High pressure processing at ambient and high temperatures and its influence on food processing contaminants, food borne diseases and bacterial spores in model- and real-food systems

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“If my efforts have led to greater success than usual, this is due, I believe, to the fact that during my wanderings in the field of medicine, I have strayed onto paths where the gold was still lying by the wayside. It takes a little luck to be able to distinguish gold from dross, but that is all.”

Robert Koch

Preface

This thesis is based on reformatted work which has been published in the following peer reviewed publications:

- Reineke, K., Sevenich, R., Hertwig, C., Janßen, T., Fröhling, A., Knorr, D., Wieler, L.H., and Schlüter, O. (2015). Comparative study on the high pressure inactivation behavior of the Shiga toxin-producing *Escherichia coli* O104:H4 and O157:H7 outbreak strains and a non-pathogenic surrogate. *Food Microbiology* 46**,** 184-194.
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- Sevenich, R., Hecht, P., Reineke, K., Fröhling, A., Schlüter, O.K., Rauh, C., and Knorr, D. (2015). Impact of different water activities (aw) adjusted by solutes on high pressure high temperature inactivation of *Bacillus amyloliquefaciens* spores. *Frontiers in Microbiology* 6.
  doi: http://dx.doi.org/10.3389/fmicb.2015.00689
- Sevenich, R., Bark, F., Crews, C., Anderson, W., Pye, C., Riddellova, K., Hradecky, J., Moravcova, E., Reineke, K., and Knorr, D. (2013). Effect of high pressure thermal sterilization on the formation of food processing contaminants. *Innov. Food Sci. Emerg. Technol.*
  doi: http://dx.doi.org/10.1016/j.ifset.2013.07.006
- Sevenich, R., Kleinstueck, E., Crews, C., Anderson, W., Pye, C., Riddellova, K., Hradecky, J.,Moravcova, E., Reineke, K., and Knorr, D. (2014). High-Pressure-Thermal Sterilization: Food safety and food quality of baby food puree. *J. Food Sci.* 79**,** M230-M237.
  doi: http://dx.doi.org/10.1111/1750-3841.12345
- Sevenich, R., Bark, F., Kleinstueck, E., Crews, C., Pye, C., Hradecky, J., Reineke, K., Lavilla, M., Martinez-De-Maranon, I., Briand, J.C., and Knorr, D. (2015). The impact of high pressure thermal sterilization on the microbiological stability and formation of food processing contaminants in selected fish systems and baby food puree at pilot scale. *Food Control* 50**,** 539-547.
  doi: http://dx.doi.org/10.1016/j.foodcont.2014.09.050.

Zusammenfassung

Hochdruck bei 600 MPa in Kombination mit milden Temperaturen macht es möglich, sichere und qualitativ hochwertige Lebensmittel herzustellen. In den letzten Jahren gab es grundlegende Arbeiten zur Klärung des Inaktivierungsmechanismus von Sporen (*Bacillus subtilis* etc.) und vegetativen Mikroorganismen wie *Escherichia coli* und *Lacotbacillus subsp.*, die zum größeren Verständnis der Wirkungsweise dieser Technologie beitrugen. Ein weiteres Anwendungsgebiet der Hochdrucktechnologie ist die schonende Dekontamination von Lebensmitteln, die mit resistenten pathogenen Keimen, wie dem *Escherichia coli* (EHEC) Stamm O104:H4, der für den Infektionsausbruch in Deutschland 2011 verantwortlich war, verunreinigt sind. Die Kombination von Drücken zwischen 100-400 MPa und Temperaturen zwischen 40-70°C führten zu einer schnelleren Inaktivierung im Vergleich zur thermischen Behandlung. Um eine Haltbarkeit von Lebensmitteln bei Raumtemperatur zu gewährleisten, wird das Lebensmittel mittels Hitze sterilisiert. Dieser Prozess ist sehr intensiv und birgt eine hohe thermische Belastung des Produktes, wodurch zwar Sporen abgetötet werden, es aber zur Bildung von unerwünschten Prozess-Kontaminanten wie z.B. Furan, 3-MCPD (monochlorpropandiol)-ester und Acrylamid, und den Verlust von wertgebenden Inhaltsstoffen kommen kann. Deshalb soll im Rahmen dieser Arbeit die Hochdrucksterilisation als Alternative zur thermischen Sterilisation hinsichtlich der Inaktivierung von Sporen und Reduzierung der Prozesskontaminanten getestet werden. Es gibt bis heute jedoch keinen festgelegten Leitkeim für die Hochdrucksterilisation und keine Kenntnisse über das Verhalten möglicher Leitkeime wie *Clostridium sporogenes*, *Bacillus amyloliquefaciens* oder *Geobacillus stearothermophilus*, in lebensmittelnahen oder echten Lebensmittelsystemen. In den letzten Jahren gab es grundlegende Arbeiten zum Verständnis des Inaktivierungsmechanismus von Sporen (*Bacillus subtilis* etc.), aber diese wurden hauptsächlich in Puffersystemen durchgeführt. Deshalb wurden die vorhergenannten Sporenbildner in einem breiten Druck-Temperatur Spektrum getestet, um ihre Resistenz zu bestimmen. Die Inaktivierung unterschiedlicher Sporen (*Clostridium sporogenes*, *Bacillus amyloliquefaciens* und *Geobacillus stearothermophilus*) in einem breiten Druck- (100-600 MPa) und Temperaturbereich (40-100°C) in ACES-Puffer zeigte, dass unter diesen getesteten Bedingungen die *Bacillus amyloliquefaciens* Sporen, die höchste Resistenz besaßen und er somit als Leitkeim für die Hochdrucksterilisation in Frage kommen könnte. Zum besseren Verständnis der Inaktivierung in komplexeren Systemen und dem möglichen Schutzeffekt von gelösten Stoffen wurden *Bacillus amyloliquefaciens* Sporen

in unterschiedliche Zucker-und Salzkonzentration (0.83-1.7 mol/L bzw. 1.2-2.7 mol/L) mit variierenden a_w-Werten (0.9-1) inokuliert und hinsichtlich Inaktivierung, Dipicolinsäure (DPA)-Ausschleusung und Veränderung an der Membranstruktur mittels FCM, in einem Temperaturbereich von 105-115°C bei 600 MPa untersucht. Je höher die Konzentration der gelösten Stoffe war, desto schlechter ist die Inaktivierung und dementsprechend die DPA-Ausschleusung, wobei Zucker einen höheren Schutzeffekt auf die Sporen hatte als Salz. Für beide Stoffe zeigte sich ein ausgeprägter, schützender Effekt bei einem a_w von $\leq$ 0.94. Ab einer Temperatur von 115°C verschwindet der schützende Effekt und es existieren kaum Unterscheide hinsichtlich der Inaktivierung oder der DPA-Ausschleusung. Die FCM-Analyse zeigte, dass für Modelsysteme, die eine hohe Konzentration an gelösten Stoffen enthielten, die Färbung optimiert werden muss. Allerdings zeigten die Ergebnisse Tendenzen, die auf einen Einfluss der gelösten Stoffe auf die innere Sporenmembran schließen lassen.

Noch komplexer sind echte Lebensmittelsysteme und deren Einfluss auf die Sporeninaktivierung und möglichen chemischen Reaktionen während der Behandlung, die zur Bildung von Prozesskontaminanten führen können. In den letzten Jahren wurde die Reduzierung der krebserregenden Food process contaminants (FPCs), wie Furan oder 3-MCPD-ester, in Lebensmitteln diskutiert und es gilt Alternativen, wie die Hochdrucksterilisation, zur herkömmlichen Prozessierung nach ihrem Potential zu untersuchen. Deshalb wurde *Bacillus amyloliquefaciens* in ausgewählte Lebensmittelsysteme (Babynahrung auf Gemüsebasis und Rohprodukte für Fischkonserven) inokuliert, um das Potential der Hochdrucksterilisation auf die Inaktivierung von Sporen und die Reduzierung von FPCs zu untersuchen. Es zeigte sich außerdem, dass eine Inaktivierung von *Bacillus amyloliquefaciens* Sporen in den Lebensmittelsystem und Modelllebensmittelsystemen im Bereich 90-110°C stark von der Zusammensetzung/Konzentration der Inhaltsstoffe abhängig war. Ab 115°C existierten nur marginale Unterschiede zwischen den Systemen, da hier die treibende Kraft der Inaktivierung die Temperatur ist. Die gewonnen Inaktivierungskinetiken für Babynahrung auf Gemüsebasis und Rohprodukte für Fischkonserven, konnten zur Modellierung einer extrapolierten 12 $\log_{10}$ Inaktivierung, mittels eines n^{ten} –Ordnung Ansatzes verwendet werden. Es konnte ein Scale up vom Labormaßstab (4 mL) auf Pilotmaßstab (55 L) durchgeführt werden. Die durchgeführten Lagerversuche zeigten, dass ein sicheres Produkt in einem Temperaturbereich von 110-115°C und einer Haltezeit zwischen 6.5-28 min bei 600 MPa gewährleistet werden kann. Die Furananalyse zeigte, dass die Bildung von Furan nur in Lebensmittelsystemen auftritt, die

Vorläufer (sog. Precursor wie Zucker, mehrfach ungesättigten Fettsäuren und Aminosäuren) enthalten. Bei den Produkten Sardine in Olivenöl und Babynahrung auf Basis von Gemüse konnte eine Reduzierung im Vergleich zur thermischen Sterilisierung (F_0=7) von 72-97 % (ausgehend von 57.88 $\mu g\, kg^{-1}$) bzw. 81-96 % (ausgehend von 30 $\mu g\, kg^{-1}$) in einem Temperaturbereich von 90-121°C bei 600 MPa erzielt werden. Bei den 3-MCPD-estern erfolgte keine Bildung während der thermischen oder der hochdruck-hochtemperatur Behandlung in den getesteten Systemen. Nur in Thunfisch in Sonnenblumenöl wurden größere Mengen an 3-MCPD-estern gefunden. Diese sind allerdings auf die Verwendung von raffiniertem Sonnenblumenöl zurückzuführen.

Die Daten dieser Arbeit können dazu beitragen, diese neue alternative Sterilisationstechnologie zu kommerzialisieren und somit die Lebensmittelsicherheit und Lebensmittelqualität zu erhöhen.

Abstract

High pressure pasteurization, typically at 600 MPa in combination with mild temperatures, makes it possible to produce safe and high quality foods. In the last few years groundbreaking research was conducted to understand the inactivation mechanisms of vegetative microorganism such as, *Escherichia coli* and *Lactobacillus subsp.,* under high pressure conditions, which led to a better understanding of the principles of this technology. Another application field of high pressure technology is the mild decontamination of foods, such as seeds (Neetoo et al., 2008), which are contaminated, with resistant pathogen bacteria such as *Escherichia coli* (EHEC) strain O104:H4, which was responsible for an outbreak of HUS (Hemolytic Uremic Syndrome) in Germany in 2011. The combination of pressures between 100-400 MPa and temperatures between 40-70°C showed an accelerated inactivation as in comparison to the thermal treatment. The kinetic data from this study and the suggested mechanisms can aid in optimizing this promising sterilization technology and will contribute to enhancing food safety.

To obtain long storage periods for foods at ambient temperatures, sterilization by heat is one of the main technologies used in the food industry. The process is quite intense with a huge thermal load applied to the product, leading not only to the inactivation of spores but also to the formation of undesired chemical byproducts as also termed food processing contaminants (FPCs) such as for example furan, 3-MCPD (monochloropropanediol)-ester and acrylamide, accompanied in some cases by a loss of nutrients. This is why the use of the high pressure thermal sterilization (HPTS) may be an alternative for thermal retorting leading to spore inactivation in real and model food systems and possibly concomitantly to the mitigation of FPCs. To this day there is no indicator microorganism for high pressure thermal sterilization and knowledge about the behavior of possible target microorganisms, such as *Clostridium sporogenes*, *Bacillus amyloliquefaciens* or *Geobacillus stearothermophilus*, in food model systems or real food system is scarce. In the last few years groundbreaking research was conducted to understand the inactivation mechanisms of spores (*Bacillus subtilis* etc.) but mainly in buffer systems. Therefore the aforementioned spore strains need to be tested over a broad pressure-temperature range to evaluate their resistance. The inactivation of different spore strains (*Clostridium sporogenes*, *Bacillus amyloliquefaciens* and *Geobacillus stearothermophilus)* over a wide pressure (200-600 MPa) and temperature (40-100°C) range in ACES-buffer showed that the most resistant organism under these conditions is *Bacillus amyloliquefaciens*. Therefore, this spore strain could be used a possible indicator

microorganism for the HPTS. To gain a better understanding of the inactivation of spores and possible baroprotective effects of solutes in complex food systems *Bacillus amyloliquefaciens,* spores were inoculated in different sucrose and NaCl solutions (0.83-1.7 moll/L respectively 1.2-2.7 moll/L) with a_w-values ranging from 0.9-1. Investigated were inactivation, DPA-release and possible inner membrane changes via Flow Cytometry Measurement (FCM) in a temperature range of 105-115°C at 600 MPa. The higher the solute concentration the more pronounced was the impact on a retarded inactivation and dipilonic acid (DPA)-release. The impact of sucrose as a baroprotective solute was stronger than NaCl. Both solutes showed their potential as a baroprotective solute at $a_w \leq 0.94$. At temperatures of 115°C and higher the baroprotective effect of the solutes diminishes and the differences in inactivation and DPA-release are nominal at all a_w. The FCM-analyses needs to be optimized for high concentrated solutions since the dyeing of all spores was not always possible. Nevertheless, the results indicated that the solutes impacted on the inner spore membrane and thereby led to a retarded inactivation and DPA-release. Even more complex are real food systems and their influence on the inactivation of spores and possible chemical reactions during food processing which can lead to the formation of FPCs. *Bacillus amyloliquefaciens* was inoculated in selected food systems: baby food on the basis of vegetables and raw products for fish cans and tested at lab scale. Spore inactivation as well as the formation of FPCs, such as furan and 3-MCPD-esters under HPTS conditions. The treatment was conducted in a temperature range of 80-121°C at 600 MPa (Annex II for parameters) with dwell times up to 28 minutes. It showed that the inactivation in food of *Bacillus amyloliquefaciens* at 600 MPa 90-110°C and food model systems is strongly dependent on the composition/concentration of the ingredients. At 115 °C and 600 MPa the difference in terms of inactivation was nominal since the driving force of inactivation was the temperature. The collected inactivation data, for the baby food puree and the raw products for fish cans, was used to model with an n^{th}-order approach extrapolated optimal treatment conditions for a 12 $\log_{10}$ inactivation. Based on these calculations from lab scale (4 mL) a scale up approach for a 55 L vessel was conducted. The storage trials revealed that in a temperature range of 110-115°C and dwell times between 6.5-28 min a safe product can be guaranteed. The analyses of furan showed that the formation of furan was only present in those foods (baby food puree and sardine in olive oil), which contained precursors of furan, such as amino acids, sugar and poly unsaturated fatty acids. For the products sardine in olive oil and baby food puree a reduction of furan in comparison to the initial content in the thermally treated sample was possible.

The reduction ranged from 72-97 % (based on 57.9 µg kg^{-1}) respectively 81-96 % (based on 30.0 µg kg^{-1}) in a temperature range of 90-121°C at 600 MPa. No 3-MCPD-esters were formed either under thermal or under HPTS conditions. Only high amounts of 3-MCPD-esters were found in tuna in sunflower oil which is most likely due to the use of refined sunflower oil.

The kinetic data from this study and the suggested mechanisms aid in optimizing this promising sterilization technology leading to food products with improved quality, lower amounts of undesired process contaminants, and ensuring an equivalent degree of microbial food safety as currently existing technologies.

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List of abbreviation

Abbreviation	Description
ACES	N-(2-Acetamido)-2-aminoethanesulfonic acid
ALARA	As low as responsible achievable
A_w-value	Water activity [-]
AZTI	Spanish Food Technology Research Center in Bilbao, Spain
BfR	Bundesinstitut für Risikobewertung
bw	body weight [kg]
Ca	Calcium
Ca-DPA	Calcium- pyridine-2,6-dicarboxylic acid
d	day
DCP	Dichloropropene
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DPA	Pyridine-2,6-dicarboxylic acid or Dipicolinic acid
dV	reaction volume
D-value	decimal reduction time [min]
EFSA	European Food Safety Authority
EHEC	Entero-haemorrhagic *Escherichia coli*
Eq.	Equation
EPA	Eicosapentaenoic acid
F_0	Overall lethality effect of a heat process
FCM	Flowcytomerty
FDA	Food and Drug Administration of the United Sates
Fig.	Figure
FPC	Food processing contaminants
GeR	Germinant receptors

IARC	International Agency for Research on Cancer
3-MCPD	3-Monochloropropanediol
Mg	Magnesium
Mn	Manganese
MoE	Margin of exposure
MPa	Mega [10^6] Pascal
MRE	Meal ready to eat
NaCl	sodium chloride
HP	High pressure
HPP	High pressure processing
HPTS	High pressure thermal sterilization
K	Potassium
PATS	Pressure assisted thermal sterilization
PI	Propidium iodide
PUFA	Poly unsaturated fatty acid
RMSSE	Root Mean Sum of Squared Error
SASP	Small acid soluble proteins
σ	Sigma factor for sporulation
Syto 16	Green Fluorescent Nucleic Acid Stain
t	Time [min]
T	Temperature [°C]
tRNA	Transfer ribonucleic acid
UV	Ultraviolet
WHO	World Health Organization
WWF	World Wide Found for Nature

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1. Introduction

Prometheus and Epimetheus were spared imprisonment in Tartarus because they had not fought with their fellow Titans during the war with the Olympians. They were given the task of creating man. Prometheus shaped man out of mud, and Athena breathed life into his clay figure. Prometheus had assigned Epimetheus the task of giving the creatures of the earth their various qualities, such as swiftness, cunning, strength, fur, and wings. Unfortunately, by the time he got to man Epimetheus had given all the good qualities out and there were none left for man. So Prometheus decided to make man stand upright as the gods did and to give him fire. Prometheus loved man more than the Olympians, who had banished most of his family to Tartarus. So when Zeus decreed that man must present a portion of each animal he scarified to the gods, Prometheus decided to trick Zeus. He created two piles, one with the bones wrapped in juicy fat, and the other with the good meat hidden in the hide. He then bade Zeus to pick. Zeus picked the bones. Since he had given his word, Zeus had to accept this pile as his share for future sacrificed. In his anger over the trick, he took fire away from man. However, Prometheus lit a torch from the sun and brought it back again to man. Zeus was enraged that man again had fire. He decided to inflict a terrible punishment on both man and Prometheus. To punish man, Zeus had Hephaestus create a mortal of stunning beauty. The gods gave the mortal many gifts of wealth. He then had Hermes give the mortal a deceptive heart and a lying tongue. This creation was Pandora, the first woman. A final gift was a jar which Pandora was forbidden to open. Thus completed, Zeus sent Pandora down to Epimetheus, who was staying amongst the men. Prometheus had warned Epimetheus not to accept gifts from Zeus, but Pandora's beauty was too great and he allowed her to stay. Eventually, Pandora's curiosity about the jar she was forbidden to open became intolerable to her. She opened the jar and out flew all manner of evils, sorrows, plagues, and misfortunes. However, the bottom of the jar held one good thing - hope. Zeus was angry at Prometheus for three things: being tricked by the sacrifices, stealing fire for man, and refusing to tell Zeus which of Zeus's children would dethrone him. Zeus had his servants, Force and Violence, to seize Prometheus, take him to the Caucasus Mountains, and chain him to a rock with unbreakable adamantine chains. Here he was tormented day and night by a giant eagle tearing at his liver. Zeus gave Prometheus two ways out of this torment. He could tell Zeus who the mother of the child that would dethrone him was, or meet two conditions. The first was that an immortal must volunteer to die for Prometheus, and the second was that a mortal must kill the

eagle and unchain him. Eventually, Chiron the Centaur agreed to die for him and Heracles killed the eagle and unbound him. Hesiod (700 v. Chr.) (Dougherty, 2004).

