacteria. *C. botilinum* has the possibility to originate spores that, in the adequate conditions, will germinate and turn in a vegetative cell capable of produce a deadly toxin. Besides another amount of foodborne possibilities, fish and specifically, rainbow trout, is contaminated by this bacterium and its spores.

The spores are the great challenge to the HPP for being very resistant structures.

This work pretends to do some pre-experiments to know better how are the growth properties of *C. botulinum* vegetative cells and spores in different culture media and in a food model system (smoked trout) at different storage temperatures. This leaded to HPP treatments combined with temperature of inoculated smoked and fresh trout with *C. botulinum* type E spores, to reach a thermal-pressurized "safe food" and see the difference of both food model systems on those situations. The spores are dead, inactivated or injured after pressure, so with the addition of lysozyme as a recover supplement, will be seen if its work on a better germination of the spores after a submission to environmental stress.

2 Materials and Methods

2.1 Materials

2.1.1 Bacterial Strains

Table 2 - *Clostridium botulinum* type E strains used during the present studies

Strain	Alternative Name	Isolation Source	MRI* No. / Comment
TMW 2.990	Beluga	Beluga	C2 / ---
TMW 2.992	1576	---	C45 / Norway
TMW 2.994	---	Beluga	C126 / Baumgart

The stains used during the course of this study (Table 1) were obtained from the MRI* (Max Rubner-Institute; Federal Research Institute of Nutrition and Food; Kulmbach; Germany). The three strains were picked upon their putative, fish-related source of isolation. For the main part of the current study, i.e. the examination of inactivation properties of *Clostrium botulinum* Type E spores in fish products, strain TMW 2.994 was selected to be used upon its advantageous growth and sporulation characteristics.

2.1.2 Fish Samples

For these experiments fillets of smoked trout (MAP (modified atmosphere package) “Forellenfilets”, TIP, Goldhand Vertriebsgesellschaft mbH, Düsseldorf, Germany) were used (Figure 4), which obtained at a local supermarket. Approximately three years old adult females of fresh rainbow trout (*Oncorhynchus mykiss*) with an average length and weight of 30 cm and 500 g, respectively, were caught approx. 1 h before each experiment (Figure 5), obtained from the Institute for Functional Aquatic Ecology and Fish Biology of the TU Munich (Funktionelle Aquatische Ökokolgie und Fischbiologie, Weihenstephan, Technische Universität München, Germany).

Figure 4 - Smoked Rainbow Trout (author's picture)

Figure 5 - Fresh Rainbow Trout (author's picture)

2.1.3 Chemicals and Growth Media

The description of the different media and reagents used in this work is given in the following tables. The corresponding companies are labelled with superior numbers and a detailed list is provided in the attachment section.

Trypticase-Peptone-Yeast Extract (TPY):	
	g/L
Trypticase (pancreatic digest of casein) *[1]	50
Peptone (soya peptone) *[2]	5
Yeast extract *[1]	20
Sodium thioglycolate *[3]	1
Agar-agar *[4]	15
H_2O_{dest} (1000 mL)	
pH 7.0	

Trypticase-Peptone-Yeast Extract/Glucose (TPYG):	
	g/L
Trypticase (pancreatic digest of casein) *[1]	50
Peptone (soya peptone) *[2]	5
Yeast extract *[1]	20
Sodium thioglycolate *[3]	1
Agar-agar *[4]	15
H_2O_{dest} (850 mL)	
Glucose *[1]	4
H_2O_{dest} (150 mL)	
pH 7.0	

Trypticase-Peptone-Yeast Extract/Sugar (TPYC):	
	g/L
Trypticase (pancreatic digest of casein) *[1]	50
Peptone (soya peptone) *[2]	5
Yeast extract *[1]	20
Sodium thioglycolate *[3]	1
Agar-agar *[4]	15
H_2O_{dest} (850 mL)	
Glucose *[1]	4
Maltose *[1]	1
Cellobiose *[5]	1
Soluble Starch *[1]	1
H_2O_{dest} (150 mL)	
pH 7.0	

Trypticase-Peptone-Yeast Extract/Sugar/Lysozyme (TPYC+L):	
	g/L
Trypticase (pancreatic digest of casein) *[1]	50
Peptone (soya peptone) *[2]	5
Yeast extract *[1]	20
Sodium thioglycolate *[3]	1
Agar-agar *[4]	15
H_2O_{dest} (800 mL)	
Glucose *[1]	4
Maltose *[1]	1
Cellobiose *[5]	1
Soluble Starch *[1]	1
H_2O_{dest} (150 mL)	
Lysozyme (from dhicken egg white) *[5]	0.01
H_20_{dest} (50 mL)	
pH 7.0	

Differential Reinforced Clostridial (DRCM)*[1] :

	g/L
Peptone from casein	5
Peptone from meat	5
Meat extract	8
Yeast extract	1
Starch	1
D-Glucose	1
L-Cysteinium chloride	0.5
Sodium acetate	5
Sodium dissulfite	0.5
Ammonium iron (III) citrate	0.5
Resazurin sodium	0.002
Agar-agar	15
H_2O_{dest} (1000 mL)	

pH 7.1

Reinforced Clostridial (RCM):

	g/L
Meat extract *[1]	10
Peptone from casein *[1]	10
Yeast extract *[1]	3
D-Glucose *[1]	5
Starch *[1]	1
Sodium chlorure *[6]	5
Sodium acetate *[7]	3
L-Cysteinium *[7]	0.5
Agar-agar *[4]	15
H_2O_{dest} (1000 mL)	

pH 6.8

Anaerobic Egg Yolk Agar* (EGG):

	g/L
Yeast extract *[1]	5
Soya Peptone *[2]	5
Peptone from casein *[1]	20
Sodium chlorure *[6]	5
Agar-agar *[4]	15
H_2O_{dest}(1000 mL)	
2 fresh eggs	
Ethanol (70%) *[6]	
Sterile Saline (0.85%) *[6]	

pH 7.0

*adapted from BAM Media M12 (U.S. FDA [updated 2009a])

Trout Medium Agar* (TROUT):	
	g/L
Trout	500
H_2O_{dest} (1000 mL)	
pH 7.0	
*adapted from BAM Media 38 (U.S. FDA [updated 2009a])	

Peptone Water:	
Peptone from casein *[1]	10
Sodium chlorure *[6]	5
H_2O_{dest} (1000 mL)	
pH 7.2	

- Ethanol (denatured)*[8]

2.2 Methods

The whole present work was divided in four parts: Growth in different culture media culture, long-term incubation of inoculated smoked trout samples, effect of combined high hydrostatic pressure and temperature on *C. botulinum* spores in fresh and smoked trout and effect on lysozyme on the recovery of *C. botulinum* spores. The general experimental scheme is given in Figure 6.

