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High pressure temperature inactivation of *Clostridium botulinum* endospores in low-acid foods and food model systems

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Abbreviation

a_w	Water activity
B.	*Bacillus*
BADGE	Strain Blast Diagnostic Gene Finder
BM	Base matrix
BoNT	Botulinal neurotoxin
BRIG	BLAST Ring Image Generator
BS	Braised veal
C.	*Clostridium*
CFU	Colony forming units
DPA	Dipicolinic acid
D-value	Decimal reduction time
FFA	Free fatty acids
GPH	Green peas with ham
HHP	High hydrostatic pressure
HPT	High pressure thermal
IPB	Imidazole Phosphate buffer
LA	Low-acid
min	Minute
mM	Millimolar
mm	Millimeter
N	number of surviving endospores

N_0	Initial endospore count
NS	Niedrig-sauer
p	Pressure
RTE	ready-to-eat
rpm	Rounds per minute
s	Second
S+	0.85% saline + 0.1% Antifoam B Emulsion
SASP	Small, acid-soluble protein
SS	Steamed sole
ß	Curve shape factor
T	Temperature
t	Time
TMW	Technische Mikrobiologie Weihenstephan
TPYC	tryptone-peptone-yeast extract-carbohydrates
VS	Vegetable soup
wt%	Percentage in weight
XG	Xanthan gum
δ	Time of first decimal reduction

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1 Introduction

The first section of this work provides background information and basic principles regarding the high pressure temperature (HPT) inactivation of the food safety relevant organism *Clostridium* (*C.*) *botulinum*.

1.1 *Clostridium botulinum*

Clostridium botulinum is an ubiquitous, rod-shaped, gram-positive, anaerobic endospore-forming bacteria with the ability to produce human-pathogenic neurotoxins. Its initial name was *Bacillus botulinus* ("botulus", Latin word meaning sausage), which was given by van Ermgen who isolated the organism from raw salted ham and the spleen of a deceased consumer in 1897. Being the most potent neurotoxin known so far, as little as 30 ng of botulinal neurotoxin (BoNT) are sufficient enough to cause fatal illness by consuming contaminated food (Collins and East 1998) (Peck 2006). Later on, the falsely as *Bacillus* described anaerobic organism was assigned to the genus *Clostridium,* whereas *C. botulinum* was further divided into four phylogenetically different groups (Table 1). Neurotoxins produced by proteolytic group I and non-proteolytic group II organisms (toxin type A, B, E, F) are responsible for botulism in humans and represent key food safety determinants. The species *C. botulinum* cannot be considered a taxon following strict criteria of bacterial taxonomy. Instead, it consists of a very heterogeneous group of taxa and its classification depends on the ability to produce BoNT. It is therefore not surprising that other *Clostridium* species are also able to produce one or more BoNTs. This, for example, includes strains of *C. butyricum* or *C. baratii* (Suen, Hatheway et al. 1988). The high heterogeneity of the species *C. botulinum* is illustrated in Figure 1 (also see Figure 7). Notably, group I organisms are closely related to the non-toxigenic organism *C. sporogenes* whereas group II apparently does not have a non-toxigenic or any other relative, which exhibits a high homology to this group II (Lenz 2017). In general, one strain produces one certain type of BoNT and the localization of the corresponding gene (chromosome, plasmid (Marshall, Bradshaw et al. 2007), bacteriophage (Raffestin, Couesnon et al. 2009) encoded) depends on the toxin-type and -strain. The high heterogeneity can most likely be attributed to toxin gene transmissibility. For example, genes coding for toxin type E

have been found on large plasmids (Zhang, Hintsa et al. 2013) although type E toxins are generally chromosomal located. Furthermore, possible toxin gene transfer has been associated to a transduction in the presence of defective phages, e.g., transfer from neurotoxigenic *C. butyricum* to a nontoxigenic *C. botulinum* type E-like strain (Zhou, Sugiyama et al. 1993).

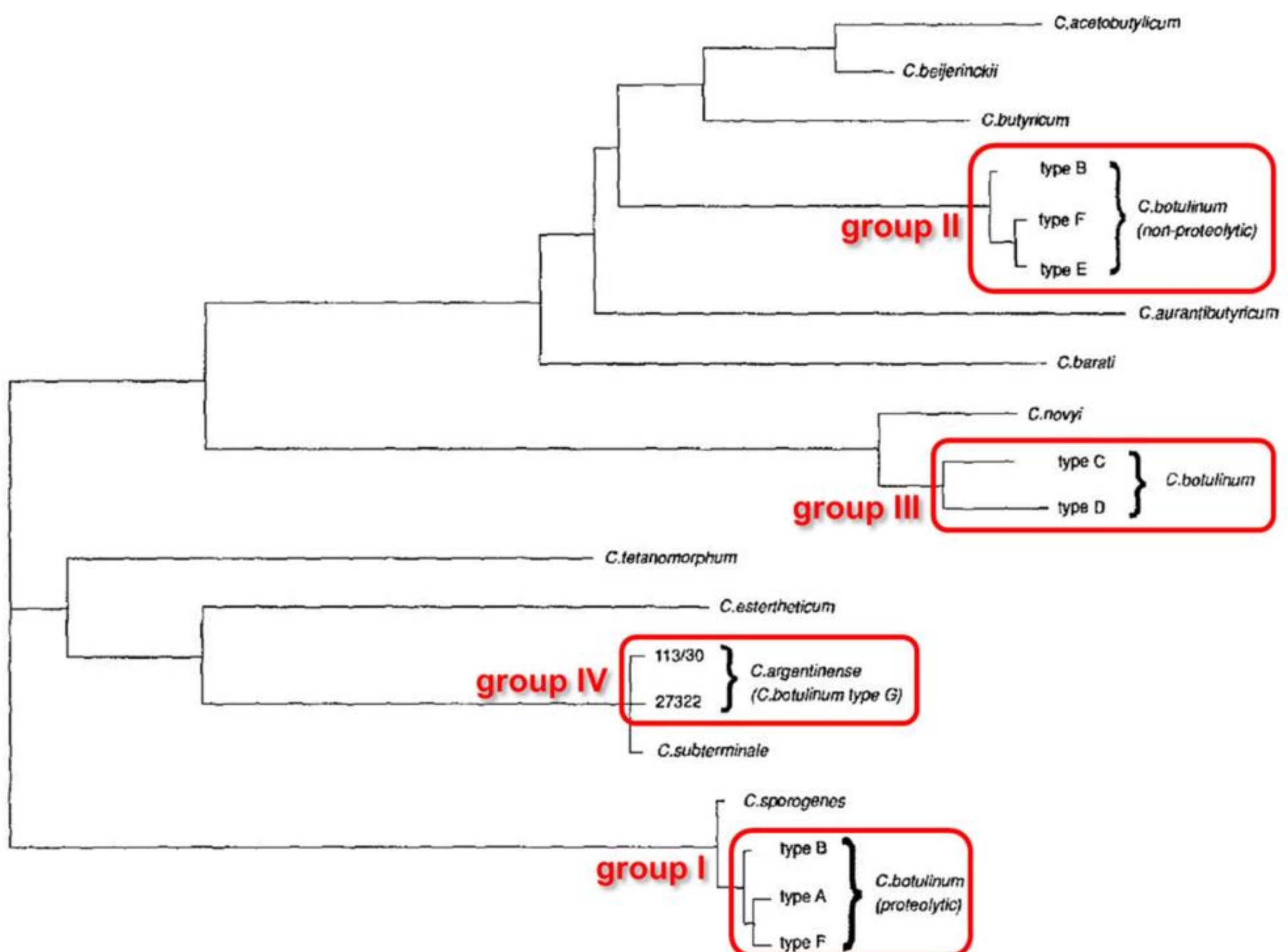

Figure 1. Phylogenetic dendrogram of *C. botulinum* species showing evolutionary distances given by the sum of the horizontal lengths (physiological *C .botulinum* groups indicated in figure adopted from Hutson, Thompson et al. (1993), Lenz (2017)).

Table 1. Phenotypic differences between four groups of *C. botulinum.*

Characteristic	Groups			
	I	II	III	IV
Toxin type	A, B, F	B, E, F	C, D	G
Proteolysis	+	-	-	+
Fermentation of:				
Glucose	+	+	+	-
Fructose	±	+	+	-
Mannose	-	+	+	-
Maltose	±	+	±	-
Sucrose	-	+	-	-
Trehalose	-	+	-	-
Lipase	+	+	+	-
Growth temperature [°C]				
$T_{minimum}$	10	2.5 – 3.3	15	12
$T_{optimum}$	35 – 40	18 – 25	35 – 40	35 – 40
$T_{maximum}$	48 – 50	45	n/a	~40
Minimum pH	4.6	5.0	n/a	n/a
Inhibitory NaCl conc.	10%	5%	3%	>3%
Inhibitory a_w	0.93 – 0.94	0.97	n/a	n/a

According to: (Collins and East 1998) (Peck 2006) (Setlow and Johnson 2007)

1.1.1 Botulism and its prevalence

As indicated above, botulism is a life threatening, neuroparalytic disease which generally results in respiratory paralysis and failure as well as cardiac arrest (Johnson 2013). Besides foodborne intoxication, botulism can also be caused by the growth and toxin production of *C. botulinum* in the intestine or in wounds. The former primarily affects humans with an undeveloped intestinal microbiota (i.e. typically infants up to 12 month of age; also referred as infant botulism), elderly with weakened intestinal microbiota or people with gastrointestinal disease (Hatheway 1990). The latter way of intoxication occurs due the colonization of *C. botulinum* in wounds with subsequent toxin production under favorable conditions (Merson and Dowell Jr 1973). Typically for foodborne botulism, BoNTs are preformed during growth phase and released in the surrounding food matrix. Botulinal neurotoxins are protein complexes of around 150 kDa, which are composed of a toxigenic and non-toxigenic unit. The non-toxigenic protein unit provides protection against detrimental environmental influences, e.g. gastric acid and proteases or heat during food processing (toxin inactivation by heat: 80 °C for 30 min or boiling for 5 min) (Hatheway 1990, Dodds 1993). Once the intoxicated food is ingested and the BoNT is in the circulation, the neurotoxin that reaches peripheral nerve endings binds onto their cell surface and penetrates them by receptor-mediated endocytosis. Inside the neuron, BoNTs cleave SNARE proteins, which are essential for exocytosis. Hence, synaptic vesicles containing acetylcholine (neurotransmitter) cannot be released into the synaptic cleft. The missing signal transduction leads to the characteristic flaccid paralysis of botulism (Arnon, Schechter et al. 2001). Fortunately, botulism is a rare disease. For example, the reported cases of foodborne botulism in the European Union in 2015 and in the United States of America in 2016 did not exceed the number of 201 and 205 cases, respectively (ECDC 2016, CDC 2018). Particularly advances in food processing, in quality of supportive care and the availability of antitoxins contributed to a reduction of the fatality rate from 60 – 70% in the 1800s and 1900s to currently 5 – 10%. Nonetheless, botulism still represents a major hazard to the food industry and determines the framework conditions of food processing and preserving methods, especially for low-acid foods.

1.1.2 Relevance in low-acid foods

Low-acid (LA), shelf-stable (storage at room temperature) foods are of special interest regarding the prevention of foodborne botulism caused by proteolytic *C. botulinum* strains belonging to the physiologic group I of this heterogeneous species (Table 1) (Collins and East 1998). This is related to the absence of common hurdles preventing growth and the production of BoNT in such food products, i.e., storage temperatures < 10°C, pH values < 4.6 and water activity values < 0.93. Consequently, the primary causes for foodborne BoNT intoxications of group I organisms are insufficient sterilization and the ineffectiveness of other growth-inhibiting measures, which facilitate the survival of *C. botulinum* endospores, their germination and outgrowth. In contrast to proteolytic strains, non-proteolytic endospores from *C. botulinum* type E strains are able to germinate and grow under refrigerated temperatures as low as 3 °C. This hazard is of special concern regarding the microbiological safety of chilled storage foods, especially in the absence of additional hurdles such as pH ≤ 5.0 or water activity ≤ 0.97. In both cases, it is important to note that growth concomitantly may result in toxin production in the food product (Graham, Mason et al. 1997, Peck 2006). Although *C. botulinum* are generally widely spread in nature, the occurrence on food of a certain organism usually reflect their prevalence in the geographical origin of the food. For example, fish products are mainly contaminated with endospores of non-proteolytic *C. botulinum* type E since their origin is generally associated with aquatic environments. On the other hand, foods originating from continental soil (e.g. vegetables, meats) are often contaminated with endospores of proteolytic *C. botulinum* type B. In general, the natural contamination levels on food samples are very low (approx. 10 to 1000 endospores/kg) (Dodds 1993, Lindström and Korkeala 2006, Peck 2006, Lenz 2017). However, any type of food can be contaminated with *C. botulinum* endospores. This is due to complex formulations of food products with different ingredients, plant hygiene or human error (Pflug 2010, Sachdeva, Defibaugh-Chávez et al. 2010).

1.1.3 Bacterial endospores of *C. botulinum*

Clostridium species are able to transform from a vegetative into an extremely robust and dormant state. This transformation process is called sporulation and is generally induced due unfavorable conditions, e.g. starvation for carbon and/or nitrogen sources and/or a lack of moisture. Formed bacterial endospores can withstand stress factors such as heat, radiation, high pressures and pH extremes and are able to survive harsh conditions for hundreds of years (Setlow 2007, Setlow 2011). Once sporulation is initiated, an asymmetric cell division takes place whereby a smaller forespore and a larger mother cell are formed. As sporulation proceeds, the mother cell grows around the forespore, which leads to a double-layer membrane engulfed forespore. During maturation, a thick peptidoglycan cortex is synthesized between the outer and the inner forespore membrane. This process is accompanied by a drastic decrease in volume and water content of the forespore. In addition, large amounts of dipicolinic acid (DPA) and small, acid-soluble proteins (SASPs) are synthesized and Ca^{2+}-ions are accumulated in the endospore core compartment. This enhances ongoing dehydration even further and contributes to the typical increased heat resistance. Finally, when maturation is complete (approx. 6 – 8 h), the fully developed endospore (Figure 2) is released by lysis of the mother cell (Stragier and Losick 1996, Errington 2003, Setlow 2007).

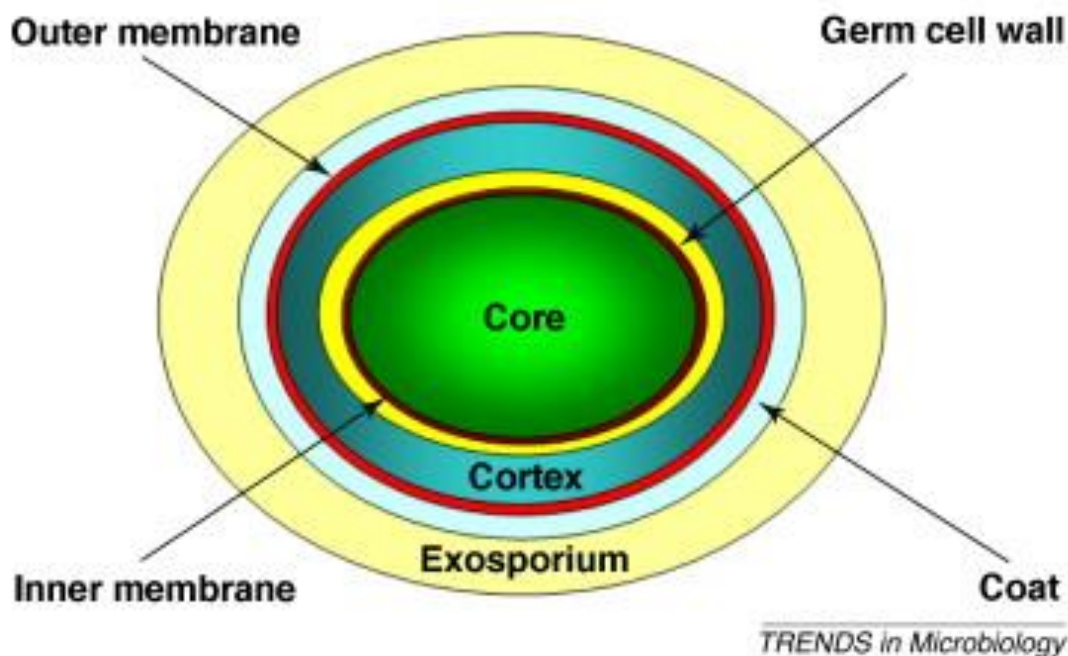

Figure 2. Schematic structure of a bacterial endospore. The different layers are highlighted by different colors and are not drawn to scale. Note, that an exosporium is not present in all endospore-forming species (Paredes-Sabja, Setlow et al. 2011).

Even though endospores can remain in a metabolically dormant state over years, they can also return to vegetative cells by undergoing germination and outgrowth. Germination can be triggered by penetrating of specific nutrients (e.g. amino acids or sugars) or by non-nutrient factors (e.g. high pressure), which bind to or activate specific germinant receptors. This triggers the release of monovalent (H^+, K^+) and divalent cations (Zn^{2+}) and DPA. The loss of these ions is counterbalanced by the uptake of water, simultaneously resulting an increase of pH, which is necessary for the functionality of enzymes and finally, metabolism. The decrease in DPA-Ca chelates concentration inside the endospore core further enhances core rehydration, resulting in decreased wet-heat resistance. This first stage of germination is then followed by a second one which includes cortex peptidoglycan hydrolyses, ongoing core rehydration, core expansion, reactivation of enzymes and protein mobility. This finally leads to outgrowth (metabolism, SASPs degradation, synthesis of macromolecules, emerging from remaining endospore structures) which is culminating in active cell division (Setlow 2007).

1.2 Thermal processing of food

The systematic preservation of foods by heat goes back more than 200 years and is connected to food supply problems of Napoleon's enormous armies marching through Europe. In 1810, the Frenchman Nicolas Appert successfully described in detail a new method to preserve foods in hermetically sealed containers (glass bottles sealed with cork and wax) by boiling them in water for a particular amount of time (Appert 1810). Although this method performed well, the exact mechanisms behind achieving shelf-stable foods remained unknown until Luis Pasteur revealed in 1860, that microorganisms are responsible for food spoilage. Besides microorganism responsible for spoilage, food-borne pathogens are the major food safety issue to be controlled. In particular, bacteria with the ability to form highly resistant endospores play a key role in designing and performing proper heat treatments regarding the production of safe foods.

To date, the decimal reduction time (D-value) is predominantly used to determine the resistance of specific microorganisms. The D-value specifies the time necessary to inactivate 90% or 1-log cycle of the initial endospore or cell count at a defined temperature in a certain medium. The D-value is based on simple log-linear models and allows the prediction and design of safe commercial processes. However, conventional thermal processes cause severe food quality losses due to significant temperature gradients in the product (Reineke 2013) and low heating rates at ambient pressure. The appropriate utilization of official recommendations for the production of safe, LA food products mainly depend on the intended storage conditions, in particular, chilled storage or storage at ambient temperature. Food products allowing the growth of non-proteolytic *C. botulinum* strains are assumed safe when exposed to a so called 6D process (a reduction of six orders of magnitude). In general, a thermal treatment at 90 °C for 10 min or equivalent lethality is recommended to achieve a 6-log cycle reduction of endospores. Such a treatment should ensure proper food safety for up to 10 days at $T \leq 5$ °C, provided that a constant and adequate cooling chain is maintained (ACMSF 1995, Peck 2006, FSA 2008). On the other hand, the current practice of thermal processing to inactivate proteolytic *C. botulinum* endospores and achieve commercial sterility of LA foods stored at ambient temperature is still based on the

12-D concept, i.e., the application of a thermal process sufficient to theoretically result in a 12-log cycle reduction in the numbers of viable endospores. This concept is based on an early study published in 1922 which investigated the endospore heat resistance of over one hundred strains of *C. botulinum* in phosphate buffer (Esty and Meyer 1922). Based on this data, a $D_{121.1°C}$-value of 0.21 min was established for heat sterilization of food products. Hence, a thermal process at 121.1 °C for ~2.5 min would result in a 12-log cycle reduction, however, this is hardly possible to proof in practice and has never been demonstrated.

1.3 High pressure processing of food

The application of high hydrostatic pressures (HHP) at ambient temperatures to preserve foods is a non-thermal food processing method. The pressure that is applied in commercial operations usually ranges from 200 up to 600 MPa. Its application regarding the inactivation of food-borne microorganisms was first described by Hite and co-workers more than 100 years ago, in 1899. They strived to find alternative preservation methods for heat sensitive foods such as milk, meats or fruit juices. Despite the difficulties to generate and hold such high pressures, they managed to prolong the shelf-life of milk compared to untreated milk as well as reduce sensorial quality losses occurring due to heat treatments (Hite 1899). However, the industrial response to the growing demand for minimally processed, high quality food products took 93 more years. In 1992, Meidiya Food Company introduced HHP-treated fruit jams, jellies and sauces with almost natural, fresh-like appearances into the Japanese food market (Hayashi 1996). Altering original flavor, texture, nutrient content and color of foods to a lesser extent is a crucial advantage of this technology compared to conventional heat treatments. Furthermore, post-processing recontamination with microbial pathogens can be prevented since food products are usually pressure-treated in packed form (Huang, Wu et al. 2017). To date, over 500 million kg of diverse HHP-treated food products are produced worldwide. Those include fish and seafood products (~12%), meat products (~27%), vegetable products (~27%), juices and beverages (~14%) and other products like sauces, dips and dressings (~20%) (Elamin, Endan et al. 2015). Regarding the HHP processing of vegetative bacteria (e.g. *Listeria monocytogenes*), significant inactivation can be achieved within minutes at moderate

temperatures and pressures between 400 and 600 MPa, although HHP resistance can vastly vary among different species (Gänzle and Liu 2015). However, the main constraint of implementing HHP processing for the production of safe LA foods is its insufficiency to inactivate relevant, highly resistant bacterial endospores at ambient temperatures (Black, Setlow et al. 2007), for which current available industrial HHP units are designed and constructional limits are reached.

1.3.1 General principles of high pressure processing

The pressure distribution follows the isostatic rule, which states that HHP transmission is constant and instantaneous throughout the entire product and by implication, independent of its size and shape (Norton and Sun 2008). Thus, different packaged food products of varying volumes can be pressurized in the same batch. The applied pressure is mechanically generated (e.g. due to an intensifier pump) and transferred by a low-compressibility liquid (e.g. water or fluids containing silicon oil, glycol or ethanol) (Hugas, Garriga et al. 2002). During the pressure build-up phase, a rise of temperature occurs inside the HHP vessel. This effect is known as adiabatic heating. The applied work of compression results in a temperature increase without any heat exchange (ideally) (Norton and Sun 2008). The reverse effect is called adiabatic cooling and occurs during decompression. Adiabatic effects ideally lead to highly homogeneous and rapid temperature changes within the food product and, thereby, constitute another main advantage of HHP processing (Knoerzer, Buckow et al. 2010). Besides the geometry of the system and the pressure built-up speed, the temperature increase of the food product is highly dependent on the food matrix composition. Water at 25 °C for example, exhibits an increase in temperature of ~3 °C per 100 MPa whereat olive oil at 25 °C can increase up to ~9 °C per 100 MPa (Ting, Balasubramaniam et al. 2002). Consequently, the food composition can markedly influence the extent of increase and decrease in temperature during compression and decompression. Therefore, the process control (p/T/t), i.e., the establishment of isothermal/isobaric holding times is crucial for the assessment of defined HHP/HPT treatments (Figure 3).

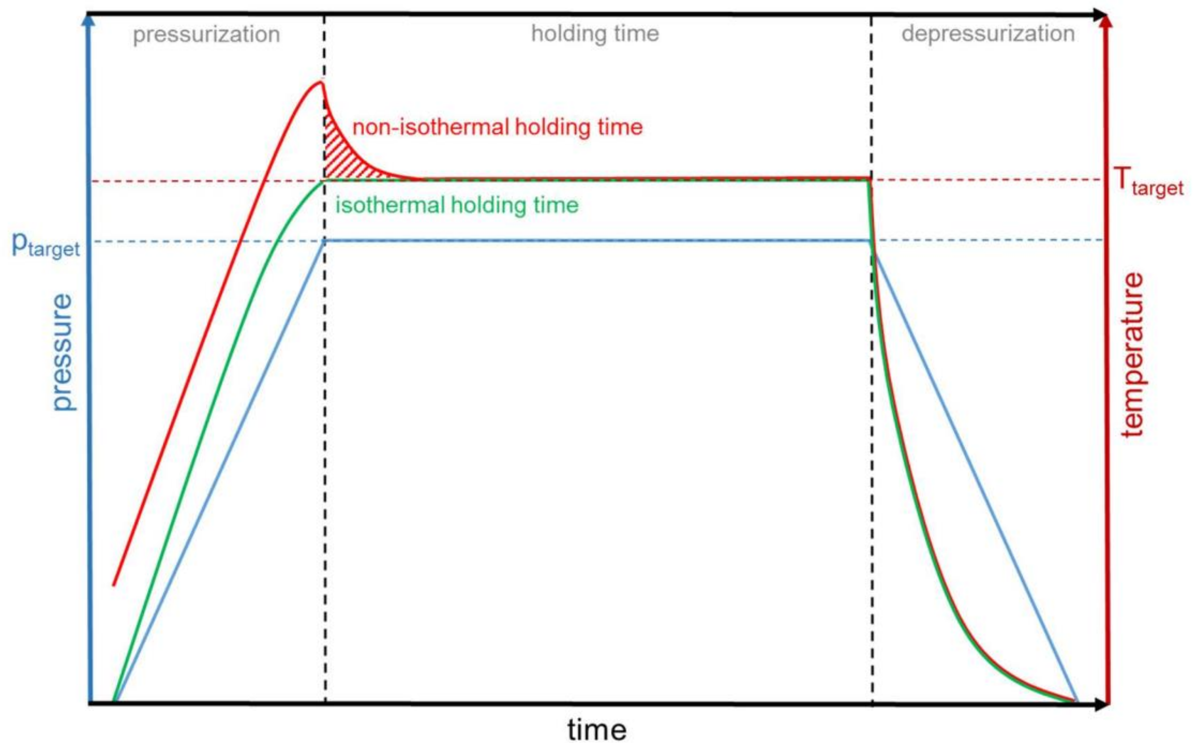

Figure 3. Isothermal/isobaric holding time vs. non-isothermal holding time. The temperature profile of an isobaric/isothermal holding time, i.e. taking the adiabatic heating into account, is depicted in green. The non-isothermal holding time is highlighted as the red shaded area, showing the exceedance of the desired target temperature (T_{target}) and the equilibrium over time (Lenz 2017).