If mankind obtained the fire as the Greek mythology tells it, is of course up for discussion or if it was just pure coincidence as many of great inventions in the history of mankind. However, the ”taming” of fire certainly cannot be considered a negligible discovery and what it meant for mankind. Around 790,000 years ago humans were able settle down, found comfort at the fire-side. Some scholars even proclaim that language was developed whilst sitting around a fireplace and most importantly they were able to make their foods more palatable, digestible, and longer lasting. They learnt that the cooking of foods increases the amount of energy the body can metabolize. The extra energy intake gave those ancestors who cooked a biological advantage. Those genes were consequently passed on and humans rapidly adapted to eating a diet based on cooked foods. One could say humans are the cooking apes, in other words “the creatures of the flames” (Wrangham, 2009). The use and knowledge of fire became more and more important for the preparation and preservation of foods.

Over modern centuries people have tried to make food last longer and to preserve foods by salting or sugaring the foods. These preservation methods did not result in the best taste of the food. The horrific war tragedies of 18th century Europe were a key driver that accelerated the development of methods to preserve and seal the foods in their fresh-like state. In fact, more soldiers died due to malnourishment or contaminated foods than were actually killed on the battle field. In 1795 Napoleon offered 12000 Franc to the person who would develop a useful method to preserve food so that his army as he said “could march with a full stomach”. The inventor Nicolas Appert rose to the challenge and treated foods sealed airtight with a cork in glass bottles in a hot water bath. The same year he published his work in “*The Art of Preserving Animal and Vegetable Substances”*. Without knowing it he developed heat sterilization which killed vegetative microorganisms and spores. The specific connection of the influence of heat on microorganisms was made by the microbiologists Louis Pasteur, Robert Koch and John Tyndall in the mid of the 19th century. However, Louis Pasteur was the first of these researchers who developed the first autoclaving/sterilization system. The packaging in tinplate or aluminum sheet cans as we know it today was invented in England in1812 by Bryan Donkin and John Hall. The early cans had two problems one being the fact that these were sealed with lead, which caused a slinking plumbism and second botulism caused by spoilage of the cans by *Clostridium botulinum*

(Hartwig et al., 2014). Today cans are sealed by crimping to avoid plumbism and the risk of botulism has been minimized with the development of the 12 D-sterilization concept in the 20th century.

In the literature sterilization is defined as the act or process, physical or chemical, which destroys or eliminates all forms of life, especially microorganisms and spores to achieve an acceptable sterility assurance level (Block, 2001). Great primary importance to food manufacturers is the safety of their products. In the case of canning the greatest concern has been over the presence of *Clostridium botulinum* as the most dangerous spore forming organism potentially present in foods. *Clostridium botulinum* will not produce its toxin below pH 4.5, and consequently high acid products with a pH below 4.5 do not need as severe a heat treatment. Therefore, the thermal processing of low acid foods (i.e. pH > 4.5) is of great importance to guarantee a stable product. A dilemma exists in that exponential destruction never reaches zero, suggesting that there may be a surviving spore which could give rise to poisoning. The resolution of this predicament is the 12 D cook. Considered is the worst possible scenario - a can full of solid packed *Clostridium botulinum* spores; in such a situation the spore count is believed to be 10^{12} spores per mL. Thus, in this worst case, putting the food through a cooking process which achieves a 12 decimal reduction should destroy all the spores of *Clostridium botulinum* in this worst possible case. The temperature of 121°C is used as a reference temperature. At this temperature the D-value, which is the time needed to inactivate 10^{1} spores respectively reduce the population by 90 %, for *Clostridium botulinum* is 0.21 minutes; thus a 12 D cook is equivalent to 2.52 minutes. Effectively, the food is held for 3 minutes at 121°C (with z = 10 K) the outcome should be a safe and commercially sterile product. However, cans take time to reach temperatures of 121°C. The ratio of D values at any given temperature compared to the reference temperature is also referred to as the lethality and can be envisaged as the equivalent destruction achieved in minutes at 121°C. If one adds up the lethality of heat up-, dwell- and cooling time one gets the termed F_0-value (Chen and Rosenthal, 2009). $F_0^Z = \int_0^t 10^{\frac{(T-121.1°C)}{z}} dt$ with t=holding time [min], T = holding temperature [°C] and z = increase in T to achieve the same effect in 1/10 of the time. An F_0-value of 3 means that the product was treated with a thermal load equivalent to a heat treatment at 121.1°C for 3 min. This approach is based on the worst case scenario where to be on the "safe side" the F_0 sometimes is well above what is required (sometimes 40-50) to compensate for cold spots. Target F_0 4-8 are sufficient to reach sterility in canned and jarred foods.

Nevertheless, an over processing of the food is quite extremely. This could result in loss of nutrients, quality and formation of compounds within the food that are catered by heat driven reactions such as the Maillard reaction (Considine et al., 2008). Therefore other gentler alternative processes for the sterilization of packed foods are needed and must be investigated, which also result in a safe and high quality food.

The concept of utilizing high pressure processing (HPP) as a tool for pasteurization and sterilization of foods has been around for decades (Wilson and Baker, 2001). However, until now the process referred to as high pressure thermal sterilization (HPTS) has not found implementation in the food industry (Matser et al., 2004; Heinz and Knorr, 2005; Georget et al., 2015). High pressure processing at ambient temperatures for non-thermal food pasteurization was introduced to the Japanese market during 1994 and into the US market soon thereafter (Cheftel and Culioli, 1997; Hogan and Kelly, 2005). In 2014 over 252 high pressure systems produced over 500,000 metric tons of high pressure treated foods, which were put on the market worldwide. The numbers are increasing every year (Samson, 2014). The HP pasteurization needs to achieve an inactivating effect of a 6 $\log_{10}$ reduction on key vegetative pathogenic microorganisms, including *Escherichia coli, Listeria monocytogenes,* and *Salmonella sup*.. This is the case for all mentioned key pathogens at 580 MPa at ambient temperatures and holding times of 3-5 minutes (Hogan and Kelly, 2005; Considine et al., 2008; Georget et al., 2015). Especially the enterhemorrhagic (EH) *Escherichia coli* (EC) strains O157:H7 and O104:H4, which have caused EHEC outbreaks in the food chain in the last years, have a high barotolerance. Therefore in this thesis the inactivation mechanisms and inactivation behavior in different systems were tested for the aforementioned strains in a broad pressure temperature domain (p = 0.1-500 MPa and T = 20-70°C).

Even more resistant to external factors are spores: Two processes which are currently used in the food industry to achieve a sterile product are depending on the product either retort heating or the ultra-high-temperature treatment, where the long treatment times or high temperatures often have a negative impact on the nutritional value and the quality of the food (Matser et al., 2004). Due to the growing consumer demand of minimally processed low acid foods in recent years, an alternative process is essential to produce foods which are healthy, have as-fresh characteristics, a long shelf life and are produced in an economically viable manner (Heinz and Knorr, 2005; Koutchma et al., 2005; Escobedo-Avellaneda et al., 2011). In order to make high pressure processing feasible for the inactivation of spores, which is essential to achieve commercial

sterility, elevated temperatures of 90 to 121°C are required. This combined process could be an alternative over the conventional thermal sterilization processes as a means to produce shelf-stable low acid foods. Although not yet adopted by the food industry due to the lack of appropriate scale and output, two sterilization concepts have been investigated. The first one, Pressure Assisted Thermal Sterilization (PATS) (NCFST, 2009) only considers the thermal component, i. e. sterilization temperature being reached during pressure dwell time, as the lethal factor; whereas the second, High Pressure Thermal Sterilization (HPTS), also takes into account the impact of pressure on spore inactivation (Mathys et al., 2009), i.e., the synergy of combined pressure and temperature. In both cases, the process relies on adiabatic heating during pressurization to bring the product to temperatures, where microbiological spores can be inactivated. Depending on the food system and the initial temperature before compression the temperature increase can be 3 to 9°C per 100 MPa. For HPTS processing, two approaches have been suggested. The first one consists of a single pressure pulse (Heinz and Knorr, 2005) while the second consists of multiple pulses. However, multiple cycles reduce the lifetime of high pressure equipment and increase both processing and maintenance costs. This second approach is, therefore, not, recommended for both technical and economic reasons (Reineke et al., 2011).

The advantages that HPTS offer in comparison to conventional thermal retorting are: i) shorter total process times; ii) uniform pressure and "uniform" temperature distribution; iii) faster heating and cooling rates due to the additional adiabatic heat of compression and decompression. Therefore, a lower thermal load is applied to the product and higher inactivation rates of spores are achievable due to the synergistic effects of pressure and temperature (Olivier et al., 2015). Another possible key advantage is the Le Chatelier's principle which states that any phenomenon (chemical reaction, phase transition etc.) accompanied by a decrease in reaction volume is enhanced by pressure. Therefore, formation of unwanted and harmful chemical substances could also be limited, if their specific reaction volume is positive (Ramirez et al., 2009; Escobedo-Avellaneda et al., 2011; Bravo et al., 2012a). So if the reaction volume is positive the formation of the FPCs under pressure may not occur or could be limited. Also, the allergenic potential of some food allergens can be reduced, through the application of high pressure processing (Huang et al., 2014).

The inactivation of bacterial spores by HPP has also been investigated and high pressure/high temperature sterilization could produce minimally processed foods of higher quality (Mathys, 2008) than what is achievable by heat treatment alone (Heinz et al., 2010). It has, however, not

yet been successfully introduced into the food industry, possibly due to limited knowledge regarding the inactivation mechanisms of high resistant bacterial spores but mainly due to technical limitations (Reineke et al., 2012).

HPTS can combine the synergistic effect of elevated temperatures (90-121°C) and pressures above or equal to 600 MPa for a better overall inactivation of spores, of pathogenic and spoilage microorganisms as well as the retention of the food structure and overall food quality (Matser et al., 2004; Knoerzer et al., 2007; Olivier et al., 2015). In the food industry, a pressure of 600 MPa has been the common pressure applied for HPP and is also suggested for HPTS in combination with elevated temperatures (Tonello, 2010; USFDA, 2014). The temperature non-uniformity in the treatment chamber, which can vary for industrial units is roundabout 10°C, is also a factor that needs to be taken into account to ensure the microbiological safety of the HPTS process (Knoerzer et al., 2007; Grauwet et al., 2012; Martinez-Monteagudo et al., 2012). However, the rapid heating during HPTS reduces the lack of temperature uniformity that occurs in traditional thermal sterilization processes (Knoerzer et al., 2010a).

In the last decade, much research has been conducted to comprehend the underlying mechanisms in the high pressure inactivation of spores within simple aqueous systems and this is well described in the literature (Wuytack et al., 1998; Ananta et al., 2001; Paidhungat et al., 2002; Setlow, 2003; Margosch et al., 2004a; Margosch et al., 2004b; Margosch et al., 2006; Rajan et al., 2006; Black et al., 2007; Paredes-Sabja et al., 2007; Wimalaratne and Farid, 2008; Juliano et al., 2009; Mathys et al., 2009; Heinz et al., 2010; Ramaswamy et al., 2010; Yi and Setlow, 2010; Knorr et al., 2011b; Reineke et al., 2011; Olivier et al., 2012; Reineke et al., 2012; Reineke et al., 2013a; Reineke et al., 2013b; Doona et al., 2014; Luu-Thi et al., 2014; Georget et al., 2015; Olivier et al., 2015).

One proposed mechanism for the inactivation of spores under the severe HPTS-conditions is based on a non-physiological germination (Setlow, 2003; Reineke et al., 2013a). This effect results in a destruction of the inner spore membrane and/ or an opening of the dipicolinic acid (DPA)-channels. The release of dipicolinic acid (DPA), which makes up 5-15 % of the dry matter content of the spores, is thought to be the rate-limiting step of the inactivation (Reineke et al., 2013b). For a rapid and sudden inactivation of spores under pressure it is important to apply pressures ≥ 600 MPa and temperatures above 60°C to ensure the loss of heat resistance (Reineke et al., 2013b). Under these conditions (600 MPa), the driving force of the inactivation is the applied temperature (Reineke et al., 2013b; Sevenich et al., 2013; Sevenich et al., 2014).

However, most of these inactivation mechanisms are derived in simple aqueous systems, such as buffer solutions and can be affected by food constituents. From the microbiological indicator perspective, *Clostridium* spores (*Clostridium botulinum*, *Clostridium sporogenes and Clostridium perfringens*), *Bacillus* spores (*Bacillus amyloliquefaciens)* and *Geobacillus stearothermophilus* spores are mentioned by numerous research groups as being very highly pressure and temperature resistant (Margosch et al., 2006; Reddy et al., 2006; Ahn and Balasubramaniam, 2007; Wimalaratne and Farid, 2008; Juliano et al., 2009; Georget et al., 2015; Sevenich et al., 2015). Some authors report as most promising surrogate indicator, for the relevant target microorganisms, *Bacillus amyloliquefaciens* (Margosch et al., 2006; Reineke et al., 2013a). One of the main difficulties in selecting a reference indicator strain is that some of the known target microorganisms for thermal sterilization are high pressure sensitive and that some high temperature sensitive microorganisms can become more resistant under high pressure conditions (Margosch et al., 2006; Sevenich et al., 2013).

A very important component in establishing and implementing HPTS in a commercial scenario, is finding an indicator microorganism; otherwise, as long as there is no certified target microorganism, the PATS-process will be the only process approved by regulators; i.e., only the thermal component will be considered as the process variable causing inactivation of *C. botulinum* spores and the synergistic effect will not be taken into account. In addition, food matrix specific effects may have an impact on spore inactivation. Furthermore, it has been suggested that future validation schemes for high pressure high temperature sterilization should be based on product specific and relevant indicator strains (Eisenbrand, 2005).

The influence of the food matrix effect on inactivation efficiency of spores cannot be neglected. Food matrices are complex environments which may offer protection for microorganisms, even under harsh treatment conditions. Specifically, low water activity (a_w) matrices have been shown to be particularly challenging to achieve microbial decontamination by any kind of decontamination strategies, including HPP (Doona and Feeherry, 2007). Certain ingredients (e.g. sugar, salts, fat etc.) can have a baroprotective effect on spores and can cause incomplete inactivation (retarded DPA-release; influence on inner spore membrane etc.). This could represent a limitation of HPTS, therefore, in this thesis the inactivation effect in model and real food systems was studied under HPTS-conditions at lab- and pilot-scale level, to understand the underlying mechanisms in these environments. The increased resistance to temperature and high pressure of vegetative cells and spores in low a_w environments may be explained by their

partially dehydrated state. Proteins and other essential cell constituents become more resistant to thermal damage or other extrinsic stress factors in the partially dehydrated state (Barbosa-Canovas et al., 2007). These observations were already made by researchers between the years 1950 to 1980, who tried to understand the protective effect of solutes (sodium chloride, sucrose, glucose, fats, acids etc.) and the change in water activity during the thermal inactivation of microorganisms (Anderson et al., 1949; Secrist and Stumbo, 1958; Murrell and Scott, 1966; Molin and Snygg, 1967; Cook and Gilbert, 1968; Beuchat, 1974; Gould, 1977; Hätnulv et al., 1977; Senhaji and Loncin, 1977). Although these observations were made, so far there was no knowledge transfer to emerging technologies such as high pressure processing. The understanding of these mechanisms could greatly assist in the future development and eventually to the commercial implementation of HPTS.

The high temperatures needed for the inactivation of spores can lead to the formation of undesired byproducts of potential health concern in the food. Since the beginning of the new millennium more attention has been given to the mechanisms and mitigation strategies of these compounds. Research on the influence of HPTS on the formation of food processing contaminants (FPCs) such as furan or 3-monochloropropanediol-esters (3-MCPD-esters), in comparison to the thermal processing, is scarce. HPTS might be in favor to thermal processing, since these reactions are temperature driven and shorter process times and lower temperatures could be applied by HPTS (Vervoort et al., 2012; Sevenich et al., 2013; Palmers et al., 2014; Sevenich et al., 2014). A reduction of furan or other FPCs would be a significant benefit of HPTS over conventional thermal processing since this would lead to the reduction in the exposure of potentially harmful substances, which also results in a better overall product quality without compromising the food safety.

Literature data on upscaling trials, based on inactivation kinetics obtained at lab- to pilot-scale for HPTS, are rare. A research project of the Washington State University entitled “Shelf stable egg-based products processed by ultra-high pressure technology” in 2003-2007 treated different egg-based products inoculated with different high temperature resistant spore strains (*Bacillus stearothermophilus* and *Bacillus amyloliquefaciens*) in a 35 L high pressure high temperature vessel (QUINTUS Food Autoclave Type 35 L-600 Avure Technologies, Kent, WA, USA) under PATS conditions. Further, they looked at the quality parameters of the foods such as color, texture and taste. Their statement concerning the quality and spore inactivation was that the quality of the products was good but that lower processing temperatures than 121°C cannot yet

assure commercial sterility. Therefore, more research is needed; based on the current knowledge, regulatory approval can only be obtained by filing this technology as a thermal process.

To gain a broader knowledge of the influence of the HPTS process on the inactivation of *Clostridium sporogenes, Bacillus amyloliquefaciens and Geobacillus stearothermophilus* spores, the formation of food processing contaminants (furan and 3-MCPD-esters), the influence of solutes on the high pressure high temperature inactivation and upscaling potential of this technology, different food systems and model systems were tested within this study at lab and pilot scale level.

2. Theory

2.1. High isostatic pressure

Pressures currently used in the food industry for the high pressure treatment of foods are up to 600 MPa. A processing at 600 MPa and ambient temperatures is referred to in the literature as cold pasteurization (Matser et al., 2004; Knoerzer et al., 2007; Mújica-Paz et al., 2011). The use of high pressure (HP) as a technology for pasteurization of different kinds of foods, such as juices, ham, sauces and seafood is a growing trend sector in the food industry since the 1990's (Hogan and Kelly, 2005). In 2014 over 250 high pressure systems located in 200 companies produced over 500.000 metric tons of high pressure treated foods (Figure 2-1) which were put on the market worldwide. The numbers are increasing each year, especially in the juice (beverage) sector (Samson, 2014). Furthermore, there is a high acceptance of pressurized foods by consumers (Olsen et al., 2010).

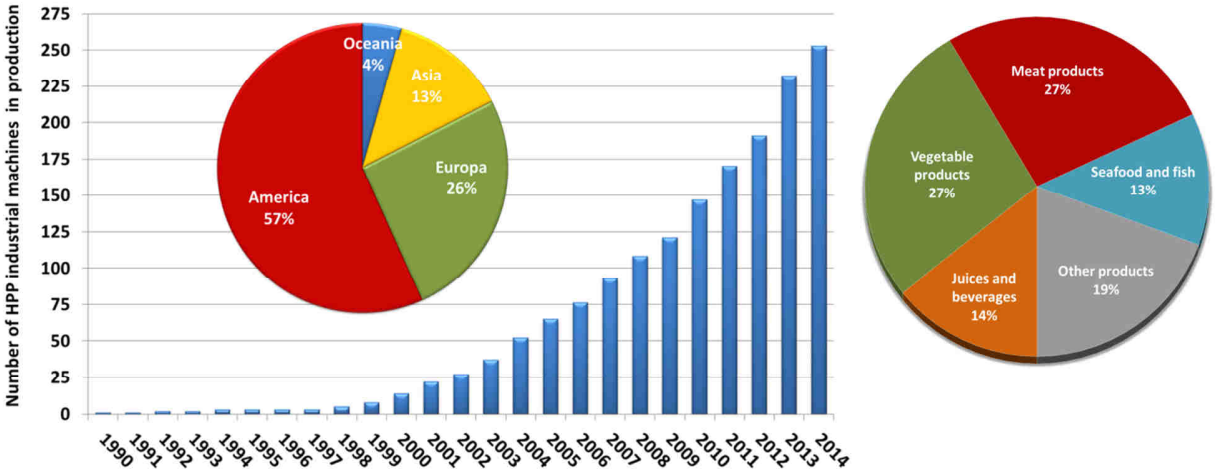

Figure 2-1: Industrial scale high pressure machines used worldwide and total vessel volume used for different food products; not including 15 dismantled machines installed before 2003 (Tonello Samson,C. 2014, NC Hiperbaric, Spain, personal communication).