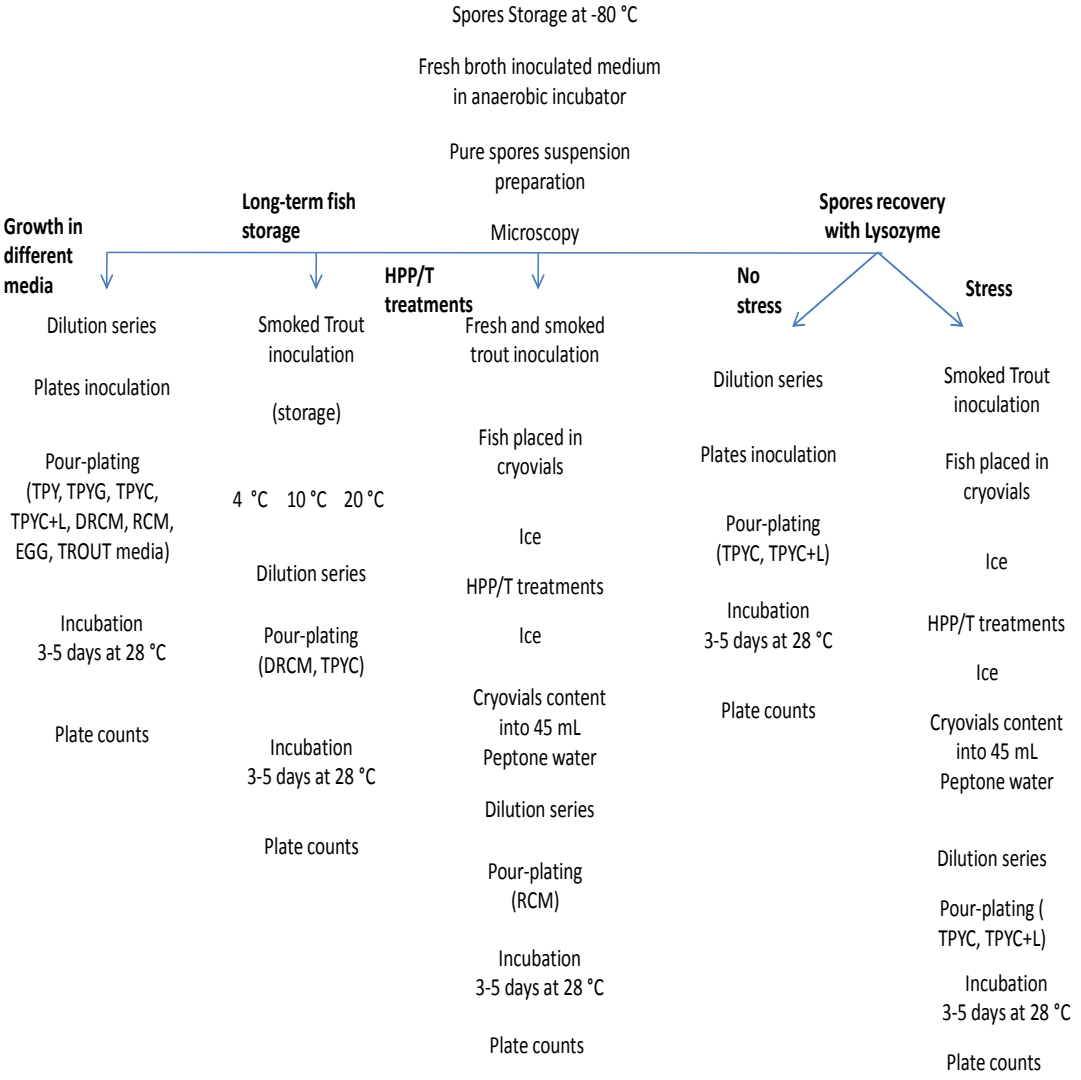

Figure 6 - General scheme of the methodology used in this study

2.2.1 Long-Term Storage of Cell Cultures

For the purpose of long-term storage, 10 mL of fresh overnight cultures were centrifuged (5000 × g; 7 min; 4 °C), resuspended in 750 μL fresh RCM medium, mixed with 750 μL sterile glycerine and stored in cryovials at -80 °C.

2.2.2 Cell Culture Preparation

For the preparation of cell cultures, 50 mL centrifuge tubes (Sarstedt AG & Co, Nümbrecht, Germany) containing RCM broth were inoculated from the -80 °C, stocks of the proper bacterial strain (1% (v/v)) and incubated in an anaerobic incubator (70 % N_2 and 30 % CO_2).

2.2.3 Preparations of Pure Spore Suspensions

Centrifuge tubes (50 mL) were filled with fresh liquid medium (TPYC broth) and inoculated with 10% (v/v) from overnight cultures. These tubes were centrifuged (10,000 × g; 15 min; 4 °C) and the supernatant was discarded. The remaining pellet was resuspended in 10 mL sterile double distilled water by vortexing. The tubes were again centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant was discarded and the pellet resuspended in 2 mL of sterile double distilled water. After a second washing step employing the same conditions, the pellet was again resuspended in 2 mL of sterile, double distilled water and an equal amount of pure, filter sterilized ethanol (filter pore size = 0.20 μm, Filtropur, Sarstedt AG & Co, Nümbrecht, Germany) was added to the tubes and mixed by vortexing to kill remaining vegetative cells. The tubes were incubated for 1h at room temperature (approx. 20 °C). Tubes were then centrifuged again (10,000 × g; 10 min; 4 °C) and the spore pellet was resuspended in 2 mL sterile double distilled water. To destroy debris and free remaining vegetative cells, the tubes were placed in an ultrasonic water-bath (Sonorex Super RK103H, Bandelin electronic, Berlin, Germany) for 2 min, followed by a final washing step using sterile, double distilled water. The prepared spore suspension was transferred to 1.5 mL reaction tubes (Sarstedt AG & Co, Nümbrecht, Germany), placed at -80 °C, for a rapid cool down, and finally stored at -20 °C until use.

2.2.4 Creation and Control of Anaerobic Conditions

Since *C. botulinum* is and strict anaerobic bacterium, all experiments were conducted inside of an anaerobic chamber (WA 6200, Heraeus instruments, Munich, Germany). The gas mixture used was composed of forming gas 95/5 (i.e. 95% N_2 and 5% H_2). On a regular basis, anaerobic indicators (Anaerobic Indicator BR0055, Oxoid, Basingstooke, England) were put inside of a Petri's dish, soaked in distilled water and placed inside of the anaerobic chamber, to verify anaerobic conditions. TPYC agar plates were let sit open and incubated inside of the chamber for at least 3 days at 28 °C, to monitor eventual contaminations inside of the chamber.

2.2.5 Determination of the Total Spore Count

To determine the total number of spores present in a spore suspension, a Thoma counting chamber (depth: 0.02 mm; area of one square: 0.0025 mm^2) was used. The counting chamber was cleaned before use with ethanol to be fat free. For the purpose of microscopic examination using a counting chamber, respective spore suspensions were diluted in sterile distilled water so that the final average number of spores ranged between 10^8 and 10^9. The cover slip was tightly attached to the counting chamber and a small aliquot of the proper dilution was transferred into the gap between cover slip and counting chamber. The number of spores was determined for at least fifteen squares by phase contrast microscopy (Axiostar Plus, Carl Zeiss, Microlmaging GmbH, Munich, Germany) (Figure 7). The total number of spores of a particular spore suspension was then calculated according to the following equation:

$$\frac{Spores}{mL} \times f_d = \frac{\left(\frac{Spores_{square}}{V_{square}}\right)}{mL} \times f_d = \frac{\left(\frac{Spores_{square}}{0.025\ mm^2 \times 0.02\ mm}\right)}{1000\ mm^3} \times f_d = Spores_{square} \times 2 \times 10^7 \times f_d$$

$Spores_{square}$ = Average number of spores counted per square

V_{square} = Volume of one square = area × depth

f_d = Dilution factor of the dilution used for microscopy

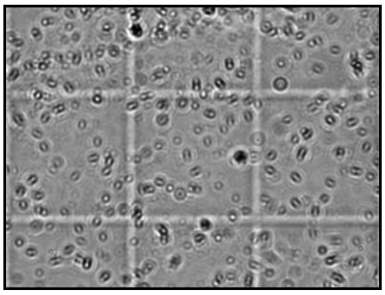

Figure 7 - *Clostridium botulinum* type E spore suspension (100 × objective and 10 × ocular) (author's picture)

Based upon that formula, the volume needed from spore suspensions to inoculate a specific spores/g concentration could be estimated for all experiments.

2.2.6 Pour Plate Cell Count

All cells counts during the present study where determined by preparing pour plates. All media were autoclaved at 121 °C for 20 min, and still liquid (approx. 50 °C), transferred into an anaerobic workbench. 0.1 mL of an appropriate dilution was transferred into a Petri's dish and mixed with approx. 20 mL of fresh, liquid culture medium containing approx. 15 g/L agar. Plates were gently moved after pouring to mix agar and sample. Incubation of the plates was done anaerobically in the integrated incubators of the anaerobic chamber for 3 to 5 days at 28 °C before counting.

For the comparison of the initial total spore count and the number of colony forming units (CFU) present in a poured plate it was presumed that spores are existent as single spores and not aggregated. The factor of possible aggregation has to be considered when evaluating results on the percentage (%) of germination. The relation between the initial total spore counts (determined using a counting chamber and phase contrast microscopy) and the number of spores that really germinate in a poured plate can be calculated. The following equation was used to calculate the percentage of germination:

$$Germination\ (\%) = \left(\frac{S}{MO}\right) \times 100$$

S = Number of spores determined as CFU using pour plate count

MO = Number of spores determined using a counting chamber and microscopy

Using this equation, the % of initial spores that will germinate can be calculated for every experiment.