1.4 Combining high pressures and temperatures

A promising alternative technique to inactivate bacterial endospores and ensure the safety of LA foods is the combination of high hydrostatic pressures with elevated temperatures. The rationale behind this merge of technologies is to use potential synergistic effects to reduce endospore counts more effectively. Thereby, initial endospore counts should be decreased to comparable levels which are achieved by traditional thermal processes. Additionally, the total thermal load applied on the product can be reduced (Sevenich, Rauh et al. 2016) by shortening the total process duration. A more comprehensive overview of benefits and limitations of HPT processing are given with the following bullet points:

Benefits:

- Pressure and temperature can act synergistically on endospore inactivation (Margosch, Ehrmann et al. 2004, Bull, Olivier et al. 2009).
- Post-processing technology. Prevention of recontaminations (Huang, Wu et al. 2017).
- Rapid and homogeneous heating and cooling resulting in reduced thermal loads (Mathys, Reineke et al. 2009, Sevenich, Rauh et al. 2016).
- Enhanced preservation of nutritional quality (Sevenich 2016).
- Mitigation of food processing contaminants, e.g. furan (41% to 98%) (Sevenich, Bark et al. 2013, Sevenich, Kleinstueck et al. 2014).
- High consumer acceptance (Olsen, Grunert et al. 2010, Jermann, Koutchma et al. 2015).

Limitations:

- High material stress due to high fluctuations of elevated temperatures and pressures. Special vessel constructions (e.g. wire-wounded high tensile steel alloy) (de Orte, B., 2017, Metronics Technologies S.L., Spain, personal communication).
- Low throughput (approx. 264 kg/h) and high processing costs (approx. 0.5 to 1.5 €/kg) (Mujica-Paz, Valdez-Fragoso et al. 2011, Sevenich 2016).
- Discontinuous process and requirement of a preheating system.
- Live monitoring of temperature profile in the vessel and food product during processing is impeded (localization of possible temperature inhomogeneity) (de Orte, B., 2017, Metronics Technologies S.L., Spain, personal communication).
- Lack of coherent strategy for process design regarding food safety. Influence of food matrix on endospore inactivation.

1.4.1 Behavior of *C. botulinum* endospores towards HPT processing

Accelerated but varying inactivation results for endospores from different strains of *C. botulinum* by HPT processing have been reported by several authors (Reddy, Solomon et al. 1999, Reddy, Solomon et al. 2003, Margosch, Ehrmann et al. 2004, Margosch, Ehrmann et al. 2006, Reddy, Tetzloff et al. 2006, Bull, Olivier et al. 2009, Lenz and Vogel 2014, Skinner, Marshall et al. 2014, Lenz, Reineke et al. 2015, Skinner, Morrissey et al. 2018). Consequently, no coherent inactivation strategy could be derived for endospores of *C. botulinum* so far due to their diverse behavior towards HPT processing. This inconsistency becomes most apparent when considering the findings of Margosch, Ehrmann et al. (2006) for proteolytic *C. botulinum* strain TMW 2.357. There it has been demonstrated that HPT treatments at 600 and 800 MPa in combination with temperatures of 100, 110 and 120°C resulted in a reduced endospore inactivation compared with equivalent thermal treatments at ambient pressure. These findings indicated a pressure stabilization of a spore fraction within the population during isobaric/isothermal holding times. Modeled isoeffect lines suggest that this effect of endospore stabilization also occurs at pressure levels below 600 MPa. However, experimental data in a lower pressure range, which could verify the accuracy of this model or disprove it, are missing. Additionally, it is difficult to predict from existing data, whether stabilization effects also occur in relevant LA food matrices.

Endospore destruction at high pressures (approx. 200 up to 600 MPa) and elevated temperatures (above 60 to 70 °C) is commonly thought to be a physiochemical process which underlies an at least two-step mechanism. Initially, sublethally injured endospores start to release DPA due to increasing pressures and temperatures. Accumulated DPA (~20% of endospore core dry weight) in the endospore's core decreases the water content and thereby contributes to wet heat resistance. Hence, a DPA release results in partial core hydration and in a concomitant loss of heat and pressure resistance (also see section 1.1.3). Thereby, DPA release is usually accelerating with increasing process intensity whereat the endospore's resistance is determined by its ability to retain DPA as well as the pressure/heat resistance of the DPA-free endospore (Margosch, Ehrmann et al. 2004, Margosch, Gänzle et al. 2004, Setlow 2011, Reineke 2013, Lenz 2017).

1.4.2 Matrix parameters affecting HPT resistance of endospores

The property of the endospore surrounding matrix can have significant influence on the outcome of HPT experiments. Primary components/parameters affecting endospore resistance during HPT experiments are fat, protein, salt and carbohydrates as well as the pH value and the water activity. A major impact has been attributed to the pH value of the surrounding matrix. Elevating pressures and temperatures cause a shift of the dissociation equilibrium, i.e., concomitant changes in pH value. This is important regarding HPT experiments conducted in water and simple aqueous solutions like buffers because not pressure- and temperature-stable solutions undergo a change in pH and might accelerate or decelerate endospore inactivation (Mathys, Kallmeyer et al. 2008). In general, lower pH values are thought to increase endospore inactivation. This has been shown for endospores of proteolytic *C. botulinum* type B in Tris-His buffer and for endospores of *B. coagulans* in citrate phosphate buffer at pH values ≤ 4.0 (Roberts and Hoover 1996, Margosch, Ehrmann et al. 2004) or for endospores of *B. cereus* in a complex matrices (sucrose, soybean protein and oil) for pH values < 7.0 (Gao and Ju 2010). Thereby, it is thought that low pH values contribute to alterations in the endospores' permeability barrier which enhance the leakage of DPA and decrease resistance during HPT treatments (Margosch, Ehrmann et al. 2004). However, changes in the pH of complex food matrices cannot be predicted or calculated and have to be measured during HPT processing.

Another important influence has been attributed the total water content and to the a_w. The a_w is strongly dependent on the on the present type of solute as well as its concentration. Generally, a low a_w is thought to increase the resistance of endospores towards high pressure and temperature (Sale, Gould et al. 1970, Sevenich, Reineke et al. 2015) which is basically connected to the fact that the presence of water is necessary for an effective inactivation of microorganisms in general. The exact mechanisms of an endospore protection due to low a_w are unknown but the protection of proteins from denaturation or impeded rehydration of the inner endospore compartments might be involved. Furthermore, one has to consider that substances which can influence the a_w to an equivalent extent might exhibit additional effects on microbial inactivation (Lenz 2017).

To date, the influence of surrounding food components (fat, carbohydrate, salt, protein) on the inactivation of endospores is not coherent. For *Bacillus* endospores, for example, a fat-rich nor a carbohydrate-rich medium exerted additional protection against high pressure and temperature inactivation compared to a less-rich medium (Moerman, Mertens et al. 2001). Gao and Ju (2010), on the other hand, investigated the resistance of *Bacillus* endospores towards the combination of high pressure and heat in complex food model systems. They found that increasing soybean protein and sucrose contents exerted a protective effect on endospores during HPT treatments. Hence, the response of suspended endospores towards their surrounding matrix during HPT processing most likely depends on factors such as the treatment intensity (p/T/t), the suspended component type and concentration and the present species.

2 Motivation and Objectives

Due to the importance of *C. botulinum* for food safety considerations, the lack of HPT inactivation data in relevant matrices presents a fundamental gap of knowledge that has to be closed to come towards a precise evaluation of the safety of HPT processes for the production of shelf-stable LA food products.

To contribute filling this gap, the present work should evaluate the impact of an industrially feasible and preferential pressure range (300 to 600 MPa) in combination with elevated temperatures (80 to 120 °C) on endospores of *C. botulinum* suspended in different LA foods and pressure/temperature-stable imidazole/phosphate buffer (IPB). In addition, conventional thermal treatments at ambient pressure should serve as reference processes in order to evaluate the effect of adding pressure on endospore inactivation. Furthermore, a model system should be developed to evaluate the HPT inactivation of bacterial endospores in complex matrices. Each primary food component should be substituted by a single stereotype component to systematically investigate their effects on the HPT inactivation (600 MPa, 80 – 110 °C). Towards a generic approach, the model system should be used to mimic four low-acid food products and evaluate its application.

Regarding proteolytic *C. botulinum* type B, two specific questions should be targeted that help to gain a better understanding of the nature of endospore stabilization effects:

(i) whether tailing effects depend of initial endospore counts, i.e., whether they occur due to (detection) limits in the experimental design or due to the presence of resistant endospore fractions,

and

(ii) whether surviving endospores retain their resistance properties upon repeated outgrowth and sporulation.

Regarding non-proteolytic *C. botulinum* type E, following main questions should be addressed:

(i) whether the HPT inactivation behavior of endospores obtained in buffer systems is representative and applicable to those obtained in LA foods,

and

(ii) whether possible matrix-dependent protective effects are associated to the aquatic origin of the endospore-former, i.e., are occurring HPT resistances related to the presence of fish oil (or free fatty acids).

3 Material and Methods

3.1 Bacterial strains

The bacterial strains used in this work are shown in Table 2. Proteolytic and non-proteolytic strains of *C. botulinum* were grown in tryptone-peptone-yeast extract-carbohydrates (4 g/L glucose, 1 g/L maltose, 1 g/L starch, 1 g/L cellobiose) (TPYC) broth (Artin, Carter et al. 2008, Lenz and Vogel 2014). Strains were cultivated under anaerobic conditions (85% N2, 10% CO2, 5% H2) at 28 °C for non-proteolytic and at 37 °C for proteolytic strains.

Table 2. Used strains.

TMW	BoNT / group	Alternative name	Origin of isolation
2.990	Type E / II	Kulmbach C2 / Beluga	Fermented whale flippers
2.997	Type E / II	REB 1718	n/a
2.357	Type B / I	REB 89[1)]	n/a

[1)] obtained from the Institut für Medizinische Mikrobiologie und Infektionsepidemologie, Leipzig, Germany

3.2 Endospore production and purification

Growth conditions and endospore purification were basically performed as previously described by Lenz and Vogel (2014). Briefly, endospore production started by inoculating 45 mL of TPYC broth with a -80°C glycerol stock culture and subsequent incubation in an anaerobic chamber (85% N2, 10% CO2, 5% H2) for 24 h at 37°C for TMW 2.357 and at 28 °C for TMW 2.990. The growing culture was then transferred

into 450 mL of fresh TPYC broth and anaerobically incubated for 12 ± 2 d at 37°C or 28 °C, respectively. The produced endospores were harvested by centrifugation (10.000 x g, 4°C, 10 min), washed three times with ice-cold deionized water and one time with S+ (0.85% saline + 0.1% Antifoam B Emulsion (Dow Corning, Germany) to reduce possible endospore agglomeration) followed by incubation in 50% ethanol for 2 h at room temperature. Afterwards the endospore suspension was washed for at least three more times with ice-cold deionized water and, finally, resuspended in deionized water or imidazole phosphate buffer (pH 7, 50 mM Na_2HPO_4 and 50 mM NaH_2PO_4 mixed 1:1 with 50 mM imidazole) obtaining a viable endospore count of 10^8–10^9 endospores/mL. Sporulation resulted in a uniform population of phase bright endospores ≥ 90% as determined by phase contrast microscopy (Figure 4). To check for vegetative cells, a heat treatment at 60 °C for 15 min was applied (appendix Figure 26). The endospore suspensions were stored at 4°C until use.

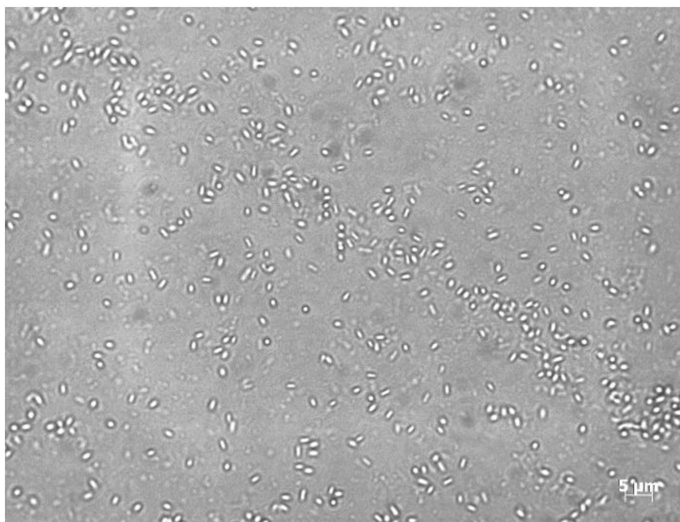

Figure 4. Representative microscopic picture of purified endospore suspension of *Clostridium botulinum* TMW 2.990.

3.3 Determination of endospore surface hydrophobicity

The surface hydrophobicity was determined as described by Kafka, Reitermayer et al. (2014). Briefly, purified endospore suspensions were washed twice (IPB, 5000xg, 5 min, 25 °C) and resuspended in IPB to an optical denisity of OD_{590} of 0.35 to 0.4. The endospore suspensions were mixed with 400 µl of *n*-hexadecane (Sigma-Aldrich

Chemie GmbH, Germany) in round-bottom glass test tubes (10 mm diameter). Following incubation at 30 °C for 10 min, the suspensions were thoroughly vortexed for 2 min and then incubated without movement at room temperature for 15 min to ensure complete separation of the organic and aqueous phases. The absorbance of the aqueous phase was measured at 600 nm before (A_0) and after (A) the treatment with *n*-hexadecane using glass pipettes (Gilford 2600 UV–VIS spectrophotometer).

Table 3. Surface hydrophobicity of *C. botulinum* endospore suspensions.

Strain	*Surface hydrophobicity (%)
TMW 2.357	51.2 ± 1.1
TMW 2.990	7.8 ± 0.3

*Percentage absorbance of the aqueous phase after treatment with hexadecane (400 µl) relative to initial absorbance (A/A_0). Each value represents the mean of three independent determinations.

3.4 Detection of *Clostridium botulinum* type E and B by toxin PCR

A standard DNA isolation kit (E.Z.N.A. Bacterial DNA kit, Omega Bio-tek, Inc., Norcross, USA) was used to extract DNA from overnight growing *C. botulinum* strains. Primers used for the detection of BoNTs are shown in Table 4. Each PCR was performed with a 25 µl mixture containing 15.15 µl HPLC-grade H_2O, 3 µl buffer + $MgCl_2$, 1.2 µl $MgCl_2$, 1 µl DNTPs, 0.75 µl of each primer (forward and reverse, (Lindstrom, Keto et al. 2001)), 0.15 Taq-polymerase and 3 µl of purified DNA template or HPLC-grade H_2O as control sample. The mixture was heated at 95 °C for 15 min and then subjected to 28 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C, and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. PCR products were dyed with loading dye and analysed by 2% agarose gel electrophorese at 90 V and a 100 bp molecular ruler (Figure 5 and Figure 6).

Table 4. Primers used for PCR amplification of type E and B BoNT genes.

Toxin type	Primer	Sequence (5' – 3')	Product size [bp]
E	$CBMLE_{for}$	CCA AGA TTT TCA TCC GCC TA	389
E	$CBMLE_{rev}$	GCT ATT GAT CCA AAA CGG TGA	
B	$CBMLB_{for}$	CAG GAG AAG TGG AGC GAA AA	205
B	$CBMLB_{rev}$	CTT GCG CCT TTG TTT TCT TG	

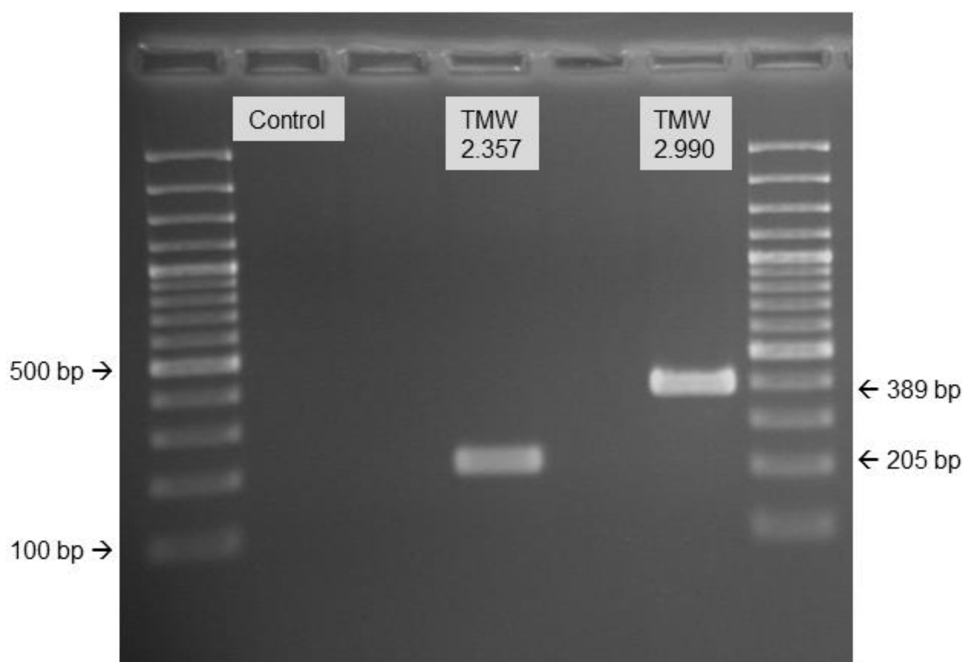

Figure 5. Toxin gene PCR detection of *C. botulinum*. Outer lanes: molecular weight markers. Control lane: negative control. TMW 2.357 lane: *C. botulinum* type B. TMW 2.990 lane: *C. botulinum* type E.

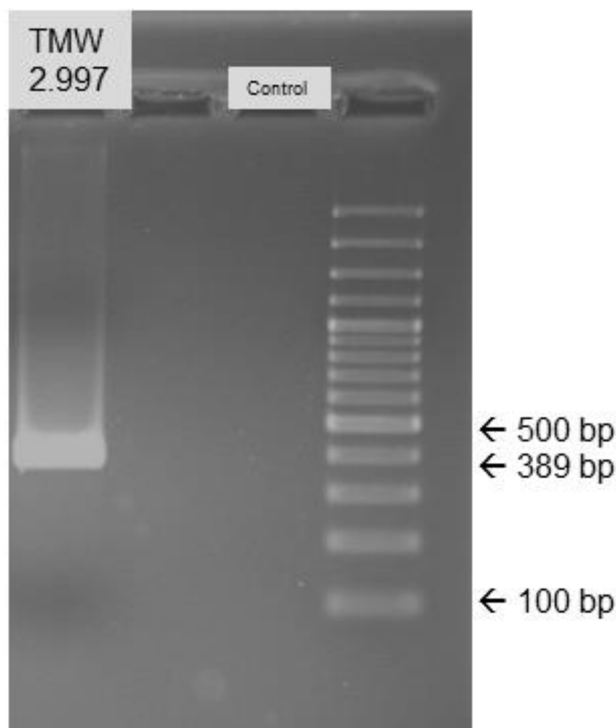

Figure 6. Toxin gene PCR detection of *C. botulinum* TMW 2.997. Outer right lane: molecular weight marker. Control lane: negative control. TMW 2.997lane: *C. botulinum* type E.

3.5 DNA extraction for genome sequencing, detection of gene encoding for botulinum neurotoxin and genomic comparison

The DNA extraction procedure was basically based on the method of Hielm et al. (Hielm, Björkroth et al. 1998). First, 4 ml of an overnight culture (16 h incubation at optimum growth temperature) were harvested (1100 x g, 25 min, 4 °C) and resuspended in 1 ml of formaldehyde mix (10 mM Tris [pH 4.5], 1 M NaCl, 4% formaldehyde solution [37% formaldehyde stabilized with 10% methanol]). After gentle agitation, the sample was incubated on ice for min. 1 h. This was followed by two washing steps with formaldehyde solution (5000 x g, 2 min, 4 °C). Subsequently, the sample was resuspended in 1 ml lysis solution (12 mM Tris [pH 7.5], 2 M NaCl, 200 mM EDTA [pH 8.0], 1% Brij 58, 0.4% deoxycholate, 1% sodium laurolyl sarcosine, 40 µl/ml RNase, 2 mg lysozyme/ml, 40 U/ml mutanolysin) and incubated at 37 °C for min.

2 h with gentle shaking every 15 min. Afterwards, 100 µg/ml proteinase K, 0.5 mM EDTA (pH 8.0) and 0.8% sodium dodecyl sulfate were added and incubated for 1 h at 60 °C. After cell lysis, the standard protocol of a DNA isolation kit (E.Z.N.A. Bacterial DNA kit, Omega Bio-tek, Inc., Norcross, USA) was followed.

Additionally to the toxin PCR, strains were classified using Strain Blast Diagnostic Gene Finder (BADGE), a tool for the for the rapid prediction of diagnostic marker genes, valuable for diagnostics, large scale strain screening or selection for desirable traits (Behr, Geissler et al. 2016). Non-proteolytic strains TMW 2.990 and TMW 2.997 were classified as *C. botulinum* type E based on the identified nucleotide sequence of the BoNT gene encoding for the type E neurotoxin (3759 bp). Likewise, proteolytic strain TMW 2.357 was classified as *C. botulinum* type B based on the identified nucleotide sequence of the BoNT gene encoding for the type B neurotoxin (3876 bp). Furthermore, a genomic comparison of the three used strains was performed by processing the data output of BADGE with the BLAST Ring Image Generator (BRIG) (Alikhan, Petty et al. 2011). The generated Figure 7 (and Figure 25) illustrates the similarity of the two type E strains and, on the other hand, the high heterogeneity within *C. botulinum*. This high heterogeneity is mainly highlighted by the small core genome, which contains just 9 genes present in all individual strains.

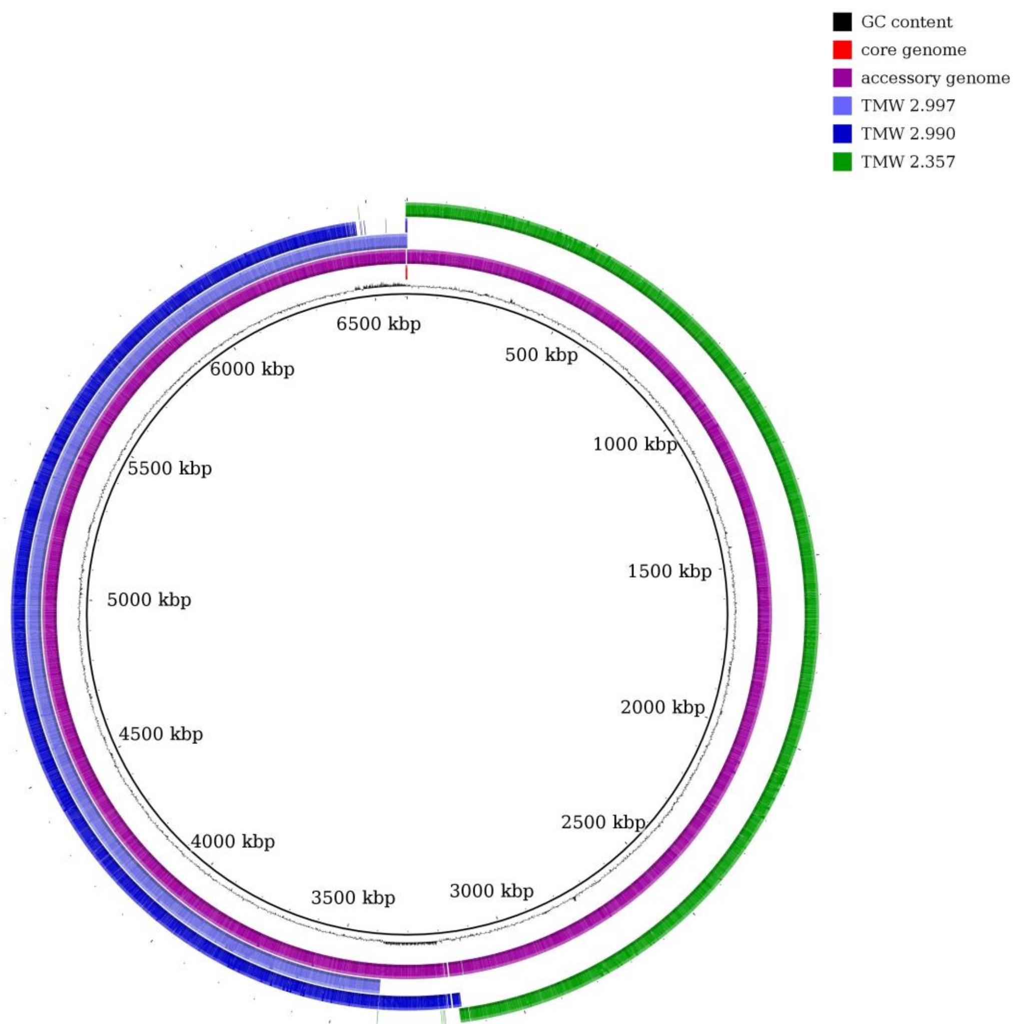

Figure 7. Genomic comparison of TMW 2.990, TMW 2.997 and TMW 2.357 based on the nucleotide sequence and visualized by using BRIG (Alikhan, Petty et al. 2011). The figure enables to show the presence of similarities and differences of all sequenced strains within the species.