The application of HP in combination with elevated processing temperatures has been evaluated as a promising food processing alternative to classical retorting technologies in several studies (Heinz and Knorr, 2005; Koutchma et al., 2005). High pressure thermal sterilization brings the benefits of an enhanced product quality and its application to packed foods. In addition, this has a huge advantage of the homogenous heating of the entire product during compression and the instantaneous cooling during decompression. This is due to the adiabatic heating, which will be discussed in chapter 2.1.1. The combination of moderate pressures and ambient temperatures

offers the possibility of a significant vegetative microorganism (Chapter 2.1.5) and spore reduction (Chapter 2.2.2). However, if a process has sterilization as its goal, very high pressures (> 500 MPa) have to be combined with elevated initial starting temperatures (> 80 °C). This will be discussed in chapter 2.2.2. In addition to the underlying inactivation mechanisms of bacterial endospores under pressure, matrix effects in the real food must also be taken into account since some may offer protection against spore inactivation (Olivier et al., 2011; Georget et al., 2015). This will be discussed in chapter 2.2.3. Further, shorter holding times needed for this technology could lead to lower formation of the carcinogenic FPCs, such as furan and 3-MCPD-esters. This will be discussed in detail in chapter 2.3.

2.1.1 Adiabatic heat of compression

The theoretically resulting adiabatic heating can be derived from the first law of thermodynamics (Bridgman, 1912) and leads to the maximal achievable temperature change. In uniform material the temperature rise happens simultaneously in the whole product. The extent of the temperature rise depends on the material properties (adiabatic heating depending on the compressibility and the specific heat of the substance) and overlapping heat transfer phenomena (non-adiabatic conditions). Different pressure transmitting media would result in a variable adiabatic heating (Ardia et al., 2004). However, due to the lack of thermo-physical data on real foods under pressure, the modeling of adiabatic heating in real system has been limited (Ardia et al., 2004). Primarily it was practical measurements which were able to demonstrate the differences of the adiabatic heating in real food systems. The relevant knowledge of adiabatic heating during HPP of foodstuff is summarized in Table 2-1.

Table 2-1: Heat of compression values of selected foods determined at initial sample temperature of 25 °C adapted from Georget et al. (2015)

Food Sample	Temperature Increase (°C) per 100 MPa
Water	3
Orange juice, tomato salsa, 2% fat milk, and other water-like substances	Ranging from 2.6 to 3.0
Linolenic acid	9.0-5.9[a]
Soybean oil (100 % oil)	9.1-6.2[a]
Olive oil (100 % oil)	8.7-6.3[a]
Crude beef fat (23 % fat content)	4.4

Extracted beef fat (85 % fat content)	8.3-6.3[a]
Beef ground	3.2
Gravy beef	3.0
Chicken fat	4.5
Chicken breast	3.1
Salmon	3.0
Egg albumin	3.0
Egg yolk	4.4
Egg whole	3.3
Mayonnaise (75 % fat content)	7.2-5.3[a]
Whole milk (3.5 % fat content)	3.2
Tofu	3.1
Mashed potato	3.0
Yoghurt	3.1
Cream cheese (34 %)	4.8
Hass avocado	4.1-3.7[a]
Honey	3.2
Water/glycol (50/50)	4.8-3.7[a]
Propylene glycol	5.3
Ethanol	10.6-6.8[a]

a Substances exhibited decreasing adiabatic heating as pressure increased

The main ingredient in most food is water and thus the thermo-physical properties of water have been utilized to estimate the temperature increase upon compression of high moisture foods. However, when working with fatty/oily matrices or emulsions, this approximation is not accurate. As shown in Table 2-1 (Ting et al., 2002; Gupta and Balasubramaniam, 2012), the compression heating in fat containing foods (fat content between 80-100 % (Table 2-1)) could be up to three times higher than for water, due to their higher expansivity and lower heat capacity (Ting et al., 2002). This highlights a potential application for HPTS or PATS. Indeed, the transfer of heat from the pressure transmitting medium into the product could be used to increase the temperature of the food system during and after the adiabatic heating, achieving therefore much faster lethal conditions. In which case, however, non-uniform temperature distributions would need to be accounted for. Further investigation and potential modeling of adiabatic heating of fat containing

as well as emulsified foodstuff would be required in order to achieve controlled heating of these challenging matrices. Furthermore, it is important to consider that oil systems exhibit nonlinear adiabatic heating with increasing pressures. Finally, adiabatic heating of oils is independent from the initial temperature, which differs from water based systems. After compression, the product temperature increases to a value that depends on the geometry of the system and on the required time for pressure build-up. Immediately after decompression the product returns to its initial temperature, or even to a lower value, as a consequence of thermal equilibration with the surroundings, established during dwell time. This high cooling capacity is very valuable for the production of high quality foods and the preservation of food ingredients. During compression there are no shear forces inside the liquids and solids, and the transport of force is practically instantaneous. Consequently, each volume element of the product is characterized by the same pressure level, and, under adiabatic conditions, by the same temperature.

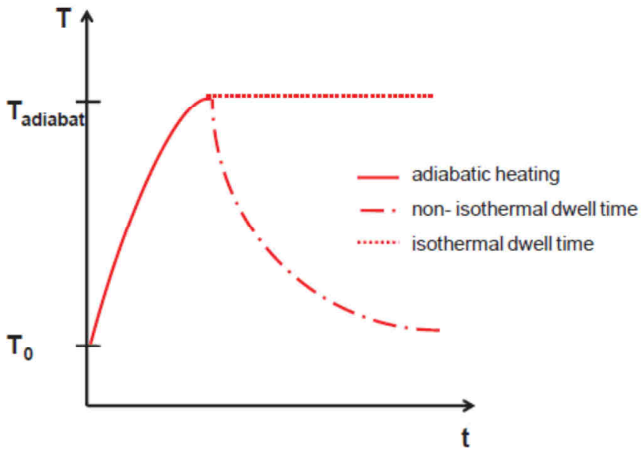

Figure 2-2: Temperature profile in high pressure vessel under non-isothermal and isothermal conditions during dwell time adapted from (Reineke, 2012).

A temperature gradient occurs when there is a heat flux across the boundary of the system (Figure 2-2). Under these conditions, the resulting transient temperature field in the product must be taken into account (Ardia et al., 2004). Consequently, under non-isothermal conditions thermal equilibration occurs during dwell time and even during the pressure build-up, ultimately causing a non-uniform microbial inactivation during the pasteurization and sterilization processes. In this context, a higher compression rate results in a higher absolute value of temperature and a more homogenous temperature distribution inside the product, since less time is given to thermal equilibration. In each case, the product is cooled soon after compression, due

to the thermal gradient between the product and the pressure vessel. If the pressure vessel is not heated or if there is no insulating layer inside the pressure vessel (Ardia, 2004).

2.1.2 Le Chatelier's Principle

Le Chatelier's principle states that an application of pressure shifts the equilibrium of the system to the state that occupies the smallest volume. Therefore, any physical or chemical change (phase transitions, chemical reactions, and molecular configuration changes) that results in a volume decrease is enhanced by the application of pressure. Therefore, the formation of unwanted and harmful substances could be limited, if its specific reaction volume is positive (Ramirez et al., 2009; Escobedo-Avellaneda et al., 2011; Bravo et al., 2012a). Reaction volumes dV for selected reactions of biological importance are summarized in table 2-2.

Table 2-2: Reaction volumes of selected reactions of biological importance adapted from Jaenicke (1983) and Wurche & Klärner (2002)

Reaction type	Example		dV [cm^3 mol^{-1}]
Protonation/* Ionic pair binding	Water dissociation:	$H^+ + OH^- \rightarrow H_2O$	+21,3
	Amino groups:	$P\text{-}NH^{3+} \rightarrow P\text{-}NH_2 + H_2O$	+20
	Carboxylic acid:	$P\text{-}COO^- + H^+ \rightarrow P\text{-}COOH$	+10
Hydrogen bond formation	Helix formation		
	L-Lysine		-1,0
	Adenin and Uracil		+1,0
Hydrophic interaction*	Lipid assemblies		-10
Hydration of polar bonds	n-Propanol → n-Propanol + H_2O		-4,5
Hydrophobic hydration*	$C_6H_6 \rightarrow C_6H_6 + H_2O$		-22,7
Protein denaturation	Myoglobin (pH 5, 20 °C)		-98,0
Cyclization of C-rings*	C_3-C_{10}-Rings		-5.5 - -32

* Reactions that might be involved in the pathways of either furan or 3-MCPD formation

2.1.3 Temperature inhomogeneity

In recent years much research was conducted in order to understand temperature distribution in high pressure vessels, since an inhomogeneous temperature distribution during high pressure processing, would be problematic in terms of process performance (Knoerzer et al., 2007; Grauwet et al., 2012). To demonstrate the potential of the process to the industry, in addition to demonstrating the quality and consumer acceptability of the product, it is necessary to prove that it can be operated uniformly, predictably, repeatedly, and safely at pilot- and eventually at commercial/industrial -scale (Knoerzer et al., 2007). Further studies by Smith et al. (2014) have shown that the orientation of the vessel, horizontal or vertical, also has a severe impact on the temperature distribution within the vessel. They concluded that the horizontal orientation would be in favor due to a better uniformity of the temperature distribution. The temperature inside a pilot scale system is difficult to measure in comparison to existing lab scale system, where the temperature can at least be measured inside one of the treated sample containers. Pressure, due to the isostatic principle, is assumed uniform and the treatment time is fixed. The temperature non-uniformity has been described by many research groups and can be explained by differences in adiabatic heating of the product, pressure medium and the heat transfer from the vessel wall (Juliano et al., 2009; Grauwet et al., 2012). Temperature inhomogeneity occurs if temperature differences between vessel wall and vessel load, or differences in the adiabatic heat of compression of pressure medium and vessel load exist. The difference in temperature results in a temperature gradient, which leads to a temperature loss. A temperature loss in this manner could compromise the effectiveness of a high pressure process by allowing the sample temperature to drop below the level of lethality necessary to ensure the inactivation of relevant microorganisms and the safety of the product to the consumer (Ting, 2007). Hence, the temperature non-uniformity in the treatment chamber, which for pilot-scale units can be in the order of ~ 10°C between the bottom and the top of a horizontal high pressure system, and even greater for vertical systems. This is a factor that needs to be taken into account to ensure the microbiological safety of the process (Knoerzer et al., 2007; Grauwet et al., 2012; Martinez-Monteagudo et al., 2012). Different modifications could allow maximizing the process temperature and minimizing possible heat losses: the choice of pressure medium (for food applications mainly water); packaging material; use of an insulated product basket; control of the temperature of the vessel wall; and, both the product and the incoming pressure medium should have the same temperature (Grauwet et al., 2010).

The temperature of the vessel wall should be set to the required final target temperature and the temperature of the pressure medium and vessel load should be set to the initial temperature at which the adiabatic heat of compression is able to reach the final target temperature for the sterilization process. This method is referred to in the literature as Dynamic Temperature Method (DTM)—Different Initial Temperatures for Sample and Vessel (Ting, 2007). By doing so, the process is near isothermal conditions and, thus, a uniform impact on the food can be achieved. The properties of the packaging material can have a significant effect, especially if the sample mass and the packaging mass are of the same relative magnitude. Plastics such as polyethylene undergo significantly greater extent of compression heating compared to that of water and foods (Knoerzer et al. 2010a). Due to that, heat can be transferred to the products during the holding time in the process called secondary heating (Ting, 2007). The mapping of the temperature inhomogeneity can be made visible by a quantitative analysis of uniformity of temperature using three-dimensional numerical simulations (Rauh et al., 2009) or by a so called pressure-temperature-time indicator (pTTi) (Grauwet et al., 2009). The pTTi is a protein based indicator that can be placed in several different positions within the vessel and the read-out can be conducted after the treatment. More promising seems to be the development of an inline tool to measure the temperature directly in the vessel and the product, such as the "Thermo-egg" a pressure resistant shell, in combination with a commercially available temperature logger, developed by CSIRO Australia (Knoerzer et al., 2010b) or a wireless measurement device developed by Hiperbaric (Tonello Samson, C., 2011, NC Hyperbaric, Spain, personal communication). Research for the development of this kind of equipment is still ongoing to this day.

2.1.4 High pressure operating systems and working principle

For food processing, two different concepts of high pressure units have been developed: batch units with an external pump and intensifier (Figure 2-3 A); and, high pressure units with an internal intensifier (Figure 2-3 B) (Rovere, 2001).

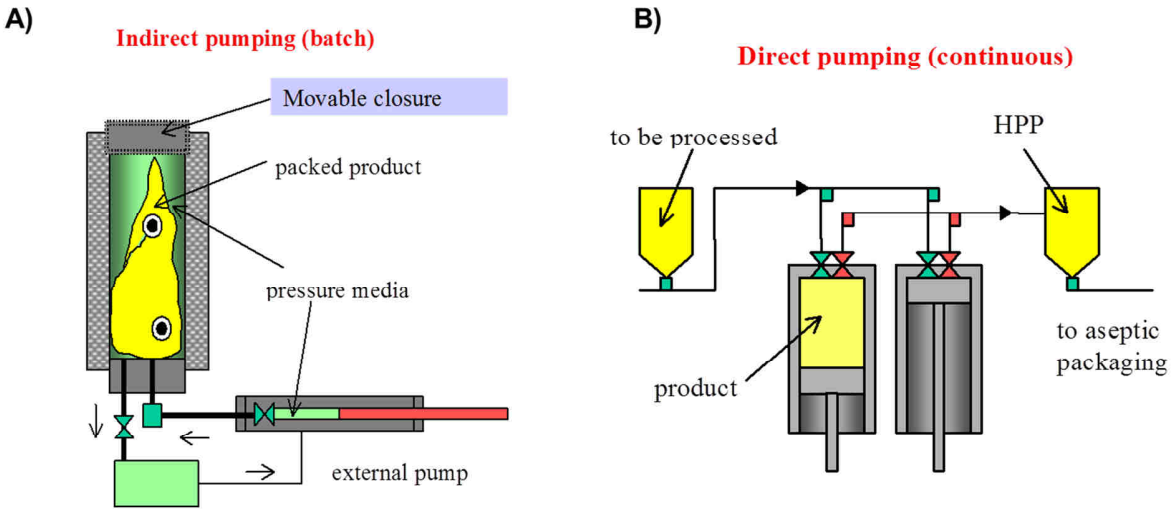

Figure 2-3: The two different concepts of HP-units A) indirect (batch) and B direct (continuous) pumping (Rovere, 2001).

Both concepts include an intensifier, which basically consist of both a low pressure and a high pressure cylinder (Figure 2-4). The simplest practical system is a single-acting hydraulic driven pump. To reach a quasi-continuous product flow, a double-acting arrangement can be used. While one high-pressure vessel is charged, the other can be processed (Reineke, 2012).

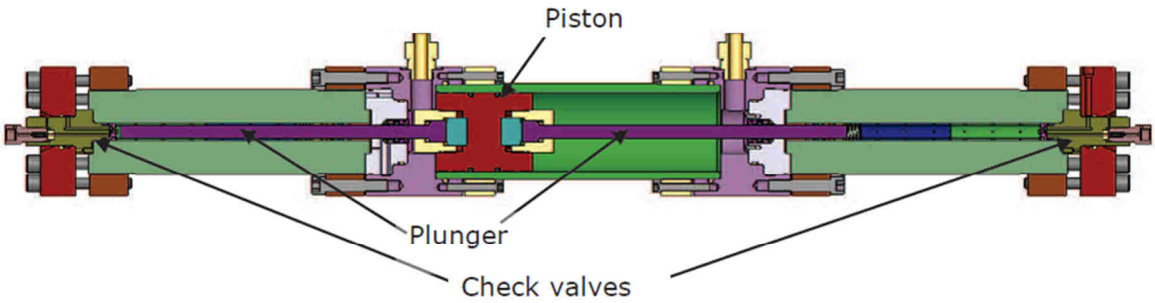

Figure 2-4: Double acting intensifier.

Both high-pressure concepts (batch and continuous) are suitable for possible high-pressure thermal sterilization. Batch units have an advantage because they can process all kinds of packed foods. Packing is necessary to avoid direct contact between the pressure transmitting media (often water or mixture of water and oil) and the processed food. The disadvantage of a batch unit is that the inflow of cold pressure media causes a temperature increase in homogeneities during pressurization (Figure 2-3 A). In a continuous high pressure unit packing is not necessary because the high pressure vessel is sealed; such that no contact between the processed food and the pressure transmitting media is possible (Figure 2-3 B).

The systems that are needed for the high pressure thermal sterilization need to be able to cope not only with high pressures but also with high temperatures. This is quite a challenging engineering task that needs to be dealt with. Nevertheless, many lab scale systems and even some pilot/semi industrial scale systems do exist, e.g. the Quintus 35 L 600 MPa Food Autoclave by AVURE and the 55 L Wave HP/HT system from Hiperbaric. A description of generic lab and pilot scale systems mostly used today follows below.

U111 - lab scale system:

The high pressure equipment (Model U111, Unipress, Warsaw, PL) consisted of five pressure vessels (V = 4 mL) made of copper beryllium, and is completely immersed in a thermostatic bath filled with silicon oil, and connected to a pressure intensifier through capillary tubes (Figure 2-5 A). This design allows a simultaneous treatment of five different samples in one pressure build-up at close to isothermal conditions and the possibility to perform reproducible inactivation kinetics up to pressures of 700 MPa and over a wide temperature range between -40°C and 120°C. Each pressure-chamber, separately connected to an oil-driven intensifier through high pressure valves, is equipped with a K-type thermocouple and a pressure sensor to monitor the temperature and pressure history of the sample during the treatment cycle (Figure 2-5 B). A hydraulic pump (Mannesman Rexroth Polska Ltd, Warszawa, Poland) produces a pressure of 70 MPa in the low pressure part of the intensifier while, due to the section reduction in the intensifier (multiplying factor ≈ 11), it is possible to reach 700 MPa on the high pressure side. The compression rate can be set constant to reach a pressure level of 600 MPa e.g. in 24 seconds. Making use of a multi-meter (Keithley, Multimeter 7001) and a computer acquisition program, the pressure and temperature readings were registered during the pressure build-up with a rate of 3.5 scans per second.

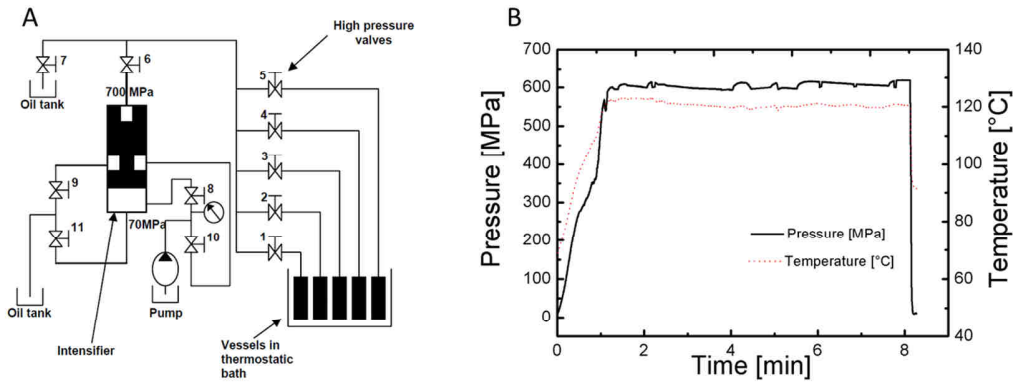

Figure 2-5: A) Schematic hydraulic diagram of multivessel high pressure apparatus U 111; B) Typical pressure temperature profile of an HPTS-process at 600 MPA measured inside the vessel.

Wave 6000/55HT – Pilot scale system:

The Wave 6000/55HT equipment (Hiperbaric, Burgos, Spain) is situated at the Pilot Plant facilities of AZTI-Tecnalia (Derio, Spain) and is one of a kind in the world (Figure 2-6 A). This machinery is specially designed (in 2009) to develop HPTS at the industrial level, capable of combining high pressure (up to 620 MPa) and high temperature (up to 117ºC) processing. Its main differential characteristic, apart from its unique volume of treatment for a HPTS equipment (55 L) and the horizontal design of the vessel (a cylinder of 20 cm in inner diameter), is that it also includes an accurate control of the vessel wall, plugs and inlet water temperature in order to avoid heat loss and consequently assure a nearly constant temperature during the treatment (Figure 2-6 B). For treatments, inlet water needs to be heated to the corresponding initial temperature, between 85-90°C, taking into account the adiabatic heating during the treatment. Vessel wall and plugs can be heated to the final temperature of each treatment (temperature under pressure).