2.2.7 Growth Properties of *C. botulinum* in Different Culture Media

The spore concentration in a given spore suspension was determined as described above. In this part of the study, the recovery potential of different types of media was determined. The initial spore concentration used was 5.28×10^8 CFU/mL. Proper dilutions were pipetted into plates and mixed with different media (TPY, TPYG, TPYC, TPYC+L, DRCM, RCM, EGG and TROUT). Pour plate counts were conducted and CFU per mL calculated for every single medium.

2.2.8 Long-term Incubation of Inoculated Smoked Trout Samples

2.2.8.1 Samples Inoculation

The spore concentration of the used spore suspensions was determined as described above using phase contrast microscopy. 53 g of smoked trout fillet was weighed out in a sterile plastic beaker and inoculated with a proper volume of spore suspension to give a final concentration of 10^5 spores/g. The inoculated fish was minced and homogenized with a sterilized hand blender. Under anaerobic conditions, 5 g of the inoculated fish were placed into 15 mL centrifuge tubes (Sarstedt AG & Co, Nümbrecht, Germany), respectively. Tubes without inoculation were included as a control.

2.2.8.2 Long-term Incubation

The inoculated tubes as well as the control tubes were placed at three different temperatures including 4 °C, 10 °C and 20 °C in anaerobic jars containing anaerobic incubation bags (Anaerocult® A mini, Merck, Darmstadt, Germany). Incubation temperatures were constantly monitored during the course of this experiment (approx. 35 days).

2.2.8.3 Dilutions and Pour-plating

For each measurement time point one tube per temperature and strain was removed from the respective anaerobic jar and the content was transferred to centrifuge tubes (50 mL)

followed by the addition of 45 mL peptone water. Samples were homogenized by shaking vigorously. Proper dilutions were used for plate counts as described above using TPYC and DRCM medium.

2.2.9 Outgrowth Potential of *C. botulinum* Spores in Smoked Trout at Different Temperatures

2.2.9.1 Preparation of Fresh Trout Fillets

For each experiment, the surface of freshly caught Rainbow trout was cleaned with ethanol (70%), to remove the surface bacterial flora. After disinfection, fillets were cut off using a sterile disposable scalpel (Aesculap AG & Co-KG, Tuttlingen, Germany) avoiding to hit any organs or the intestinal tract.

2.2.9.2 Inoculation and the Respective Controls

The total initial spore count for the respective spore suspension used was determined using phase contrast microscopy as described above. Thereafter, the homogenized fillets of both smoked and fresh trout were inoculated with a specific amount in order to obtain an initial concentration of spores of 10^8 spores/g. The inoculated samples as well as the control samples for both fish products were transferred in 2 g portions to cryogenic vials. The controls for both fishes were done as shown in the following Table 3:

C1 = Number of spores in the untreated fish sample

C2 = Number of spores after treatment in the fish sample

Cα = Number of spores after the inoculation in the untreated fish sample

Table 3 - Composition of each control used for the High Pressure experiments

Control	Inoculation	Pressure	Temperature
C1	-	-	-
C2	-	+	+
Cα	+	-	-

2.2.10 Effect of Combined High Hydrostatic Pressure and Temperature (HHP/T) Inactivation of *C. botulinum* Spores in Smoked and Fresh Trout

The cryo tubes were sealed with parafilm to avoid the penetration of pressure transmitting fluid into the respective vial and stored on ice until use. The samples were pressurized at temperatures ranging from 20 to 80 °C and pressures ranging from 200 to 800 MPa with a proper double-vessel high hydrostatic pressure unit (model specially designed for Lehrstuhl für Technische Mikrobiologie, Weihenstephan, Technische Universität München, Germany) (Figure 8), with approx. 146 cm^3 of internal volume per autoclave. Polyethylenglycol 400 mixed 1:1 with tap water was used as pressure transmitting fluid. The high pressure autoclaves were pre-heated to have the desire temperature prior to pressurization and maintained during the treatments using an external flow heater (FC 600, Julabo Labortechnik GmbH, Seelbach, Germany). The temperature was monitored by a thermal sensor inside of each vessel. The samples were placed into the pressure vessels some minutes prior to compression to equilibrate the sample temperature. A computer program (Autoklav - Steuerung V2.06 (C) 1995-1998 Peter Knam & Roland Mast) controlled pressure ramp and level as well as holding time of pressurization. The holding time for all treatments was 300 s applying a constant pressure ramp of 200 MPa × min^{-1} to limit the adiabatic heating effect. The average maximum adiabatic heating effect is shown in Table 4 and did not exceed 1 to 4°C. After treatment the samples were placed in the anaerobic chamber and the content was transferred to 50 mL centrifuge tubes and filled with peptone water. Samples were diluted and plate counts were determined as stated above using RCM as plating medium. These experiments were repeated three times in order to increase statistical significance.

Table 4 - Temperature measurements on the beginning of holding time during HHP/T treatments

Pressure (MPa)	Initial Temperature (°C)	Max. Temperature (°C)
200	20	22.3
	40	41.2
	60	61.5
	80	81.1
400	20	23.2
	40	42.2
	60	62.7
	80	82.5
600	20	23.5
	40	43.9
	60	63.8
	80	82.1
800	20	23.2
	40	43.3
	60	63.5
	80	84.1

Figure 8 - High Pressure Processing equipment used on this work (author's picture)

The inactivation effect of HPP/T treatments on the inoculated *C. botulinum* spores was monitored by a reduced cell count on pour plates. The logarithmic reduction of CFU was then calculated according to the following equations:

$$i = log\left(\frac{N}{N_0}\right) \times 100 \quad \text{and} \quad log\left(\frac{N}{N_0}\right) = log\left(\frac{S - C2}{C\alpha - C1}\right)$$

i = spores inactivation

N_0 = initial spore counts

N = counts after treatment

C1 = Number of spores in the untreated fish sample

C2 = Number of spores after treatment in the fish sample

Cα = Number of spores after the inoculation in the untreated fish sample

S = Number of spores determined as CFU using pour plate count

To know how the combined HPP/T treatments affected the spore population and how much time is needed to inactivate 90% of this population, the D value was calculated using the following equation:

$$D_{P,T} = \left(\frac{t}{\log\left({}^{N_0}/_{N}\right)}\right)$$

P = Pressure (MPa)

T = Temperature (°C)

T = Time (min)

N_0 = initial spore counts

N = counts after treatment

2.2.11 Effect on Lysozyme on the Recovery of *C. botulinum* Type E Spores

For this assay, the initial number of spores from the spore suspension was determined by phase contrast microscopy as stated above. The examine the effect of lysozyme in the recovery medium on the estimated high pressure inactivation of spores, dilutions of pressurized, inoculated samples of fresh and smoked trout fillets were pour plated as stated above using TPYC (control, with no lysozyme) and TPYC+L (with lysozyme) medium. Low temperature (20 °C) and pressure (600 MPa) were used to check the influence of

lysozyme on the recovery of a stressed spore population. Calculation of the log reduction was performed as mentioned in the previous chapter.

3 Results

3.1 Growth Properties of *C. botulinum* in Different Media

In order to determine the best conditions for the recovery of spores of the *C. botulinum* type E strains used during the course of this study several anaerobic recovery media were tested. Pour plates were prepared using *C. botulinum* spores and colony forming units (CFU) were counted after incubation at 28 °C.

With the results from plate counts it was possible to create a graph (Figure 9) and compare the recovery characteristics of spores in the different media. All of the media seem to provide similar recovery characteristics supporting *C. botulinum* germination and outgrowth. The results as provided in the graph below show the spore recovery expressed as CFU on a logarithmic scale. All of the media provided a recovery potential of around 10^7 CFU/mL with slight variations. Trout extract medium provided the lowest and Reinforced Clostrial Medium (RCM) the best recovery of spores. The highest recovery efficiency in RCM was followed by Differential Reinforced Clostridial Medium (DRCM) and EGG yolk medium. The different Tryptone Peptone Yeast Extract (TPY) media promoted similar recovery with no significant differences between TPYC (TPY with sugar mixture) and TPYC+L (TPYC with lysozyme).