3.6 Sample preparation for high pressure temperature and thermal treatments

Samples for experiments in low-acid RTE foods were basically prepared as previously described (Maier, Lenz et al. 2017, Maier, Schweiger et al. 2018). Briefly, heat-

sterilized RTE foods (Table 5) were blended into homogeneous pastes, inoculated with endospore suspension (endospores suspended in deionized water) and thoroughly mixed with a spatula and by vortexing. For experiments in IPB, endospores (endospores suspended in IPB) were homogeneously distributed in IPB. Typically, initial endospore counts were 10^6 to 10^7 endospores/g before thermal or HPT treatments. For HPT experiments, the inoculated samples were filled into custom-made PTFE tubes and closed with silicon stoppers that were fastened by screw-caps. For thermal treatments, samples were prepared in the same manner, but they were filled into custom-made stainless steel tubes, which were of the same size as the PTFE tubes used for HPT treatments. Stainless steel allowed more rapid heating and cooling rates. In addition, uninoculated samples were prepared for temperature profile measurements in the geometrical center of a test vial during processing. All samples were stored on ice before and after treatments.

Table 5. Formulations of four low-acid food products.

Food product	Fat [wt%]	Protein [wt%]	CH[1)] [wt%]	Salt [wt%]	Fiber [wt%]	pH	a_w[2)]
Green peas with ham	12.8	5.1	5.1	1.1	3.9	6.0	0.97
Steamed sole	8.6	10.2	5.7	2.9	1.7	6.75	0.98
Braised veal	6.2	7.6	6.7	3.7	1.5	6.53	0.97
Vegetable soup	2.9	1.2	5.9	1.0	1.3	5.8 – 6.0	0.98

[1)] Carbohydrate; [2)] Water activity

3.7 Food model system preparation

A food model system was used to mimic the composition of the four different food products listed in Table 5. Furthermore, single (stereotype) primary food components

were varied to study their impact on the HPT inactivation of endospores. Each primary food component (fat, protein, carbohydrate, salt) was substituted by a single ingredient, namely, rapeseed oil (commercially available) for fat, casein peptone (Carl Roth, Karlsruhe, Germany) for protein, sucrose (Sigma-Aldrich, St. Louis, USA) for carbohydrate and sodium chloride (Carl Roth, Karlsruhe, Germany) for salt. The composition of the food model system, mimicking the four foods is given in Table 6. The values were adjusted by excluding the respective amount of fiber.

Table 6. Food model system formulations (excluding fiber) mimicking food products (Table 5).

Model system	Fat [wt%]	Protein [wt%]	CH[1)] [wt%]	Salt [wt%]	pH	a_w[2)]
Green peas with ham	12.3	4.9	4.9	1.0	6.0	0.97 ± 0.008
Steamed sole	8.5	10.0	5.6	2.9	6.75	0.96 ± 0.004
Braised veal	6.1	7.5	6.6	3.6	6.53	0.96 ± 0.001
Vegetable soup	2.9	1.2	5.8	1.0	5.8 – 6.0	0.98 ± 0.003

[1)] Carbohydrate; [2)] Water activity

The formulations of the different matrices used to study the impact of varying single food components are given in Table 7. Thereby, a food model matrix with average values (10wt% fat, protein and carbohydrate; 2wt% for salt; pH 5.8) served as a base matrix (BM). Originating from the BM, only one single parameter was changed to its maximum or minimum value at a time. For example, the impact of a high fat content was studied by adjusting its value to 20wt% whereas the remaining components were held constant (10wt% protein and carbohydrate; 2wt% salt; pH 5.8). In the case of fat variation, the contents of the remaining solutes (protein, carbohydrate, salt) were calculated with regard to the aqueous phase. In doing so, their concentrations

remained constant and possible occurring effects could be contributed to the varying amount of fat.

Table 7. Food model system formulation with varying primary food components.

	Fat [wt%]	Protein [wt%]	CH[1)] [wt%]	Salt [wt%]	pH	Xanthan gum [wt%]
Maximum	20	20	20	4.0	7.0	0.3
Base matrix	10	10	10	2	5.8	0.3
Minimum	0	0	0	0	4.6	0.3

To prepare the food model systems, all components except fat and xanthan gum (XG) were dissolved in deionized water and sterilized by autoclaving (121.1 °C, 15 min) before each experiment. Carbohydrates were autoclaved separately from protein and salt. Afterwards, the sterilized solutions were mixed with the respective amount of fat and processed by homogenization at maximum speed (35.000 rpm) for 5 min (Ultra-Turrax MICCRA-D1, ART, Müllheim, Germany). Prior to homogenization, 0.3wt% of XG (Carl Roth, Karlsruhe, Germany) was added for stabilization of the two-phase system. After processing, the model system was adjusted to its respective pH value (1M HCL) and stored at 4 °C until use. The resulting aw-values of the model systems are given in Table 8.

Samples for HPT experiments including free fatty acids were prepared as followed. Oleic acid (Sigma-Aldrich, St. Louis, USA) and stearic acid (Merck, Darmstadt, Germany) were reported as being more than 90% and 98% pure, respectively. Stock solution of 100 mM were prepared by dissolving free fatty acids (FFA) in ethanol absolute. For HPT treatments, these solutions were added to IPB (pH 7) to a final concentration of 2 mM, which was lower than their critical micelle concentration

(Lekogo, Coroller et al. 2010). Subsequently, samples for HPT treatments were basically prepared as described in section 3.6.

Model emulsion consisting of 10% fish oil from menhaden (natural fat content approx. 9 to 13%; Sigma-Aldrich, St. Louis, USA), IPB (pH 7) and 0.5% Tween 80 were prepared as follows. The emulsifier tween 80 and fish oil were stirred together for at least 1 h before mixing with IPB. Afterwards, all ingredients were combined and homogenized for 5 min at 34.000 rpm (Ultra-Turrax MICCRA-D1, ART, Müllheim, Germany). Emulsions were freshly prepared and stored at 4 °C before each HPT experiment.

Table 8. Resulting a_w-values due to varying contents of fat, protein, carbohydrates and salt and pH values.

Food model system	a_w-value[2)]
Base matrix	0.95 ± 0.005
Base matrix fat[1)]	0.96 ± 0.001
0wt% fat	0.96 ± 0.006
20wt% fat[1)]	0.95 ± 0.002
0wt% protein	0.97 ± 0.002
20wt% protein	0.93 ± 0.001
0wt% carbohydrate	0.97 ± 0.002
20wt% carbohydrate	0.94 ± 0.001
0wt% salt	0.97 ± 0.002
4wt% salt	0.94 ± 0.002
pH 4.6	0.95 ± 0.002
pH 7	0.95 ± 0.002

[1)] Contents of protein, carbohydrate and salt calculated with regard to the aqueous phase

[2)] Water activity measured with Labmaster – aw, Novasina AG, Switzerland

3.8 High pressure temperature equipment and treatments

The dual vessel HP unit TMW-RB (Knam Schneidetechnik GmbH, Langenargen, Germany; V = 2 x 7 mL, p_{max} = 800 MPa, T_{max} = 70 °C) was used for initial experiments at moderate temperatures (70 °C). Temperature inside the pressure vessels was controlled via thermostating jackets, which were connected to an external circulating water bath (FC 600; JULABO Labortechnik GmbH, Seelbach, Germany). A mixture of 70% polyethylene glycol 400 (Roth, Karlsruhe, Germany) and 30% deionised water was used as pressure-transmitting fluid. Samples were filled into 0.5 mL cryotubes with an internal thread (Thermo Fisher Scientific, Bonn, Germany) and stored on ice before treatment. Prior to pressurization, samples were placed into a preheated pressure vessel and incubated for 5 min at the respective temperature. This and the fact that the temperature profile over time during pressure treatments was not measured inside the sample but in its surrounding medium led to non-isothermal conditions during HPT treatments.

The HPT equipment used for process temperatures up to 120 °C consisted of a hand pump-driven high pressure intensifier system (Unipress, Warsaw, Poland). With an inner volume of 8 mL. Two samples could be placed inside the high pressure vessel (type MV2-13, Unipress, Warsaw, Poland) at a time. Bis(2-ethylhexyl) sebacate (Nr. 84822; Sigma-Aldrich, USA) served as a pressure transmitting fluid. The double wall high pressure vessel was temperature-controlled by a circulating oil bath (witeg Labortechnik GmbH, Wertheim, Germany) with silicon oil (Sil 180, Fisher Scientific, New Hampshire, USA) as a heating fluid. The lid of the high pressure vessel was equipped with a lead-through for a type K thermocouple (Figure 8).

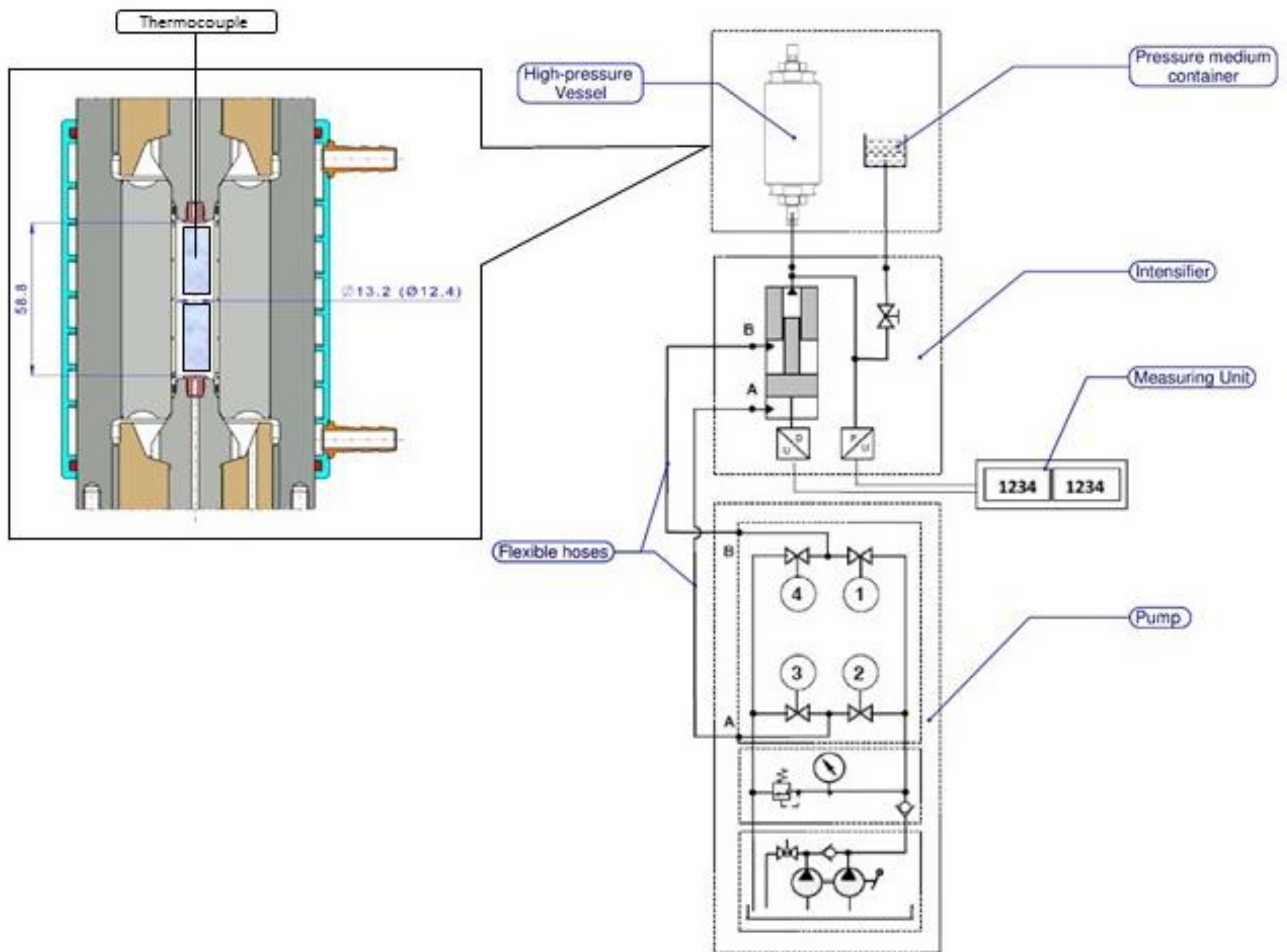

Figure 8. Schematic diagram of high pressure intensifier system including a cross section of the high pressure vessel M2-13. Adapted with permission by Unipress, Warsaw, Poland (2013) (2014).

To achieve isobaric/isothermal pressure-holding times, the start of compression began at an empirically determined target pressure, target temperature, and sample type-dependent temperature. This starting point is shown exemplarily in Figure 9. The temperature was monitored in the geometrical center of an uninoculated sample vial directly above the inoculated sample. Pressure levels applied ranged from 300 up to 600 MPa, process temperatures from 80 to 120°C and pressure-holding times from 1

to 300 s. The average compression rate was around 6.5 MPa/s and decompression took place in less than 12 s.

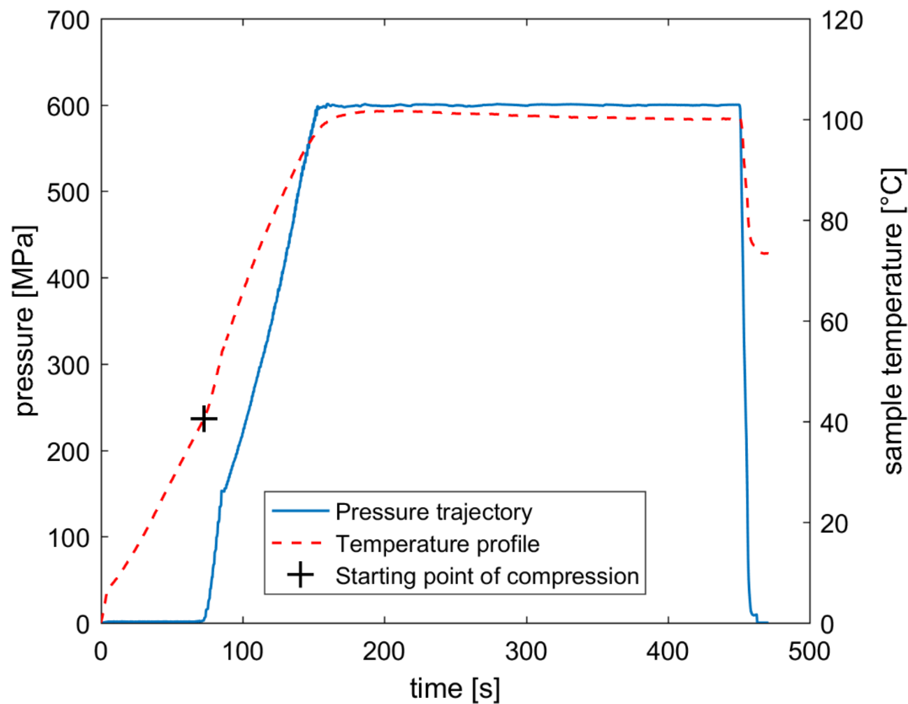

Figure 9. Typical temperature and pressure profiles during HPT treatments. Exemplarily, pressure (dashed line) and temperature (solid line) profiles of a HPT treatment with a target pressure of 600 MPa, a target temperature of 100°C and a pressure-holding time of 300 s are depicted. A cross marks the starting point of compression (40.5°C, 72.5 s).

3.9 Thermal treatments

Thermal inactivation experiments at ambient pressure were performed similar to the HPT experiments with the exceptions that samples were not pressurized and stainless steel sample tubes were used. Thermal treatments were applied at temperatures ranging from 80 to 121.1°C °C and holding times from 1 to 600 s. The average heating rate was around 0.3 °C/s (Figure 10).

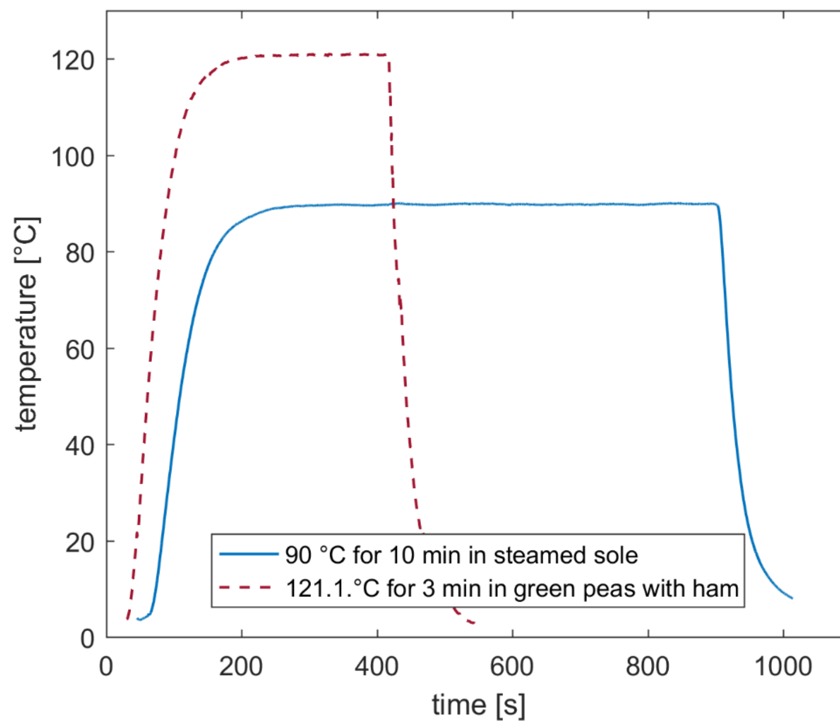

Figure 10. Temperature profile over time during thermal treatment at ambient pressure. Exemplarily, temperature profile in steamed sole (solid blue line) and in green peas with ham (red dashed line at 90 °C for 10 min and 121.1°C for 3 min, respectively.

3.10 Enumeration of surviving endospores

Surviving endospores were enumerated by pour-plating with TPYC agar (15 g/L agar–agar) immediately after treatment. Samples were opened in an anaerobic chamber (85% N2, 10% CO2, 5% H2), serially diluted in S+ solution and pour-plated in duplicate. Survivors were counted after anaerobic incubation for up to 5 d at 28 °C for non-proteolytic strains and at 37 °C for proteolytic strains. For visualization, the results are presented as $\log_{10}(N/N_0)$, where N describes the number of surviving endospores after a treatment and N_0 is the initial endospore count. The detection limit was defined at a minimum of two colony-forming units in relation to the initial cell count N_0.

3.11 Statistical analysis

All experiments were conducted at least in independent duplicates. The significance of differences between mean values from independent experiments was determined by one-way ANOVA. Tukey's HSD test at an error probability of 5 % ($P < 0.05$) served as a post-hoc analysis. If applicable, isobaric/isothermal inactivation curves, i.e. excluding the lethal effect of the pressure ramp (1s pressure holding time), were described by applying the following modified Weibull distribution (Mafart, Couvert et al. 2002):

$$Log\frac{N}{N_0} = -\left(\frac{t}{\delta}\right)^{ß} \qquad \text{Eq. 1.}$$

or

$$t = \delta\left(log\frac{N}{N_0}\right)^{\frac{1}{ß}} \qquad \text{Eq. 2.}$$

where $\log(N/N_0)$ is the decimal reduction ratio at a time t. The scale parameter δ can be considered as an equivalent to the traditional D-value (first-order kinetic) and represents the time of first decimal reduction. The factor ß describes the curve shape, where values < 1 imply upward concave (tailing) inactivation patterns and values > 1 describe downward concave (shoulder) curve shapes, and curve linearity is described by ß = 1 (Peleg and Cole 1998, Mafart, Couvert et al. 2002). The flattening of biphasic survivor curves or in other words, drastically decreased inactivation rates at longer holding times are generally described as tailing. The exact reasons remain unclear but proposed contributing factors include heterogeneous resistance properties within an endospore population, endospore clumping, adhesion to any surfaces during sample handling and protective effects of dead endospores (Cerf 1977, Margosch, Ehrmann et al. 2006, Ahn, Balasubramaniam et al. 2007, Mathys 2008, Wilson, Dabrowski et al.

2008). In contrast, an initial lag phase or shoulder effect in inactivation curves describes an initial increase in cell count from treated compared to untreated endospore samples. The disassembly of endospore agglomerates or a pressure-induced germination of superdormant endospores are two reasons often discussed in literature (Mathys, Heinz et al. 2007, Wei, Shah et al. 2010, Lenz 2017). Statistical analysis and curve fitting were performed using MATLAB software (version R2016B, Mathworks, Natick, USA).

4 Results

4.1 Inactivation of TMW 2.357 (group I / BoNT B) endospores in low-acid foods

4.1.1 HPT inactivation of TMW 2.357 endospores in green peas with ham, steamed sole, vegetable soup and braised veal

The HPT treatments at 600 MPa and 110 °C resulted in rapid endospore inactivation in all four RTE foods (green peas with ham, GPH; steamed sole, SS; vegetable soup, VS; braised veal, BS) within the first 60 s (Figure 11). A minimum log reduction of 2.6-log cycles (SS) and a maximum of 3.9-log cycles (BV) was achieved after a pressure-holding time of 1 s, i.e., after compression and instant decompression. Isobaric/isothermal pressure-holding times between 60 s and 300 s did not result in further inactivation in all four food matrices. Endospores tended to be more resistant in SS and GPH, reaching final inactivation levels of 4.8- and 5.2-log cycles, respectively. On the contrary, endospores treated in VS and BV were significantly less resistant ($p < 0.05$) to pressure treatments for 300 s, with log reduction values of 5.8 and 6.0 in VS and BV, respectively. At this final time point, minimum one out of three viable endospore counts reached the detection limit (VS: -6.0; BV: -¬5.9-log cycle reduction in CFU). Since, along with SS, endospores of *C. botulinum* TMW 2.357 showed greatest resistance in GPH, and outbreaks of foodborne botulism connected to proteolytic *C. botulinum* type B are often linked to food products involving ham and vegetables (Peck 2006), further inactivation experiments were conducted in GPH.

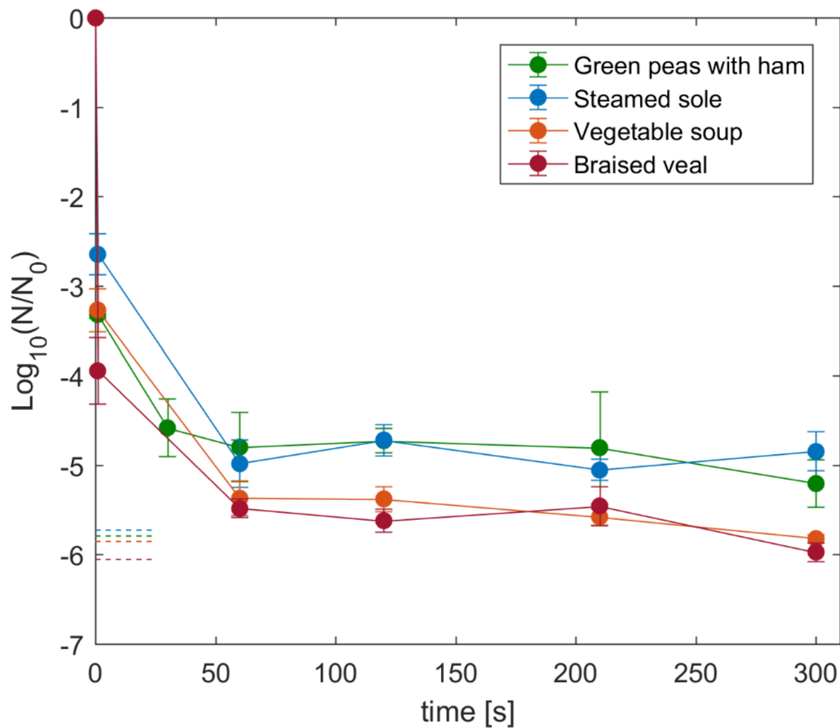

Figure 11. Inactivation of *C. botulinum* TMW 2.357 endospores in RTE foods (composition according to Table 5). Endospores suspended in green peas with ham (green circles), steamed sole (blue circles), vegetable soup (orange circles) and braised veal (red circles) after HPT treatment at 600 MPa and 110 °C for 1 to 300 s. Results are presented as $\log_{10}(N/N_0)$, where N describes the number of surviving endospores after a treatment and N_0 is the initial endospore count Dashed lines indicate the corresponding detection limits. Initial endospore count: 10^7 endospores/g. Data are shown as means ± standard deviations of triplicate independent experiments.