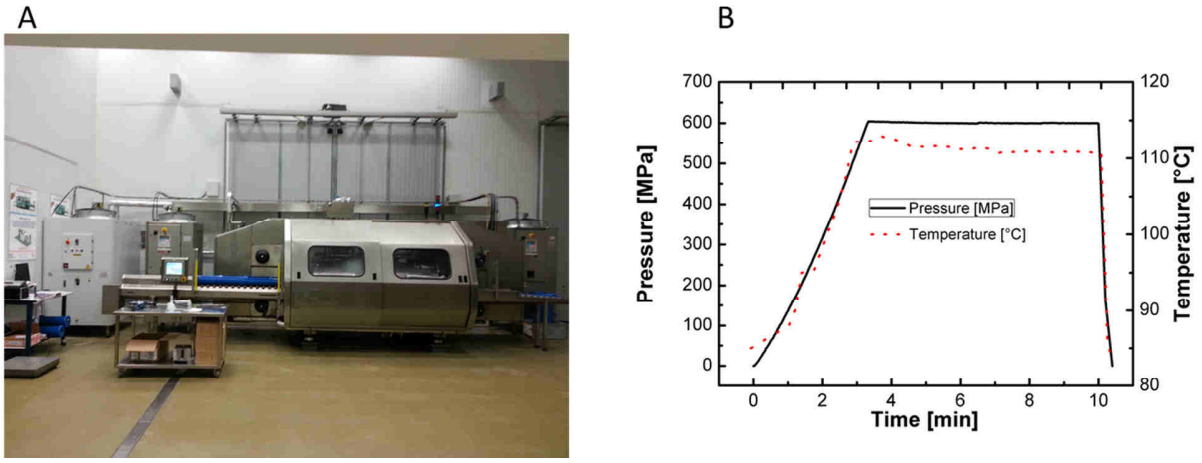

Figure 2-6: A) NC Hiperbaric Wave 600 HP/HT (55 L) System at ATZI (Bilbao, Spain); B) Temperature-pressure profile at 600 MPa measured inside the vessel.

The samples can be placed in baskets designed for the high pressure systems. There is literature data available that showed that the temperature within (top to bottom) the basket can differ up to 10 K. The temperature of the water (pressure transmitting medium) during the processing is measured by thermocouples, but not directly inside the pouches (Fig. 2-6 B). The temperature is a mean of the temperature measured by two thermocouples, which protrudes into the vessel from the center of each plug.

Packaging material for high pressure processing:

For the HPP and the HPTS-treatments flexible (important for pressure transmission), resilient and high barrier packaging need to be used. That packaging must withstand not only 600 MPa, but also cope with high temperature, not changing its protective properties towards the food. Further, the airspace of this packaging must be as low as possible, to limit oxidation reactions and also since more effort is needed to compress air in comparison to water (Balasubramaniam et al., 2015). The reduction of airspace can be accomplished through vacuum sealing. Stand up, meal-ready-to-eat (MRE) pouches, already used by the food industry for various products (ketchup, soups, sauces, fish foods and vegetable and fruit purees etc.), have the optimal geometric form to be placed in the high pressure treatment baskets to get a maximum filling ratio (Figure 2-7 A).

Figure 2-7: Multilayer pouches: A) Pouches commonly used in the food industry B) Promising material composition for HPTS treatment composed of polyethylene, aluminum foil and 4 mL polyolefin (Smurfit Stone).

The packaging materials that should be used have been discussed in many research projects or in literature (Barbosa-Canovas, 2008; Galotto et al., 2010; Juliano et al., 2010; Koutchma et al., 2010; Balasubramaniam et al., 2015). Fleckenstein et al. (2014) divided the damages of polymeric packaging materials that could occur during HPP into direct effects (caused by high pressure treatment) and indirect effects (generated by compression of other substances in the package). Direct effects are caused by high pressure treatment and indirect effects are generated by compression of other substances in the package. Direct effects have a temporal and reversible decrease in permeability not negatively on the functional properties of the packaging; morphology of the polymers may change and the delamination of the multilayer system (mainly between inorganic (e.g. Aluminum) and polymeric layers (e.g. Polypropylene). The two last mentioned effects have a negative impact on the packaging, therefore either the adhesion needs to be improved or systems without inorganic layers need to be developed. Indirect effects represent all influences of compressed substances inside the package. The higher concentration of gases in the polymeric matrix at high pressure can lead to a plasticization of polymers, followed by structural changes or an extraction of constituents from the polymer enabled by the supercritical state. These failures arise in the form of cracks, blistering or other microstructures and turn up to concentrate in weak zones of the materials. Therefore, packaging material such as EVOH (Nylon based ethylene-vinyl-alcohol-co-polymer) based materials show a high potential due to their integrity and oxygen permeability (Fleckenstein et al., 2014). A combination of a three layered

system of polyethylene, aluminum foil and 4 mL polyolefin (Figure 2-7 B) showed good results for a high pressure high temperature processing between 105-121°C at 700 MPa (Barbosa-Canovas, 2008). Nevertheless, more research is needed to evaluate the influence of HP and HPTS treatment on different packaging materials and also if there is a migration from packaging compounds into the food. Development of suitable packaging will be crucial for a wider application of HP and HPTS processing.

2.1.5 High pressure inactivation of vegetative microorganisms and food borne pathogens

The use of HPP to inactivate pathogenic vegetative microorganisms has been largely investigated for the pasteurization of commercial products for decades (Heinz and Buckow, 2010). In 1899 Hite was

first to conduct experiments using high pressure in combinations with foods to extend shelf life, and reported that milk stayed sweet longer after the treatment with high pressure (Hite, 1899). Since then, significant research efforts have focused on understanding the underlying mechanisms of the inactivation of microorganisms under high pressure conditions. HPP offers a lower thermal input into the product by comparison with conventional thermal treatment and therefore increases the quality of the food while maintaining food safety (Smelt, 1998; Hogan and Kelly, 2005; Balasubramaniam et al., 2008; Bermudez-Aguirre and Barbosa-Canovas, 2011; Barba et al., 2012). Despite the steadily increasing commercial production of high pressure pasteurized food with more than 500.000 tons per year (Samson, 2014), some important scientific and technological questions are still unresolved.

One of these issues is the impact of different intrinsic and extrinsic factors on the inactivation mechanisms of vegetative bacteria and bacterial spores under pressure. To unravel the impact of the different pressure and temperature combinations on a possible cell death or recovery, detailed analyses about the physiological state of the cells and how they are influenced by different food constitutes are needed. According to Le Chatelier's principle in a system facing a shift of equilibrium, all cellular components are affected by high pressure, including the cell membrane and its membrane proteins, enzymes and ribosomes as well as all the cell metabolism (Smelt et al., 2001; Winter and Jeworrek, 2009). In general, prokaryotic cells show a higher resistance towards pressure than eukaryotic cells. Yeast and molds are in general more pressure sensitive although ascospores of some molds such as *Byssochlamys* and *Talaraomyces* can be very

pressure resistant (Smelt, 1998; Chapman et al., 2007; Considine et al., 2008). Within prokaryotes, gram positive microorganisms such as *Bacillus, Listeria, Staphylococcus* and *Clostridium* have a thicker peptidoglycan layer and are therefore more pressure resistant than gram-negative microorganisms such as *Escherichia coli* (Smelt, 1998; Chapman et al., 2007; Considine et al., 2008). The mechanisms leading to cell death have been investigated in several bacterial species (Smelt et al., 2001). However, the particular events leading to inactivation are not well understood (Cheftel and Culioli, 1997; Heinz and Buckow, 2010; Klotz et al., 2010). High pressure between 300-800 MPa at ambient temperatures can lead to the unfolding and denaturation of important cell enzymes and proteins in vegetative microorganisms (Rastogi et al., 2007; Knorr et al., 2011b), but the specific pressure effects on microorganism are more complex and several different mechanisms leading to cell death can interact when high pressures are applied. Primarily, pressure at a sufficiently high level, can induce enzyme inactivation, membrane proteins denaturation and cell membrane rupture caused by a phase transition of the membrane and change in its fluidity (Molina-Gutierrez et al., 2002; Van Opstal et al., 2003; Molina-Höppner et al., 2004; Winter and Jeworrek, 2009; Klotz et al., 2010). The pressure level needed to achieve a 5 $\log_{10}$ reduction of pathogenic microorganism in different food-products ranges from 300 to 800 MPa (Hendrickx and Knorr, 2002) and often synergism between pressure and temperature is observed (Buckow and Heinz, 2008). By increasing the process pressure, it is possible to decrease the temperature needed to achieve the same inactivation. According to Smelt et al. (Smelt et al., 2001) the pressure induced effects leading to cell death of vegetative microorganisms can be attributed to four factors:

i) Protein and enzyme unfolding, including partial or complete denaturation
ii) Cell membranes undergoing a phase transition and change of fluidity
iii) Disintegration of ribosomes in their subunits
iv) Intracellular pH changes related to the inactivation of enzymes and membrane damage (Molina-Gutierrez et al., 2002; Knorr et al., 2011a)

In the recent years a common world-wide concern has been foodborne outbreaks with pathogens like EHEC (O157:H5; O104:H4). The contamination of the food supply with spoilage and pathogenic microorganisms continues to be a global problem despite the wide range of preservation methods employed (Juneja, 2003). Despite significant advances in food processing technologies (hurdle concept, new innovative non-thermal technologies), an annual estimated 76

million cases of foodborne illnesses occur in the U.S. resulting in approximately 5,000 deaths (CDC, 2011). In the year 2000 e.g. , approximately 2.4 million pounds of beef were recalled due to possible contamination with *Escherichia coli* O157:H7 (Juneja, 2003). Newest studies from the WHO (2015) on foodborne diseases caused by pathogenic microorganisms, such as *Salmonella*, *Campylobacter*, *EHEC* and *Norovirus*, show that worldwide 1 in 10 people fall ill every year from eating contaminated food and 420.000 die as a result. Here the application of high pressure in combination with mild temperatures could possibly be used to inactivate vegetative pathogenic microorganisms as well as several enzymes, which cause food deterioration at pressures between 300 and 700 MPa. The resistance towards temperature of the pathogenic *Escherichia coli* strains is usually higher than its non- pathogenic counterpart (García-Graells et al., 1999). Hence a more intense heat treatment needs to be applied. The more complex and resistant the microorganism the more intense must be the treatment. In comparison to the pathogenic *Escherichia coli* higher temperatures and higher pressures must be applied to inactivate spores (Reineke et al., 2011; Reineke et al., 2013a). This becomes even more complex if this is conducted in a real food system since here baroprotective effects can occur and the severe heat treatment can lead to unwanted changes in the food matrix, leading to the formation of unwanted and possibly unhealthy compounds in the foodstuff. All of the above mentioned challenges and research aspects will be discussed in more detail in the following chapters.

2.1.6 Thermal sterilization and high pressure thermal sterilization

The term *sterilization/sterility* can be separated into three different forms of sterility: i) biological sterility inactivation of all microorganisms spores and enzymes, ii) bacteriological sterility inactivation of all microorganisms, spores and partial enzyme inactivation and iii) commercial sterility to render the product free of microorganisms capable of growing in the product at non-refrigerated conditions (over 50°F or 10°C) at which the product will be held during distribution and storage (usually 6-12 month). In the food industry usually the concept of commercial sterility is applied. Therefore, to achieve commercial sterility, a canned, jarred or pouched low-acid food (pH > 4.5 and aw > 0.85) is processed by heat, or other treatments which have the same effect, to achieve commercial sterility. Commercial sterility implies the inactivation of all microorganisms that endanger public health to a very low probability of survival. For canned foods, the critical organism is *Clostridium botulinum*. The 12 D concept as a minimum process for inactivation of *C. botulinum* in canned foods is accepted, in principle, by regulatory agencies and the food

industry. However, its interpretation has undergone a process of evolution, from a literal 12 decimal reduction, to what is generally accepted as a probability of survival of 10^{-12}. The latter interpretation signifies a dependence of minimum processes according to the 12 D concept on initial spore loads. In the canning industry, the F_0 value is often used as a basis of the thermal inactivation effect on spores for the low-acid canned foods and refers to the sterilization value (F^{Z}_{Tref} value) with a z value of 10°C and a reference temperature (T_{ref}) of 121.1°C (Eq. 1)).

Eq.1) $$F^{Z}_{Tref} = \int_0^t 10^{\frac{(T-T_{ref})}{z}} dt$$

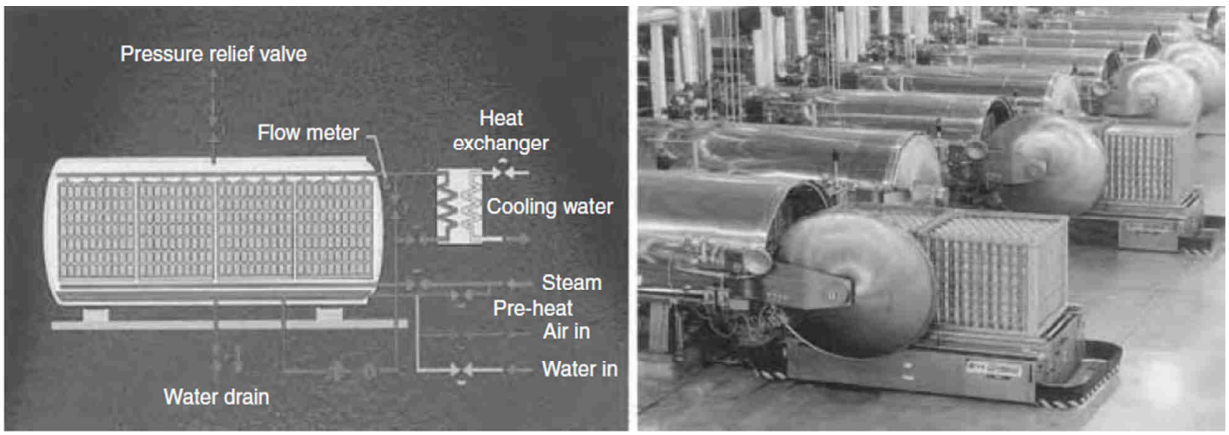

Figure 2-8: Steam Water Batch Retort System. Left: System concept view. Right: Fully automated system with automatic loading unloading system. Adapted from Jun-Weng (2006).

For the industrial production of sterilized products usually rotary pressure sterilizers (Figure 2-8) (continuous and discontinuous versions) are used, which offer good heat conductivity and have good throughput.

As already discussed in detail in Chapter 1, the inactivation of bacterial spores by HPP has also been investigated and high pressure/high temperature sterilization could produce more uniform, minimally processed foods of higher quality (Mathys, 2008) than heat treatment alone (Heinz et al., 2010). It has, however, not yet been successfully introduced into the food industry, possibly due to limited knowledge regarding the inactivation mechanisms of high resistant bacterial spores as well as technical limitations (Reineke et al., 2012). High pressure thermal sterilization (HPTS) may offer an alternative to retort processing, and a means by which high quality products are achieved. HPTS can combine the synergistic effect of elevated temperatures (90-121°C) and pressures above or equal to 600 MPa for a better overall inactivation of spores and pathogenic microorganism as well as the retention of the food structure and quality (Matser et al., 2004;

Knoerzer et al., 2007; Olivier et al., 2015). In the food industry a pressure of 600 MPa has been the common pressure applied and is also suggested for HPTS in combination with high temperatures (Tonello, 2010; USFDA, 2014).

In Figure 2-9, a classical temperature profile of a possible HPTS-process is shown in comparison to a conventional thermal retort process. Before the start of the treatment, the product needs to be preheated to approximately 70 to 90°C (I in Figure 2-9) and through adiabatic compression heating, during pressure build-up, the process temperature can reach 90 to 130°C (II in Figure 2-9). After the holding time (III in Figure 2-9), the pressure is released, which reverses compression heating and providing an decompression cooling effect (IV in Figure 2-9), followed by further cooling in, e.g., an ice or water bath (V in Figure 2-9). It is important that preheating and pressure come-up time are fine-tuned to guarantee optimal treatment conditions (Barbosa-Canovas and Juliano, 2008) as discussed in chapter 2.1.3. However, being able to use lower process target temperature and lower thermal load applied to the product without endangering the food safety would make it possible to reduce unwanted changes such as nutritional loses and taste.

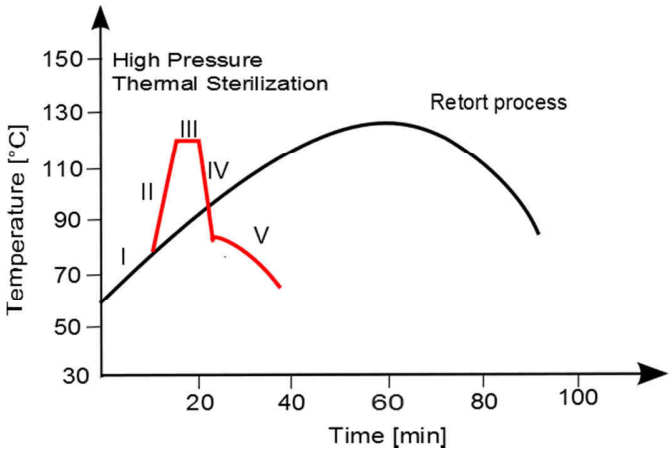

Figure 2-9: Schematic comparison of an HPTS-process and a retort process in a temperature-time diagram adapted from (Matser et al., 2004).

2.2 Spores

Bacterial endospores are resistant, well adapted and dormant structures produced by stressed vegetative cells (Setlow, 2007; Torred et al., 2012). Due to their intrinsic resistance to environmental stresses, such as heat, radiation, chemicals, and desiccation spores can survive thousands of years (Traag et al., 2013). It is even believed by some Biblical scholars that an infection in Exodus, Chapter 9 may reference animal anthrax. Today we know that this infection

is caused by *Bacillus anthracis*. Therefore, this outbreak was probably the first written record of a true epizootic outbreak caused by spores (Torred et al., 2012). Endospore formation is unique to the low-G+C group of Gram-positive bacteria (*Firmicutes*). For the most part, it is restricted to the family *Bacillaceae* and the class *Clostridia* (Traag et al., 2013). Further, endospores can monitor their surroundings and if the external conditions are in their favor they can germinate and cause infections. Bacterial endospores were first studied by Cohn and Koch independently in 1876 and both described a 'germ' that could survive boiling. Further Cohn might have been the first researcher to describe the sporulation process: after growing; some of the *bacilli* would swell at one end and become filled with refractive little bodies. Cohn believed that these bodies represented a life stage of the bacilli and those real spores could survive boiling and afterwards form new bacteria.

Although spore dormancy and resistance have been much studied since then, questions still remain concerning the basic mechanisms and the kinetics of heat inactivation and other new innovative decontamination technologies in particular (Gould, 2006). Botulism, an often fatal disease of the nervous system caused by *Clostridium botulinum*, is another example of well adapted spores to an extreme heat treatment applied to foods. First cases of this disease were reported in Europe in 1735, around the time people started to study and learn to deal with food preservation and storage of foods. In Latin '*botulus*' means sausage and this describes the origin of this food spoiling organism which is associated with spoiled German sausages (Torred et al., 2012). By 1925, outbreaks of Botulism from industrial canned foods essentially ceased due to the widespread adaption of standards for the necessary heating times and temperatures for industrial-scale food canning (Reineke, 2012). Inactivation by heat remains the premier method of spore control, the basis of a huge worldwide industry. This still relying on the basic kinetics of inactivation of *Clostridium botulinum* spores (12 D concept/12 D cook), and the reasoning regarding safety first evolved by Bigelow et al. (1920) in 1920. 'Newer' processes such as treatment with ionizing radiation (first proposed in 1905) and high hydrostatic pressure (first proposed in 1899) may be introduced if consumer resistance and some remaining technical barriers could be overcome (Gould, 2006).