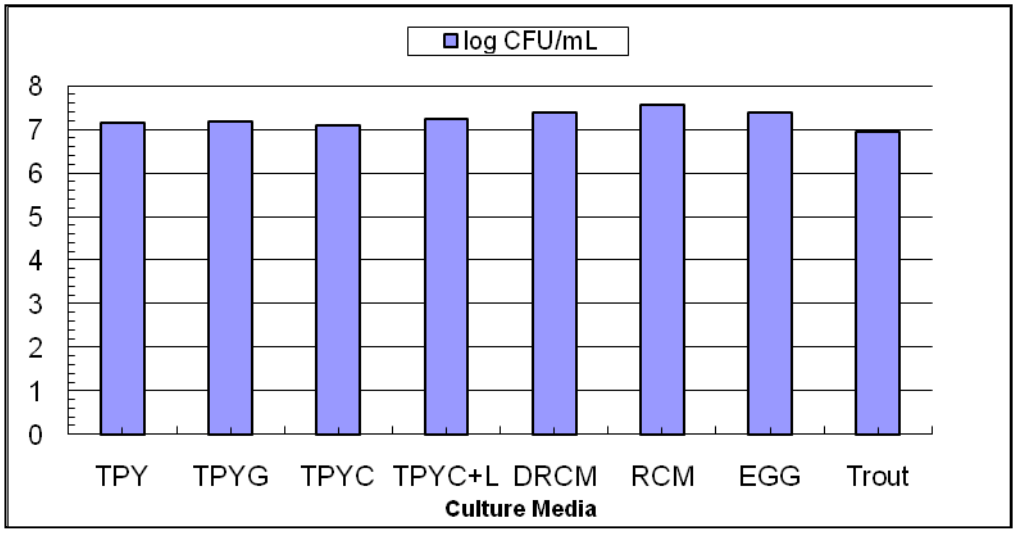

Figure 9 - Outgrowth of *C. botulinum* type E spores in different culture media

Furthermore, the total spore count as determined by phase contrast microscopy using a counting chamber was compared to the CFU appearing on the plates containing different recovery media and the percentage (%) of germination was calculated. Corresponding to the log CFU results shown above RCM showed the highest germination percentage. Results for the overall % of germination in different media are given in Table 5.

Table 5 - Germination rate of *C. botulinum* type E spores for the different culture media used

Culture media	Germination (%)
TPY	2.74
TPYG	2.89
TPYC	2.32
TPYC+L	3.2
DRCM	4.53
RCM	6.7
EGG	4.5
TROUT	1.7

3.2 Outgrowth Potential of *C. botulinum* Spores in Smoked Trout at Different Temperatures

Homogenized smoked trout fillet was inoculated with *C. botulinum* spores and incubated at 4 °C, 10 °C and 20 °C over a period of 31 days. This experiment was conducted using three different *C. botulinum* type E strains (TMW 2.990, TMW 2.992 and TMW 2.994) which showed similar outgrowth properties over time. Results for strain TMW 2.994 are shown in Figure 10.

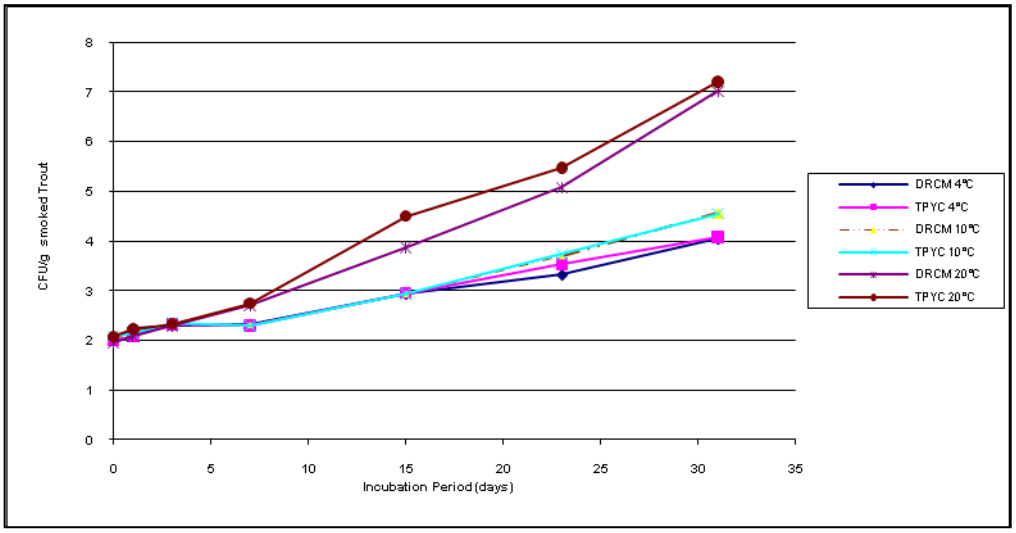

Figure 10 - Growth of *Clostridium botulinum* type E spores in DRCM and TPYC culture media at 4 °C, 10 °C and 20 °C upon 31 days

Fish

samples were inoculated with a total spore concentration of 10^5 spores/g which yielded an amount of colony forming units of approximately 10^2 CFU/g on day 0. The lag phase of growth in the homogenized fish sample last approximately 3 days at 20 °C and one week at 4 and 10 °C. After that, slow growth occurs showing a similar growth rate at 4 and 10 °C reaching approximately 10^4 CFU/g on day 31. In the other hand, a more rapid growth was observed at 20 °C present reaching 10^7 CFU/g on day 31. TPYC medium provided a slightly faster growth than the differential medium (DRCM).

3.3 High Pressure-Temperature Inactivation of *C. botulinum* Type E Spores by in Smoked and Fresh Rainbow Trout

The first visible result is a color change of the samples. The color of fresh trout changes from a translucent orange-pink color into a whitish color, while the color of smoked trout fillets did not change significantly after treatment (Figure 11). During handling the samples it has also been noticed that the fillets' compactness increased after treatments for both fishes

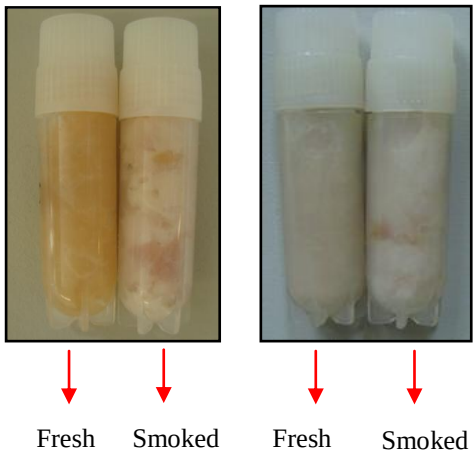

Figure 11 - Images of fresh and smoked trout before (left) and after (right) HPP/T treatment at 800 MPa and 40 °C

Treatments of inoculated trout fillets at 200 MPa resulted in a no significant spore inactivation at 20 °C in both fish homogenates and reached a 3 log reduction at 80 °C. The logarithmic (log) inactivation of outgrowing spores at 400 MPa follows similar pattern as it was observed at 200 MPa, although the inactivation was slightly more effective. At 600 MPa the combined inactivation effect of pressure and temperature starts to be significantly more effective. Here, the log reduction exceeded 4 log at 80 °C. The highest inactivation is achieved at 800 MPa, where the log reduction of recovering spores reached a level of 5 log and more at 80°C. In summary it can be stated that the lowest log reduction can be found at

200 MPa treatments and the highest at 800 MPa. In terms of temperature it could be noticed that for all pressure levels the inactivation efficiency at 20 and 40 °C reaches relatively similar levels. The application of temperatures in the range of 60 °C and above, the inactivation is way more effective, although depending on the pressure applied. The most effective spore inactivation level of all experiments was achieved at 800 MPa and 80 °C in fresh trout fillet homogenate. After any HPP treatment at any temperature the inactivation efficiency was higher in fresh trout. Results for both trout are displayed in Figure 12. Error bars indicate the standard deviation.