4.1.2 HPT inactivation of TMW 2.357 endospores in green peas with ham with extended p/T range

To evaluate the impact of HPT processing in an industrial and economical feasible pressure range, pressure levels of 300, 450 and 600 MPa in combination with 100, 110 and 120 °C were applied on endospores in GPH for up to 300 s (Figure 12). A considerable effect on endospore inactivation was observed after compression and decompression (1 s pressure-holding time) at all pressure/temperature combinations. This effect increased with increasing process temperatures, having its maximum

impact at 120 °C. Prolonged pressure-holding times (> 1 s) exhibited reduced endospore inactivation, where differences became less pronounced with increasing process temperatures. Hence, isobaric/isothermal pressure-holding times had only marginal impact on further endospore inactivation, especially for process temperatures ≥ 110 °C. Remarkably, a combination of 300 MPa and 100, 110 or 120 °C resulted in higher inactivation levels than pressure levels of 450 and 600 MPa and the same process temperatures. At a pressure level of 300 MPa in combination with 120 °C and 110 °C, the detection limit could already be reached after 30 s and after 210 s, respectively. Treatments at pressure levels > 300 MPa were not sufficient to reduce the endospore count below the detection limit at any pressure/temperature combination. For example, the maximum inactivation after 300 s pressure-holding time at 120 °C was 4.9-log cycles at 450 MPa and 5.3-log cycles at 600 MPa. Thus, endospores of C. botulinum TMW 2.357 exhibited increased HPT resistance at pressure levels >300 MPa resulting in pronounced tailing effects.

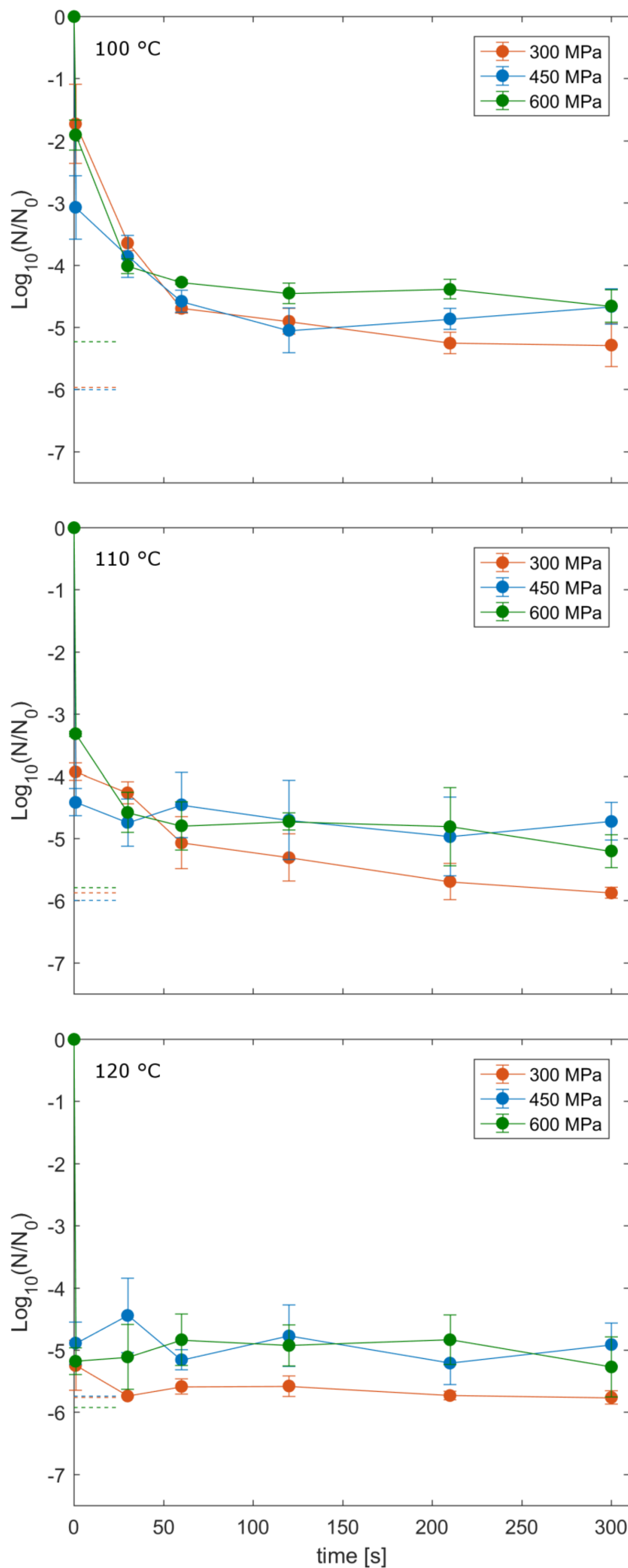

Figure 12. Inactivation of *C. botulinum* TMW 2.357 endospores in green peas with ham. Target pressure levels of 300 MPa (orange circles), 450 MPa (blue circles) and 600 (green circles) in combination with process temperatures of 100 °C, 110 °C and 120 °C were applied for 1 – 300 s. Results are presented as $\log_{10}(N/N_0)$, where N describes the number of surviving endospores after a treatment and N_0 is the initial spore count. Dashed lines indicate corresponding detection limits. Initial endospore count: 10^7 – 10^8 endospores/g. Data are shown as means ± standard deviations of triplicate experiments.

4.1.3 Thermal inactivation of TMW 2.357 endospores in green peas with ham at ambient pressure

Thermal treatments of ~10^8 endospores/g *C. botulinum* TMW 2.357 endospores in GPH at 121.1 °C for 3 min resulted in an average of 5.4 ± 0.2-log cycles reduction (Figure 13). The detection limit was not reached in any of the three experiments conducted. This indicates that the theoretical 12-log cycle reduction was not achievable by this standard heat sterilization treatment assuming a D-value of 0.25 min (green pea soup; (Nelson 2010)).

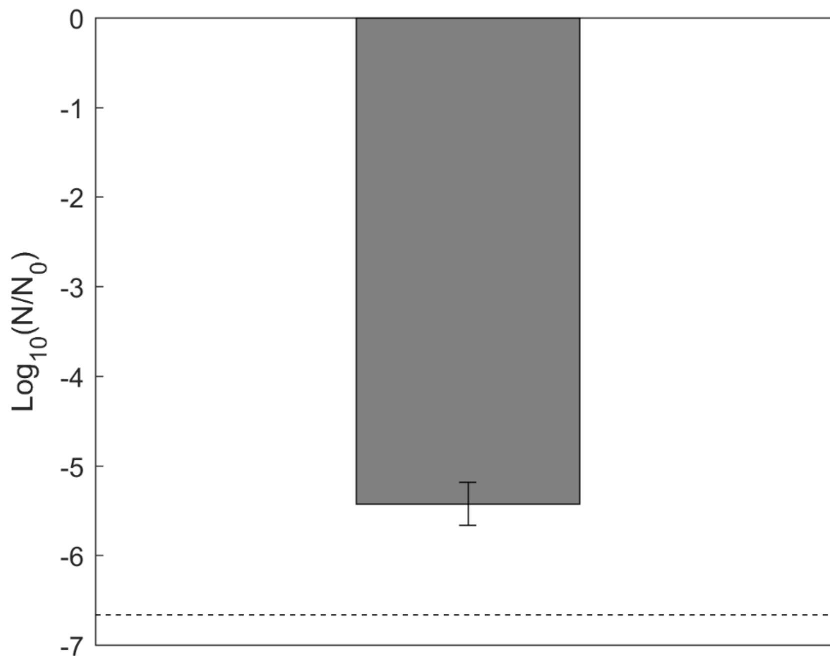

Figure 13. Thermal inactivation of TMW 2.357 endospores in green peas with ham at 0.1 MPa and 121.1 °C for 3 min. Results are presented as $log_{10}(N/N_0)$, where N describes the number of surviving endospores after a treatment and N_0 is the initial endospore count. The dashed line represents the detection limit (-6.7-log cycles). Data are shown as means ± standard deviations of triplicate independent experiments.

4.1.4 Impact of initial endospore count on HPT inactivation of TMW 2.357

The impact of varying initial endospore counts on the inactivation at 600 MPa and 110 °C is shown in (Figure 14). The curve shapes of the inactivation kinetics were similar and only shifted along the y-axis depending on the initial endospore count. A minimum reduction to 4.5 log CFU/g was achieved after 30 s of pressure-holding time independent of the initial endospore count. Longer isothermal/isobaric holding times resulted in slow inactivation. With initial endospore counts of 10^6 CFU/g, the detection limit was reached within 1 s. However, inactivation curves starting with 10^7 and 10^8 CFU/g indicate that approximately one out of 10,000 endospores survives HPT treatments up to 300 s.

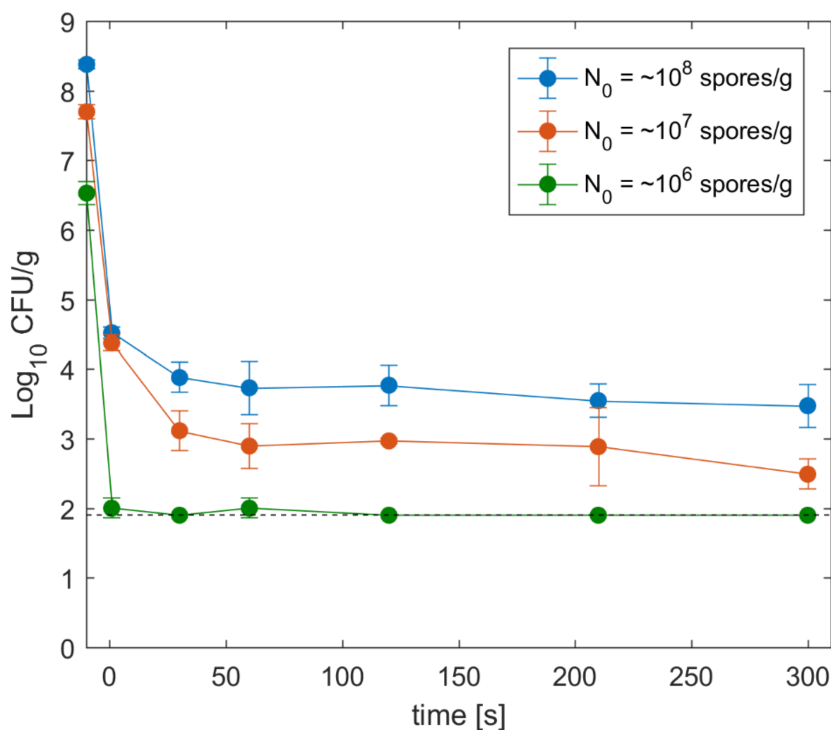

Figure 14. Viable endospores (CFU/g) of *C. botulinum* TMW 2.357 at 600 MPa and 110°C in green peas with ham. Different symbols indicate different initial endospore counts, i.e., 10^6 (green circles), 10^7 (orange circles) and 10^8 (blue circles) endospores/g. Results are presented as $\log_{10}(N/N_0)$, where N describes the number of surviving endospores after a treatment and N_0 is the initial endospore count. The

dashed line represents the detection limit (log_{10} CFU/g: 1.9). Data are shown as means ± standard deviations of triplicate independent experiments.

4.1.5 HPT treatment of surviving fraction of TMW 2.357 endospores

Endospores that survived HPT treatments at 600 MPa, 110 °C for 300 s were picked from agar plates and used to produce a fresh endospore suspension. The comparison of the HPT resistance of spores grown from glycerol stocks (standard procedure) and spores produced from the HPT resistant fraction is shown in Figure 15. No significant differences in the HPT resistance of both endospore batches was observable. Rapid inactivation took place within the first 60 s and final (300 s) log reduction values were around 5-log cycles for both endospore batches. Tailing of the inactivation curves occurred in the same way as observed before.

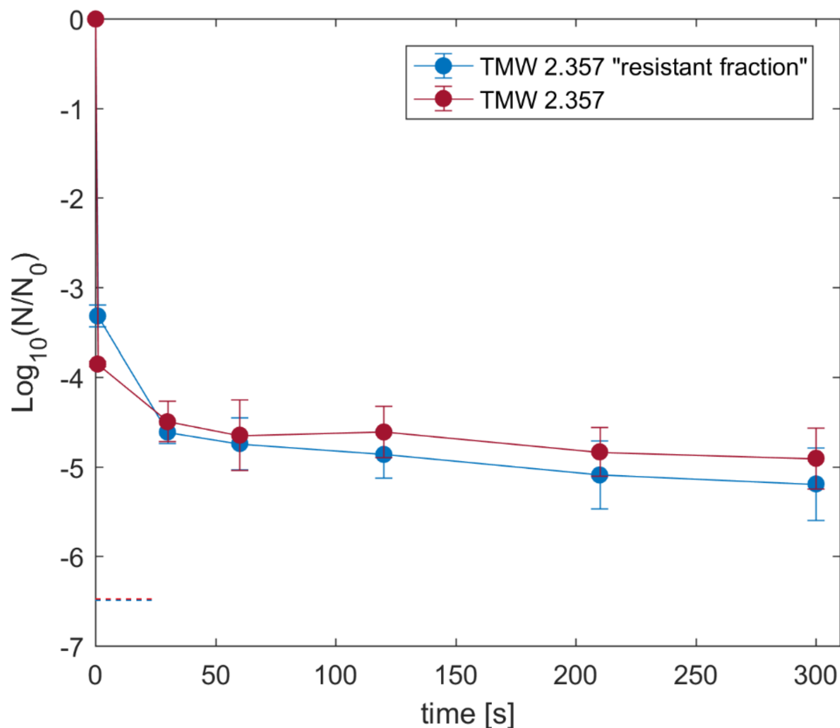

Figure 15. Inactivation of *C. botulinum* TMW 2.357 endospores at 600 MPa and 110°C in green peas with ham. Blue circles indicate endospores produced from the resistant fraction surviving HPT treatment at 600 MPa, 110 °C for 300 s. Red circles indicate endospores produced using the standard procedure. Results are presented

as $\log_{10}(N/N_0)$, where N describes the number of surviving endospores after a treatment and N_0 is the initial endospore count. Data are shown as means ± standard deviations of at least duplicate independent experiments.

4.2 Inactivation of TMW 2.990 (group II / BoNT E) endospores in low-acid foods and buffer

4.2.1 HPT inactivation of TMW 2.990 endospores in green peas with ham, steamed sole, vegetable soup and braised veal

In general, HPT treatments at 600 MPa and 90 °C resulted in similar endospore inactivation in all four foods (Figure 16). Endospore inactivation due to compression and instant decompression (1 s holding time) were > 1-log cycles in all foods tested. Further pressurization for up to 300 s under isothermal/isobaric conditions increased endospore inactivation by at least 3.0-log cycles. Inactivation levels reached the detection limit in green peas with ham, vegetable soup and braised veal (detection limits: green peas with ham: at -5.2-log cycles; vegetable soup: at -5.0-log cycles; braised veal: at -4.8-log cycles) after the maximum pressure holding time of 300 s. The Weibull function (Equation 1.) was applied to describe and fit non-linear inactivation kinetics during isobaric/isothermal holding times, i.e. excluding lethal effects of the pressure ramp. Generated parameters are shown in Table 9. All ß values were < 1, which reflects the observed upward concave inactivation pattern. Based on the obtained model parameters, the required pressure holding times to achieve a 6-log cycle reduction at 90 °C and 600 MPa were calculated and resulted in values between 9.6 and 14.5 min. Since endospores of non-proteolytic *C. botulinum* type E strains were more resistant in steamed sole and their habitats are primarily associated with aquatic environments (Hielm, Bjorkroth et al. 1998, Hyytiä-Trees 1999), further HPT inactivation experiments were performed using steamed sole as a food sample matrix.

Table 9. Endospore count reduction due to pressure ramp and kinetic Weibull model parameters for isothermal/isobaric conditions at 600 MPa and 90 °C.

Food product	Pressure ramp / heating phase reduction [$\log_{10}(N/N_0)$]	ß	δ [s]	R^2	6D value [min]
Green peas with ham	-1.4 ± 0.1	0.6271	33.24	0.98	9.6
Steamed sole	-1.5 ± 0.2	0.6083	45.59	0.97	14.5
Braised veal	-1.2 ± 0.2	0.5396	24.8	0.96	11.4
Vegetable soup	-1.5 ± 0.1	0.6196	34.61	0.98	10.4

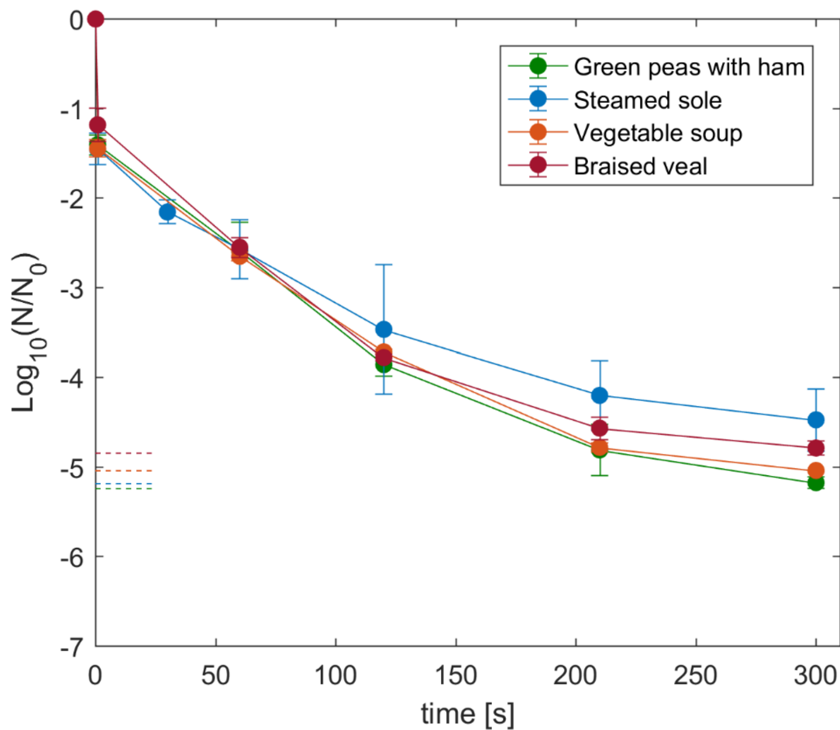

Figure 16. Inactivation of *C. botulinum* TMW 2.990 Endospores in RTE foods (composition according to Table 5). Endospores suspended in green peas with ham (green circles), steamed sole (blue circles), vegetable soup (orange circles) and braised veal (red circles) after HPT treatment at 600 MPa and 90 °C for 1 to 300 s. Results are presented as $\log_{10}(N/N_0)$, where N describes the number of surviving

endospores after a treatment and N_0 is the initial endospore count Dashed lines indicate the corresponding detection limits. Initial endospore count: 10^7 endospores/g. Data are shown as means ± standard deviations of triplicate independent experiments.

4.2.2 HPT and heat-only inactivation of TMW 2.990 endospores in steamed sole and imidazole phosphate buffer

Endospores of the non-proteolytic type E strain TMW 2.990 were treated in steamed sole and IPB at pressures ranging from 0.1 to 600 MPa and process temperatures of 80 to 100 °C (Figure 17). In general, endospore inactivation increased with increasing process intensities in both matrices.

Endospores suspended in steamed sole and pressurized at 300, 450 and 600 MPa at 80 °C had similar curve shapes and did not exhibit major differences total inactivation after a maximum holding time of 300 s, which resulted in final log reduction values between 3.1 and 3.5. Thermal treatment at 80 °C and ambient pressure did not result in significant endospore inactivation (0.4-log cycles) after 5 min of holding time. Increasing the process temperature to 90 °C, endospores pressurized at 450 MPa exhibited less resistance (5.9-log cycles) compared to those treated at 300 (4.8-log cycles) and 600 MPa (4.5-log cycles). Only a slight inactivation of 0.8-log cycles was achieved by thermal processing at 90 °C for 300 s. Further thermal treatment up to 10 min at 90 °C (not depicted in Figure 17) increased endospore inactivation to 1.8 ± 0.1-log cycles which generated a $D_{90°C}$ value (linear function) of 6.4 minutes. At 100 °C on the other hand, the highest inactivation was achieved by applying a pressure level of 300 MPa, partially reaching the detection limit (-5.7-log cycles) after 300 s of pressure holding time. Furthermore, treatments at 0.1, 450 and 600 MPa resulted in decelerated endospore count reduction after 120 s, approximating the respective detection limit and resulting in similar final inactivation levels.

Regarding the results of HPT treatments of endospores suspended in IPB, a more apparent tendency could be observed. At every applied process temperature (80 °C, 90 °C, 100 °C), endospore count reduction could be enhanced by increasing the pressure level stepwise from 300 MPa, to 450 MPa and, finally, to 600 MPa. Thermal

treatments at ambient pressure generally resulted in an improved endospore inactivation with a gradual increase in temperature. At 80 and 90 °C, pressures < 600 MPa were not sufficient to reduce the initial endospore count by more than 3.1-log cycles after a maximum holding time of 300 s. Furthermore, thermal treatments at 0.1 MPa and pressurization at 300 MPa resulted in similar endospore count reduction (max. 1.6-log cycles) after 300 s holding time with just marginal benefits of applying pressure. The conventional thermal treatment at 90 °C for 10 min just slightly increased endospore count reduction to 1.7 ± 0.2-log cycles (not depicted in Figure 17) compared to 300 s holding time and resulted in a D90°C-value of 20.8 min (calculated at isothermal holding time). On the other hand, the final inactivation level was more than doubled by the addition of pressures ≥ 450 MPa compared to thermal treatments at ambient pressure. At 100 °C, the previous described order of inactivation did not fully apply to thermal and HPT treatments. A thermal treatment at ambient pressure was more effective (4.7-log cycles) than HPT treatments at 300 MPa (2.3-log cycles) or 450 MPa (4.3-log cycles). However, the most effective inactivation in IPB was achieved by applying 600 MPa in combination with 100 °C. This treatment resulted in a final log reduction of 6.4-log cycles, with endospore counts falling below the detection limit at the same time.

By comparing the HPT inactivation in both matrices, no definite food matrix-dependent protective effect could be observed for endospores suspended in steamed sole in general. Endospores tended to be less resistant when pressurized in steamed sole, i.e., embedment in this matrix resulted in predominantly lower or at least similar final endospore count reduction. The only exception from this general trend was observed at 600 MPa and 100 °C, where the highest total endospore inactivation was achieved in IPB. For process temperatures up to 90 °C, the addition of defined pressure levels clearly accelerated endospore inactivation in both matrices. In IPB, pressures ≥ 450 MPa the final inactivation result was at least 2.2-fold higher compared with thermal treatments at ambient pressure. Such a synergistic effect of pressure and temperature was also observed in steamed sole, resulting in at least 5.5-fold higher endospore count reduction. Regarding the inactivation effect of the pressure ramp (1 s of pressure holding time), a more pronounced and pressure-dependent impact was observed in IPB, almost reaching a 3-log cycle reduction at 600 MPa and 90 and 100 °C (Table 10

and Table 11). Subsequent endospore count reduction in IPB during isobaric/isothermal holding times resulted in much flatter curve progressions. Consequently, obtained pressure holding times to achieve a 6-log cycle reduction were generally higher in IPB except for the already mentioned combination of 100 °C and 600 MPa. Based on the calculated model parameters, a desired 6-log cycle reduction within 10 min could be reached in steamed sole at 90 °C with 300 or 450 MPa or at 100 °C in combination with 300 MPa (Table 10).