In this study, *Bacillus amyloliquefaciens* was investigated. The genus *Bacillus* includes in total 139 species and 11.537 occurrences world-wide (GBIF, 2014).

Out of the entire spore forming *Bacillus* genuses, *Bacillus amyloliquefaciens* is reported to be very high pressure/high temperature resistant and therefore predestinated to be tested as a possible target microorganism for the high pressure thermal sterilization.

2.2.1 Sporulation and structure of endospores

The well-characterized process of endospore development is initiated by environmental conditions that limit growth. Sporulation leads to two cell compartments, which in the end results in the lysis of the mother cell and the release of the spore. This development program is completed in 8-10 hours, can be divided into eight states (0-VII), and is controlled by five sigma (σ) factors (Robleto et al., 2012). An enzyme-gene-cascade (mainly SpoO-genes and kinases) is responsible for the start of the sporulation sequence, which is triggered by environmental cues (Phase 0). This is followed by the pre-asymmetric division (Phase I) which leads to an inhomogeneous and unequal constriction of the cytoplasm membrane. The asymmetric compartment splits the originating cell into a small (forespore) and large (mother cell) compartment. In addition 48 genes are transcribed into the forespore (Phase II) by certain sigma factors, which are keys to the gene expression. The plasma membrane of the mother cell then grows around the forespore, generating an engulfed forespore surrounded by two apposed membranes (Phase III). After engulfment, σ-factor from the compartment of the mother cell controls the gene expression required for cortex formation. This factor regulates more than 260 genes and influences further the formation of the spore coat and the mother cell's metabolism (Robleto et al., 2012). In stage IV, the mother cell membrane grows around the forespore, which is already surrounded by an intact membrane. The synthesis of a thick peptidoglycan cortex between the outer and inner forespore membranes instigates the formation of the cortex and the germ cell wall (stage IV). At the same time as the cortex is formed the forespore loses water and ions like potassium (K^+), prompting a large decrease in volume and a simultaneous decrease in pH by approximately one unit. The loss of water is a response to the uptake of cations like Ca^{2+}, Mg^{2+} or Mn^{2+}, and pyridine-2,6-dicarboxylic acid (Ca-DPA or DPA), all of which are synthesized in the mother cell. Within stage IV-V small acid-soluble proteins (SASPs) are synthesized, which remodel the forespore nucleotide into a ring. Furthermore, at stage V, the protoplast becomes increasingly resistant to high pressure, heat, radiation and the attack of chemical agents or enzymes. This resistance is due mainly to the spore´s high degree of compartmentalization. During sporulation a complex proteinaceous coat, with coat proteins (exosporium) from the

mother cell, is layered on the outer surface of the spore (stage VI), affecting the specific hydrophobicity of bacterial spores. This proteinaceous coat could cause a possible spore agglomeration (Mathys, 2008) and is also responsible for the magnitude of adhesion forces to packing materials and surfaces, for example.

After the mother cell lyses, the spore is released (stage VII). The spores formed have no active metabolism and enable the microorganism to survive over long periods of time and under extreme environmental conditions (Gould, 2006). The formed spore is multilayered, well-structured and this morphology is the basis of the high resistant of spore towards all kinds of extrinsic factors. Starting from the outside the spore layers include, exosporium, coats, outer membrane, cortex, germ cell wall, inner membrane and the central spore core (Figure 2-10).

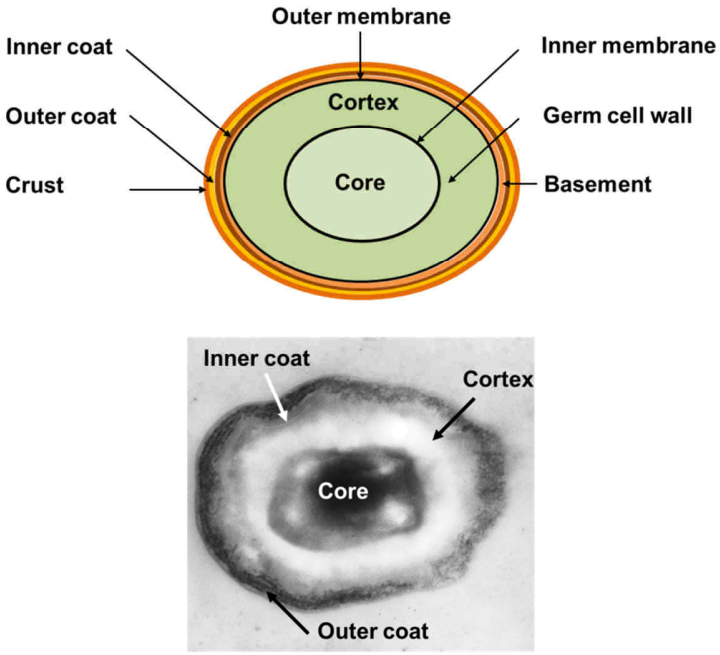

Figure 2-10: Schematic structure of an endospore adapted from Setlow (2006) and Reineke (2012).

The spore consists of different compartments, which will be described and their function within the multilayered structure of the spore will be explained in the following. The exosporium is the most external multilayered structure which surrounds the spore but is not attached to the spore. It consists of specific glycoproteins and the thickness of this layer varies from microorganism to microorganism (Pedraza-Reyes et al., 2012). It is thought that the function of the exosporium is the interaction with surfaces/microorganisms (Setlow, 2006). Next is the spore coat which consists of many layers composed of ≥ 50 different proteins. The coat is important in spore

resistance to some chemicals, to exogenous lytic enzymes that can degrade the spore cortex and to predation by protozoa, but has little or no role in spore resistance to heat, radiation and some other chemicals (Setlow, 2006; Pedraza-Reyes et al., 2012). Further, it plays an important role in the germination process since it is permeable to nutrients into the inner spore membrane (Pedraza-Reyes et al., 2012). Beneath the coat lies the outer membrane which has no particular function, it is thought to be essential during the process of the spore forming but studies have even shown that the resistance of spores is not altered if the outer membrane is removed (Pedraza-Reyes et al., 2012). The cortex layer is composed of peptidoglycan and serves as a retaining structure to withstand turgor pressures generated by high concentrated solutions and to keep the water content constant in the spore core. Connected to the cortex is the germ cell wall which is also made of peptidoglycan structures similar to vegetative microorganisms and as the initial cell wall during the outgrow of the spore (Setlow, 2006; Pedraza-Reyes et al., 2012). The inner and final layer of protection that protects the core is the inner spore membrane. Due to its vital role to protect the DNA in the core it is a very strong but permeable surface, which is thought to be in gel like state. Furthermore, the inner spore membrane is the seat of the nutrient receptors which play a key role in the germination process of the spore (Setlow, 2003). The final spore layer/part is the core, the analogue of the growing cell's protoplast. The core contains most spore enzymes as well as DNA, ribosomes and tRNAs. In almost all cases, the spore's enzymes and nucleic acids are identical to those in growing cells, although there are some unique features and macromolecules in the core, which increase the spores resistance. Only 27-55 % of the wet weight of the spore core is water (responsible for dormancy of the spore); further, free water is also scarce and therefore restricts the movement of macromolecules (Pedraza-Reyes et al., 2012). Pyridine-2,6-dicarboxylic acid (dipicolinic acid, DPA) which is accumulated into the spore core during sporulation and makes up 5-15 % of the dry matter content of the spore. The total concentration of Ca-DPA in individual spores differs in its concentration (> 800 mM to 1 mM). This leads to low water content in the spore. The core is believed to be in a glassy state. The last of the unique molecules are the small, acid-soluble spore proteins (SASP)(Christie, 2012). These small proteins are highly concentrated in the spore core and constitute 3–6 % of the total spore protein. The α / β SASP bind to and saturate the spore DNA causing structural changes in the DNA and altering the DNA's photochemistry. Spore mutant strains that lack α- and β- type SASPs show a reduced UV-light resistance as well as a higher sensitivity to heat, peroxides,

ionizing radiation, and other sporicidal treatments (Setlow, 2003; Setlow, 2006; Pedraza-Reyes et al., 2012; Reineke, 2012)

2.2.2 Inactivation of spores by heat and high pressure

The inactivation of spores under heat and high pressure have been studied over the last decades and knowledge of the mechanisms involved have been growing although still not all is well understood (Härnulv et al., 1977; Ababouch et al., 1995; Ananta et al., 2001; Paidhungat et al., 2002; Setlow, 2003; Margosch et al., 2004b; Gould, 2006; Black et al., 2007; Setlow, 2007; Mathys et al., 2009; Heinz et al., 2010; Olivier et al., 2011; Reineke, 2012; Reineke et al., 2013a; Doona et al., 2014; Georget et al., 2015; Olivier et al., 2015). Nevertheless, several studies showed that the heat resistance of various spore strains does not correlate with high resistance to pressure (Margosch et al., 2004a; Margosch et al., 2004b; Olivier et al., 2011). Furthermore, Margosch et al. (2004b) supposed that the resistance of spores to combined pressure and temperature treatments depends on their ability to retain Ca-DPA. The main key for eradication is germination. The germination can be triggered by physiological or non-physiological events (Reineke, 2012). Both pathways of spore germination will be described and discussed in the following chapter since their understanding plays a vital role for the dimensioning of inactivation regimes.

2.2.2.1 Physiological germination of spores

In order to initiate germination and restore vegetative growth when conditions become favorable, bacterial spores must be able to monitor their external environment (Moir et al., 2002); therefore, the natural germination process is usually initiated by the presence of nutrients, in this context called germinants. These germinants are a mixture of asparagine, glucose, fructose, ribosides and K^+, which need to penetrate through the spore layers to get to the germinant receptors located at the inner spore membrane (Moir et al., 2002; Setlow, 2003; Gould, 2006; Moir, 2006; Christie, 2012). If the commitment, the first event during germination and irreversible, is made due to exposure to germinant molecules and binding of those to the germinant receptors, then the result is a rapid loss of resistance properties (Setlow, 2003; Yi and Setlow, 2010; Christie, 2012). Under physiological conditions, two germination pathways have been identified in spores of Bacillus species. The best understood is the binding of nutrients to the nutrient receptors that are bound to the inner spore membrane (Christie, 2012). The second and more recently discovered pathway is mediated by serine/threonine kinase (PrkC), which initiates germination after the binding of

defined peptidoglycan fragments. The germination induced by nutrients can be divided into two stages (Figure 2-11): Stage I where the permeability of the inner spore membrane changes. This resulting in the movement of selected ions and other small molecules through the membrane from the spore core, with water moving the opposite direction; and Stage II, which encompasses later degradation steps of spore layers and the outgrowth of the vegetative cell (Setlow, 2003; Yi and Setlow, 2010; Christie, 2012).

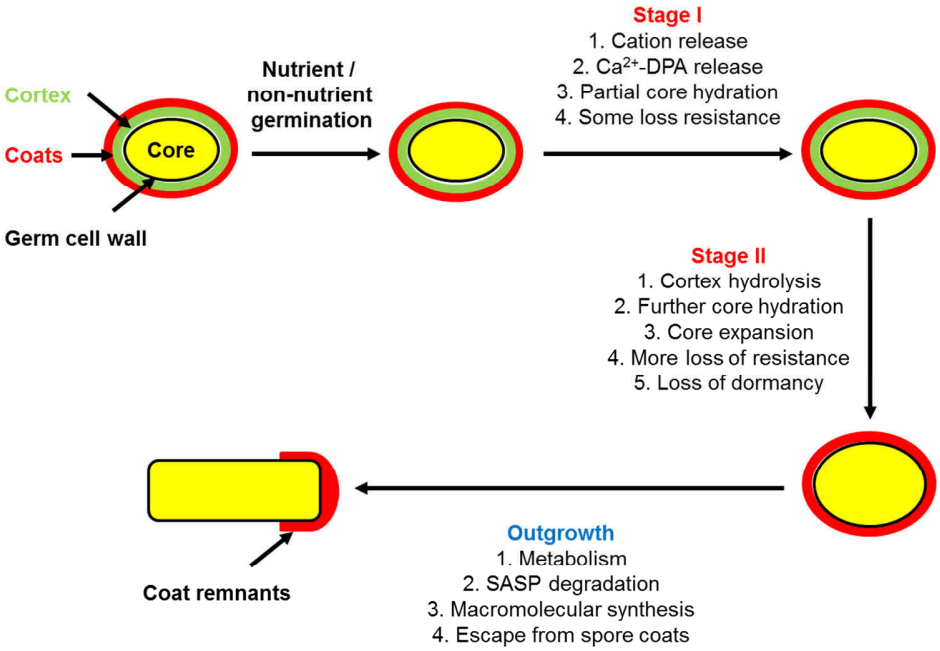

Figure 2-11: Stages of spore germination adapted from Reineke (2012).

As mentioned the germination process starts irreversibly if germinant receptors (Ger) (GerA, GerB, GerK, GerN and GerD) are activated by nutrients after the commitment monovalent ions (H^+, K^+ and Na^+) are released from the spore core denoting the beginning of Stage I of the spore germination process. Due to the ion release the internal pH increases, which is important for later enzyme activity within the spore structure. DPA is released from the spore core with simultaneous rehydration of the spore core by water. At the end of stage I the resistance to extrinsic factors is reduced but diffusion and mobility of molecules in the core remains restricted (Setlow, 2003; Yi and Setlow, 2010; Christie, 2012; Reineke et al., 2013a). Stage II is responsible for the degradation of the thick spore specific cortex. Here two important cortex lytic enzymes (CLEs), Sleb located in the inner spore membrane and CwlJ located at the junction of cortex and coat junction, play a key role during the degradation process. The enzymes are triggered by

changes in the germinating spore. CwlJ is activated if the DPA-concentration in the spore core reaches a certain level (Christie, 2012). The activation of Sleb is still unknown (Moir et al., 2002; Setlow, 2003; Moir, 2006; Yi and Setlow, 2010; Christie, 2012; Reineke et al., 2013a). Further factors affecting enzyme activity are the water content and the pH-value of the core.
The loss of the cortex results in further water uptake and swelling of the core. Protein mobility can resume at this point permitting enzyme action marking the end of germination and the transition to vegetative metabolism. The next step is that SASPs, which saturate the spore DNA, are degraded and hydrolyzed by the germination protease, Gpr, in the outgrowth phase, followed by the synthesis of macromolecules and the escape from spore coats (Setlow, 2003). This follows a macromolecular synthesis and enables them to escape from the spore coats. For an individual spore these events may take only a few minutes, but because of the significant variation of spores, these mechanisms may take much longer for the whole population (Setlow, 2007).

2.2.2.2 Initiation of non-physiological germination/inactivation by high pressure

The inactivation of bacterial endospores by pressure is generally considered to rely on pressure-induced spore germination, followed by inactivation of germinated spores (Margosch et al., 2004b). In the past decades other possible non-physiological pathways of the spore germination have been detected. Non-nutrient germination can be further categorized into a (recently discovered) second physiological and several non-physiological routes. The physiological routes include germination initiated by eukaryotic-like serine/threonine kinase, which is located in the inner spore membrane like nutrient receptors. This kinase, which is present in Bacillus and Clostridium species, recognizes peptidoglycan fragments. Non-physiological germination pathways initiate spore germination by bypassing individual germination steps. This could be stimulated by physiochemical agents, such as exogenous Ca-DPA (Paidhungat et al., 2002; Moir, 2006), which directly activates the CLE CwlJ, or cationic surfactants such dodecylamine (Setlow, 2003), which interferes with the inner spore membrane and causes a direct release of Ca-DPA. The pathways of the high pressure stimulated germination are summarized in Figure 2-12. At pressures between 100-400 MPa it has been shown that the nGeR of *Bacillus subtilis* and *Bacillus cereus* are triggered. The spores germinated quite well between pressures of 100-200 MPa and lead to a maximum 4 $\log_{10}$ inactivation but the pressure induced physiological germination decreased for higher pressures, indicating that other pathways must be active (Wuytack et al.,

1998; Paidhungat et al., 2002; Reineke et al., 2013a). A treatment of 200-400 MPa at 40°C for 30 minutes showed germination of the spores but negligible inactivation (Wuytack et al., 1998).

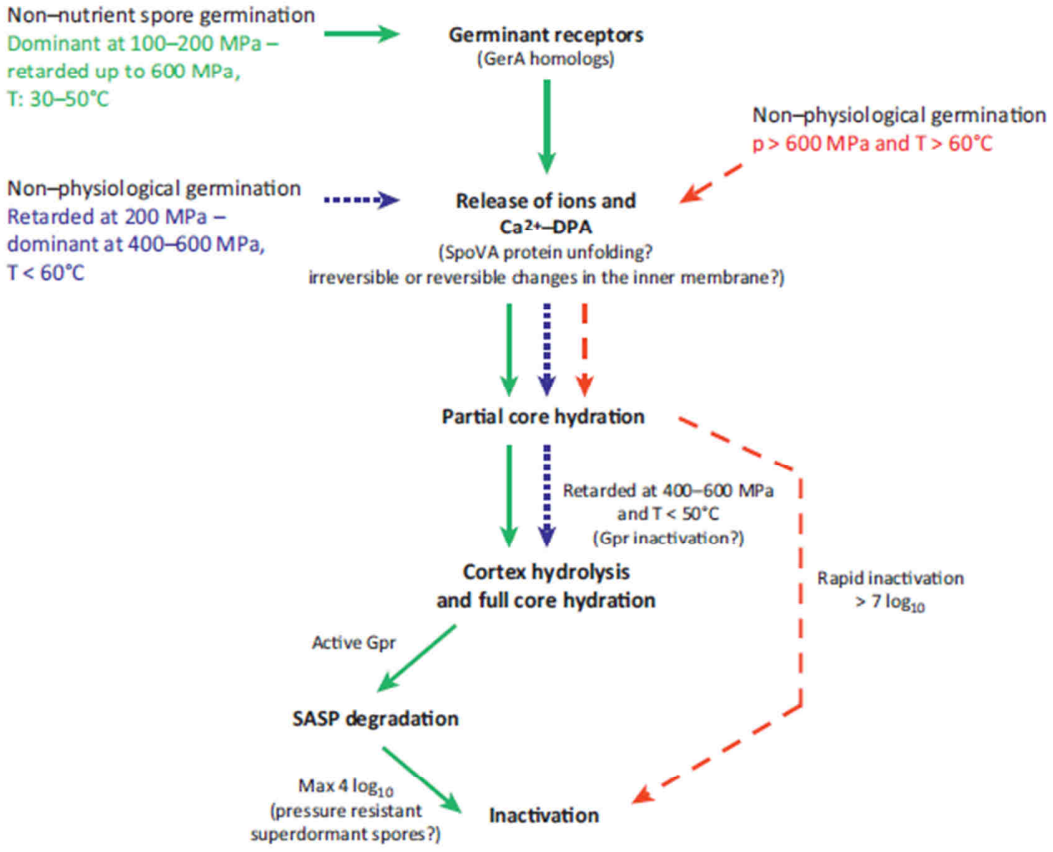

Figure 2-12: Proposed germination and inactivation pathway of Bacillus subtilis dependent on the applied pressure (p) and temperature (T) based on Reineke et al. (2013a).