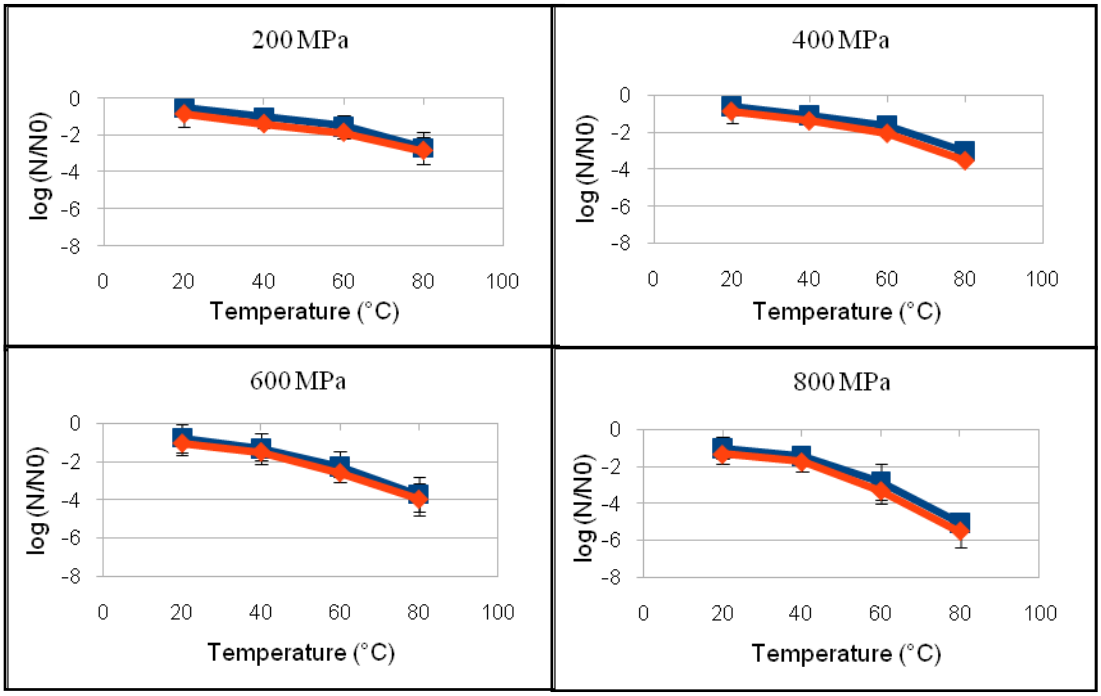

Figure 12 – Inacttivaction of *C. botulinum* type E spores by HPP/T treatments at 200, 400, 600 and 800 MPa and 20, 40, 60 and 80 °C. Blue line represents smoked trout and red line the fresh trout.

The Figure 13 presents the results of the HHP/T treatments to inoculated smoked trout for all pressure levels. On this graphic could be seen that 200 and 400 MPa behaves in a similar way at all temperatures, less at 80 °C where 400 MPa shows a higher log reduction, approx. 2.5 and 3 log, respectively. The 600 MPa inactivation curve is distinct from the ones of lower pressure levels, since at 20 °C the inactivation value is already higher that the ones from 200 and 400 MPa. The slope of line for 600 MPa inactivation curve is higher also for the rest of the temperatures, reaching approx. 3.5 log reduction at 80 °C. For 800 MPa curve, it acts like the 600 MPa one till 40 °C, but has higher inactivation values for 60

and 80 °C, achieving on the last > 5 log reduction value. The standard deviation bars are bigger for 60 and 80 °C for all pressure levels.

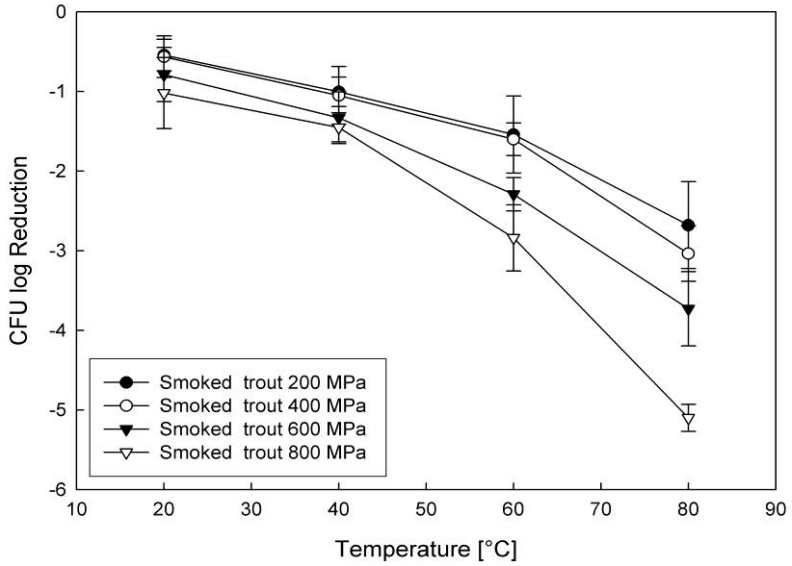

Figure 13 – High Pressure Inactivation curves of *C. botulinum* type E TMW 2.994 spores in smoked trout fillets

The inactivation curves of inoculated fresh trout are presented in Fig. 14. For 20 and 40 °C all pressure levels act at same way, with low inactivation values. The inactivation curves are very similar with the ones presented on Figure 13, with the difference on the higher log reduction values for 60 and 80 °C, for all pressure levels. Here, the most striking treatment (800 MPa, 80 °C) shows an inactivation value of approx. 5.5 log. The standard deviation bars appear also bigger for 60 and 80 °C, principally for the higher pressure level treatments.

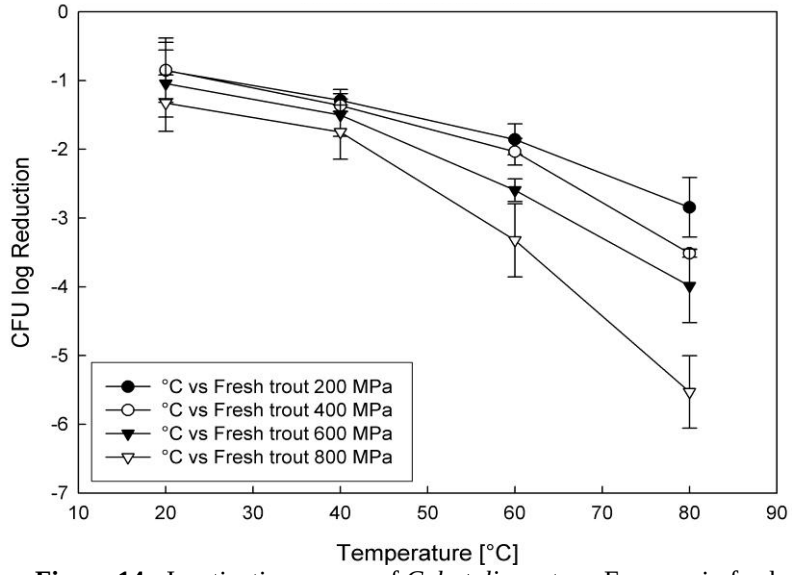

Figure 14 - Inactivation curves of *C. botulinum* type E spores in fresh trout at all used pressure levels and temperature

The overall results of the high pressure-temperature inactivation effects on *C. botulinum* TMW 2.994 spores are summarized in figure 15. It is shown to have a better perception of the results together. Can be seen that, in general, higher pressure levels treatments combined with a higher temperature originates a higher log reduction value.

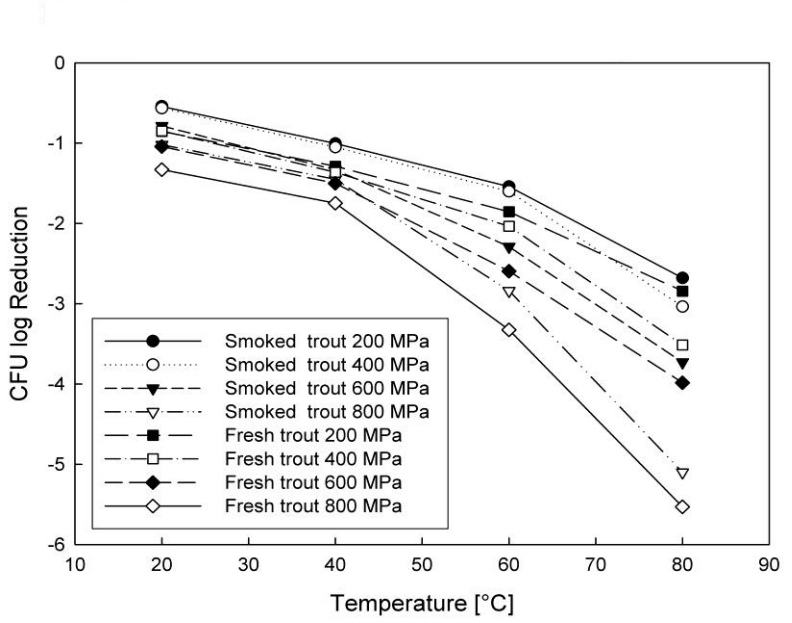

Figure 15 - Combination of all inactivation curves of *C. botulinum* type E spores in smoked and fresh trout at all used pressure levels and temperature. Smoked trout curves are presented in dashed line and fresh trout in full line

The inactivation values of the spores are in log reduction scale for smoked trout and shown in Figure 16. Is possible to see that to obtain a 1 log reduction is needed higher (40 °C) temperature in lower pressure levels (200 and 400 MPa) and lower temperatures (30 and 20 °C) for higher pressure levels (600 and 800 MPa, respectively). The 2 log reduction is just found at much higher temperature than the one found to reach 1 log. The same effect of pressure/temperature happens for the rest of the log reductions. 4 log reduction is achieved at 800 MPa over 70 °C. The maximum inactivation, 5 log, is just achieved at 800 MPa and 80 °C.