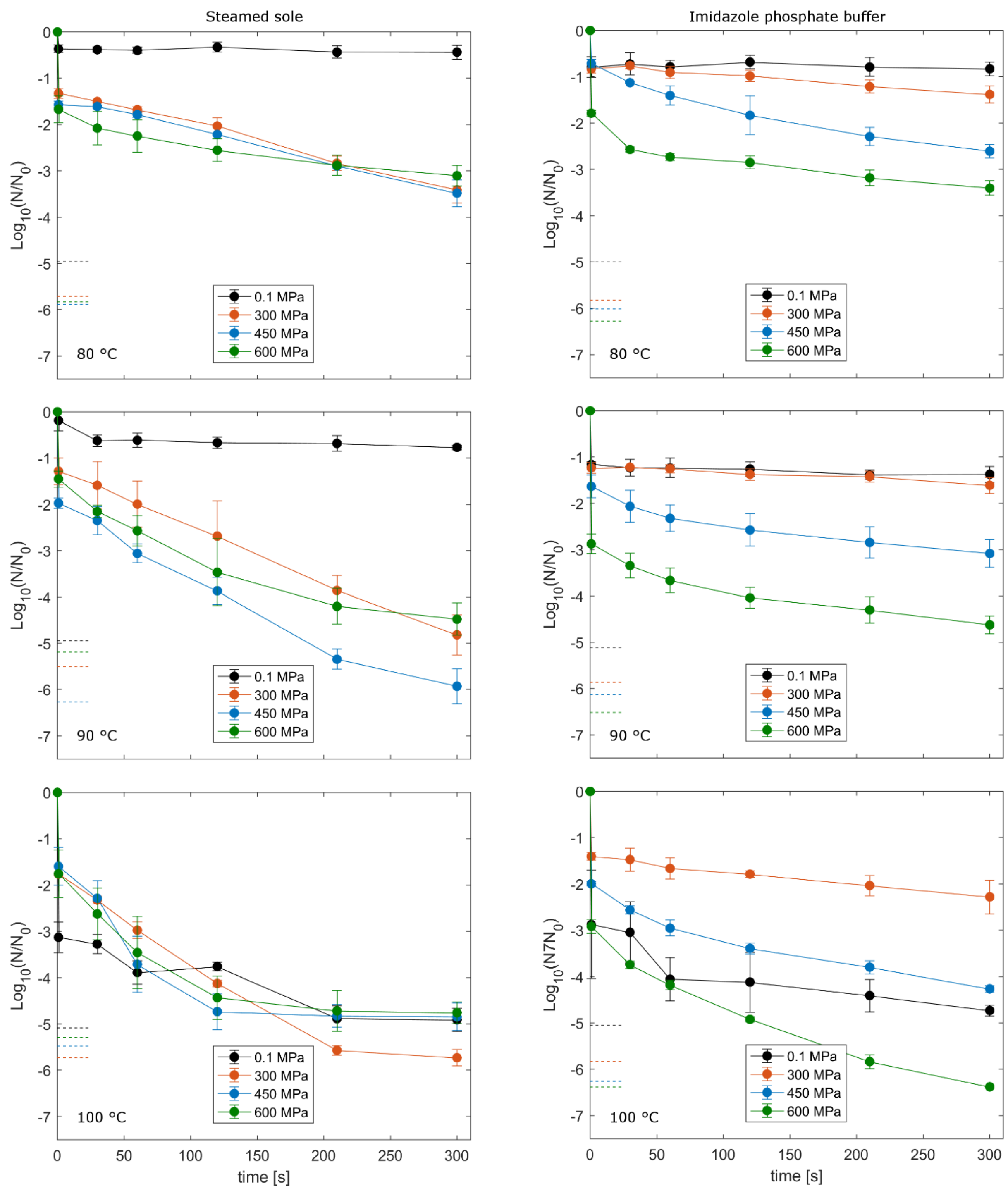

Figure 17. Inactivation of *C. botulinum* TMW 2.990 endospores suspended in steamed sole and IPB after treatments at 80 – 100 °C and 0.1 – 600 MPa for 1 – 300 s. Results are presented as $\log_{10}(N/N_0)$, where N describes the number of surviving endospores after a treatment and N_0 is the initial endospore count. Dashed lines indicate the corresponding detection limits. Initial endospore count: ~10^7

endospores/g. Data are shown as means ± standard deviations of three independent experiments.

Table 10. Endospore inactivation (TMW 2.990) due to pressure ramp and kinetic Weibull model parameters for isothermal/isobaric conditions in steamed sole.

Temperature [°C]	Pressure [MPa]	Pressure ramp / heating phase reduction [$\log_{10}(N/N_0)$]	ß	δ [s]	R^2	6D value [min]
80	0.1	-0.4 ± 0.1	-	-	-	-
80	300	-1.3 ± 0.1	1.0360	153.7	0.99	14.4
80	450	-1.6 ± 0.1	1.1400	178.6	0.99	14.3
80	600	-1.7 ± 0.3	0.5379	160.4	0.99	74.8
90	0.1	-0.2 ± 0.2	0.777	365.9	0.84	61.2
90	300	-1.3 ± 0.3	0.9107	81.6	0.99	9.7
90	450	-2.0 ± 0.1	0.8108	54.0	0.98	8.2
90	600	-1.6 ± 0.2	0.6524	73.1	0.97	19.0
100	0.1	-3.1 ± 0.3	0.7980	123.6	0.91	19.5
100	300	-1.8 ± 0.1	0.7193	39.4	0.97	7.9
100	450	-1.9 ± 0.1	0.4111	13.9	0.85	18.0
100	600	-2.3 ± 0.1	0.3854	17.5	0.91	30.4

*Conventional first-order kinetic D values during isothermal holding time were calculated for 90 and 100 °C at ambient pressure: $D_{90°C}$ = 6.4 min; $D_{100°C}$ = 2.5 min.

Table 11. Endospore inactivation (TMW 2.990) due to pressure ramp and kinetic Weibull model parameters for isothermal/isobaric conditions in IPB.

Temperature [°C]	Pressure [MPa]	Pressure ramp / heating phase reduction [$\log_{10}(N/N_0)$]	ß	δ [s]	R^2	6D value [min]
80	0.1	-0.8 ± 0.2	-	-	-	-
80	300	-0.8 ± 0.1	1.3950	448.7	0.96	27.0
80	450	-0.7 ± 0.1	0.6356	106.3	0.99	29.7
80	600	-1.8 ± 0.1	0.3837	85.7	0.96	152.4
90	0.1*	-1.2 ± 0.1	0.8347	1467.0	0.97	209.2
90	300	-1.2 ± 0.1	1.5350	576.0	0.96	30.8
90	450	-1.6 ± 0.3	0.5076	141.1	0.99	80.2
90	600	-2.9 ± 0.2	0.5291	102.5	0.99	50.5
100	0.1*	-2.9 ± 1.2	0.4969	82.8	0.92	50.8
100	300	-1.4 ± 0.1	0.876	350.5	0.99	45.2
100	450	-2.0 ± 0.1	0.5699	71.0	0.99	27.5
100	600	-2.9 ± 0.2	0.6262	39.9	0.99	11.6

*Conventional first-order kinetic *D* values during isothermal holding time were calculated for 90 and 100 °C at ambient pressure: $D_{90°C}$ = 20.8 min; $D_{100°C}$ = 2.3 min.

4.2.3 Comparison of HPT and thermal treatment conditions

An exemplary temperature-, pressure- profile is shown in Figure 18. Isothermal holding times were overlapped in order to illustrate differing lengths of process duration (60 s thermal treatment vs 60 s HPT treatment).

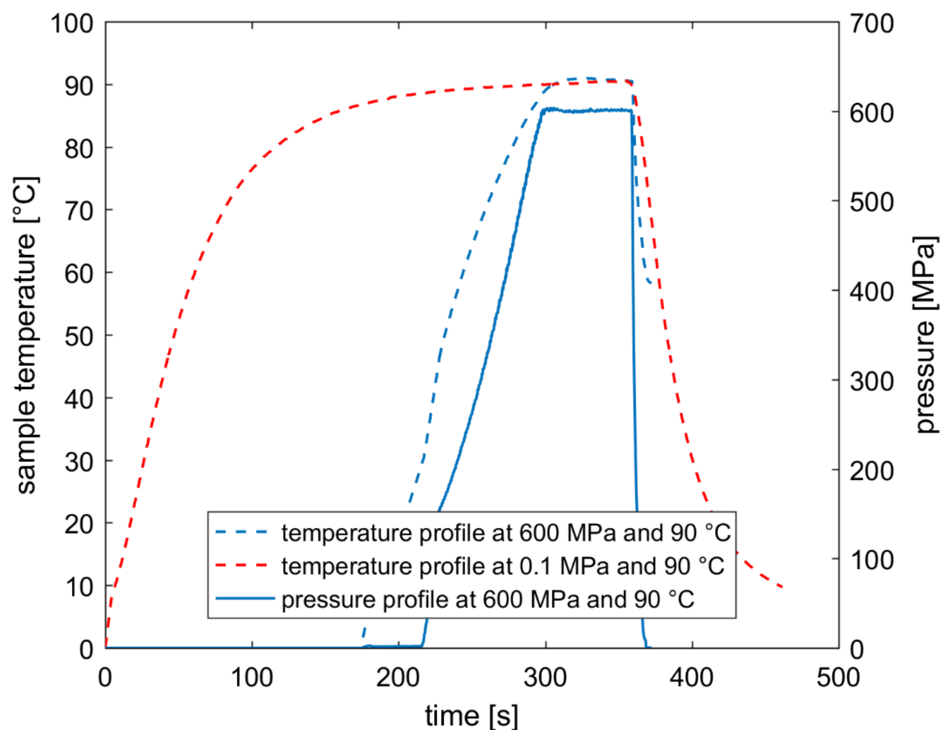

Figure 18. Typical temperature and pressure profiles during HPT and thermal treatments. Exemplarily, the temperature profile (red dashed line) of a thermal treatment (0.1 MPa, 90 °C) and the pressure (blue line) and temperature (blue dashed line) profile of a HPT treatment (600 MPa, 90 °C) with a holding time of 60 s in steamed sole are depicted. HPT heating time: ~124 s. Thermal heating time: ~298 s.

4.3 Endospore inactivation in food model systems by HPT

To date the basis of HPT studies of microbial endospores underlie a case-by-case strategy. One major reason can be found in the differential impact of various food components on the resistance of relevant microorganisms.

Towards a generic approach, a model system was developed to evaluate the HPT inactivation of bacterial endospores. Each primary food component (fat, carbohydrate, protein and salt) was substituted by a single stereotype component (rapeseed oil, sucrose, tryptone/peptone and NaCl, respectively) to systematically investigate their effects on the HPT inactivation (600 MPa, 80 – 110 °C) of food safety-relevant *Clostridium* (*C.*) *botulinum* endospores (proteolytic TMW 2.357 and non-proteolytic

TMW 2.990). Additionally, inactivation results in model systems mimicking the composition of four specific low-acid food products were compared with those obtained in the real food products.

4.3.1 HPT inactivation of proteolytic TMW 2.357 endospores in food model systems simulating RTE foods

The HPT treatments at 600 MPa and 110 °C resulted in rapid endospore inactivation in all four RTE foods (Figure 11) and their equivalent food model systems (Figure 19) within the first 60 s. The overall negative exponential curve shapes of the inactivation kinetics derived in both systems were similar but total inactivation levels partially differed. For example, initial inactivation due to 1 s pressure holding time was significantly different in green peas with ham compared to its equivalent FMS but no significant difference in inactivation occurred after 300 s of pressurization. In sum, log reduction values at respective time points of HPT-treated endospores in FMS matched 60%, 40%, 20% and 20% with the log reduction values obtained in green peas with ham, steamed sole, vegetable soup and braised veal, respectively (Table 12). Isobaric/isothermal pressure holding times between 60 s and 300 s did not result in further inactivation in RTE food nor in FMS matrices. Endospores tended to be more resistant in the RTE foods steamed sole and green peas with ham, reaching final inactivation levels of 4.8 and 5.2-log cycles, respectively. In contrast, endospores treated in model systems showed highest resistance in FMS vegetable soup (4.3-log cycles) and FMS green peas (4.7-log cycles). At the final time point of 300 s, minimum one out of three viable endospore counts reached the detection limit in vegetable soup at -6-log cycles, in braised veal at -5.9-log cycles and in FMS steamed sole at -5.5-log cycles.

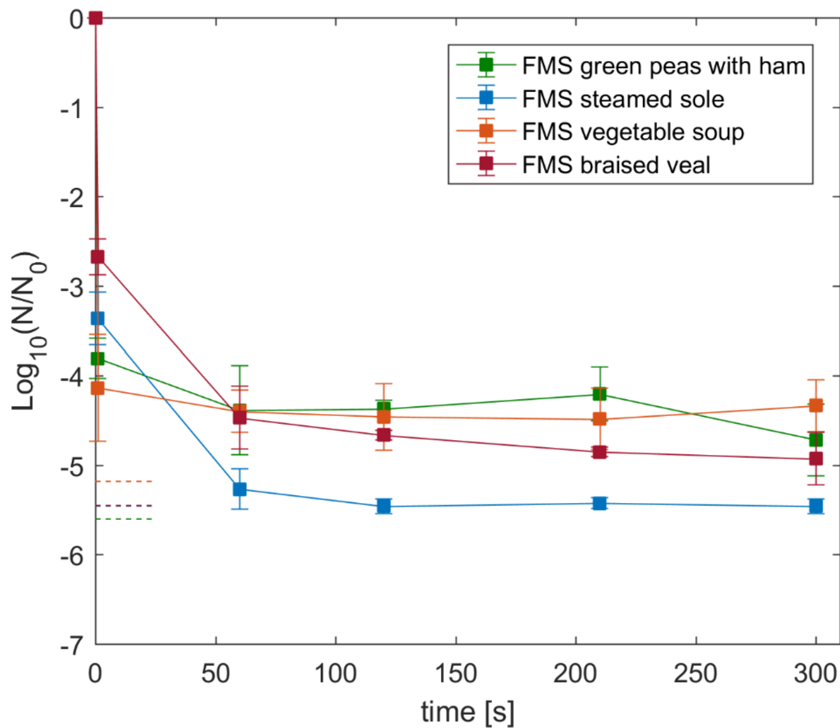

Figure 19. Inactivation of *C. botulinum* TMW 2.357 endospores in food model systems (FMS) (according to Table 6). Endospores suspended in FMS green peas with ham (green squares), FMS steamed sole (blue squares), FMS vegetable soup (orange squares) and FMS braised veal (red squares) after HPT treatment at 600 MPa and 110 °C for 1 to 300 s. Results are presented as $\log_{10}(N/N_0)$, where N describes the number of surviving endospores after a treatment and N_0 is the initial endospore count. Dashed lines indicate the corresponding detection limits. Initial endospore count: 10^7 endospores/g. Data are shown as means ± standard deviations of triplicate independent experiments.

4.3.2 Impact of primary food components on the HPT inactivation of proteolytic TMW 2.357 endospores in food model systems

The effect of varying primary food components on the HPT inactivation of endospores at 600 MPa, 100 and 110 °C for 60 s is displayed in Figure 20.

The **fat** content was varied between 0 and 20% whereas rapeseed oil served as substitute. To prevent a concentration shift of solutes when incorporating fat into the

FMS, its content was calculated based on the amount of the aqueous phase. In this way, the concentration of the solutes remained constant in the aqueous phase. Regarding the HPT endospore inactivation at a process temperature of 100 °C, the absence of fat resulted in a significantly ($p < 0.05$) decreased endospore resistance (5.5-log cycle reduction). A fat content of 10% resulted in an endospore inactivation of 4.3-log cycles but a further increase by 10% (in total 20%) did not enhance endospore protection (4.25-log cycles). At 110 °C, endospore resistance properties were independent of the fat content whereas the increase in temperature seemed to overcome possible protective effects. However, the detection limit (-5.2 log cycles) could be reached in FMS containing 20% of fat (due to a slightly lower initial endospore count).

At 100 °C, the inactivation results of varying **protein** contents are all significantly different ($p < 0.05$) from each other. In general, protein contents ≥10% promoted increased endospore inactivation by HPT treatments. Surprisingly, a content of 10% resulted in the highest inactivation (4.6-log cycles). HPT treatments at 110 °C basically showed a similar trend. However, the observed effect was much less pronounced exhibiting no substantial differences between varying fat contents but total endospore inactivation could be increased.

The absence of **carbohydrates** resulted in similar inactivation results as compared to FMSs with 20% of carbohydrates, namely 3.5- and 3.6-log cycles, respectively. In FMSs containing 10%, a significantly higher inactivation level was achieved (4.6-log cycles). A temperature increase of 10 °C, promoted the endospore inactivation in total which, however, was independent of the carbohydrate content.

A variation of the salt content had no significant impact on the HPT inactivation at 100 °C or 110 °C. As expected, a higher temperature (110 °C) resulted in a more effective endospore inactivation per se and a reduction below the detection limit in FMSs with no salt present.

As observed for varying salt contents, no significant differences were detected at 100 and 110 °C between a pH of 4.6, 5.8 and 7. The total inactivation was just slightly

higher at 110 °C (5.1- to 5.5-log cycles) than at 100 °C (4.6- to 5.1-log cycles) and a medium high pH (5.8) seemed to slightly increase endospore resistance.

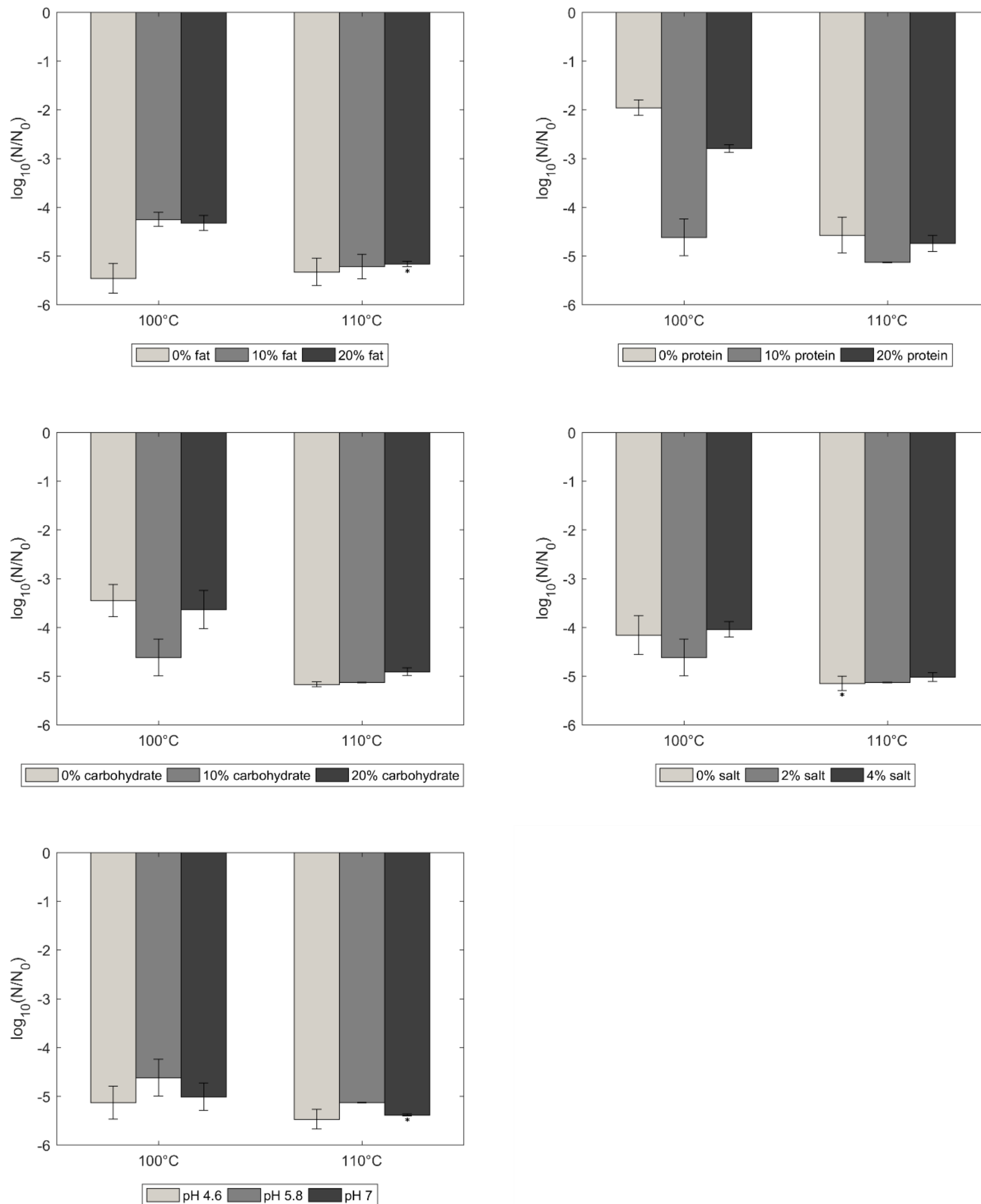

Figure 20. Inactivation of *C. botulinum* TMW 2.357 endospores in food model system with varying fat, protein, carbohydrate and salt content as well as pH value. Endospores were treated at 600 MPa at 100 and 110 °C for 60 s. Results are

presented as $\log_{10}(N/N_0)$, where N describes the number of surviving endospores after a treatment and N_0 is the initial endospore count. Initial endospore count: 10^6 – 10^7 endospores/g. Log reduction below the detection limit is indicated by an asterisk (*). Data are shown as means ± standard deviations of triplicate independent experiments.

4.3.3 HPT inactivation of non-proteolytic TMW 2.990 endospores in food model systems simulating RTE foods

In general, HPT treatments of endospores of non-proteolytic TMW 2.990 type E at 600 MPa and 90 °C resulted in similar inactivation results in all four foods. Log reduction values between 1.2 and 1.4 log units were achieved within a pressure holding time of 1 s, i.e., compression and instant decompression (Figure 16). Further pressurization under isothermal/isobaric conditions increased endospore inactivation by at least 3.5-log cycles. This resulted in inactivation levels reaching the detection limit (green peas with ham: -5.2-log cycle; steamed sole: -5.2-log cycle; vegetable soup: -5.0-log cycle; braised veal: -4.8-log cycle) in all four foods after a maximum pressure holding time of 300 s. On the other hand, generated inactivation kinetics in FMS at the same HPT conditions were not as uniform as observed in foods. Initially, inactivation due to compression and decompression resulted in similar inactivation, namely between 1.0- (FMS steamed sole) and 1.5-log cycles (FMS vegetable soup). With progressing pressure holding time, curve shapes of endospores treated in FMS varied from each other and resulted in different log reduction values. At the final time point (300s), log reduction of endospores suspended in FMS green peas with ham and FMS vegetable soup reached their detection limits of -4.7- and -4.5-log cycles, respectively. Hence, endospores treated in FMS steamed sole and FMS braised veal exhibited higher HPT resistance (FMS steamed sole: 4.2-log cycles; FMS braised veal: 3.5-log cycles). The highest conformity in log reduction of endospores treated in food and FMS was achieved in green peas and ham (80%), whereas the residual log reduction values achieved a conformity of 60% (steamed sole) or less.

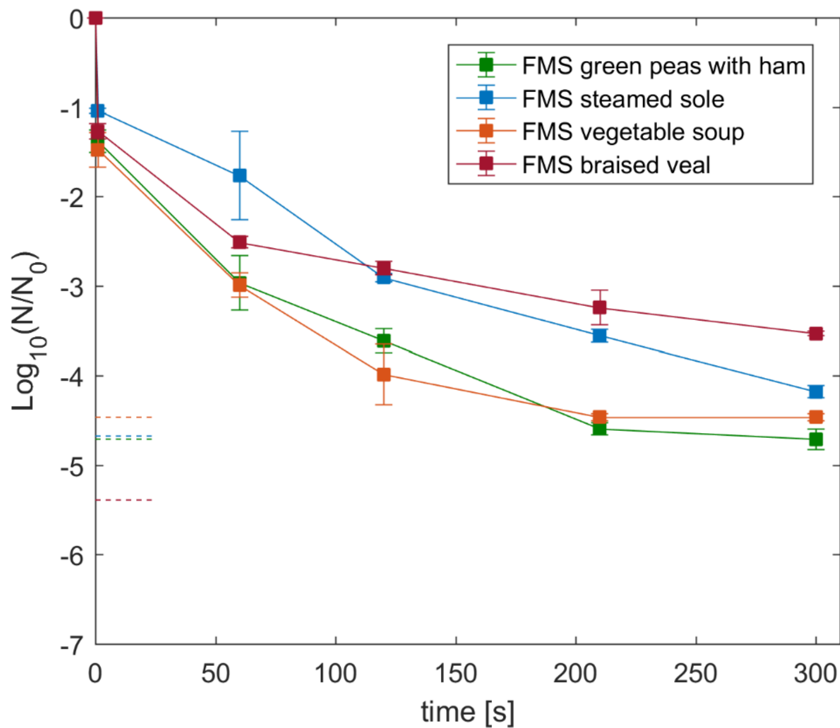

Figure 21. Inactivation of *C. botulinum* TMW 2.990 endospores in food model systems (FMS) (according to Table 6). Endospores suspended in FMS green peas with ham (green squares), FMS steamed sole (blue squares), FMS vegetable soup (orange squares) and FMS braised veal (red squares) after HPT treatment at 600 MPa and 90 °C for 1 to 300 s. Results are presented as $log_{10}(N/N_0)$, where N describes the number of surviving endospores after a treatment and N_0 is the initial endospore count. Dashed lines indicate the corresponding detection limits. Initial spore count: 10^7 endospores/g. Data are shown as means ± standard deviations of triplicate independent experiments.

4.3.4 Impact of primary food components on the HPT inactivation of non-proteolytic TMW 2.990 endospores in food model systems

The effect of varying primary food components on the HPT inactivation of TMW 2.990 endospores at 600 MPa, 80, 90 and 100 °C for 60 s is shown in Figure 22.

A treatment at 80 °C resulted in increased inactivation ($p < 0.05$) in FMS containing **fat** > 0%. Though, the difference in inactivation was rather modest (10% fat: Δlog_{10} = 0.4-

log cycles; 20% fat: $\Delta\log_{10}$ = 0.7-log cycles). Furthermore, the observed trend did not proceed by applying a higher process temperature of 90 °C since there was no significant difference between varying fat contents observable. The same trend applied to HPT treatments at 100 °C, however, total inactivation could be increased stepwise by elevating temperatures.

In general, **protein** contents > 0% enhanced endospore protection at all temperatures applied. However, contents of 10 or 20% resulted in similar inactivation results, hence protein contents ≥ 10% did not promote further barotolerance of endospores suspended in FMS. Nevertheless, the difference in inactivation due to the absences or presence of protein increased stepwise with increasing temperatures.