To achieve a quick and sudden inactivation of spores, which is relevant for food processing, pressures above 500 MPa must be applied in combination with temperatures above 60°C (red line in Figure 2-12). Under these conditions, Paidhungat et al. (2002) were able to germinate *B. subtilis* spores that lack all major nutrient receptors. This suggests a direct opening of the spores' Ca–DPA channels, a germination mechanism identified as active at 200 MPa and moderate temperatures (<50°C) using *B. subtilis* mutant strains that lack the nGeR. These findings were also verified by Reineke et al. (Reineke et al., 2012) for pressures ≥ 600 MPa and temperatures ≥ 60°C. The following step, which is rapid release of Ca–DPA under pressure, is accompanied by core hydration. This step of germination is the crucial step with regard to loss of resistance, and it is therefore of great interest for a variety of sterilization techniques and research (Reineke et al., 2013a). Therefore, the ability of the spore to retain the DPA as long as possible under these

conditions becomes the rate limiting step of the spore inactivation (Margosch et al., 2006; Reineke et al., 2013b). This suggests that the structure most susceptible to HP (600 MPa) and high temperatures (60°C) is the inner spore membrane or its associated membrane proteins (Reineke et al., 2013a; Reineke et al., 2013b). At pressures above or equal to 400 MPa when an opening of the Ca - Dipicolinicacid (DPA) - channels occur the following happens: i) DPA is released from the spore core; ii) the spore core gets hydrated; and iii) the spore becomes thermo- and pressure sensitive and can be inactivated (Reineke et al., 2012). Further a threshold pressure of 600 MPa was established at and above this pressure level the DPA-release is dominated by the temperature. To guarantee a successful inactivation of spores by pressures of 600 MPa many researchers recommend a treatment at 90-121°C (Margosch et al., 2004b; Margosch et al., 2006; Barbosa-Canovas and Juliano, 2008; Wimalaratne and Farid, 2008; Mathys et al., 2009; Heinz et al., 2010; Knorr et al., 2011b; Reineke et al., 2013a; Georget et al., 2015) due to the synergistic effect pressure and temperature have on the spore inactivation (Olivier et al., 2015). In comparison to a conventional retorting, this could reduce the thermal load applied to the product in comparison to a conventional retorting without affecting the safety or the quality of the food. Two sterilization approaches can be derived from this which both needs the adiabatic heat of compression to reach the target temperature:

- Pressure assisted thermal sterilization (PATS): pressure is neglected and only seen as the method to reach the end temperature faster (Sizer et al., 2002; Technology, 2009)
- High Pressure Thermal Sterilization (HPTS) which takes into account the impact of pressure on the spore inactivation (Mathys, 2008; Reineke et al., 2011)

A broad overview of the work already conducted on high pressure induced germination or inactivation of spores is given in Annex 1. The table in Annex 1 is a data collection of 159 publications from 1903-2015, containing the basic information on what kind of spore strain the researches worked on, what conditions were applied, what was the inoculation medium and what was the inactivation.

2.2.2.3 Inactivation of spores by heat

One of the main characteristics of bacterial spores is their resistance to heat when suspended in an aqueous environment (Setlow, 2006). The mechanism of heat inactivation is not completely understood but when spores are heated there is also a progressive loss of DPA. It is thought that

the leakage is due to damages to the inner spore membrane and rupture of other spore compartments (Coleman et al., 2007).

2.2.3 Spores in complex food systems and its influence on the inactivation

Certain ingredients of foods such as fats, sugars, salts and the resultant water activities could lead to a retarded or reduced inactivation under these conditions. The so called baroprotective effect is not well studied yet for spores in combination with high pressure and high temperatures. Water activity (a_w) is a physical chemistry concept which quantifies the relationship between moisture in foods and the ability of microorganisms to grow on them. Water activity is defined as shown in Eq.2) : $a_w = \frac{p}{p_0}$ where p is the partial pressure of the water vapor in the test material and p_0 is the water vapor pressure of pure water under the same conditions (Pitt and Hocking, 1997). Figure 2-13 is a schematic impression of combined influence of pH and a_w on microbial growth is shown.

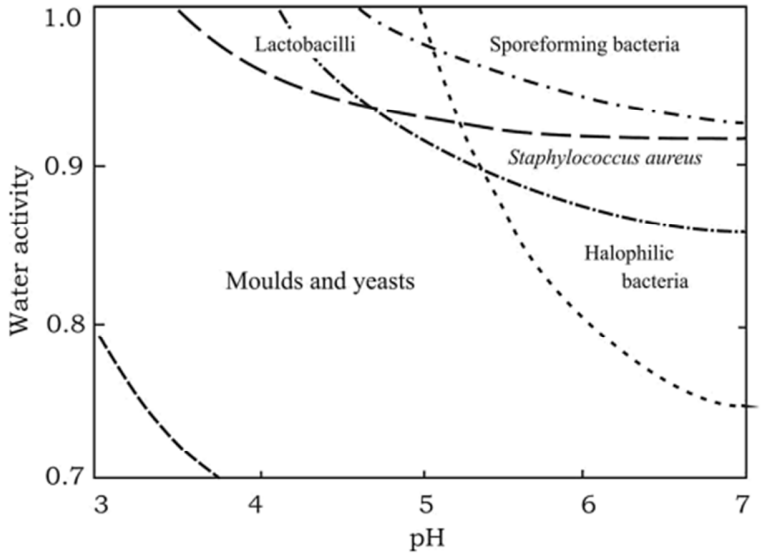

Figure 2-13: A schematic diagram of the combined influence of pH and aw on microbial growth from Pitt & Hocking (1997).

The basis for the survival and death of microorganisms as influenced by a_w is complex. Multiple factors, intrinsic and extrinsic, influence this relationship but can greatly differ within food types, processes and different forms of microorganisms (Lenovich, 1987).

Water activity is a major factor in preventing or limiting growth (bacteria causing food borne diseases will not grow under a_w of 0.85) but it can also lead to an increased resistance of microorganisms and spores. This may not be a safety problem at first since spores and

microorganism may not grow in low a_w but if these systems are diluted or other extrinsic factors come into play, spoilage could occur. The influence of salts or sugars on the water activity (a_w) of foods or suspension media does not explain the marked baroprotective effects of these solutes and it has been suggested that specific interactions between solutes and biological macromolecules contribute to their baroprotective effects (Fujii et al., 1996; Molina-Höppner et al., 2004). The increased resistance to temperature and high pressure of vegetative cells and spores in low a_w environments could be explained by their partially dehydrated state. Proteins and other essential cell constituents become more resistant to thermal damage or other extrinsic stress factors in the partially dehydrated state (Barbosa-Canovas et al., 2007). These observations were already made by researchers between 1950 and 1980, which tried to understand the protective effect of solutes (sodium chloride, sucrose, glucose, fats, acids etc.) and the precipitated change in water activity during the thermal inactivation of microorganisms (Anderson et al., 1949; Secrist and Stumbo, 1958; Murrell and Scott, 1966; Molin and Snygg, 1967; Cook and Gilbert, 1968; Beuchat, 1974; Gould, 1977; Hätnulv et al., 1977; Senhaji and Loncin, 1977). These researchers indicated that a protective effect of solutes/a_w-value exists and can lead to an increase of the D-value for the tested microorganisms. Some further stated that the protective effect is depending on the concentration of solute, the solute itself (ionic e.g. salt or non-ionic e.g. sugar), the selected microorganism and the temperature. However, the protective mechanism of a reduced a_w-value is still unclear. Molin and Snygg (1967) reported in their studies that fats/oils increased the heat resistance of *Bacillus cereus* and *Bacillus subtilis* spores. They stated that the protective effect is not only due to the low heat conductivity or the water free environment. A more apparent possibility for that protective effect might be that the free fatty acids present in some oils could have a stabilizing effect on spores.

The use of an emerging technology such as high pressure processing might prove valuable to overcome the protective effect of the solutes. And by doing so, achieve shorter process times. Some interesting studies concerning the influence of high pressure on baroprotecive solutes exist. Oxen and Knorr (1993) showed that high pressure inactivation of *Rhodotorula rubra* in different a_w solutions was more effective than by heat alone. Molina-Höppner et al. (2004) stated that due to the osmotic up-shock microorganisms (*Lactococcus lactis, Escherichia coli*) accumulate solutes (NaCl and sucrose), which then can interact with biomolecules, as per the example of phospholipid bilayers. Sucrose preserved the metabolic activity and membrane integrity of the

cells during the high-pressure treatment, whereas salt preserved the membrane integrity but not the metabolic activity.

Due to the accumulation, the membrane stays in a more fluid state during the treatment and therefore shows a higher high pressure resistance. However, the baroprotection of salt (ionic solutes) requires higher concentrations than the same baroportection by disaccharides.

No such detailed research was conducted at this point for spores but it is plausible that also spores could accumulate solutes by diffusion in the inner compartments, e.g. inner spore membrane, where these could interact with biomolecules. Some prior works already indicated that such a phenomenon could exist in food systems (cacao mass, fish systems, baby food puree, food concentrates) for high pressure/high temperature processes as well (Ananta et al., 2001; Sevenich et al., 2013; Sevenich et al., 2014; Georget et al., 2015; Sevenich et al., 2015). Black and Gerhardt (1961; 1962) stated, that the central core of a spore is kept relatively dry but that the rest of the compartments are free to extensive water and solute permeation. Other authors mentioned that the predominant compartment, which plays an important role in the uptake of solutes, is the spore cortex. The cortex possesses a negative net charge. The low degree of cross-linking supposes that the spore cortex is able to change volume in response to changes as a result of balancing the electrostatic interaction (Kazakov, 2008; Kazakov et al., 2008). These induced changes in the different spore compartments could have an impact on the inactivation mechanism under high pressure. Another possible postulated impact of the baroprotective of solutes could be the influence on the rate limiting step of the spore inactivation under pressure, which is the release of DPA out of the spore core and the followed rehydration of the core by water. Lower a_w means that most of the outside water is bound to solutes and cannot freely move. Therefore, one could assume that the exchange of water and DPA is slowed down due to changes in the osmotic pressure or the solubility of DPA in high concentrated solutions might differ, which both could result in retarded and delayed inactivation. Further, Hofsetter et al. (2013) showed that by adding reutericyclin to *Clostridium spp.* resulted in more-fluid membranes during and after high-pressure thermal processing but did not consistently enhance spore inactivation. In addition, a third postulated impact could be that high concentrations of solutes close to the oversaturation point can inhibit the pressure transmission of water to some degree and therefore generate an insufficient and in-homogenous pressure distribution within the system (Eder and Delgado, 2007; Min et al., 2010). The spores might, if the threshold pressure (500-600 MPa) is not reached, not undergo the postulated pathways of germination/inactivation.

2.2.4 Modeling of spore inactivation

The description of the inactivation kinetics of spores in food is of great importance since it shows the goodness of the applied treatment in terms of possible achievable inactivation. In the field of food technology it is essential to know the quantity of surviving microorganism to guarantee food quality and therefore minimize the risk for the consumer to eat spoiled food.

According to general and food microbiology textbooks, microbial inactivation follows first-order kinetics. Hence, it can be characterized by a single rate constant k or its reciprocal, the D-value, considered a measure of resistance to an applied lethal agent. There are three problems with methods based on these models: (i) Substantial evidence indicates that microbial inactivation, usually is not a process which follows first-order kinetics. (ii) Even if it were, neither of the above two temperature-dependence models would be a good choice because both give an inappropriate relative "weight" to low temperatures, where little activation takes place, at the expense of high temperatures, where most of the inactivation actually occurs. (iii) The standard formula to calculate the F-value has a reference temperature. But since the F-value can be translated into a residual survival ratio, the latter will be independent of the reference temperature if the relationship between log D and temperature is linear over the whole pertinent temperature range.

Therefore two main basic approaches—mechanistic and vitalistic—are used to study the inactivation of bacterial spores which are based on the findings of Watson (1908) early in the 20th century.

The vitalistic approach:

As already described the first order kinetic implies that all microorganisms have the same probability of dying. Hence it is more unlikely that all microorganisms behave the same towards extrinsic factors (heat, chemicals, high pressure etc.), due to their heterogeneity. If one with this point of view looks at inactivation kinetics the survival curve can be seen as a cumulative form of the underlying distribution of individual inactivation times (van Boekel, 2002b). Therefore it can describe the often occurring shouldering effects (spore agglomeration) or tailing effects (more resistant subpopulation, single hit theory, insufficient treatment etc.). These non-linear curve progressions with tailing or shouldering can be described e.g. by Weibull distribution (resident in reliability engineering), which is a powerful tool appropriate for the analysis of inactivation data for all kind of innovative non-thermal processes (van Boekel, 2002b; Mathys, 2008; van Boekel,

2009; Reineke, 2012). The equation of the Weibull distribution is shown in Eq. 3, with k as the scale parameter, b as the shape parameter.

Eq.3) $\log\left(\frac{N}{N_0}\right) = -(\frac{t}{k})^b$

The shape parameter, b, determines the curve progression of the inactivation curve, and simultaneously gives information about the dying behavior of the respective microorganisms. For values of b smaller than 1 the decreasing curve becomes increasingly flatter. This implies that the microorganisms, not yet killed, are more resistant to the treatment than the microorganisms that have already been killed. If b equals 1 the graph is linear and therefore corresponds to a first order inactivation. For values of b greater than 1 the inactivation curve decreases progressively; it can be deduced that the remaining cells exhibit increasing sensitivity against the applied treatment (van Boekel, 2002a).

The mechanistic approach:

The mechanistic approach is based on the fact that the inactivation of microorganisms is similar to chemical reaction which is time dependent. This dependency is shown in Eq. 4. Here the number of surviving microorganisms versus time is plotted and the inactivation curves become linear with the rate constant *k* (inactivation rate).

Most inactivation kinetics does not follow a first order reaction and often tailing could occur. This leads to a modification of Eq.4 into Eq.5. The use of an empirical reaction order *n* leads to an n^{th}-order reaction model, with which non-linear $\log_{10}$ reductions can be calculated and fitted for n= 0-3. The reaction order *n* has no mechanistic background and can be determined by regression. For isorate calculations a functional dependency of *k* (T,p) can be derived. The n^{th}-order approach can describe the tailing effect of inactivation kinetics quite well but no statement about shouldering can be made (van Boekel, 2009).

Eq. 4) for n = 1 $log\frac{N}{N_0} = \log(e^{-k*t})$

Eq.5) for n≠1 $log\frac{N}{N_0} = \log((1 + k * t * (n-1))^{\frac{1}{1-n}})$

The comparison of Weibull (b = 0.94) and an n^{th}-order (n = 1.1) approach is shown in Figure 2-14 for the same inactivation data of *Clostridium sporogenes* in sardine in olive oil. What is obvious is that both approaches describe the tailing effect quite nicely. In this case no difference in terms of progression of the fit is present and the RMSSE with 0.240 respectively 0.221 shows that both fits could be used to describe the inactivation kinetic for the described case.

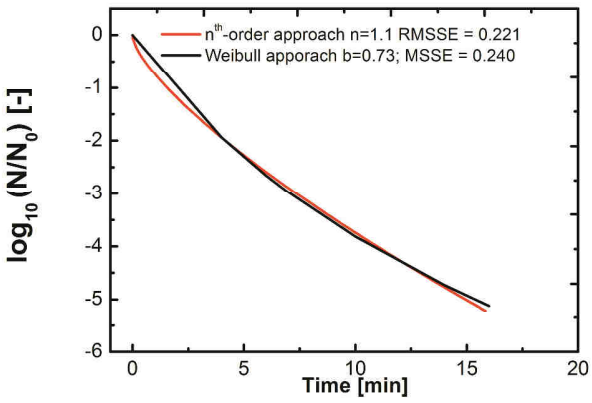

Figure 2-14: Comparison of Weibull and nth order approach for inactivation kinetics at 600 MPa 85 °C for *Clostridium sporogenes* in sardine in olive oil.

2.3 Food processing contaminants (FPCs)

The high temperatures needed for the inactivation of spores lead to the formation of unhealthy compounds within the food. Since the beginning of the new millennium more attention has been given to the mechanisms and mitigation strategies of these compounds. As already described in Chapters 1 and 2 the way of our foods from farm to fork or from raw material to better digestible safe foods is made possible by processes such as the heat treatment. Those treatments often result in an over-processing and are due to chemical changes in the product, giving rise to the food processing contaminants (FPCs) other synonyms are, process-induced toxicants and neo-formed contaminants. FPCs only involve those compounds formed during the processing (heating, roasting, frying, baking, grilling etc.) of the food. By definition FPCs are substances present in food as a result of food processing / preparation that are considered to exert adverse physiological (toxicological) effects in humans, i.e., substances which pose a potential or real risk to human health. Food in this definition also includes beverages and nonalcoholic drinks such as coffee and tea. Ingredients commonly occurring in food formulations are excellent substrates for chemical reactions occurring under the conditions encountered in food processing. The reaction products formed depend on the processes and conditions used, such as fermentation, irradiation, or heat processing (Lineback and Stadler, 2009). The compounds formed play a vital role in food properties such as flavor, aroma and color. One of the key reactions is the Maillard reaction, involving reducing sugars and amino acids, which is responsible for 330 known volatile compounds associated with cooked food (Lineback and Stadler, 2009). Roundabout 200 other

compounds arise from heated lipid systems. Precursors of FPCs are sugars and amino acids; other reaction pathways can involve poly-unsaturated fatty acids (PUFAs; linoleic acid), ascorbic acid, sugars (glucose, fructose), glycerol, chloride or carotenoids (Crews and Castle, 2007; Vranova and Ciesarova, 2009; Lachenmeier and Kuballa, 2010; Bravo et al., 2012b; Crews, 2012). Therefore changing the recipe of foods is not an option since some of the precursors of FPCs are essential nutrients. Compounds formed during the processing of food are, for example, acrylamide, furan, 3-MCPD-esters etc. These showed carcinogenic, mutagenic (genotoxic), or neurotoxic properties at high doses in animal studies (Stadler et al., 2002; Märk et al., 2006; Bolger et al., 2009; Jestoi et al., 2009; Larsen, 2009b; Lineback and Stadler, 2009; Vranova and Ciesarova, 2009; Lachenmeier and Kuballa, 2010; BfR., 2012; Bravo et al., 2012a; Palmers et al., 2014; Sevenich et al., 2015). Therefore a risk for humans especially for infants, the elderly and immune suppressed person is not negligibly. The risk of the exposure to FPCs is not a new one, since humans always have been exposed to these kinds of compounds from the moment “they caught fire”. Nevertheless there is a public concern and those risks must be minimized (Curtis et al., 2014). For genotoxic and carcinogenic substances such as furan and acrylamide the ALARA principle (as low as reasonably achievable) is applied to foods as a possible risk assessment (Crews and Castle, 2007). To prioritize risks of foodborne-genotoxic-carcinogens the margin of exposure (MoE) is used. The MoE is usually calculated as a range, taking in most cases the BMDL 10 value (the lower confidence limit on the benchmark dose associated with a 10 % cancer incidence) and the upper - and lower - bound human exposure estimate (Lineback and Stadler, 2009). In Table 2-3 some of the most important FPCs are depicted with their resulting MoE. A MoE is of > 10000 is interpreted as unlikely of concern. The interpretation of the MoE is difficult due to scarcity of amounts found in foods and difference in analytical methods.

Table 2-3: Risk assessment of selected food-borne toxicants adapted from Lineback and Stadler (2009)

Food borne toxicant	Estimated daily dietary uptake [µg*kg^{-1} bw*d^{-1}]	MoE
1,3-DCP/2,3 DCP	3-200	2,100,000-32,000
Heteroyclic aromatic amines	4.8-7.6	260,000-164,000
Polyaromatic hydrocarbons	4-10	25,000-10,000
N-nitrosamines	3.3-5.0	18,200-12,000
Ethylcarbamate	33-55	9,000-5,460
Furan	260-610	3,900-1,600
3-MCPD	360-1,380	3,055-800
Acrylamide	1,000-4,000	300-75

Nevertheless, the data shown here clearly indicates that the FPCs of major concern in foods are acrylamide, furan and 3-MCPD and their esters. The European Commission and the European Food Safety Authority have been monitoring different FPCs especially acrylamide, furan and 3-MCPD-esters in all kinds of foods over the last years and have already issued indicative levels for acrylamide in 2011 (EFSA, 2011; 2012; Curtis et al., 2014). The creation and introduction of new guidelines in the future is most certain and will be a difficult task for the food industry. Therefore other technologies and research is needed to find mitigation strategies which lead to the same quality without affecting the safety of the product. Here the high pressure thermal sterilization, ohmic heating and vacuum baking, just to name a few innovative technologies, could be powerful tools to achieve this aim. Research in all these areas is progressing at a rapid pace and these selected examples show that process toxicants have in the past few years gained significant attention on a global scale in terms of potential human health risk (Lineback and Stadler, 2009).