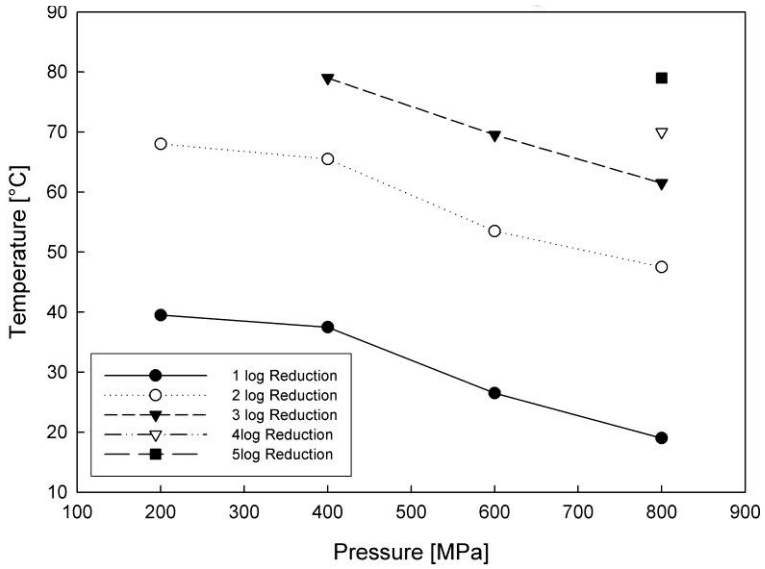

Figure 16 - Isoeffect curves for inactivation of *C. botulinum* TMW 2.994 spores in smoked trout fillet homegenates

For fresh trout the results are similar (Figure 17), with the detail that it needs lower temperatures to have the same reduction compared with smoked trout. 1 log reduction is found at 20 °C for lower pressure levels and this temperature decreases abruptly when 800 MPa is achieved. There is also a big difference on the inactivation temperature from 1 to 2 log. 5 log is reached at 800 MPa at 80 °C.

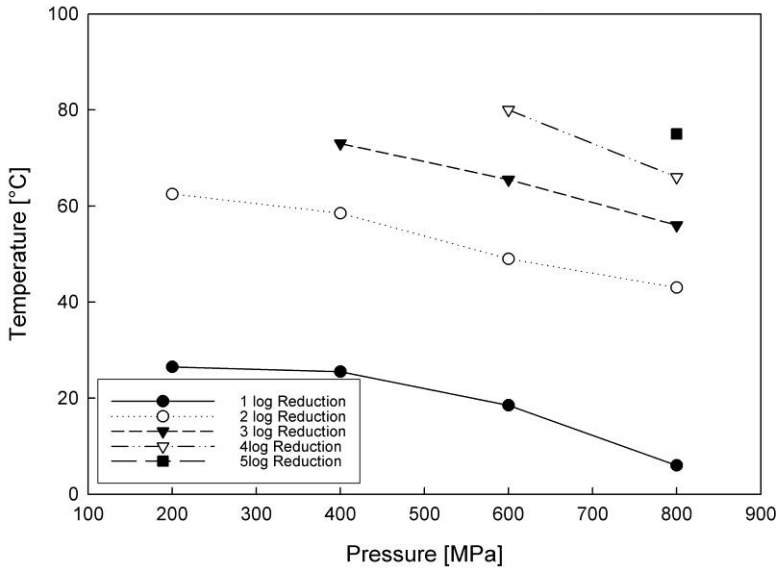

Figure 17 - Isoeffect curves for inactivation of *C. botulinum* TMW 2.994 spores in fresh trout fillet homegenates

The conjugation of Figures 16 and 17 are shown on Figure 18. Here can be seen the log reduction values achieved after treatments in order to temperature and pressure. The results for 1 log reduction were obtained for all pressure levels at pressures of 20 °C for ft (fresh trout) and 40 °C for st (smoked trout). 2 log reduction for ft were obtain near to 60 °C for lower pressure levels (200 and 400 MPa) and above 60 °C for higher pressure levels (600 and 800 MPa) and for st beginning in a little higher temperature but acting identically to ft. For 3 log the values are situated near to 80 °C to lower pressure levels, decreasing this temperature when the pressure raises. 4 log happened to ft at 600 and 800 MPa for temperatures around 80 °C, meanwhile for st it just occur at 800 MPa also near 80 °C. The higher log reduction, 5 log, is reached for both trout at 800 MPa at 80 °C. Can be notice in this graphic is that for the same log reduction order, st always need a higher temperature than ft. Another deduction is that log reduction needs a lower temperature when the pressure is higher.

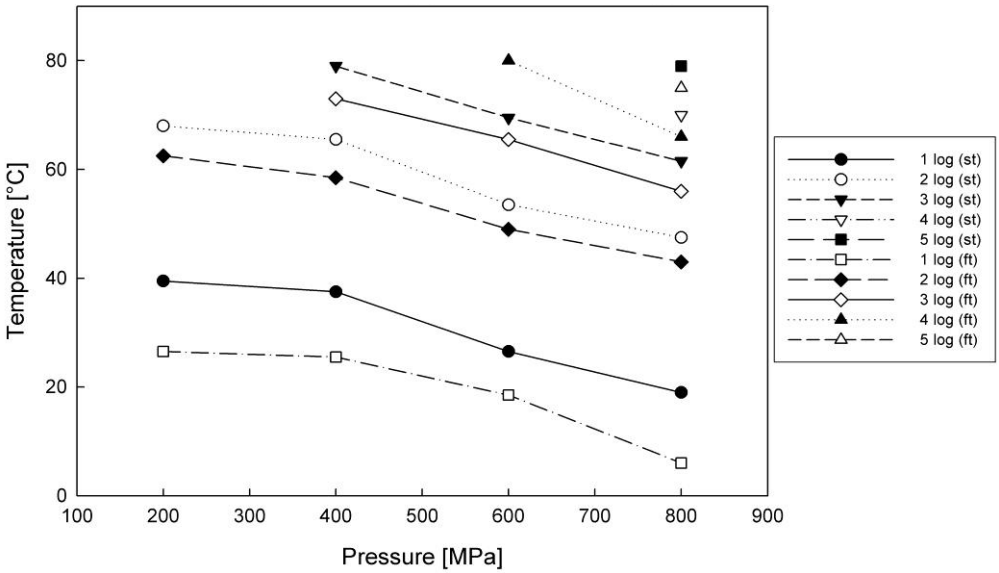

Figure 18 - Isoeffect curves for the log reduction of *C. botulinum* TMW 2.994 spores in Smoked (st) and Fresh (st) Trout Fillet Homogenates

For all HHP/T treatments D value was calculated and it is presented on Table 6. The higher D values are presented on the lower HHP/T combination and the lower D values on the higher HHP/T combinations. Exceptions can be found as in 400 MPa 20 °C as the lowest D value compared with the rest of pressures at the same temperature; and at 200 MPa 40 °C the value is lower than the rest of the higher pressures at equal temperature and at 600 MPa 80 °C the D value is higher than the pressure level before it. Strangely, the treatment at 200 MPa 60 °C has a higher value than the homologue at 40 °C. The rest of the results shown a coherent decrease of D value when the HHP/T combinations increase.