At 80 °C, the presence or absence of **carbohydrates** had no impact on the HPT inactivation of endospores of TMW 2.990 (between 1.8 to 1.9-log cycles). At 90 °C on the other hand, marginal but significant ($p < 0.05$) differences in HPT inactivation were detectable whereat higher carbohydrate contents seemed to promote endospore protection. A further increase in temperature by 10 °C (up to 100 °C) revealed a protective effect of carbohydrate contents ≥ 10% (10% carbohydrates: 3.1-log cycles; 20% carbohydrates: 3.3-log cycles). In contrast, no carbohydrates present resulted in 3.8-log cycles reduction.

At 80 °C, no apparent inactivation trend could be derived for differing **salt** contents since inactivation results varied in a small range (between 1.8 to 2.2-log cycles). However, an increase in temperature by 10 or 20 °C revealed a baroprotective effect of salt contents ≥ 2%. Furthermore, the maximum amount of salt (4%) resulted in even slightly higher endospore protection. The major impact on endospore inactivation due to an increase in temperature was achieved in FMS with no salt present. Here, the maximum inactivation was achieved at 100 °C, resulting in a $\log_{10}$ reduction of 4.6-log cycles.

At every applied temperature (80, 90, 100 °C), a **pH** of 5.8 resulted in the lowest HPT inactivation of endospores of TMW 2.990. Regarding the total inactivation, HPT treatments at 90 °C did not majorly improve endospore inactivation compared to 80 °C.

Overall, endospores suspended in FMSs with a pH of 4.6 resulted in the highest inactivation, being most effective at 100 °C. At this temperature, endospore counts fell below the detection limit (at -4.8-log cycles).

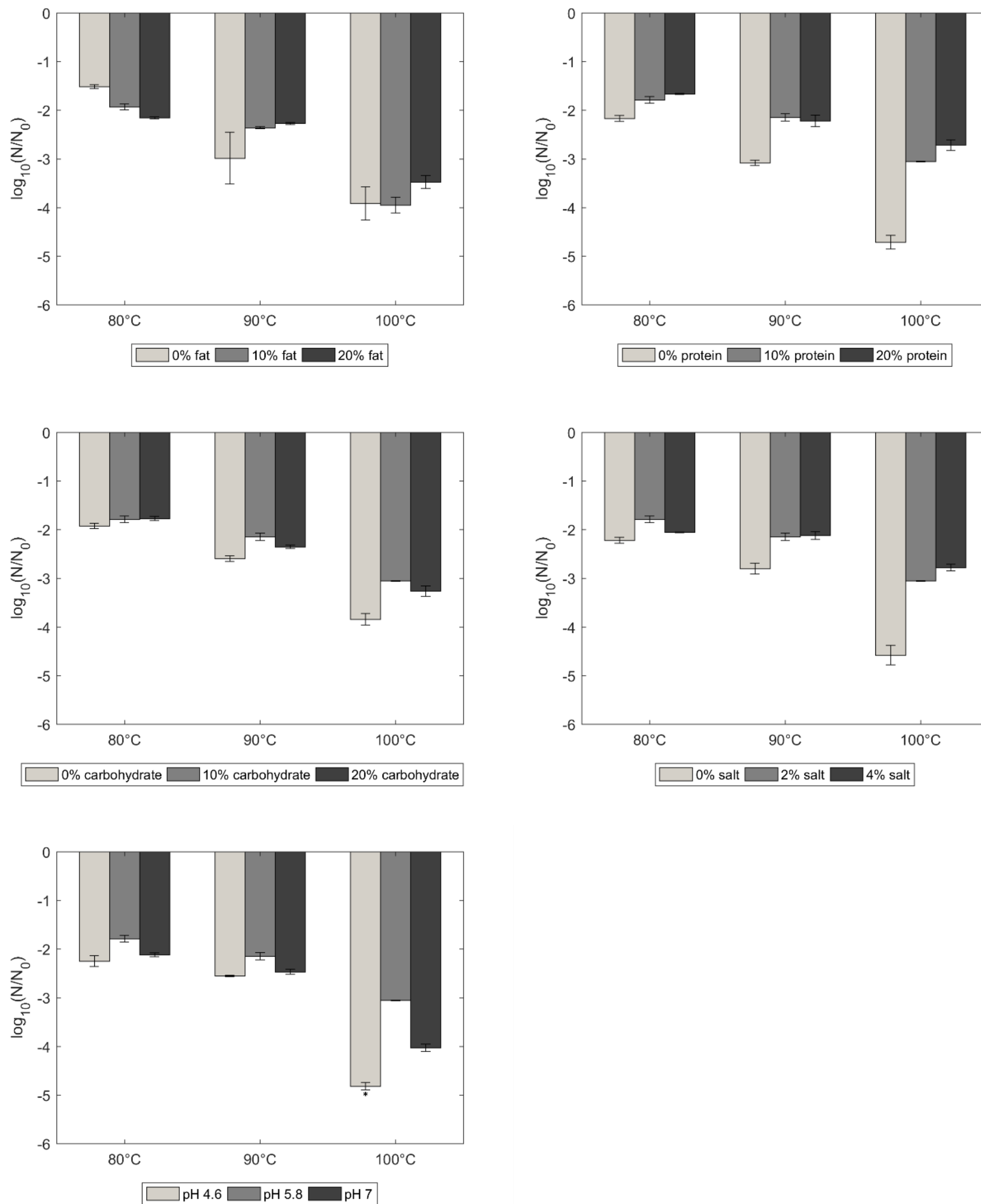

Figure 22. Inactivation of *C. botulinum* TMW 2.990 in food model system with varying fat, protein, carbohydrate and salt content as well as pH value.

Endospores were treated at 600 MPa at 80, 90 and 100 °C for 60 s. Results are presented as $log_{10}(N/N_0)$, where N describes the number of surviving endospores after a treatment and N_0 is the initial endospore count. Initial spore count: 10^6 – 10^7 endospores/g. Log_{10} reduction below the detection limit is indicated by an asterisk (*). Data are shown as means ± standard deviations of triplicate independent experiments.

4.3.5 Impact of free fatty acids on the HPT inactivation of non-proteolytic TMW 2.990 endospores

Since endospores of TMW 2.990 tended have a more hydrophobic surface profile (Table 3), they are more likely to be located in the fat phase. Obtained results in section 4.3.4 also suggest, that fat itself might influence resistance properties of endospores. Therefore, the impact of oleic acid (C18:1) which is a major free fatty acid present in oils and fats (Lekogo, Coroller et al. 2013), and its saturated chemical equivalent stearic acid (C18:0) on the HPT resistance of endospores was determined. The results shown in Figure 23, imply that the presence (2 mM) of free fatty acids more likely reduce the HPT resistance of endospores. This effect was independent of the applied temperature, although the overall inactivation increased with elevating process temperatures. Maximum detected differences in endospore inactivation between no fatty acids presents and 2 mM of fatty acids did not exceed more than 0.5-log cycles. At 80 and 90 °C, endospore inactivation was independent of the degree of unsaturation of the carbon chains. At 100 °C however, only saturated fatty acids (stearic acid (C18:0)) increased inactivation whereby unsaturated fatty acids had no impact on endospore inactivation.

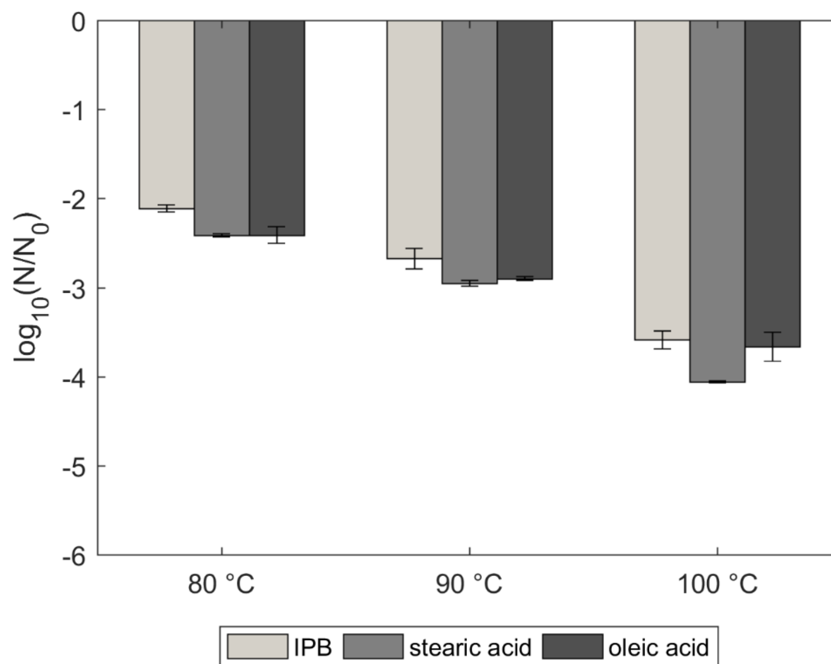

Figure 23. Inactivation of *C. botulinum* TMW 2.990 in the presence of 2 mM stearic acid (C18:1) or oleic acid (C18:0) in IPB (pH 7) at 600 MPa and 80, 90 and 100 °C for 60 s. Results are presented as $\log_{10}(N/N_0)$, where N describes the number of surviving endospores after a treatment and N_0 is the initial endospore count. Initial spore count: 10^7 – 10^8 endospores/g. Data are shown as means ± standard deviations of triplicate independent experiments.

4.3.6 Impact of fish oil on the HPT inactivation on various non-proteolytic *C. botulinum* type E endospores

The impact of 10% fish oil from menhaden (natural fat content approx. 9 to 13%) on non-proteolytic type E TMW 2.990 and TMW 2.997 is depicted in Figure 24. In comparison to HPT treatments in IPB, the strains were not protected by the presence of fish oil in the applied p/T/t range. However, slightly strain-dependent HPT resistance properties occurred. Derived from this experiment, TMW 2.990 exhibited a higher resistance towards HPT processing in IPB and in the emulsion containing 10% fish oil.

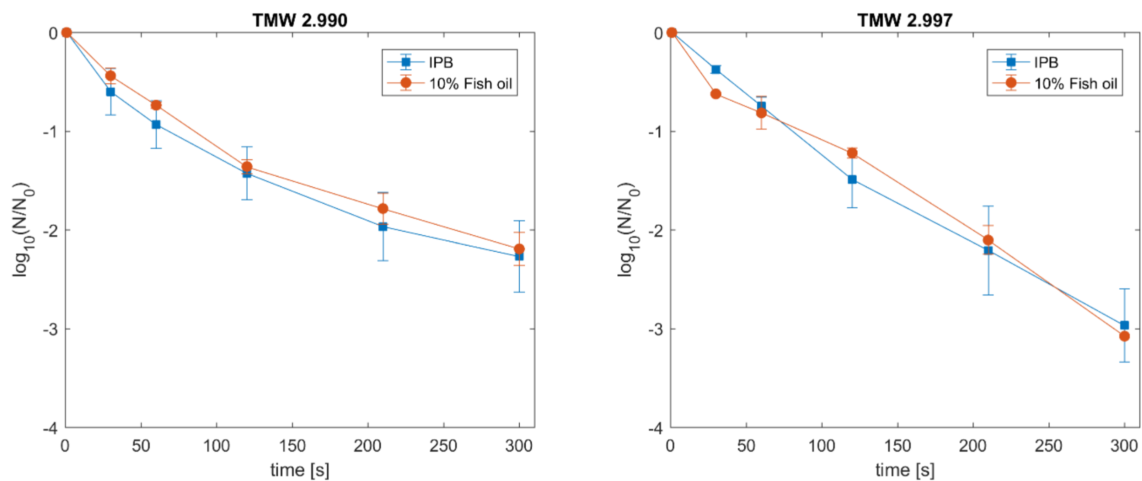

Figure 24. Inactivation of *C. botulinum* TMW 2.990 and TMW 2.997 during isothermal/isobaric conditions at 600 MPa, 90 °C for 1 to 300 s. Endospores were suspended in 10% fish oil (menhaden) and IPB buffer, stabilized by 0.5% Tween 80 and homogenization (5 min, 34.000 rpm). Results are presented as $log_{10}(N/N_0)$, where N describes the number of surviving endospores after a treatment and N_0 is the initial endospore count. Initial endospore count: 10^7 endospores/g. Data are shown as means ± standard deviations of triplicate independent experiments.

5 Discussion

Based on the obtained results, the initial questions raised (section 2) can be refined to the following theses.

Regarding endospores of TMW 2.357:

- Tailing effects of endospores of TMW 2.357 are related to the presence of a small resistant endospore fraction within the population.
- The resistance properties of a resistant endospore fraction are not related to intrinsically different properties, e.g., due to genetic variations.

Regarding endospores of TMW 2.990:

- The HPT inactivation behavior of endospores obtained in buffer systems is not applicable to those obtained in food matrices.
- The presents of endospore origin related matrix components, in particular fish oil, does not significantly influence endospore resistance.

Additionally, the following new and more general theses towards food safety can be posted:

- HPT processing can achieve equivalent product safety at lower process temperatures and/or shorter treatment durations compared to standard thermal sterilization.
- The maxime “the more pressure, the better the endospore inactivation” is not always the best choice for future HPT process design (non-linear pressure/temperature dependence).
- Extrapolation of inactivation kinetics is impeded due to biphasic inactivation curves.
- The pressure ramp has a significant impact on endospore inactivation and can serve as an additional safety margin.
- A generic approach to replace case-by-case studies is impeded due to the strong heterogenic behavior towards HPT treatments within and between strains of *C. botulinum*.

- Prediction of spore inactivation from simple component food compositions is not possible.

5.1 HPT inactivation of TMW 2.357 endospores in low-acid foods

To date, few studies investigated the HPT inactivation of endospores from the important food intoxicating organism, *C. botulinum*, in food matrices and food model systems (Reddy, Solomon et al. 1999, Reddy, Solomon et al. 2003, Margosch, Ehrmann et al. 2004, Bull, Olivier et al. 2009, Schnabel, Lenz et al. 2015). Moreover, the comparison of inactivation data is not only difficult due to varying process parameters but also because of different thermodynamic properties of HPT units, in particular controlling and monitoring adiabatic heating effects. Data reported here (section 4.1) extend the knowledge of HPT inactivation of a relatively resistant proteolytic *C. botulinum* type B strain (TMW 2.357; (Margosch, Ehrmann et al. 2004, Margosch, Ehrmann et al. 2006)) in relevant matrices, i.e., low-acid ready-to-eat (LA RTE) foods under matrix-independent isothermal isobaric conditions.

In principle, HPT treatments at 600 MPa and 110°C resulted in similar curve shapes in all four LA foods whereby endospores treated in GPH (green peas with ham) and SS (steamed sole) showed greater resistance (Figure 11). Bull, Olivier et al. (2009) found that endospores of proteolytic *C. botulinum* exhibited increased heat tolerance when treated in a model product with a higher fat content (~7.4% fat). However, this effect was less distinct when endospores were HPT-treated. For endospores of non-proteolytic *C. botulinum* type E, it has been shown that increasing fat contents can result in reduced HPT and thermal inactivation (Molin and Snygg 1967, Schnabel, Lenz et al. 2015). Both GPH and SS have a higher fat content than BV (braised veal) and VS (vegetable soup), namely 12.8% and 8.6%, respectively. From the current setup it is impossible to spot specific food components that are responsible for the slightly different inactivation results in the different RTE foods. However, results obtained here together with previously published data suggest that, besides other food components, the overall fat content and/or probably the fat composition are likely to play some role in the HPT inactivation of *C. botulinum* endospores.

Previous studies showed the increased HPT resistance of proteolytic *C. botulinum* TMW 2.357 endospores in a pressure range between 600 and 1400 MPa (Margosch, Ehrmann et al. 2004, Margosch, Ehrmann et al. 2006). Since the maximum pressure that is applied in commercial operations is about 600 MPa (Norton and Sun 2008), the focus of this study was on equal or lower pressure levels. Our results shown in Figure 12 complement and, in general, support the findings reported by Margosch, Ehrmann et al. (2006) even though earlier experiments were conducted in a different HPT unit, endospores were treated in Tris-His buffer and sporulation conditions were different. We could also observe drastically decreased inactivation rates at longer isothermal/isobaric holding times, i.e., a significant tailing effect in survivor curves. Such tailing effects appear to be significantly less pronounced for *C. botulinum* strains belonging to the non-proteolytic group II (Lenz, Reineke et al. 2015, Schnabel, Lenz et al. 2015, Lenz 2017). Most importantly, this demonstrates that it can be difficult to completely inactivate endospores from proteolytic *C. botulinum* strains in relevant food matrices by HPT treatments in an industrially feasible range, and that an extension of the holding time is not always a suitable approach to reach commercial product sterility. Interestingly, the efficiency of endospore inactivation increased applying lower pressure levels of 300 MPa at process temperatures of 100, 110 and 120°C. This, in turn, demonstrates the non-linearity of the effectiveness of different pressure levels and the importance of finding effective p/T combinations when designing HPT processes to inactivate proteolytic *C. botulinum* strains. At 300 MPa, inactivation results with equal or even higher log reduction values (≥ 5.4-log cycles) than after standard thermal sterilization treatments at ambient pressure (121.1°C, 180 s) became achievable. This was the case at 110°C within 210 s and at 120°C within 30 s and demonstrates the potential of replacing standard retorting processes by HPT processes achieving product safety at lower process temperatures and/or shorter treatment durations.

Based on the widely accepted parameters for heat sterilization ($D_{121.1\,°C}$ ≈ 0.21 min, z = 10°C) (Ball and Olson 1957, Pflug and Odlaug 1978, Pflug 1987, Stumbo 2013), the thermal treatment applied (121.1°C, 3 min; Figure 13) should have resulted in a theoretical inactivation of > 14-log cycles of *C. botulinum* endospores (12-log cycles when 0.25 min are used for calculation), which is far away from the achieved 5.4-log

cycles for *C. botulinum* TMW 2.357 in GPH. This is not surprising since thermal log inactivation curves can be non-linear and, in addition to matrix effects, large strain-dependent differences in the heat resistance of *C. botulinum* endospores were reported, i.e., $D_{120\ °C}$ / 121.1 °C values of up to 1.2 min (Casolari 1994, Margosch, Ehrmann et al. 2004). In line with previous argumentations (Margosch, Ehrmann et al. 2004, Margosch, Ehrmann et al. 2006), this indicates that the 12-D concept generally recommended for the inactivation of *C. botulinum* in shelf-stable LA RTE food is in fact only a 4-D concept for resistant *C. botulinum* endospores. The fact that standard heat sterilization processes are sufficient to ensure proper consumer safety is putatively mainly related to relatively low contamination levels of *C. botulinum* in food samples in general (10 to 1000 endospores/kg) (Dodds 1993, Lindström and Korkeala 2006, Lenz 2017).

Since all obtained HPT inactivation curves exhibited tailing effects subsequent to a generally high inactivation effect within the first seconds, experiments with varying initial endospore counts were conducted (Figure 14). One major reason was to exclude that inaccuracies as cell counts approach the detection limit play a role in the observed tailing effects. Generally, survivor curve tailing has been frequently reported, but the exact reasons still remain unclear. Proposed contributing factors include heterogeneous resistance properties within an endospore population, endospore clumping, adhesion to any surfaces during sample handling and protective effects of dead spores (Cerf 1977, Margosch, Ehrmann et al. 2006, Ahn, Balasubramaniam et al. 2007, Mathys 2008, Wilson, Dabrowski et al. 2008). Microscopic analysis of aqueous endospore suspensions indicated that there is no great tendency of *C. botulinum* endospores to aggregate, which could be observed regardless of the endospore concentration. Although it cannot be excluded that this is different in the food matrices used, results suggest that tailing effects are related to the presence of a small resistant endospore fraction accounting for approximately 0.01% of an endospore population.

The fact that endospores produced from HPT survivors exhibited similar inactivation kinetics as endospores produced from glycerol stocks indicates that the resistance

properties of the resistant endospore fraction are not related to intrinsically different properties, e.g., due to genetic variations (Figure 15).

Our results suggest that HPT processes based on the maxim “the more pressure, the better” are not always the best choice and should be kept in mind for future HPT process design and food safety considerations. While non-linear pressure/temperature-dependence of the HPT endospore inactivation efficiency is likely also for other strains, respective quantitative effects may differ. Therefore, more HPT inactivation studies with other, differently pressure (and heat) resistant strains of *C. botulinum* are necessary to draw any general conclusion on how the phenomenon observed for the strain used in this study affects the overall suitability of HPT for commercial implementations.

5.2 HPT inactivation of TMW 2.990 endospores in low-acid foods and buffer

The thermal as well as the HPT tolerance of a resistant non-proteolytic *C. botulinum* type E strain (TMW 2.990) (Lenz and Vogel 2014, Lenz, Reineke et al. 2015) was determined in four different low-acid foods and in pressure/temperature-stable imidazole/phosphate buffer (section 4.2) and will be discussed in the following paragraphs. All HPT experiments were conducted under isothermal/isobaric conditions to eliminate temperature inconsistencies due to adiabatic heating. Therefore, comparable results among different process parameters (e.g. matrix, target pressure, target temperature) were generated. As mentioned above, for an industrial implementation of HPT technology, current maximum feasible and economical pressure levels have to be considered. Therefore the focus of this study was on pressure levels ≤ 600 MPa (Norton and Sun 2008).

Endospore inactivation by HPT treatments in all four different foods, which covered a broad range of intrinsic properties and would enable growth of non-proteolytic *C. botulinum*, was characterized by similar negative exponential curve shapes with a slightly but significantly ($p < 0.05$) increased spore resistance in steamed sole (Figure 16). Taking the conventional thermal treatment into account (90 °C, 0.1 MPa, 10 min),

in three out of four food matrices, a theoretical 6D endospore count reduction was not achieved during isobaric/isothermal holding times within 10 min (based on Weibull parameters; Table 9). However, the heating rate could considerably be increased by applying pressure (approx. 2.4-fold faster; Figure 18) and more importantly the final endospore counts fell below the detection limit in three out of four foods. Hence, the addition of pressure substantially increased endospore inactivation, shortened the overall process duration and concomitantly reduced the applied thermal load. Furthermore, considerable lethal effects of the compression and decompression phase (> 1-log cycle) were not included into the calculated times for 6D reductions. Therefore, the compression and decompression phase could serve as additional safety margins or directly be integrated into the total lethal effect of HPT treatments. This observed non-negligible impact is in line with previous studies, which investigated the HPT inactivation on endospores of several *Clostridium* and *Bacillus* species (Margosch, Ehrmann et al. 2006, Patazca, Koutchma et al. 2006, Rajan, Ahn et al. 2006, Ahn, Balasubramaniam et al. 2007, Maier, Lenz et al. 2017). Thereby, the impact of varying compression and decompression rates should also be taken into account, since they can largely affect endospore inactivation. This has been shown especially for endospores of *Bacillus* species, whereby slow pressurization rates enhanced endospore count reduction for pressure holding times < 5 min (Ratphitagsanti, Ahn et al. 2009, Syed, Reineke et al. 2012). Typical industrially applied pressurization rates can be up to 3.3 MPa/s (Syed, Reineke et al. 2012). The times to reach certain levels of pressure reported in our study were almost twice as fast. Considering this, finding a balance between sufficient inactivation due to pressurization and economic feasibility with regard to total process time could be an additional challenge for future HPT applications.

Available studies on the HPT inactivation of endospores of *C. botulinum* type E comprise a broad pressure range between 300 to 1200 MPa but commonly do not exceed temperature levels > 80 °C (Reddy, Solomon et al. 1999, Rodriguez, Larkin et al. 2004, Skinner, Marshall et al. 2014, Lenz, Reineke et al. 2015, Lenz and Vogel 2015, Schnabel, Lenz et al. 2015). This can probably be attributed to the fact that spores of non-proteolytic strains of *C. botulinum* typically exhibit less resistance towards conventional thermal processing when compared to other food safety relevant

endospore-forming bacteria (Peck 2006, Van Asselt and Zwietering 2006). Nevertheless, existing data on thermal inactivation of endospores of *C. botulinum* type E indicate a broad spectrum of resistance properties. Lindström, Nevas et al. (2003) for instance, reported highly biphasic destruction curves, which resulted in thermal D-values of up to 7.1 min (at 90 °C) for *C. botulinum* type E endospores suspended in whitefish medium and recovered in medium containing lysozyme, which can possibly assist in germination of heat-stressed endospores. Regarding this, in a worst-case scenario, a 6-log cycle reduction would theoretically take 42.6 min of thermal holding time, also meaning, that the recommended 10 min thermal treatment would basically just result in a 1.4-log cycle reduction. The fact that properly performed conventional thermal preservation processes are sufficient to ensure consumer safety is putatively mainly related to low contamination levels of *C. botulinum* in food samples in general (Lenz 2017). In our study, we obtained similar endospore count reduction values in steamed sole (approx. 1.8-log cycles) and IPB (approx. 1.7-log cycles) after 10 min at 90 °C but quite different linear thermal D-values for isothermal holding times. The heating phase in IPB had a much more severe impact on endospore inactivation than in steamed sole, which resulted in a smaller curve slope during isothermal holding time and hence, a higher D-value. Temperatures below 90 °C at ambient pressure resulted in marginal endospore count reduction (< 1-log cycles) in both matrices (Figure 17), which indicated insufficient process intensities. Below 100 °C, the addition of pressure clearly enhanced endospore inactivation, acting synergistically with heat in steamed sole and partially in IPB (for $p > 300$ MPa). Such synergisms of pressure and temperature on HPT inactivation for several pathogenic and non-pathogenic endospore-formers in various buffer and relevant food systems have previously been reported and are basically in line with our observations (Margosch, Ehrmann et al. 2004, Koutchma, Guo et al. 2005, Rajan, Ahn et al. 2006, Rajan, Pandrangi et al. 2006, Ahn, Balasubramaniam et al. 2007, Bull, Olivier et al. 2009, Maier, Lenz et al. 2017). However, opposite effects, i.e. pressure-protection at certain p/T combinations, have been reported for endospores of proteolytic *C. botulinum* (Margosch, Ehrmann et al. 2006, Maier, Lenz et al. 2017) and recently, for endospores of non-proteolytic *C. botulinum* strains (Skinner, Morrissey et al. 2018). Such pronounced pressure-mediated protective effects (resulting in strong tailing) could be related to a small but highly resistant fraction within a heterogeneous endospore population. In general,

several factors can contribute to a variability in endospore resistance properties and alter the outcome of HPT inactivation studies. Such factors include, among others, sporulation conditions (e.g. medium mineral content, sporulation temperature), endospore purification (spore stock purity), spore surrounding matrix (e.g. water content, pH, fat content) process control and intensity (e.g. temperature monitoring, p/T/t conditions) as well as recovery conditions (e.g. incubation time, medium) (Lenz 2017).