2.3.1 Furan in Foods

Furan (C_4H_4O) is a heterocyclic compound mostly associated with the flavor of foods with a low boiling point of 31°C. The name comes from the Latin *furfur*, which means bran. Technically furan can be obtained by distillation of bran with sulfuric acid. In the chemical industry furan is hydrated to Tetrahydrofuran, which is a solvent. First found in heat treated foods in 1979, this has been followed by many surveys of heat processed foods. In the mid 1990's furan was classified as possibly carcinogenic to humans (group 2 B) by the International Agency of Research on

Cancer (IARC, 1995), since it induced tumors and liver toxicity in experimental trials with animals (mice and rats) (IARC, 1995; Blank, 2009; Bolger et al., 2009). This is why the ALARA (as low as reasonably achievable) principle is applied to foods for furan. The furan-induced toxicity is due to cytochrome P450 activity (liver enzyme CYP2E1) which converts furan into cis-2-butene-1,4-dial. The compound formed can bind with proteins and nucleosides, which causes severe DNA damage and a deficit of function in the affected compounds (Bolger et al., 2009). The pathways of furan formation are quite complex since many precursors and intermediates can lead to the formation of furan. Further, information on the mechanisms of furan formation under industrial food process conditions or domestic cooking are scarce (Blank, 2009). In model studies possible precursors and pathways have been identified (Hasnip et al., 2006; Blank, 2009; Vranova and Ciesarova, 2009). Hence, multiple sources of furan formation (Figure 2-15) are 1) thermal degradation/Maillard reaction reducing sugars, alone or in the presence of amino acids, 2) thermal degradation of certain amino acids, and thermal oxidation of 3) ascorbic acid, 4) carotenoids and 5) poly-unsaturated fatty acids (PUFAs) (Hasnip et al., 2006; Märk et al., 2006; Blank, 2009; Vranova and Ciesarova, 2009).

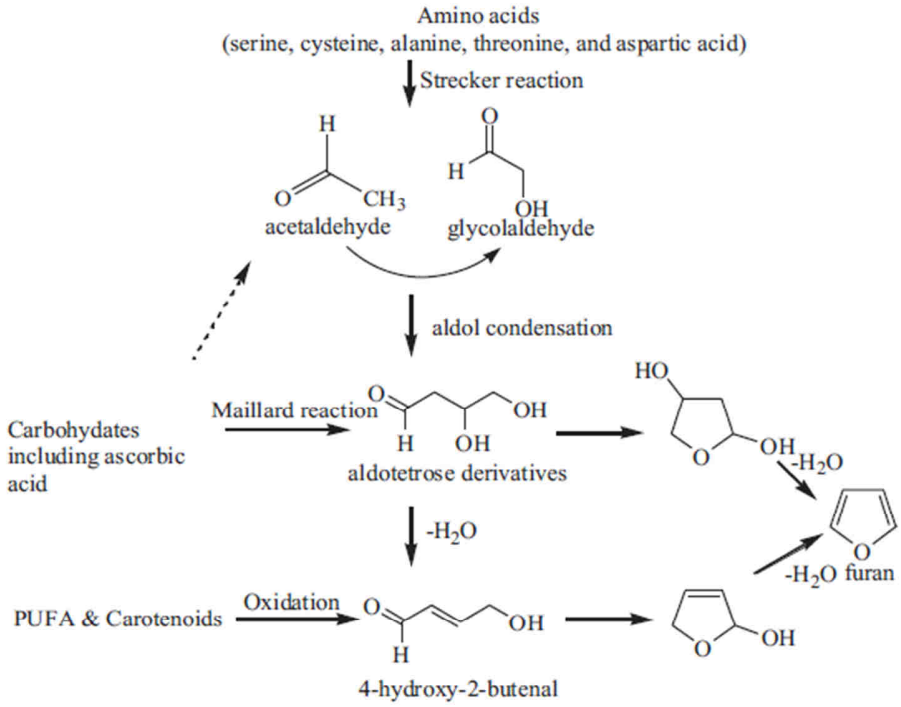

Figure 2-15: Different origins of furan formation [O] = Oxidation. Adapted from Perez Locas and Yaylayan (2004).

Therefore possible precursors of furan are the reduction of sugars (glucose and fructose) and amino acids; other reaction pathways can involve poly-unsaturated fatty acids (PUFAs; especially linoleic acid), ascorbic acid or carotenoids. Märk et al. (2006) reported that the potential (molar yield) to form furan is dependent on the precursors by the following order: ascorbic acid > PUFAs (linoleic acid) > sugars (glucose, fructose). Further Märk et al. (2006) concluded that the more complex the matrices are the lower is the furan formation. Moreover, Crews and Castle (2007) mentioned that when ascorbic acid is present in real foods and model systems, less furan is produced, as if ascorbic acid is heated alone. Based on this it can be concluded that the formation of furan is not only dependent on the treatment conditions but also on the formation potential of the precursors in foods. High amounts of precursor do not automatically lead to high amounts of furan. A key role in the formation of furan in foods seems to be the oxidative degradation of PUFA's (Perez Locas and Yaylayan, 2004; Märk et al., 2006; Blank, 2009). Furan is present in many foods. In Table 2-4 some selected foods are listed with their corresponding furan concentration after heat treatment. The amount of furan in the samples/foods can be measured by two different analytical approaches. *The automated headspace gas chromatography-mass spectrometry* (HS-GC-MS) is the most common approach.

Table 2-4: Levels of furan in foods (mostly canned and jarred)

	mean	Min-Max values found in the surveys	Source
Food systems	Furan [µg kg^{-1}]	Furan [µg kg^{-1}]	
Baby food with chicken	23	-	(Hasnip et al., 2006)
Baby food puree	28	1;112	(Crews and Castle, 2007)
Baby food puree	48	14;125	UK survey 2014
Baby food puree vegetable	30	-	(Sevenich et al., 2014)
Baby food puree meat	49.6	-	(Jestoi et al., 2009)
Baby food puree vegetable	37	-	(Jestoi et al., 2009)
Baked Beans canned	122	-	(Blank, 2009)
Canned fish/sardine in olive oil	57.88	-	(Sevenich et al., 2013)
Coffee (beans)	2272	4895	(Bakhiya and Appel, 2010)
Coffee (drink)	74	13;146	(Vranova and Ciesarova, 2009)
Gravies	48	13,174	(Crews and Castle, 2007)
Soups containing meat	88	125,43	(Bakhiya and Appel, 2010)
Stew	25	-	(Hasnip et al., 2006)
Vegetable puree	48.6	93, 153	(Blank, 2009)

Usually Furan begins formation at temperatures ≥ 110°C. It was observed that temperature (≤ 110°C) may not lead to furan formation regardless of pH, but pH had a significant effect on thermally induced furan formation at a temperature greater than 110°C. At pH 7.00 the solution produced significantly more furan than that prepared in solution at pH 9.40 and pH 4.18, suggesting that pH is an important factor influencing furan formation (Nie et al., 2013). However, the study conducted by Nie et al (2013) was only carried out in one model solution (glucose–glycine). As depicted in Table 2-4 furan is present in many varieties of heat treated food and therefore the exposure to this compound could occur quite often. Exposure estimates are the highest in toddlers (0.05–0.31 μg kg^{-1} bw/day) and in adults (0.03-0.59 μg kg^{-1} bw/day) with jarred baby foods and coffee being the major contributors, respectively. In general furan contents between 20-70 μg kg^{-1} can be described as moderately high. Due to its volatile character (boiling point temperature 31°C) one could think that furan might evaporate out of the food during the normal heating practices that precede consumption. As Hasnip et al. (2006) have shown for fruit purees, baby food and other food systems, that is not the case. Furan seems to have accumulated well into the matrix if the food was sterilized in cans or jars prior to the preheating. Possible long-term effects of furan to the health of humans, especially children and the elderly, is unknown and real data on the exposure due to diet are scarce. However, the presence of furan in baby foods is a major concern because of their high sensitivity to carcinogens in addition to the larger amounts (relative to body weight) of certain foods that are consumed (Fan et al., 2008). Levels of furan found in baby food can vary depending on the food's ingredients. The mean concentration of furan in baby food products reported by Jestoi et al. (2009) were 9.2, 37.0 and 49.6 μg kg^{-1} for fruit-, vegetables- and meat-containing baby foods respectively. For the near future, as mentioned in Chapter 2.3, mitigation strategies are needed if EFSA or the U.S. FDA bring out guidelines for amounts of furan that are allowed to be present in foods. Changing the recipe of the products to mitigate the furan formation should not be an option since some of the precursors are also of high nutritional value. Other possible mitigation strategies discussed by Blank (2009) are:

1) Optimize the thermal processing by increasing the heat conductivity to the product without affecting the food safety
2) Intervention of the reaction pathway by the presence of free radical scavengers, such as ethanol and mannitol →Although doubtable due to carcinogenicity of ethanol
3) Reducing O_2 in the heating system to avoid auto-degradation of PUFAs

4) Probably most promising, the use of alternative technologies as Ohmic heating and high pressure thermal sterilization which lower the thermal load without affecting the food safety

All of the above mentioned possibilities could lead to a reduction of furan. However, what also is of great importance is to have reliable, validated and standardized analytical methods. Furan is very volatile (boiling point temperature 31°C), which makes the use of a headspace sampling the obvious method for furan. The samples were incubated at low temperature (40°C) to equilibrate furan into the headspace, which was sampled by an injection loop. Furan was quantified by comparison of the peak area of the furan response with that of deuterium labeled furan added at low level to the sample. The method used is described in detail elsewhere (Crews and Castle 2007). ii) Another approach to measure the volatiles (furan) is the *solid phase micro extraction* (HS-SPME) and then run through *gas chromatography-mass spectrometry* (GC-MS), also explained in more detail elsewhere (Kim et al., 2010). The SPME offers the possibility to analyze even really low levels of furan, however at 5 µg kg^{-1} with a 29 % of uncertainty (Jestoi et al. 2009)

2.3.2 3-mono-chloropropane-1,2-diol (3-MCPD) and 3-MCPD-esters

The formation of 3-MCPD/3-MCPD-esters is mostly associated with heated fat-containing food systems where refined fats or oils are used. The single positional isomer, 2-chloro-propane-1,3-diol (2-MCPD) can also be formed the distribution of both these isomers (approximately present in the ratio of 2:1) but was not analyzed within this study (Hamlet and Sadd, 2009). These ingredients can already contain high amounts of 3-MCPD/3-MCPD-esters due to prior steps during the refining of these fats/oils (Larsen, 2009a). During the deodorization step of the refining process, the oil is exposed to high temperatures (> 200°C to 270°C) and changes in the lipid matrix may occur, leading to the formation of 3-mono-chloropropane-1,2-diol (3-MCPD) esters and possibly other processing by-products (Hrncirik and van Duijn, 2011). Precursors are glycerol, glycerides and chloride (Figure 2-16) (Hamlet and Sadd, 2009).

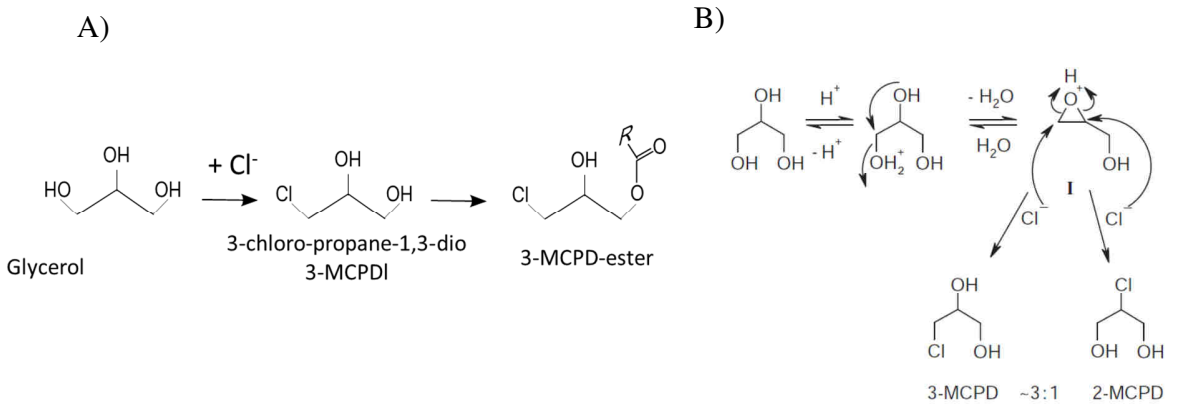

Figure 2-16: A) Schematic formation of 3-MCPD and -esters from Glycerol B) Formation of 3-MCPDs from glycerol via the intermediates (Hamlet and Sadd, 2009).

3-MCPD/3-MCPD-esters are known to be an animal carcinogens (Tomar et al., 2010) but studies concerning the toxicity of 3-MCPD-esters are limited. Since both compounds are thought to be carcinogenic, it is uncertain to what amount the 3-MCPD-esters are hydrolyzed within the digestive system by gut lipase and toxic 3-MCPD is set free (Hamlet and Sadd, 2009; BfR., 2012; Crews, 2012). Although experts from the BfR and EFSA proceed on the assumption that nearly 100 % of the 3-MCPD-esters are segregated into free 3-MCPD (Larsen, 2009a; BfR., 2012). Recent studies in an intestinal model with pancreatic lipase and an in vivo study in rats showed that almost 100 % hydrolysation of 3-MCPD-esters to 3-MCPD occurred. Therefore, the toxicokinetic behavior of 3-MCPD-esters and 3-MCPD is 1:1 (Lampen, 2014). There is evidence of kidney and testicular damage similar to the effects of free 3-MCPD (Crews, 2012). Many internationally recognized authorities (Bundesinstitut für Risikoforschung (Bfr), European Food Safety Authority (EFSA), U.S. Food and Drug Administration (FDA)) see a demand for action to reduce amounts of these compound in foods (Hamlet and Sadd, 2009; Larsen, 2009a; BfR., 2012). The amounts of MCPD-esters found in refined edible vegetables oils range from 0.3 to 10 mg kg^{-1}, in other foods e.g. French fries (0.04-0.40 mg kg^{-1}) and jarred foods (0.011 mg kg^{-1}) are lower but still reasonably high (Crews, 2012). In table 2-5 MCPD-ester amounts found in different foods are shown. Palm oil (1.1-10 mg kg^{-1} Table 2-5) is one of the worst case scenarios for MCPD-esters and due to its cheap production (58 Mio t in 2014) it is present in many foods such as bakery products, infant formula, margarine and sweets. Further, the palm oil industry is currently mainly working in a non-sustainable way, since for its production the rain forests in Indonesia and Malaysia are cut down. This raised the concern of WWF and the general public (WWF, 2015).

Table 2-5: Levels of 3-MCPD-ester in foods

Food systems	mean of 3-MCPD-ester [mg kg^{-1}]	Source
Biscuits	0.25-0.70	(Crews, 2012)
Doughnuts	0.42-1.21	(Larsen, 2009a)
Refined palm oil	1.1-10.0	(Crews, 2012)
Salami	0.80-6.40	(Crews, 2012)
refined sunflower oil	0.673	(Sevenich et al., 2013)

The ADI of free 3-MCPD is established at 2 µg of free 3-MCPD per kg body weight (EFSA, 2013). The need to reduce these FPCs exists because at this point not much is known about possible long-term accumulative effects of these compounds in the human body. Possible mitigation strategies have been proposed by Stadler (2012):

1) Lower desoderation temperature during refining

2) Lowering the amounts of chloride or chlorine in the treated oils, since the concentration of these compounds is rate limiting for 3-MCPD-ester formation

3) Removing glycerol by acid treatment

4) Filtering of the oil to extract chlorinated compounds which could break down during the processing of the oil and react

5) Changing the recipe towards unrefined oils

The free 3-MCPD can be determined by GC-MS the exact method being described elsewhere (Zelinková et al., 2008; Hamlet and Sadd, 2009). For the 3-MCPD-esters determination two approaches are possible: one involves the conversion of the 3-MCPD-esters to free 3-MCPD by either a base-catalysed transmethylation (bct), where a loss of 3-MCPD can occur, or an acid-catalysed trans-methylation (act), where the formation of additional 3-MCPD is possible; the other approach is the direct analysis as esters by LC-MS (liquid chromatography-mass spectrometry) or DART-MS (direct analysis in real time- mass spectrometry) (Hajslova et al., 2011; Moravcova et al., 2012).

A reduction of furan and 3-MCPD-esters, due to their carcinogenic potential, would be of highly valued benefit and would lead to the reduction of the toxicological potential of harmful substances, which results in a better overall product quality. Here the two advantages of the high pressure thermal sterilization can play a key role: lower thermal load applied to the product in comparison to the thermal retorting; and, the Le Chatelier principle, which states that under pressure only reactions with a negative reaction volume are favored. If the reaction volume of the FPCs is positive the formation may not occur or could be limited.

3. Comparative study on the high pressure inactivation behavior of the Shiga toxin-producing *Escherichia coli* O104:H4 and O157:H7 outbreak strains and a non-pathogenic surrogate

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Comparative study on the high pressure inactivation behavior of the Shiga toxin-producing *Escherichia coli* O104:H4 and O157:H7 outbreak strains and a non-pathogenic surrogate

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ABSTRACT

Enterohemorrhagic *Escherichia coli* strains cause each year thousands of illnesses, which are sometimes accompanied by the hemolytic uremic syndrome, like in the 2011 outbreak in Germany. For preservation thermal pasteurization is commonly used, which can cause unwanted quality changes. To prevent this high pressure treatment is a potential alternative.

Within this study, the 2011 outbreak strain O104:H4, an O157:H7 and a non-pathogenic strain (DSM1116) were tested. The cells were treated in buffer (pH 7 and pH 5) and carrot juice (pH 5.1) in a pressure temperature range of 0.1–500 MPa and 20–70 °C. Flow cytometry was used to investigate the pressure impact on cell structures of the strain DSM1116.

Both pathogenic strains had a much higher resistance in buffer and carrot juice than the non-pathogenic surrogate. Further, strains cultivated and treated at a lower pH-value showed higher pressure stability, presumably due to variations in the membrane composition.

This was confirmed for the strain DSM1116 by flow cytometry. Cells cultivated and treated at pH 5 had a stronger ability to retain their membrane potential but showed higher rates of membrane permeabilization at pressures <200 MPa compared to cells cultivated and treated at pH 7. These cells had the lowest membrane permeabilization rate at around 125 MPa, possibly denoting that variations in the fatty acid composition and membrane fluidity contribute to this stabilization phenomenon.

1. Introduction

The presence of different genera of moulds, yeasts and spoiling bacteria and occasionally pathogens on fresh produce has been recognized for many years. Several outbreaks of human gastroenteritis have been linked to the consumption of contaminated fresh vegetables and, to a lesser extent, fruits (Beuchat, 1996; EFSA, 2013). Between the different pathogens, enterotoxigenic *Escherichia coli* are the most common cause of traveler's diarrhea. Each year 75,000 cases of illnesses in the United States are associated with the enterohemorrhagic *E. coli* (EHEC) strain of serotype O157:H7 (Bavaro, 2012). Since cattle appear to be the primary reservoir of O157:H7, the vast majority of single infections or outbreaks have been associated to the consumption of undercooked beef or dairy products (Bavaro, 2012; Wieler et al., 2011). However, more recent outbreaks are caused by the consumption of water and fresh produce, for e.g. lettuce, celery, spinach, carrots and sprouts (Bavaro, 2012; Beuchat, 1996). In some cases EHEC infections are accompanied by the hemolytic uremic syndrome (HUS), as in the 2011 outbreak in Germany, which was caused by an unusual Shiga toxin-producing *E. coli* (STEC) strain of serotype O104:H4 associated with the consumption of contaminated produce presumably sprouts (Robert Koch Institute, 2011). From May to July 2011 855 cases of HUS and 2987 cases of acute EHEC gastroenteritis were reported resulting in one of the largest non-O157 *E. coli* outbreaks worldwide (Bavaro, 2012; Robert Koch Institute, 2011). In the majority of cases adults were affected and death was reported for 35 of the patients identified with HUS and 18 of the patients with EHEC gastroenteritis (Robert Koch Institute, 2011). The EHEC serotype O104:H4 involved in this outbreak was up to this point not

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described in animals and only rarely in humans. However, due to that the transmission route of this outbreak was clearly food-borne; food processors have to be aware of this risk for minimally processed foods, like fresh cut products or fresh juices to ensure the consumer's safety.