Table 6 - D value for the HHP/T inactivation of *C. botulinum* Type E spores for both trout, including standard deviation (S.D.) values

Temperature (°C)	Pressure (MPa)	Smoked		Fresh	
		D value (min)	S.D.	D value (min)	S.D.
20	200	10.72	5.47	5.46	2.42
	400	5.58	3.11	9.12	8.82
	600	10.87	10.25	7.25	4.95
	800	8.64	8.94	4.45	3.61
40	200	4.66	1.08	3.40	0.42
	400	6.81	4.55	7.21	4.65
	600	5.90	0.62	3.96	0.69
	800	3.75	0.69	3.46	0.33
60	200	8.55	8.55	4.80	3.36
	400	3.46	0.11	2.58	0.42
	600	3.42	0.28	2.90	1.20
	800	2.73	0.59	1.71	0.27
80	200	2.17	1.00	2.01	0.66
	400	1.78	0.32	1.44	0.01
	600	2.05	0.62	1.43	0.09
	800	1.13	0.63	0.88	0.28

Percentage of germination of *C. botulinum* spores type E on smoked and fresh trout were calculated with an average for these HHP/T treatments, and contrarily of the results obtained for general outgrowth of spores into vegetative cells in culture media showed on Table 5, the germination percentage specifically in fish has higher number. The results between smoked and fresh trout are quite similar as can be seen on Table 7.

Table 7 - Germination (%) of *C. botulinum* spores in smoked and fresh trout after submission to HHP/T treatments

Trout	Germination (%)
Smoked	22.23
Fresh	22.30

3.4 Effects of Lysozyme on the Recovery of *C. botulinum* Type E Spores after HHP Treatment in Fresh and Smoked Rainbow Trout

Since the % of germination for the previous assays were quite low, lysozyme was added to TPYC medium to examine the effect on the recovery of the high-pressure stressed *C. botulinum* spores. First assay was done without any environmental stress, to see possible differences in the outgrowth in presence or absence of lysozyme. As can be seen on Table 8, there is no significant difference between the CFU/mL from what is called control (no lysozyme) and with it. On the second assay, inoculated smoked and fresh trout were subjected to HHP/T and then TPYC media with and without addition of lysozyme was platted.

As can also be seen on Table 8, there is no significant difference on the CFU/mL values between the two conditions, with or without stress. The only distinction here is between the fishes: smoked fish has more CFU/mL than the fresh one.

Table 8 - Spores of *C. botulinum* type E germination counts with no submission to pressure (600 MPa) stress and with it

Stress	**CFU/mL**			
no	Control		Lysozyme	
	1.6×10^7		1.3×10^7	
yes	Smoked	Fresh	Smoked	Fresh
	1.5×10^5	3.0×10^4	1.2×10^5	3.2×10^4

The D values for HHP/T treatments were calculated for both trout as can be found on Table 9. Smoked trout takes more time to reduce the spores than the fresh. Control has higher values that the lysozyme ones for both trout.

Table 9 - D values of *C. botulinum* spores inactivation at HHP/T treatments (600 MPa 20 °C) in absence or presence of lysozyme

	D value (min)	
	Smoked	Fresh
Control	9.17	4.17
Lysozyme	7.09	1.97

The results for % of germination of this experiment (Table 10) correspond to the CFU/mL results: despite being all low, higher values for the assay without stress than with it. With

pressure, the values are lower but similar between them, distinct on the fish type (higher for smoked trout).

Table 10 – Germination (%) *of C. botulinum* type E spores with no submission to pressure stress and with it (600 MPa)

Stress	**Germination (%)**			
no	Control		Lysozyme	
	0.8300		1.0000	
yes	Smoked	Fresh	Smoked	Fresh
	0.073	0.0015	0.0061	0.0016

4 Discussion

4.1 Growth Properties of *C. botulinum* in Different Media

All media used shown a similarity to let *C. botulinum* grow but RCM and DRCM media showed a bit higher CFU/mL of outgrowth spores, it is may due to their composition. RCM differs with the extra presence of meat extract that make proteins available in the medium; L-cysteinum to enhance Maillard reaction and Sodium acetate to stabilize the pH value. DRCM, in the other hand, has additionally sodium dissulfite to preserve meat, and also ammonium iron (III) citrate and resazurin sodium but these last do not have a direct influence on the growth but act more like a clostridia marker. Both, RCM and DRCM showed good CFU/mL results when the spores grown directly on the media at 28 °C. EGG yolk had high CFU/mL results of outgrown spores. This medium serves for isolation and presumptive differentiation of *Clostridium* spp. and was shown that *C. botulinum* type E spores can grow well there. On the TPY's media *C. botulinum* spores grown similarly, it seems that the use of sugars or not in the medium does not have big positive difference, as it is shown on Figure 9, the lowest CFU/mL value between these medium type is for TPYC, the one that is joined Glucose, Maltose, Cellobiose and Starch sugars. Besides, the addition of lysozyme, it does not make such difference at these conditions, maybe because there is no additional stress on the growing environment. With the use of Trout as a medium, can be proved the growth of type E spores at the conditions given, what means that what is present in the media is enough for spores outgrowth. It gives the suggestion that when spores will be inoculated directly on fish fillets, they also grow, at least for the same conditions as used for this case. Trout showed the lowest CFU/mL results among the culture media used, but is acceptable that in a medium based on a food system, the spores germinate worse than in a specific culture medium.

The germination percentage calculated for this experiment Table 5, reveals very low percentages, it is may be due the possible spores aggregation, to the not ability of all spores to outgrowth, since part of that could be on a very resistant dormancy and maintain the normal appearance when observed in microscope; beyond this, eventually mistakes on manipulation aspects could also have influence on the % of germination low values.

4.2 Outgrowth Potential of *C. botulinum* Spores in Smoked Trout at Different Temperatures

C. botulinum type E spores are able to increase their CFU/g number over time at 4 °C, 10 °C and 20 °C. From the 10^5 spores/g inoculated into the smoked trout, just 10^2 spores/g germinated from the three temperatures on day 0, this means that, besides of the viability and spores' structure, this fish do not promote the spores growth as well as media culture for the three temperatures, as shown in "Growth Properties of *C. botulinum* Spores in Different Media". As expected, refrigeration temperatures detain the spores of a rapid outgrowth, meanwhile at the higher temperature the spores shown a faster germination and multiplication as vegetative cells as is near to the optimal spores outgrowing temperature. For all temperatures, the spores germinated as vegetative cells spend more than 7 days to increase in 1 log CFU/g values. The moderately slow growth of the vegetative cells may be due to the nonproteolytic character of the outgrown spores, that do have enzymes to act on the proteins lyses, that in this case could interfere with their growth, but also due to the inhibition influence of competing microflora, also the microenvironments that could had been created for eventually non homogenized distributions of the inoculated spores and the NaCl concentration of the smoked trout could avoid the spores outgrowth, unless the NaCl could be also not well spread for all the trout homogenously, creating space and possibility for spores germinate. The increase of growth by vegetative cells could had been facilitated by the excretion of CO_2 by the another possible bacteria already present on the smoked trout. Such inoculated amount is not realistic since the highest *C. botulinum* type E spore load had counts of 5.3 CFU/g in a Danish farmed trout (1974) (Hyytiä et al. 1999), but was an assurance that something was going to grow, seeing that the % of germination shown for previous works were quite low. In the opposite to what was reached for the grown of *C. botulinum* spores in different media, where DRCM shown a better ability to let the spores grown than TPYC, in this assay this last had enhanced the spores outgrowth and the vegetative cells multiplication at all temperatures. Maybe it happens for the necessity of sugars to outgrowth and then for the cells multiplication, as sugars means available energy as it is a need for the spores can germinate in anaerobic conditions. Can be seen a saccharolitic action by *C. botulinum* spores.

The same explanation as the one above could be given for the lower values found for the % of germination.

4.3 High Pressure-Temperature Inactivation of *C. botulinum* type E Spores by in Smoked and Fresh Rainbow Trout

In detriment of reach a 6 log reduction, what is considered to be the reduction needed to have a safe food product **(**Peck et al. 2006), smoked and fresh trout fillets were inoculated and then submitted to HPP/T treatment.