In contrast to endospores suspended in IPB, varying high pressure levels did not seem to be the driving force for endospore inactivation in steamed sole at any applied temperature. In general, endospores tended to be less resistant in steamed sole by exhibiting a much more rapid endospore inactivation, which was also indicated by shorter treatment times required to obtain a 6D reduction (Table 10 and Table 11). In terms of matrix dependent protective effects, Reddy et al. (Reddy, Tetzloff et al. 2006) found that non-proteolytic endospores of *C. botulinum* suspended in crabmeat blend were not protected by the surrounding food matrix in comparison to endospores pressurized in phosphate buffer (0.067 M, pH 7). This is basically in agreement with our findings shown in Figure 17 with an exception for HPT experiments conducted at 100 °C and 600 MPa. Here, the overall endospore inactivation was lower and the detection limit was not reached for endospores suspended in steamed sole. As indicated above, Skinner et al. (Skinner, Morrissey et al. 2018) observed protective effects of non-proteolytic endospores of *C. botulinum* in ACES buffer (0.05 M, pH 7) due to the addition of high pressures (600 to 700 MPa) to elevated temperatures (83 to 91 °C). In fact, we only observed such a pressure protective effect for $p > 300$ MPa and, in contrast, solely for endospores suspended in steamed sole rather than in IPB. Interestingly, Lenz et al. (Lenz, Reineke et al. 2015) observed an accelerated DPA-release of endospores of TMW 2.990 after treatments at 300 MPa at 30 °C compared to treatments at higher pressure levels (450 MPa, 600 MPa, 750 MPa) at which the endospore's ability to retain DPA may be favored under certain conditions and result in an increased resistance. HPT processing in such defined parameter range could also be related to the endospore surrounding food matrix, however, such a complex food matrix did not allow to spot specific responsible food components. So in principle, a protective effect of pressure towards temperature inactivation seems to be

identifiable for all endospore formers (Margosch, Ehrmann et al. 2006, Maier, Lenz et al. 2017, Skinner, Morrissey et al. 2018), however, the parameter combinations vary and such defined conditions cannot be met in industrial applications of today.

Endospore inactivation by HPT obtained in IPB gradually increased with increasing process intensities and are generally supporting and complementing inactivation kinetics reported by Lenz et al. (Lenz, Reineke et al. 2015) for TMW 2.990 conducted at 30 to 75 °C and 300 to 1200 MPa. By exploiting even higher pressures, it was shown that pressures > 600 MPa noticeably increased endospore count reduction. For instance, inactivation results obtained at 75 °C and 750 MPa (approx. -4.5-log cycles) reached equivalent endospore count reduction to those at 90 °C at 600 MPa (-4.6-log cycles) in our study. However, data reported here demonstrate that a direct transfer of inactivation results obtained in buffer systems to industrial applications, i.e. refrigerated low-acid foods, is difficult, since pressure/temperature dependencies in IPB were not applicable to steamed sole. With almost equal inactivation results at 100 °C and ambient pressure in both matrices, the impact of heat drastically increased and partially obliterated the synergism of pressure and heat. Inactivation during the heating and cooling phase with no holding time reached up to 3-log cycles in both matrices, probably due to slower heating rates involving extended temperature exposure close to 100 °C.

Similar to proteolytic *C. botulinum* endospores, natural contamination levels of non-proteolytic *C. botulinum* endospores are normally very low (Lenz 2017). This and the relatively low effectiveness of the conventional thermal processing conditions that are recommended to inactivate endospores from non-proteolytic *C. botulinum* strains in a worst-case scenario as described above suggests that HPT processing can serve as an suitable alternative preservation technology to obtain safe chilled foods with extended shelf-life. Possible process parameters for this purpose may be found at p/T/t combinations of 600 MPa, ≥ 90 °C and 300 s (≥ 4.5-log cycles for all four foods tested) and 300 to 600 MPa, ≥ 80 °C and 300 s (≥ 3-log cycles for steamed sole). However, taking into account the high variability of endospore resistance properties and possible protective p/T combinations, additional safety margins such as strict and constant storage below 3 °C should be considered for low-acid chilled storage products.

5.3 High pressure thermal inactivation of endospores in food model systems

To date the basis of high pressure thermal inactivation (HPT) studies of microbial endospores underlie a case-by-case strategy. One major reason can be found in the differential impact of various food components on the resistance of relevant microorganisms. Towards a generic approach, a model system was developed to evaluate the HPT inactivation of bacterial endospores. Each primary food component (fat, carbohydrate, protein and salt) was substituted by a single stereotype component (rapeseed oil, sucrose, tryptone/peptone and NaCl, respectively) to systematically investigate their effects on the HPT inactivation (600 MPa, 80 – 110 °C) of food safety-relevant *C. botulinum* endospores (group I/type B and group II/type E). Additionally, inactivation results in model systems mimicking the composition of four specific low-acid food products were compared with those obtained in the equivalent real food product.

5.3.1 Applicability of FMSs to simulate the HPT inactivation of endospores in real foods

To date, few studies investigated the impact of single food components on the HPT inactivation of relevant bacterial endospores in complex food models systems and fewer, if none, investigated the application of food model systems simulating real food products. Towards a more generic approach, endospores of TMW 2.357 and TMW 2.990 were HPT treated in FMSs at 600 MPa for up to 300 s at 110 or 90 °C, respectively. Generated curves followed similar negative exponential shapes for both strains in foods and FMSs but total numbers of survivors were partially significantly ($p < 0.05$) different (Table 12 and Table 13). So in general, obtained curve shapes in FMSs were similar to those obtained in real foods. Hence, the model system could serve as an early tool during product development for the prediction of endospore resistance towards HPT processing. However, a potential application would be the responsibility of the operator and depend on its safety margin.

5.3.2 Impact of different primary food components and pH value on HPT inactivation of TMW 2.357

Endospores of *C. botulinum* belonging to the proteolytic group I are known to exhibiting increased resistance properties towards common bactericidal treatments (e.g. wet and dry heat, disinfectants or UV light) including high pressures (Gänzle, Margosch et al. 2008). As shown in several studies, this is in particular true in the case of TMW 2.357 (Margosch, Ehrmann et al. 2004, Margosch, Ehrmann et al. 2006, Maier, Lenz et al. 2017). Therefore, relatively high process temperatures (100, 110 °C) in combination with high pressure were applied to gain insights into the HPT inactivation of those endospores suspended in various food model systems.

At 110 °C, total HPT inactivation values of endospores were thoroughly high in all food model matrices, achieving around 5-log cycles. Partially, the viable endospore count fell below the detection limit (20% fat, 0% salt, pH 7). At such a high temperature, no significant differences ($p > 0.05$) between varying contents of different primary food components or pH values could be detected. Possible endospore resistance enhancing or reducing effects, which could be triggered by single food components in a complex model matrix seemed to be neutralized by the intensive heat. This is in line with recently published data on the impact of different a_w on the HPT inactivation of *B. amyloliquefaciens* (Sevenich, Reineke et al. 2015). Occurring protective effects of solutes at lower temperatures (600 MPa, 105 and 110 °C) were overcome by certain pressure and temperature combinations (600 MPa, ≥ 115 °C). Regarding this for the practical implementation of HPT processing, one could eliminate possible endospore protective effects due to the food matrix by increasing the process intensity, i.e. by increasing the process temperature. This would obviously also involve decreased food quality (e.g. color, consistency, taste) compared to HPT processes at lower process temperatures. However, in the case of shelf-stable LA foods, temperatures below the typical thermal processing temperature (121.1 °C) and/or the reduction of the overall process duration due to the addition of pressure would still result in improved food quality compared to foods produced by conventional retorting.

The equivalent treatment at 100 °C, on the other hand, partially revealed significant differences ($p < 0.05$) regarding the impact of single primary food components on the endospore resistance which are shown in Figure 20 and discussed in the following sections.

In this work, **fat** contents ≥ 10% significantly enhanced the resistance of suspended endospores of TMW 2.357 towards HPT processing. However, this protective effect seemed to stagnate since it did not increase with a higher fat content of 20%. A protective effect of fat during HPT processing has previously also been described for *Geobacillus stearothermophilus* (Ananta, Heinz et al. 2001). Thereby, endospores suspended in a fat rich medium (cocoa mass with varying fat contents) exhibited increased resistance towards pressure and heat (e.g. 90 °C at 600 MPa). The authors' results indicate, that the observed protective effect of increasing fat contents was solely caused by a consequently decreasing moisture content. This is partially in line with our results shown in Figure 27 (appendix), where the lowest endospore inactivation was observed at the highest fat content, exhibiting a water activity as low as 0.93. However, this setup did not allow to spot the impact of fat within a complex matrix since the concentration of solutes (e.g. salt) shifted due to varying amounts of fat. By ensuring constant solute concentrations (resulting in only slightly varying water activities; Table 8), the observed protective effect shown in Figure 20 could directly be assigned to increasing fat contents. Assuming a direct endospore protection by fat, free fatty acids penetrating interior parts of the endospore might stabilize key molecules from heat denaturation to a certain extent. This suggestion was derived for several other endospore-forming species which were exposed to heat treatments and suspended in various lipid materials with consistent water contents (Molin and Snygg 1967, Ababouch and Busta 1987). However, a direct protection of fat due to the agglomeration of endospores onto the surface of dispersed lipid droplets seems unlikely since the surface hydrophobicity of endospores of TMW 2.357 was classified as moderate to highly hydrophilic (Table 3). This implicates a stronger tendency of endospores to migrate towards the aqueous phase of the matrix. A possible protective effect of free fatty acids is further discussed in section 5.3.3.

Further results indicate that endospores of TMW 2.357 are sensitive towards HPT processing at intermediate concentrations of **proteins** and **carbohydrates**. Higher concentrations (20%) did not promote this effect, resulting in similar or just slightly higher inactivation compared to no proteins or carbohydrates present. Those results are in contrast to other studies which evaluated the impact of proteins and/or carbohydrates on bacterial resistance properties towards heat and/or pressure (Gaillard, Leguerinel et al. 1998, Kalchayanand, Sikes et al. 1998, Raso, Gongora-Nieto et al. 1998, Mazas, Martínez et al. 1999, Ramaswamy, Jin et al. 2009, Gao and Ju 2010, Sevenich, Reineke et al. 2015). Most available studies on this topic investigated the impact of single food components on the inactivation of vegetative microorganisms and generally concluded that nutrient-rich media (particularly proteinaceous media) promote enhanced pressure-protection. Gao and Ju (2010), on the other hand, investigated the resistance of endospores of *B. cereus* towards the combination of high pressure and heat (540 MPa, 71 °C, 16.8 min) in complex food model systems (soybean oil, soy bean protein, sucrose). They found that increasing soybean protein and sucrose contents exerted a protective effect on *B. cereus* endospores during HPT treatments. However, exact mechanisms of protein-induced pressure-protection still remain unclear. Microbial endospores are naturally in a metabolically dormant state whereat the activation of the germination pathway can be triggered by available nutrients (e.g. proteins or sugars). Regarding the results obtained in this work, the time period prior to the actual HPT treatment and adequate conditions could have induced nutrient-triggered (partial-)germination even though samples were stored on ice. However, besides temperature, the lag of germination strongly depends on the endospore-forming species as well as the degree of heterogeneity within the population (inter alia, levels of germinant receptors) (Black, Setlow et al. 2007). For instance, endospores of proteolytic *C. botulinum* type A did not germinate within 75 min (time for first germination; 1.7% of the population) at 20 °C after being introduced into germination media (Billon, McKirgan et al. 1997). On the other hand, endospores of non-proteolytic *C. botulinum* type E germinated most rapid at temperatures as low as 2 °C (induced germination but no outgrowth detected) (Grecz and Arvay 1982). Hence, possible (partial-)germination could have resulted in a proportion of pressure- and heat-sensitive endospores within the population. This might also be an explanation for the pronounced inactivation due to the pressure ramp

(1 s holding time) revealed by inactivation kinetic (e.g., Figure 11, Figure 12, Figure 14 and Figure 15). As stated above, increasing sucrose contents (0%, 10%, 20%) and thereby corresponding decreasing a_w values (0.97, 0.95, 0.94) did not protect the suspended endospores during HPT treatments. These findings are basically in line with Sale, Gould et al. (1970), who found that sucrose as a non-ionic solvent had no protective effect on the inactivation of endospores (*Bacillus coagulans*) by pressure (100 MPa, 65 °C). Hence, one could conclude that HPT resistance of endospores does not solely depend on the water activity per se, as stated in various other studies, but on the type of solute. Raso, Gongora-Nieto et al. (1998) and Sevenich, Reineke et al. (2015) on the other hand, reported a decreased inactivation of *B. cereus* and *B. amyloliquefaciens* endospores by high pressure due to low a_w adjusted by sucrose. Those observations were made for endospores suspended in one-component-systems (solutes solved in water or buffer) and hence maybe not applicable for more complex surroundings such as the applied model system and the used strain in this work.

Varying **salt** concentrations had little effect on the HPT resistance of suspended endospores. By solving increasing amounts of NaCl, i.e. 0%, 2% and 4%, the a_w decreased from 0.97, to 0.95, to 0.94, respectively. A direct correlation between endospore inactivation and the a_w could not be identified. This is contradictory to results reported by other authors who identified a direct impact of the ionic solvent on the inactivation of various endospores of *Bacillus* species (Sale, Gould et al. 1970, Sevenich, Reineke et al. 2015). As mentioned before, their inactivation studies were conducted in buffers or water (aqua dest.) where interference of additional components can not alter the outcome of the inactivation experiment. Furthermore, in simple aqueous systems such as buffers, both pressure and temperature can cause a decisive shift in pH (Smelt 1998). This can alter the behavior of endospores towards HPT processing depending on the intensity of the treatment. Hence, a direct transfer of inactivation results obtained in not pressure-stable buffers into real foods is impeded since the behavior of the pH under high pressure and temperature in complex food matrices is unpredictable.

As for the results obtained at different **pH values**, no significant impact on the endospore resistance towards HPT processing could be detected. However, endospores tended to be more sensitive at the lowest applied pH value (pH 4.6). Here, more than 5-log cycles of inactivation could be achieved. This is in compliance with previous HPT (800 MPa, 80 °C) results obtained for TMW 2.357 in Tris-His buffer within pH between 4.0 to 6.0. No significant difference was detected between 5.15 and 6.0 but HPT inactivation increased markedly at pH 4.0 (Margosch, Ehrmann et al. 2004). Compared to all other results shown in Figure 20, the achieved endospore inactivation at different pH values was fairly high. One possible explanation could be the induction of sensitized endospores (also referred to as H-spores). Thereby, a transient pH shift towards the acidic can alter the inner endospore membrane, increase permeability barriers and finally lead to decreased HPT resistance (Marquis and Bender 1985, Paredes-Sabja, Gonzalez et al. 2007). The extent of a pH shift during HPT treatments can depend on several factors (e.g. temperature and pressure level, buffer system, food matrix). For example, in aqueous solutions buffered with phosphates or carboxylic acids, the pH can be reduced by about 1.0 pH unit when pressurizing up to 300 MPa (Molina-Gutierrez, Stippl et al. 2002). Hence, the pH in foods with pH ≥ 4.6 can easily drop below pH 4.6 during pressurization and influence endospore resistance properties. On the other hand, foods or food model systems can contain various numbers of constituents (e.g. organic and amino acids) which can act as buffering agents, and hence also compensate a potential inactivation effect due to decreased pH values.

As for HPT-treated endospores of TMW 2.357 in food model systems, the influence of varying primary food components and pH values did not reveal coherent inactivation results. The impact of those primary food components strongly depended on the specific component and its concentration as well as the process intensity (p/T). For now, this impedes a generic strategy to involve the impact of various components on the HPT resistance and summarize in a single factor, such as e.g. the water activity or the pH value.

5.3.3 Impact of different primary food components and pH value on the HPT inactivation of TMW 2.990

In this section the impact of fat, protein, carbohydrates, salt and the pH on the inactivation of endospores of TMW 2.990 by HPT will be discussed. A broad temperature range (80 – 100 °C) was applied in order to reveal the influence of heat on the response of endospores towards those primary food components and varying pH values.

The protective effect of **fat** seemed to be dependent on the applied process temperature. At 80 °C, increasing fat contents slightly enhanced endospore inactivation by HPT treatments. At 90 and 100 °C on the other hand, this effect diminished or partially turned into a protective effect. This temperature-dependent behavior of endospores applied for both matrices, whether the amount of fat was adjusted based on the aqueous phase (Figure 22) or on the total two-phase system (Figure 28). As mentioned and discussed in previous sections (section 5.1 and 5.3), a protective effect of fat during heat and high pressure treatments has been reported for endospores of several species (Molin and Snygg 1967, Ananta, Heinz et al. 2001, Bull, Olivier et al. 2009, Schnabel, Lenz et al. 2015). However, other reports state that a varying amount of fat has a minor impact on the endospore (*B. cereus*) resistance during HPT processing (Gao and Ju 2010). Hence, the presence of fat and possible resulting protective effects are difficult to generalize. Since the adiabatic heat during compression was considered in the experimental setup and isothermal pressure holding times were maintained, an increased inactivation of endospores due to higher occurring temperatures in the fat phase can be excluded. Endospores of TMW 2.990 seemed to have a more hydrophobic surface (Table 3). This implicates, that endospores tend to migrate towards the fatty phase, and therefore could directly interact with the boundary layer of oil droplets or be penetrated by free fatty acids, which might protect inner key molecules from denaturation. By contrast, several authors reported decreased heat resistance of endospores in the presence of free fatty acids or monoglycerides (Kimsey, Adams et al. 1981, Tremoulet, Rabier et al. 2002, Lekogo, Coroller et al. 2013).To date, no data is available reporting the behavior of *C. botulinum* endospores in the presence of free fatty acids during HPT processing. In

line with available data on heat resistance, our data suggests that free fatty acids do not increase HPT resistance of endospores (Figure 23). For instance, Lekogo, Coroller et al. (2013) investigated the impact of C18 free fatty acids on the heat inactivation of several endospores. They showed, that resistance properties of endospores of *C. sporogenes* were reduced in the presence of oleic and stearic acid during thermal processing (90, 95, 100 °C). This suggests, that free saturated and unsaturated fatty acids rather act as antimicrobial agents during HPT and heat processing. The number of injured endospores usually increases along with extended isothermal/isobaric holding time. Since the applied process duration in Figure 23 did not exceed 60 s, the impact of FFA on endospore resistance might improve with longer processing times. Our results and results published by others, however, indicate a protection of fat which is maybe related to its source. As mentioned earlier, outbreaks of type E botulisms are generally associated with fish product consumption (e.g. cooked, fermented, smoked, dried, salted). Hence, the impact of fish oil in the presence of another type E endospores has been additionally evaluated (Figure 24). No significant impact of 10% fish oil could be detected in the applied p/T/t range for any strain. However, slight strain-dependent HPT resistance properties occurred.

In this respect, it is difficult to compare results of various studies but following factors have to be taken into consideration for HPT treatments of fat-rich products: the process intensity (p/T/t), the type and amount of fat, the residual food components and finally, the present type of food-safety relevant endospore.

The variation of **protein**, **carbohydrate** and **salt** content revealed similar HPT inactivation patterns for endospores of TMW 2.990. Contents >0% resulted in increased resistance at every applied process temperature. Generally, the behavior of TMW 2.990 towards HPT processing in model systems with varying protein, carbohydrate and salt concentrations is in compliance with most other studies on this matter (Sale, Gould et al. 1970, Raso, Gongora-Nieto et al. 1998, Ananta, Heinz et al. 2001, Sevenich, Reineke et al. 2015) but in contrast with results obtained for TMW 2.357. This is not surprising since different groups of *C. botulinum* differ widely in genetic and metabolic characteristics and hence, also in resistance properties. For TMW 2.990, the often postulated correlation between water activity and endospore

resistance verifies to a certain extent. Decreasing a_w resulted in increasing HPT resistance but an $a_w < 0.95$ had relatively marginal impact on further enhancing HPT resistance. This tendency was independent of the used solute (ionic or non-ionic). Similar behavior has been shown for HPT-treated endospores of *B. cereus* (540 MPa, 71 °C, 16.8 min). Increasing concentrations of sucrose and soybean protein significantly protected those endospores against HPT treatments within a complex model matrix. However, maximum concentrations applied in this study (20%: protein and carbohydrate; 4%: salt) did not improve endospore resistance decisively. Overall, the occurring protective effects were most pronounced at 100 °C (highest difference in log reduction). Recently, it has been shown that the HPT-protection of solutes decreases with increasing temperatures (Sevenich, Reineke et al. 2015). This observation was made for HPT treatments at 600 MPa and for T ≥ 115 °C with endospores of *B. amyloliquefaciens* and could be linked to a retention of DPA inside the endospore. Possible mechanisms for the DPA-retention due to solutes might be related to the lack of free water (a_w) which is thought to be a main factor for rapid inactivation and DPA-release (Sevenich, Reineke et al. 2015). However, it seems that every relevant endospore-forming organisms has a specific threshold temperature at which protective effects of solutes are enhanced or overcome by the application HPT processing. For TMW 2.990, this threshold temperature could not be detected within the experimental setup but might just be for T > 100 °C in combination with extended process times.

Although inactivation results in response to varying **pH** values were partially significantly different from each other, total differences in log reduction values were very small (<0.5-log cycles) (Figure 22). The difference in endospore reduction became most apparent at 100 °C whereat viable endospore counts fell below the detection limit at pH 4.6. Hence, temperatures ≥100 °C seemed to trigger pH-related HPT-resistance. In general, a low pH is thought to decrease HPT endospore resistance (Roberts and Hoover 1996, Gao and Ju 2010). Factors contributing to this assumption are, for example, the induction of H-spores (described in section 5.3.2). As mentioned before, the occurrence of pressure-sensitive endospores seems to strongly depend on the species, specific process intensities and matrix composition. For TMW 2.357, a pH between 4.6 and 7.0 had little effect on endospore resistance (Figure 20) in the FMS

but inactivation could markedly be increased at pH 4.0 in Tris-His buffer at lower temperatures (800 MPa, 80 °C). The increase in inactivation was concomitantly accompanied by a drastic loss of DPA due to changes in the endospore permeability barrier. Subsequent rehydration of the endospore core would lead to a decrease in wet-heat and pressure resistance (Margosch, Ehrmann et al. 2004).

The presence and absence of primary food components partially revealed significant impact on the resistance of endospores of TMW 2.990. Overall, the presence of any food component as well as low pH values rather increased the endospore resistance. Furthermore, differences in log reduction increased with increasing temperatures. Those findings are predominantly in line with results reported for other endospore-forming species. The obtained results could help minimizing potential hazards occurring due to HPT treatment of low-acid foods intended for cold-storage.

6 Summary

The production of safe, low-acid (LA) foods depends on the control of resistant bacterial endospores, in particular those of the foodborne pathogen *Clostridium* (*C.*) *botulinum*. The current practice to inactivate bacterial endospores and achieve commercial sterility of LA foods is still based on conventional thermal retorting. However, the application of harsh thermal treatments provokes a significant decrease in quality and often results in overprocessing of food products. These negative effects on food quality can primarily be attributed to low heating rates at ambient pressure. A potential technology to shorten the total process duration and, simultaneously, inactivate microbial endospores is the combination of high pressure and high temperature. Due to a lack of knowledge regarding the inactivation of *C. botulinum* endospores in relevant food matrices, high pressure thermal (HPT) processing has not yet been implemented on an industrial level. Theses derived from this work could contribute filling this gap and provide guidance for future HPT process designs. Our results particularly highlight the suitability of HPT processing as an alternative for traditional thermal treatments by achieving equivalent food safety at lower process temperatures and shorter process durations. Notably, the maxime "the more pressure, the better" is not always the best choice for effective endospore inactivation. Furthermore, case-by-case studies cannot be replaced by generic approaches including simple food component compositions to predict the endospore inactivation by HPT processing. To generate reliable and comparable inactivation data, HPT experiments were performed under isothermal and isobaric conditions during pressure holding time using two resistant strains of *C. botulinum* as a worst-case scenario.

Inactivation curves of proteolytic type B *C. botulinum* strain TMW 2.357, relevant for shelf-stable LA foods, exhibited rapid inactivation during compression and decompression followed by strong tailing effects. At high treatment temperatures, endospore inactivation was more effective at lower pressure levels (300 vs. 600 MPa), which indicates a non-linear pressure/temperature-dependence of the HPT endospore inactivation efficiency. Further experiments using varying endospore inoculation levels suggested the presence of a resistant fraction comprising approximately 0.01% of an endospore population as reason for the pronounced tailing effects in survivor curves.

In addition, the loss of the high resistance properties upon outgrowth and re-cultivation indicates that those differences develop during sporulation and are not linked to permanent modifications at the genetic level.

Inactivation of endospores of non-proteolytic type E *C. botulinum* strain TMW 2.990, which is mainly relevant in low-acid chilled foods (storage <10 °C), was more effective by combining pressure with heat compared to only heat. In general, endospore reduction was more rapid and the overall process duration could be shortened due to accelerated heating and cooling rates during compression and decompression phase. Traditional thermal processing of endospores at 90 °C for 10 min did not result in an estimated 6-log cycle reduction. Processing at 90 °C and 600 MPa on the other hand, resulted in inactivation below the detection limit after 5 min in all foods except the fish product. Additional HPT treatments in the fish product and imidazole phosphate buffer (IPB) did not reveal pronounced food matrix dependent protective effects. Here, varying pressure levels did not appear to be the driving force for endospore count reduction in steamed sole at any temperature (80 – 100 °C). Compression and decompression phase (1 s pressure holding time) had a considerable impact on endospore count reduction (max. -2.9-log cycles) in both, LA foods and IPB.

Overall, the combination of pressure and temperature to inactivate endospores was more effective which illustrates the potential of HPT processing to replace conventional retorting. It could also been shown that the overall process duration can considerably be shortened due to accelerated heating rates (adiabatic effect) which avoids prolonged holding times and potential overprocessing.

Towards a generic approach, a food model system was developed to evaluate the HPT inactivation of bacterial endospores. Each primary food component (fat, carbohydrate, protein and salt) was substituted by a single stereotype component (rapeseed oil, sucrose, tryptone/peptone and NaCl, respectively) to systematically investigate their effects on the HPT inactivation (600 MPa, 80 – 110 °C) of TMW 2.357 (proteolytic type B) and TMW 2.990 (non-proteolytic type E). Additionally, inactivation results in food model systems mimicking the composition of four specific low-acid food products were compared with those obtained in the real food products.

Experiments evaluating the variation of single food parameters did not result in coherent conclusions neither within nor between the two strains. Generated inactivation kinetics in real foods and the respective model systems exhibited similar curve shapes, but total numbers of survivors were different. This has three major implications for future food safety considerations: The effect of single primary food components (i) can largely depend on the target organism, (ii) is not always linear, which impedes extrapolation attempts, and (iii) underlies a complex interplay when food components are combined to mimic complex food matrices. In summary, this currently impedes the implantation of a generic approach to replace case-by-case evaluations. However, a closer look on the impact of single components of primary food fractions, such as specific proteins or fatty acids, and the characterization of their interplay could contribute to a better understanding of the HPT inactivation of *C. botulinum* endospores and probably pave the way for future generic approaches.

7 Zusammenfassung

Die Herstellung von sicheren, schwach-sauren Lebensmitteln ist von der Kontrolle resistenter bakterieller Endosporen, insbesondere des lebensmittelpathogenen Erregers *Clostridium* (*C.*) *botulinum* abhängig. Die derzeitige Praxis zur Inaktivierung von bakteriellen Endosporen und zur Erreichung kommerzieller Sterilität beruht nach wie vor auf konventionellen thermischen Verfahren. Jedoch haben starke thermische Belastungen erhebliche Qualitätsverluste zur Folge. Diese Qualitätsverluste sind den überwiegend langsamen Aufheizraten unter Umgebungsdruck zuzuschreiben. Eine Möglichkeit, die Prozessdauer zu verkürzen und gleichzeitig bakterielle Endosporen zu inaktivieren beruht auf der Kombination von hohen Drücken mit hohen Temperaturen. Aufgrund von fehlenden Erkenntnissen bezüglich der Inaktivierung von *C. botulinum* Endosporen in relevanten Lebensmittelmatrices, konnte das Hochdruck-Temperatur-Verfahren (HDT) noch nicht im industriellen Maßstab implementiert werden. Die abgeleiteten Thesen aus dieser Arbeit könnten dazu beitragen, diese Lücken zu füllen und eine Hilfestellung für zukünftige HDT-Prozessdesigns darstellen. Unsere Ergebnisse heben insbesondere die Anwendbarkeit des HDT-Verfahren als eine Alternative für konventionelle thermische Verfahren dadurch hervor, dass äquivalente Lebensmittelsicherheit bei niedrigeren Prozesstemperaturen und kürzeren Prozesszeiten erreicht werden kann. Bemerkenswert ist, dass der Leitsatz „je mehr Druck, desto besser“ nicht immer die beste Wahl für eine effektive Endosporen Inaktivierung ist. Des Weiteren, können Fall-zu-Fall Studien nicht durch generische Ansätze, welche einfache Lebensmittelzusammensetzungen zur Voraussagung der HDT-Endosporen Inaktivierung beinhalten, herangezogen werden. Um vergleichbare Ergebnisse zu erhalten, wurden sämtliche HDT Versuche unter isothermen und isobaren Bedingungen mit zwei resistenten *C. botulinum* Stämmen durchgeführt.

Der proteolytische Stamm TMW 2.357 vom Toxintyp B ist hauptsächlich in schwach-sauren Lebensmitteln relevant, welche bei Raumtemperatur gelagert werden. Die erhaltenen Inaktivierungskurven wiesen eine starke Inaktivierung während der Druckrampe (Kompression und Dekompression) auf, gefolgt von starken Tailing-Effekten. Bei hohen Prozesstemperaturen war die Endosporeninaktivierung unter niedrigeren Drücken (300 vs. 600 MPa) deutlich effizienter, was einen nichtlinearen

Druck/Temperatur-Zusammenhang impliziert. Weitere Experimente mit variierenden Anfangskeimzahlen ließen auf eine resistente Endosporenfraktion (0,01%) innerhalb der Gesamtpopulation schließen, welche für die beobachteten Tailing-Effekte verantwortlich zu sein schienen. Ausgebildete Resistenzeigenschaften gingen während der Wiederkultivierung von überlebenden Endosporen verloren. Dies deutet darauf hin, dass diese nicht mit permanenten Modifikationen auf genetischer Ebene verlinkt sind.

Der nicht-proteolytische Stamm TMW 2.990 vom Typ E ist hauptsächlich in kühl gelagerten (Lagerung <10 °C), schwach-sauren Lebensmitteln relevant. Dessen Endosporeninaktivierung war wesentlich effizienter durch die Kombination von hohen Drücken mit hohen Temperaturen. Die Inaktivierung verlief grundsätzlich schneller und die gesamte Prozessdauer konnte mit Hilfe von beschleunigten Aufheiz- und Abkühlraten verkürzt werden. Eine klassische thermische Behandlung von Endosporen bei 90 °C für 10 min lieferte nicht die erwartete 6-log Reduktion. Eine Kombination von 90 °C und 600 MPa resultierte dagegen in einer Inaktivierung bis unter die Detektionsgrenze innerhalb von 5 min in allen behandelten Lebensmitteln außer im Fischprodukt. Zusätzliche HDT-Versuche in Imidazol-Phosphat-Puffer offenbarten jedoch keine durch die Matrix hervorgerufenen protektiven Effekte. Unabhängig von der Temperatur, schienen dabei variierende Drucklevel nicht die treibende Kraft für die Endosporeninaktivierung zu sein. Die Kompressions- und Dekompressionsphase (1 s Haltezeit) hatte einen erheblichen Einfluss auf die Endosporeninaktivierung (max. -2.9 log) in Lebensmitten als auch in IPB.

Insgesamt war eine Kombination von Druck und Temperatur zur Endosporeninaktivierung effizienter und verdeutlicht somit das Potential von HDT-Verfahren gegenüber klassischen thermischen Verfahren. Es konnte außerdem gezeigt werden, dass die Prozessdauer mit Hilfe von beschleunigten Heizraten (adiabatischer Effekt) deutlich verkürzt werden kann, was wiederum positiv auf die Produktqualität auswirkt.

Um einen generischen Ansatz zu ermöglichen, wurde eine Lebensmittelmodellmatrix entwickelt. Dabei wurden primäre Lebensmittelkomponenten (Fett, Kohlenhydrate,

Protein und Salz) mit einzelnen definierten Komponenten (Rapsöl, Sucrose, Trypton/Pepton und NaCl) ersetzt, um deren Einfluss auf die HDT-Inaktivierung (600 Mpa, 80 -110 °C) von TMW 2.357 und TMW 2.990 zu evaluieren. Zusätzlich wurden die schwach-sauren Lebensmittel durch das Modellsystem imitiert und die Inaktivierungsergebnisse mit denen aus den tatsächlichen Produkten verglichen.

Die Variation von einzelnen Lebensmittelparametern ermöglichte weder innerhalb eines Stamms noch zwischen den beiden Stämmen eine einheitliche Schlussfolgerung. Generierte Inaktivierungskinetiken zwischen echtem Lebensmittel und Modelsystem wiesen ähnliche Kurvenverläufe auf, jedoch waren die absoluten Werte (log-Reduktion) unterschiedlich. Dies lässt folgende drei Schlussfolgerungen in Bezug auf zukünftige Lebensmittelsicherheitsrelevante Ansätze zu: Die Auswirkung von einzelnen Lebensmittelkomponenten (i) ist abhängig vom Zielorganismus, (ii) ist nicht immer linear, was Extrapolationen erschwert, und (iii) unterliegt komplexen Wechselwirkungen von verschiedenen Lebensmittelkomponenten innerhalb einer Modelmatrix. Zusammenfassend scheint dadurch der Einsatz eines generischen Ansatzes als Ersatz für Fall-zu-Fall Betrachtungen erschwert. Jedoch könnte beispielsweise eine genauere Untersuchung von spezifischen Proteinen oder Fettsäuren, sowie deren Einfluss und Zusammenspiel auf die HDT-Inaktivierung von *C. botulinum* Endosporen wertvolle Erkenntnisse liefern, und den Weg für zukünftige generische Ansätze liefern.

8 References

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9 Appendix

9.1 Additional figures and tables

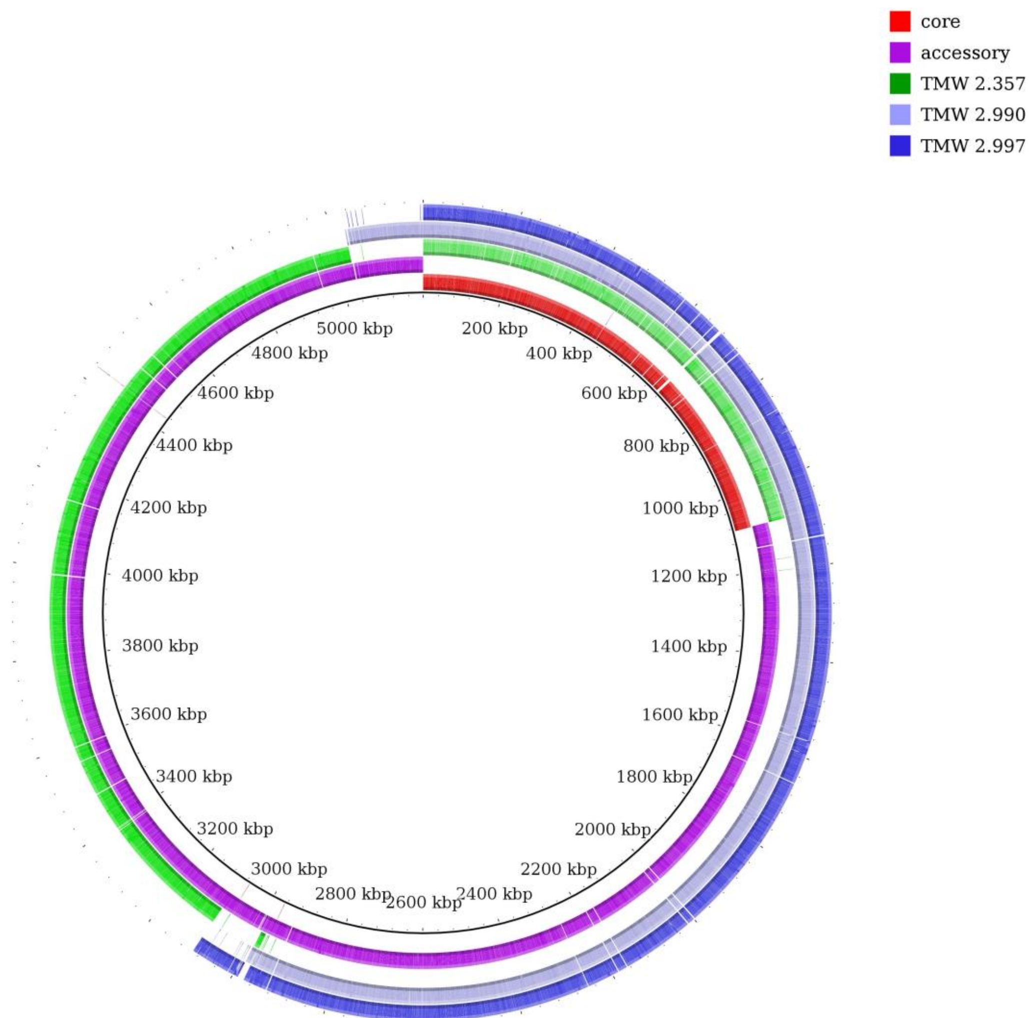

Figure 25. Genomic comparison of TMW 2.990, TMW 2.997 and TMW 2.357 based on the amino acid sequence (protein level) and visualized by using BRIG (Alikhan, Petty et al. 2011). The figure enables to show the presence of similarities and differences of all sequenced strains within the species.

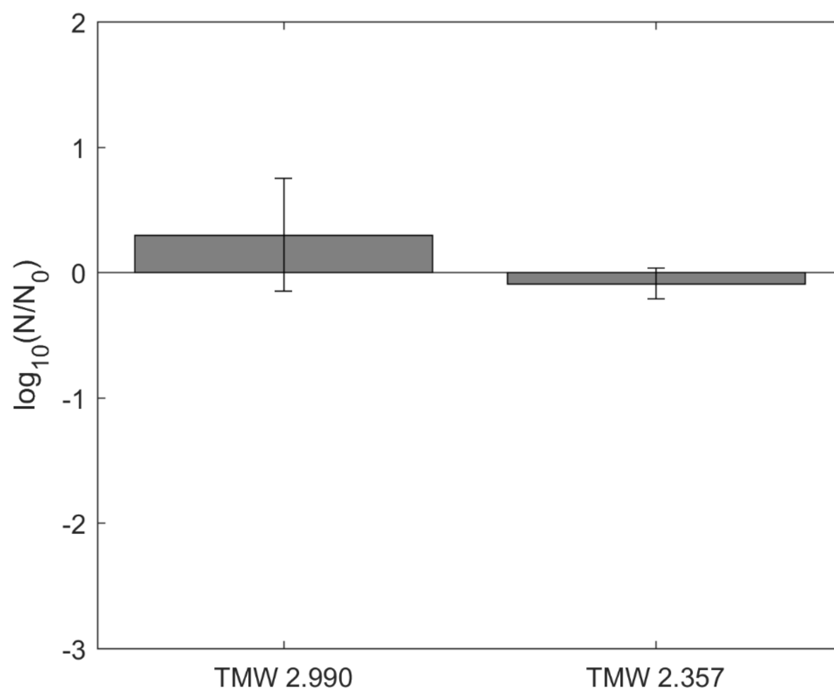

Figure 26. Heat treatment of purified spore suspensions at 60 °C for 15 min. Results are presented as $Log_{10}(N/N_0)$, where N describes the number of surviving spores after a treatment and N_0 is the initial spore count. Data are shown as means ± standard deviations of duplicate independent experiments.

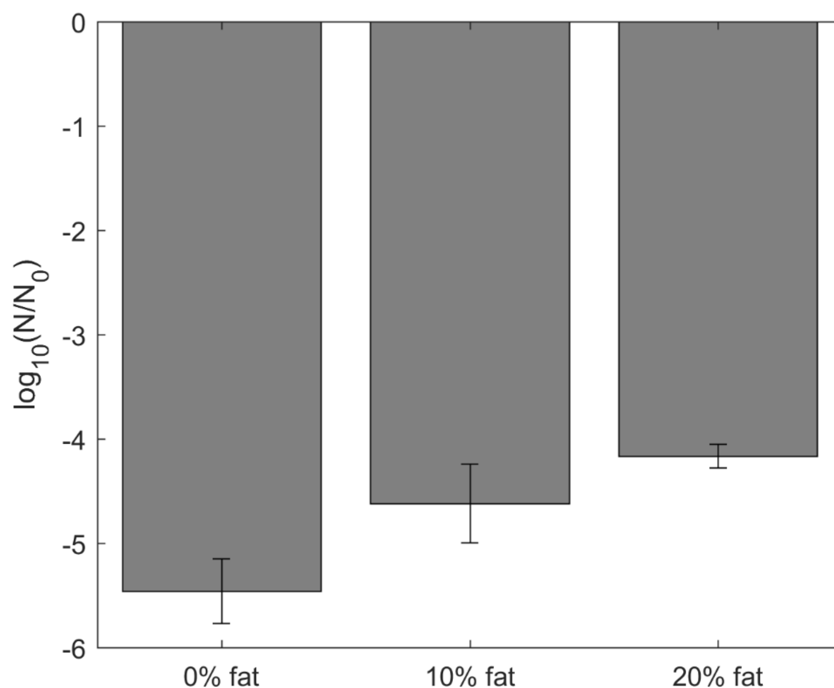

Figure 27. Inactivation of *C. botulinum* TMW 2.357 endospores in food model system with 0%, 10% and 20% of fat (note that the concentration of solutes increase with higher fat contents). Endospores were treated at 600 MPa at 100 for 60 s. Results are presented as $Log_{10}(N/N_0)$, where N describes the number of surviving spores after a treatment and N_0 is the initial spore count. Initial spore count: 10^7 spores/g. Water activity at 0% and 10% fat: 0.95; water activity at 20% fat: 0.93. Data are shown as means ± standard deviations of triplicate independent experiments.

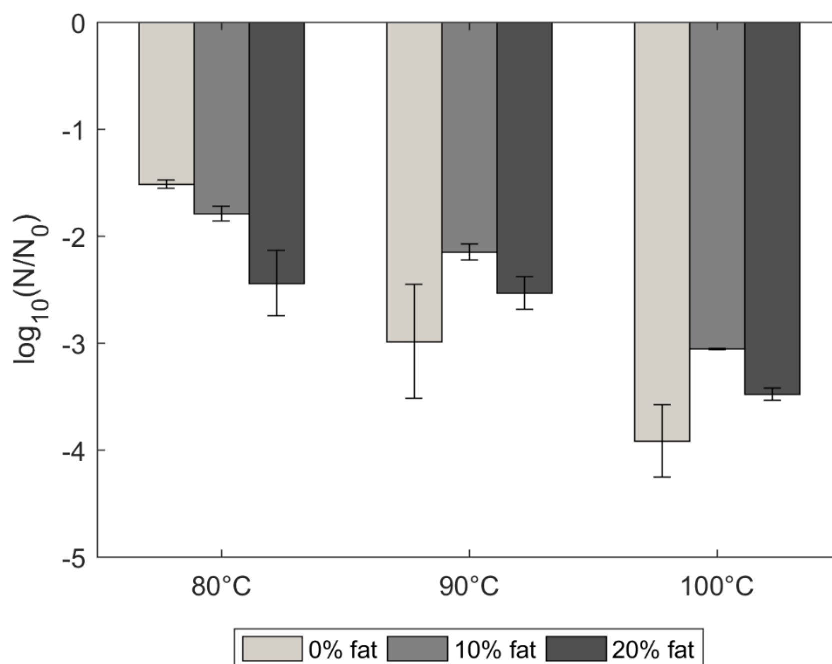

Figure 28. Inactivation of *C. botulinum* TMW 2.990 endospores in food model system with 0%, 10% and 20% of fat (note that the concentration of solutes increase with higher fat contents). Endospores were treated at 600 MPa at 100 for 60 s. Results are presented as $Log_{10}(N/N_0)$, where N describes the number of surviving spores after a treatment and N_0 is the initial spore count. Initial spore count: 10^7 spores/g. Water activity at 0% and 10% fat: 0.95; water activity at 20% fat: 0.93. Data are shown as means ± standard deviations of triplicate independent experiments.

Table 12. Data on statistical significant differences of HPT inactivation of spores of TMW 2.357 in foods and equivalent FMS at defined time points of pressure holding time.

Time [s]	GPH - FMS GPH	SS - FMS SS	VS - FMS VS	BV – FMS BV
1	Yes (p = 0.0402)	No (p = 0.0517)	No (p = 0.1301)	Yes (p = 0.0127)
60	No (p = 0.4106)	No (p = 0.3105)	Yes (p = 0.0108)	Yes (p = 0.0018)
120	Yes (p = 0.0386)	Yes (p = 0.0059)	Yes (p = 0.0312)	Yes (p = 0.006)
210	No (p = 0.2856)	Yes (p = 0.0172)	Yes (p = 0.0121)	No (p = 0.0569)
300	No (p = 0.2277)	Yes (p = 0.0211)	Yes (p = 0.002)	Yes (p = 0.0089)

*GPH: Green peas with ham; SS: Steamed sole; VS: Vegetable soup; BV: Braised veal; FMS: Food model system.

**Statistical significant difference ($p < 0.05$): yes (green); No statistical significant difference ($p > 0.05$): no (red).

Table 13. Data on statistical significant differences of HPT inactivation of spores of TMW 2.990 in foods and equivalent FMS at defined time points of pressure holding time.

Time [s]	GPH - FMS GPH	SS - FMS SS	VS - FMS VS	BV – FMS BV
1	No (p = 0.821)	Yes (p = 0.0085)	No (p = 0.8472)	No (p = 0.58)
60	No (p = 0.2924)	No (p = 0.0507)	Yes (p = 0.32)	No (p = 0.663)
120	No (p = 0.1378)	No (p = 0.2718)	No (p = 0.4464)	Yes (p ⋘ 0.05)
210	No (p = 0.885)	Yes (p = 0.0381)	Yes (p = 0.0025)	Yes (p = 0.0012)
300	Yes (p = 0.0071)	Yes (p = 0.2356)	Yes (p ⋘ 0.05)	Yes (p ⋘ 0.05)

*GPH: Green peas with ham; SS: Steamed sole; VS: Vegetable soup; BV: Braised veal; FMS: Food model system.

**Statistical significant difference ($p < 0.05$): yes (green); No statistical significant difference ($p > 0.05$): no (red).

10 List of publications derived from this work

Peer-reviewed journals

Maier, M. B., C. A. Lenz and R. F. Vogel (2017). "Non-linear pressure/temperature-dependence of high pressure thermal inactivation of proteolytic *Clostridium botulinum* type B in foods." PLOS ONE **12**(10): e0187023.

Maier, M. B., T. Schweiger, C. A. Lenz and R. F. Vogel (2018). "Inactivation of non-proteolytic Clostridium botulinum type E in low-acid foods and phosphate buffer by heat and pressure." PLOS ONE **13**(7): e0200102.

Oral presentations (speaker is underlined)

Vogel, R. F., **Maier, M. B.** and Lenz, C. A. (2018). „High pressure temperature inactivation of *Clostridium botulinum* endospores in low-acid foods and food model systems". 10th International Conference on High Pressure Bioscience and Biotechnology (HPBB), Numazu, Shizuoka, Japan.

Maier, M. B. and Vogel, R. F., (2017). "High Pressure Thermal Processing - Adequate Food Safety?". Workshop for food industries (organized by CNTA and Metronics), Pamplona, Spain.

Maier, M. B., Lenz, C. A., and Vogel, R. F., (2017). "Non-linear pressure/temperature-dependence of high pressure thermal inactivation of proteolytic *Clostridium botulinum* type B in foods". 31st EFFoST International Conference, Sitges, Spain.

Poster presentations

Maier M. B., Lenz, C. A. and Vogel, R. F., (2017). "Comparison of high pressure thermal inactivation of Clostridium botulinum spores in real foods with models with varying primary food components". 31st EFFoST International Conference, Sitges, Spain.

Maier M. B., Lenz, C. A. and Vogel, R. F., (2016). "Towards a generic approach for the evaluation of high pressure temperature inactivation of *Clostridium botulinum*". 9th International Conference on High Pressure Bioscience and Biotechnology (HPBB), Toronto, Canada.

Supervised student theses

Biebl, Patrick (2016)."Einfluss verschiedener Lebensmittelkomponenten auf die Hochdruck-Hochtemperatur-Inaktivierung von *Clostridium botulinum* Sporen". Master thesis in the course of study "Technologie und Biotechnologie der Lebensmittel".

Schweiger, Tobias (2017). "Hochdruck-Hochtemperatur Inaktivierung von *Clostridium botulinum* Typ E Sporen". Master thesis in the course of study "Technologie und Biotechnologie der Lebensmittel".