The synergical use of pressure combined with temperature shown an enhanced effect on the inactivation of *C. botulinum* spores in smoked and fresh trout, since the spores were not killed or reached less than 2 log for temperatures ranging from 50 to 80 °C at ambient pressure (Doona et al. 2007). As expected, the highest HHP/T combinations provided a better efficiency to inactivate the spores on food model system. The lower temperatures (20 and 40 °C) even at 800 MPa, do not reduce the spores counts as happens to lower pressure levels, what indicate that the main factor to inactivate spore in this situation is pressure. At higher temperatures (60 and 80 °C) the combined effect of pressure is better notice, since the log reduction has an increased value. Reddy et al. (1999) also used spores from *C. botulinum* type E to see the effects of pressure, and reached 5 log reduction for treatments of 827 MPa at 40 °C for 10 min and 827 MPa at 50 °C for 5 min (Reddy et al. 2007). The denomination of a safe product as was mentioned before is just approx. achieved at 800 MPa 80 °C for 5 min with a ≥ 5 log reduction. 800 MPa were chosen as highest pressure level for the equipment limitation but also because pressures over 800 MPa showed almost no variations to a faster spore reduction (Margosch et al. 2006). The high-pressure leads to the release of the DPA from the spores, turning them sensitive to heat. This is one of the reasons that for higher temperatures, spores have major log reduction (Margosch et al. 2004). The low values of log reduction (e.g. 1 log) happen at low temperatures, while upper values happen at much higher values and more close to each other in terms of the inactivation temperature.

Has to be remembered that *C. botulinum* are the most pressure resistant spores known; additionally they were inoculated in a medium that has big influence on the protection or injury of the spores, for its heterogeneity, structure and composition (Doona et al. 2007). Can be seen that for all treatments smoked trout appears with lower log reduction values than the fresh. A possible explanation for that could be the process of smoking that the smoked trout is submitted: during the process the trout is subjected to high temperatures

that made the percentage of water be reduced and the tenor of fat content increases as trout is a oily fish (Moore et al. 2006). This fat can remains into the fish and have, during the HHP/T treatments, a baroprotective effect for the spores as it has for vegetative cells (Gervilla et al. 2000; Escriu and Mor-Mur 2009), avoiding them to suffer the effect of the treatment in such drastically form. In other hand, the NaCl and the smoke particles content of the smoked trout could act in the way to damage the spores or do not let the germination occur, what does not seem to have any visible effect on these treatments. This can be de reason for the fresh trout had highest inactivation values upon the treatments. This trout for its freshness still has its minerals like Calcium sulfate ($CaSO_4$), Zinc sulfate ($ZnSO_4$) or Manganese sulfate ($MnSo_4$) in a normal percentage, which can cause the decrease of spores resistance. Margosch et al. (2004) verified that spores *Bacillus subtillis* spores obtained from cultures at 30 C in the presence of 5 mM of those minerals, spores were inactivated in 4 orders of magnitude.

Beside punctual errors, D values obtained reveal coherent results seeing that they reduce their value for an increment of pressure and temperature. Compared to spores of the same type subjected with only temperature for different heating medium the results were 0.07 to 6.6 min at 82 °C, showing that the pressure enhance the decrease inactivation time when conjugated with temperature, since for similar temperature (80 °C) were obtained results in a range of 1.13 to 2.17 min in smoked trout and 0.88 to 2.01 in fresh trout.
For these assays, the % of germination were also quite low and the reason why could be given for the same reason as above.

For the same strain, Rovere et al. (1998) obtained at 800 MPa/88 C/9 min 3 log reduction in a beef broth, in this work for similar conditions (800 MPa/80/5 min) were obtained 5.5 log in fresh trout. Besides the obvious differences between the two media: one broth, another solid, meat and the other fish; can be said that was reached a very effective result on inactivating C. botulism type E spores on a model system food, despite as was said, the textural and color properties had change for the original product.
The % of germination shown specifically for trout (Table 7) is much higher than the one found for “Growth Properties of *C. botulinum* in Different Media” (Table 5), whereas the initial inoculation concentration was also higher, allowing more spores to outgrowth, but

also because in trout germination was taking into account the Cα (number of spores after the inoculation in the untreated fish sample) and the S (Number of spores determined as CFU using pour plate count), meanwhile in the other was taking into account the S value and microscopy numbers, becoming the % of germination bigger for trout.

4.4 Effects of Lysozyme on the Recovery of *C. botulinum* Type E Spores after HHP Treatment in Fresh and Smoked Rainbow Trout

Even in extreme treatments, some of the spores that were damaged by the applied stress conditions could grow if the environmental conditions could pass through the injury condition. lysozyme is known as a facilitator of the heat-damaged spores of non-proteolytic *C. botulinum*, thereby increasing measured spore heat resistance. On the first assay was tried to see if the lysozyme stimulates germination even in absence of environmental stress. But was no evidence of this, the CFU/mL results were similar between the control medium with no addition of lysozyme and the other with it. Since is known that lysozyme has influence on recovery the heat-damaged spores, in this assay was tried to see if pressure-damaged spores act in the same way, using temperature of 20 °C. The obtain results did not show this effect. Was just noticed a difference on the counts higher for smoked trout than for the fresh one, as notice in the "HHP/T treatments". One factor that has to be considered is the natural presence of lysozyme on fish (Lindström et al. 2003). So, maybe the germination results are already influenced by the help on the spores recovery and if some more is added, no more influence could be given and the results CFU results are not enhanced.

In terms of D values the results should be similar, since the lysozyme was just added to the culture medium where spores were inoculated to germinate.

Once again, the % of germination results were extremely low, being that the assay with no stress with higher values than the one where pressure was applied, this because many of inoculated spores were inactivated/kill by pressure, became less spores able to germinate.

5 Conclusions

The *C. botulinum* toxins are so harmful that all efforts in food processing (and manipulation, distribution, storage, etc.) are needed to prevent such dangerousness. High Hydrostatic Pressure has been shown to be an advantage in the food microbiology safety.

Seeing that few data and knowledge is known about spores of *C. botulinum* type E principally it contamination on rainbow trout and its behavior when subjected to HHP. This work has contributed for the enhancement of the information showing that:

- *S*everal different artificial culture media as well as trout extract medium provide effective outgrowth of *C. botulinum* type E spores;
- Smoked trout permits the outgrowth of *C. botulinum* spores in a relatively short period of time at normal and abusive refrigeration temperatures (4 and 10 °C), with significantly higher growth rates at 20 °C;
- *C. botulinum* spores persistent in the trout model system can be inactivated by HHP/T showing a ≥ 5 log reduction at 800 MPa and 80 °C for 5 min;
- Smoked trout shows protective properties during HHP/T treatments regarding the inactivation of *C. botulinum* type E spores compared to fresh trout; and
- The addition of lysozyme to the medium appears not to influence the recovery of spores subjected to high pressure stress.

6 Future Perspectives

Is almost endless the knowledge missing in food applied by High Hydrostatic Pressure. The spores are hard to kill even at high pressure levels, and if are so, the food properties are changed from the original product. If in one way it could be undesirable, they way the HPP could origins new food products could lead to an interesting manner to taking advantage of this technology.

In this particular work some new objectives could be traced:

- Determination of the lowest temperatures that allow germination and outgrowth of *C. botulinum* spores in smoked or fresh trout products;
- Determination of possible alterations in terms of growth characteristics of HHP/T treated spores (e.g. prelonged lag phase of a sublethally injured spore population)
- Evaluation of the effect of gas atmosphere on the inactivation of *C. botulinum* spores in fish (e.g. hypercritical carbon dioxide);
- Comparison of the effect of commercially applied thermal processing and HHP/T processing on organolopetic, nutritional properties of fish products;
- Examination of the effect that oil has on the inactivation effectiveness comparing the results of the present study with oily suspensions as model systems or other fish products showing lower or higher fat contents; and
- Evaluation of pressure cycling processes to reach higher inactivation levels e.g. pre-use of moderate pressure to provoke spore germination combined with a higher pressure treatment at moderate temperatures to kill germinated cells.

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8 Attachment

Table 11 - Companies selling the products referred on 2.1.3 section

Code number	Companies names	City	Country
3	AppliChem	Darmstadt	Germany
4	BD	Le Pont de Claix	France
6	BDH Prolabo, VWR International	Fontenay sous Bois	France
7	Carl Roth GmbH + Co. KG	Karlsruhe	Germany
8	CLN GmbH	Freising	Germany
1	Merck	Darmstadt	Germany
2	Oxoid	Basingstoke, Hampshire	England
5	Serva Electrophoresis GmbH	Heidelberg	Germany