The common method, to inactivate EHEC in low acid food, is a mild heat treatment, which can cause changes in the color, flavor and texture of the processed product. To overcome these unwanted side-effects non-thermal technologies like irradiation, pulsed electric fields, pulsed light, cold atmospheric plasma or isostatic high pressure are investigated for its use for solid and liquid food (Knorr et al., 2011). The isostatic high pressure treatment is often regarded as one of the recent technological innovations in food preservation and has been extensively studied over the past decades. Food spoiling and vegetative pathogenic microorganisms as well as several enzymes, which cause food deterioration can be inactivated at ambient temperature and pressures between 300 and 700 MPa (Buckow and Heinz, 2008; Knorr et al., 2011). Despite the steadily increasing commercial production of high pressure pasteurized food (more than 350.000 t/a), some important scientific and technological questions are still unsolved. With respect to the microbial safety, reliable quantitative data for the inactivation of pathogens in different food matrices are missing. Furthermore, the impact of different intrinsic and extrinsic factors that influence the inactivation of these pathogens and the underlying inactivation mechanisms have to be explored.

In this regard, most studies used surrogates for the corresponding pathogenic microorganism, which are often only certified for thermal but not for high pressure processing. Furthermore, large variations for the barotolerance within a species (Benito et al., 1999; Van Opstal et al., 2003) or the generation of extremely barotolerant strains from pressure sensitive strain after multiple rounds of high pressure treatment and selection of survivors (Hauben et al., 1997) are reported for different strains of *E. coli*. Some *E. coli* strains are relatively pressure sensitive and can be easily inactivated at pressures as low as 200 MPa (Buckow and Heinz, 2008; Klotz et al., 2010; Robey et al., 2001), whereas *E. coli* strains belonging to the O157-Group are among the most pressure-resistant vegetative cells (Benito et al., 1999).

One of the extrinsic factors with a high impact on the pressure resistance of microorganisms is the food matrix. Bacteria with a relatively low pressure resistance in buffers, can become highly pressure resistant in complex matrices like milk (Garcia-Graells et al., 1999), carrot juice (Van Opstal et al., 2005) or tomato sauce (Vercammen et al., 2012). Consequently, the pathogenic strains of concern for the consumer's safety have to be tested in the product, because kinetic data can often not be transferred from buffer systems or non-pathogenic surrogates.

Furthermore, detailed investigations under isothermal and isobaric conditions during the pressure dwell time over a broad pressure and temperature range are needed, to provide a comprehensive picture of the kinetic behavior of microorganisms under the applied process conditions (Reineke et al., 2013a). To unravel the impact of the different pressure and temperature combinations on a possible cell death or recovery, detailed analysis about the physiological state of the cells are needed.

Satisfying to Le Chatelier's principle in a system facing a shift of equilibrium all cellular components are affected by high pressure, including the cell membrane and its membrane proteins, enzymes and ribosomes as well as all the cell metabolism (Heremans, 2002; Smelt et al., 2001; Winter and Jeworrek, 2009). The general mechanisms leading to cell death have been investigated in several bacterial species. However, the particular events or cellular structures leading to an inactivation are not known (Klotz et al., 2010). For the inactivation of *E. coli* under pressure, three mechanisms seem to be important: (i) protein denaturation, (ii) permeabilization of the cell membrane and (iii) oxidative stress (Mackey and Manas, 2008). Klotz et al. (2010) investigated the relationship between membrane damage, release of proteins and the loss of viability of two *E. coli* strains after high pressure treatment. They concluded that after the loss of culturability an irreversible loss of membrane integrity occurred for the tested *E. coli* strains indicated by the uptake of the fluorescent dye propidium iodide (PI). Furthermore, they attributed the difference in pressure resistance of the two tested *E. coli* strains to the differences in the ability of their membranes to withstand disruption by pressure.

The objective of this study was to collect a comprehensive data set on inactivation of the pathogenic EHEC strains O157:H7 and the O104:H4 outbreak strain from Germany in buffer (ACES-buffer) versus a vegetable juice with a high pH value and a high industrial relevance (carrot juice) over a wide pressure and temperature domain. The results gained were used to calculate iso-response contour plots for cell inactivation in dependence of pressure and temperature.

Furthermore, different fluorescent dyes were used to investigate the physiological state of a non-pathogenic *E.* coli strain by flow cytometry analysis, to evaluate the impact of different extrinsic factors on the inactivation mechanisms under pressure.

2. Material and methods

2.1. Bacterial strains and growth conditions

Within this study the Shiga toxin- (stx1 and stx2) producing *E. coli* (STEC) strain EDL933 (serotype O157:H7) isolated in 1982 from a HUS patient (USA) and the Shiga toxin- (stx2) producing, enteroaggregative (EAEC) and multiresistant (ESBL) *E. coli* HUS-outbreak strain RKI II-2027 (serotype O104:H4) isolated in 2011 from a HUS patient (Germany) where used. Furthermore, a non-pathogenic *E. coli* reference strain (DSM1116), used for quality control according to DIN 58959-7 standards and the European Pharmacopeia (739 & 6924) was tested for its high pressure resistance. All strains were stored at −80 °C in cryo bead vials (Roth, Karlsruhe, Germany). For each strain a single frozen bead was transferred into 5 mL sterile nutrient broth (Roth, Karlsruhe, Germany) with pH 7 and incubated without shaking for 16 h at 37 °C. Afterward, the optical density of the pre-culture was measured at 620 nm (OD_{620}) and 100 mL of nutrient broth (pH 5 or pH 7) was inoculated with *E. coli* cell suspension at a calculated OD_{620} of 0.07. The cell culture was incubated for 24 h at 37 °C under continuous shaking (125 rpm) to achieve *E. coli* cell cultures in the stationary phase. Each day, a fresh prepared *E. coli* culture of the respective strain was used.

2.2. High pressure treatment

A Multisizer™ 3 Coulter Counter (Beckman Coulter, Krefeld, Germany) was used to estimate the cell concentration of each prepared culture, to allow reproducible treatments with a final concentration of 1×10^8 cells mL^{-1}. Each sample was measured in triplicate.

E. coli cell cultures cultivated in nutrient broth with pH 7 were used for the suspension in pressure stable N-(2-acetamido)-2-aminoethanesulfonic acid (ACES) −buffer solution (Mathys et al., 2008) with pH 7 (0.05 M). For the inoculation in ACES-buffer with pH 5 (0.05 M) or pasteurized commercial carrot juice with pH 5.1 (Bio Moehrensaft, Voelkel GmbH, Hoehbeck, Germany) *E. coli* cell cultures incubated in nutrient broth with pH 5 (adjusted with 1 M hydrochloric acid) were used, to avoid a pH shock after inoculation. The strains O157:H7 (EDL 933) and O104:H4 (RKI II-2027) were filled into plastic tubes with screw caps (CryoTube

Vials, Nunc Brand Products, Roskild, Danemark). To enable a rapid analysis of individual aliquots of the pressure treated non-pathogenic *E. coli* strain (DSM1116) the sample volume was reduced to 4 × 300 µl, which enabled a rapid analysis of three samples by cytometry, without contaminating the fourth which was used for determination of viable cell counts. Therefore samples were pipetted into shrinking tubes (Schrumpfschlauch 3/1, DSG-Canusa, Meckenheim, Germany) and hermetically sealed with a soldering iron.

For the pressure trials a U111 high pressure system (Unipress, Warsaw, Poland) was used. The kinetic data sets for the two pathogenic strains (O157:H7 and O104:H4) were derived in a multivessel setup with five 4 mL vessels, where low viscous silicon oil (M40.165.10, Huber GmbH, Offenburg, Germany) served as the pressure-transmitting medium. For the pressure treatment of the DSM1116 strain in shrinking tubes, the pressure equipment setup was slightly modified but with now effect on the compression rate. Here a single high pressure vessel with 3.7 mL vessel volume was used.

The high pressure vessel(s) were preheated to the designated treatment temperature with a thermostat (CC 245, Huber GmbH, Offenburg, Germany). All samples were treated under isothermal and isobaric conditions during pressure dwell time. A representative pressure-temperature profile for both setups is published elsewhere (Reineke et al., 2011). For temperature measurement during a treatment, a dummy container (a CryoTube Vials or a shrink tube) – equipped with a thermocouple in the geometrical center of the container – was used to determine the heat of compression during pressure build-up. The cooled samples and the dummy sample (approximately 2 °C) were preheated to an empirically determined starting temperature in the HP vessel(s).

Treatment pressure and temperature were in the range of 0.1–500 MPa and 20–70 °C. Prior to and after the pressure treatment all samples were stored on ice. For all conditions three independent kinetics were done.

2.3. Viable cell counts

The viable cell count after the high pressure treatment was determined by cell culture methods in duplicate. The bacterial cell suspensions were serially diluted in phosphate buffer solution (0.05 M), with the respective pH-value of the treatment matrix and plated on standard-1-agar (Roth, Karlsruhe, Germany). The plates were incubated at 37 °C for 24 h and the colony forming units (CFU) were counted.

2.4. Flow cytometry analysis

For the determination of the physiological state of treated *E. coli* cells, samples of the strain DSM1116 were analyzed immediately after decompression. To analyze the membrane potential after the treatment a $DiOC_2(3)$ [3,3′-diethyloxacarbocyanine iodide, Sigma Aldrich, Hamburg, Germany] a staining method according to Fröhling et al. (2010) was used. In detail, 10 µl of cell suspension were diluted in 490 µl phosphate buffer (PBS-buffer) (pH 7, 0.05 M) containing 20 µM D-glucose and 30 µM $DiOC_2(3)$ was added and incubated for 15 min in the dark at room temperature. Afterward, the stained cell-suspension was centrifuged at 7000 × g and 4 °C for 5 min. The pelleted material was re-suspended in PBS-Buffer to a cell density of ~10^6 cells mL^{-1} and immediately measured in the flow cytometer.

For the staining no Ethylenediaminetetraacetic acid (EDTA) was added to permeabilize the outer membrane of the *E. coli* cells. This reduced the total fluorescence intensity of intact cells, due to the poor permeability of the $DiOC_2(3)$ but did not further act as an additional stressor/possible lethal factor on high pressure treated cells.

However, the used setup enables a simultaneous measurement of the red/green $DiOC_2(3)$-fluorescence intensity and allows a calculation of the fluorescence intensity ratio. It was assumed that the red/green ratio of untreated *E. coli* cells represents the membrane potential of intact cells. Hence, a reduction of the red/green ratio denotes a loss of cell membrane potential. The red/green ratio of completely depolarized cells (cells depolarized with carbonyl cyanide m-chlorophenylhydrazone) was ≤1 as previously assessed and published by Froehling (2011).

A cFDA [5(6)-carboxyflourescein diactate mixed isomers, Sigma Aldrich, Taufkirchen, Germany] staining was used to measure the esterase activity of the *E. coli* cells. Unspecific intracellular esterases can hydrolyze the non-fluorescent cFDA into carboxyflourescein (cF) and hence, indicate if intracellular enzymes are effect by the high pressure or thermal treatment (Ananta et al., 2005). In detail, a final concentration of 0.83 mM cFDA was added to treated cell suspension and incubated for 45 min at 37 °C. After the incubation, the reaction mixture was centrifuged at 7000 × g for 5 min at 4 °C to remove surplus of cFDA. The cell pellet was re-suspended in 0.05 M PBS-buffer (pH7) to a final cell concentration of ~10^6 cells mL^{-1}. The addition of the membrane-impermeant dye PI (propidium-iodide, Sigma Aldrich, Taufkirchen, Germany), after the cFDA staining, could give further information about the status of the cell membrane. Therefore, 30 µM of PI was added to stained *E. coli* cells and incubated for 10 min at 4 °C in the dark before flow cytometric analysis (Fröhling et al., 2012). This double staining enables discrimination between cells with and without esterase activity and/or intact or permeabilized cell membranes.

To confirm the status of the cell membrane after the treatment a double staining with TO (Thiazole orange, Sigma Aldrich, Taufkirchen, Germany) and PI was used. Therefore, according to Fröhling et al. (2012), 0.42 µM TO and 30 µM PI were added to the *E. coli* suspension, containing ~10^6 cells mL^{-1} and incubated in the dark for 10 min at room temperature before flow cytometric analysis. In this regard, a positive TO staining indicates intact cell membranes whereas a PI staining indicates permeabilized cell membranes.

All experiments were performed using a Cytomics FC500 flow cytometer (Beckman Coulter, Krefeld, Germany) equipped with a 20 mW argon ion laser emitting at a wavelength of 488 nm. The field stop was set on 1–8° and the discriminator to reduce the background noise was set on the side scatter (SS = 2). The fluorescence of the dyes carboxyfluorescein, thiazole orange and green $DiOC_2(3)$ was measured with the photomultiplier FL1 and a band pass filter with 525 nm ± 25 nm cut off. For the fluorescent of propidium iodide and red $DiOC_2(3)$ the photomultiplier FL3 and a short pass filter with 620 nm cut off (fluorescence intensity is recorded between 615 and 620 nm), were used. The parameters were collected as logarithmic signals and the obtained data was analyzed using CXP Analysis software (Beckman Coulter, Krefeld, Germany). 10,000 events were measured at a flow rate of approximately 1000 events s^{-1}.

2.5. Modeling of iso-response contour plots

For the calculation of iso-response contour plots for a fixed inactivation level in dependence of pressure and temperature, the average of the three viable cell counts for each kinetic point was used (at least 80 independent kinetic points for each tested *E. coli* strain). To exclude the impact of the compression and decompression on the total achieved inactivation, the viable cell count after a 1 s (N_{1s}) pressure dwell time for the respective pressure and temperature level was used as the initial cell count for the

modeling. This procedure enabled an exclusion of the impact of compression and decompression on cell inactivation and hence a comparability of the results (Reineke et al., 2013b). The inactivation level was calculated according to Eqn. (1).:

$$S(t) = \log_{10}\left(\frac{N_t}{N_{1s}}\right) \quad (1)$$

with $N(t)$ as the viable cell count after the treatment and $S(t)$ as the logarithm of the survival ratio. However, to ensure a better comparability with the flow cytometric results, the initial cell count of the untreated cell suspension (N_0) was used for the calculation of the isorate lines of the *E. coli* strain DSM1116. Hence, these isorate lines represent the achieved inactivation for the entire high pressure cycle, including compression and decompression.

The inactivation kinetics for each pressure and temperature level were fitted with the Weibullian power law:

$$\log_{10}S(t) = -b \cdot t^n \quad (2)$$

with b as the scale and n as the shape parameter. By using OriginPro (Version 8.0724; OriginLab Corporation, Northampton, MA, USA) and Eqn. (2) a non-linear regression fit was done for each individual inactivation kinetic and the scale and shape parameter were determined. To get a functional relationship of the scale parameter b with pressure and temperature, it was assumed that the shape parameter n is constant with varying pressure and temperature (Van Boekel, 2009). Therefore, the average of all values for the shape parameter was calculated (n_{av}) and the non-linear regression fit of each inactivation kinetic was repeated with this fixed average value for the shape parameter.

To get a functional relationship of the scale parameter b with pressure and temperature [$b(p,T)$], empirical equations have often been suggested (Ardia et al., 2004; Buckow and Heinz, 2008; Margosch et al., 2006). Thus, a taylor series expansion was used to calculate the shape parameter $b(p,T)$ in Eqn. (2) and by using MathCAD 15 (Mathsoft Engineering & Education, Inc., USA) the calculation of pressure and temperature dependent isorate lines (which can also be regarded as iso-time lines) for a fixed inactivation level was possible.

The same mathematical approach was used to calculate isoresponse contour plots for the loss of membrane potential, esterase inactivation and cell membrane permeabilization. Therefore, the flow cytometric data were used. To ensure a comparability of these data, all data were normalized. The respective references for the standardization were the values for the untreated culture from each cultivated batch. For the calculation of esterase inactivation only the reduction of cF-fluorescence intensity compared to the untreated sample was taken into account. The calculation of membrane permeabilization is based on data from the TO – PI staining and all detected cells with PI-fluorescence emission were regarded as permeabilized.

3. Results

3.1. High pressure resistance of pathogenic and non-pathogenic E. coli strains at pH 7

The three *E. coli* strains tested within this study showed a large intrinsic variation of pressure resistance (Fig. 1). The strains were cultivated in nutrient broth and inoculated in ACES buffer (both pH 7) prior the isothermal high pressure treatment. The extrinsic factors prior and during the treatment were equal, but the achieved inactivation differed from strain to strain. The highest pressure resistance showed *E. coli* O157:H7, where the cell inactivation at 50 °C started at 200 MPa resulting in a 3.79 ± 0.10 $\log_{10}$ reduction after 10 min. Under equal process conditions *E. coli* O104:H4 was inactivated by 3.97 ± 0.13 $\log_{10}$ and the non-pathogenic *E. coli* DSM1116 by 5.25 ± 0.40 $\log_{10}$. With increasing pressure, the inactivation rate accelerated for all strains. Whereas a rapid inactivation (>5 $\log_{10}$ in less than 2 min) at 40 °C for the strain DSM1116 was achieved at 300 MPa, 500 MPa were needed for both strains O104:H4 and O157:H7, whereby a slight higher pressure resistance was found for the last-mentioned strain (Fig. 1). Furthermore, the two pathogenic strains O104:H4 and O157:H7 showed only a reduction of ≥1 $\log_{10}$ during compression and decompression (1 s pressure dwell time) at treatment temperatures above 60 °C and 100 MPa, whereas the non-pathogenic strain DSM1116 was inactivated by 2.15 ± 0.14 $\log_{10}$ at 50 °C and 200 MPa (Fig. 1A) or up to 1.78 ± 0.28 $\log_{10}$ at 400 MPa and 30 °C under dynamic pressure temperature conditions.

Therefore and to allow a better comparability with the results from the flow cytometric analysis the kinetic modeling for the strain DSM1116 was done for the entire pressure cycle. However, to ensure a transferability of the inactivation model for the pathogenic STEC strains to other high pressure units, the kinetic modeling of the strains O157:H7 and O104:H4 was done for isothermal isobaric conditions (Reineke et al., 2013b).

After the determination of an average shape parameter n_{av} (Table 1), in accordance with Van Boekel (2009), all individual kinetics of each strain were fitted with the Weibullian power law (Eqn. (3)). The goodness of fit for each model was evaluated by plotting the data for experimental inactivation versus the calculated values, followed by linear regression of these values. The adjusted coefficient of determination and the residual sum of squares for each model are listed in Table 1 and show a good correlation of modeled and measured data. The residuals of the different models were nearly randomly distributed and hence, it is assumed that no heteroscedastic error is present in the models. The average shape parameter n_{av}, which were calculated for isothermal isobaric conditions (strain O157:H7 and O104:H4) are close to 1, indicating that the inactivation during the pressure dwell time is nearly a first-order inactivation. The average shape parameters for the strain DSM1116 are much lower because most of the inactivation kinetics of this strain showed a slight upward concavity and the parameters include the initial inactivation occurring during the compression and decompression (Fig. 1 A).

The isorate lines for a 3 $\log_{10}$ inactivation depict the much higher pressure and temperature resistance of the two STEC strains. Furthermore, with increasing process pressure the treatment temperature or treatment time could be reduced to achieve the same inactivation (Fig. 1A&B). For longer pressure dwell times (≥8 min) this effect diminishes but is still present.

3.2. High pressure resistance of pathogenic and non-pathogenic E. coli strains at pH 5

As outlined in Fig. 2 the isorate lines for a 3 $\log_{10}$ inactivation in carrot juice (pH 5.1) for the STEC strain O104:H4 and the non-pathogenic *E. coli* strain DSM1116 in ACES buffer (pH 5.0) show a similar shape compared to the inactivation at pH 7 (Fig. 1). Conversely to the inactivation behavior at pH 7, the strain O104:H4 showed a reduced inactivation rate at 100 MPa compared to a treatment at 0.1 MPa. This higher pressure resistance at between 0.1 and 100 MPa is also described by the 2 min isorate line for the O104:H4 strain. Moreover, the pressure-temperature resistance of both *E. coli* strains was significantly higher at the lower pH value. The increased resistance was more distinct for the O104:H4 strain in carrot juice but is also present in ACES buffer for the DSM1116 strain.

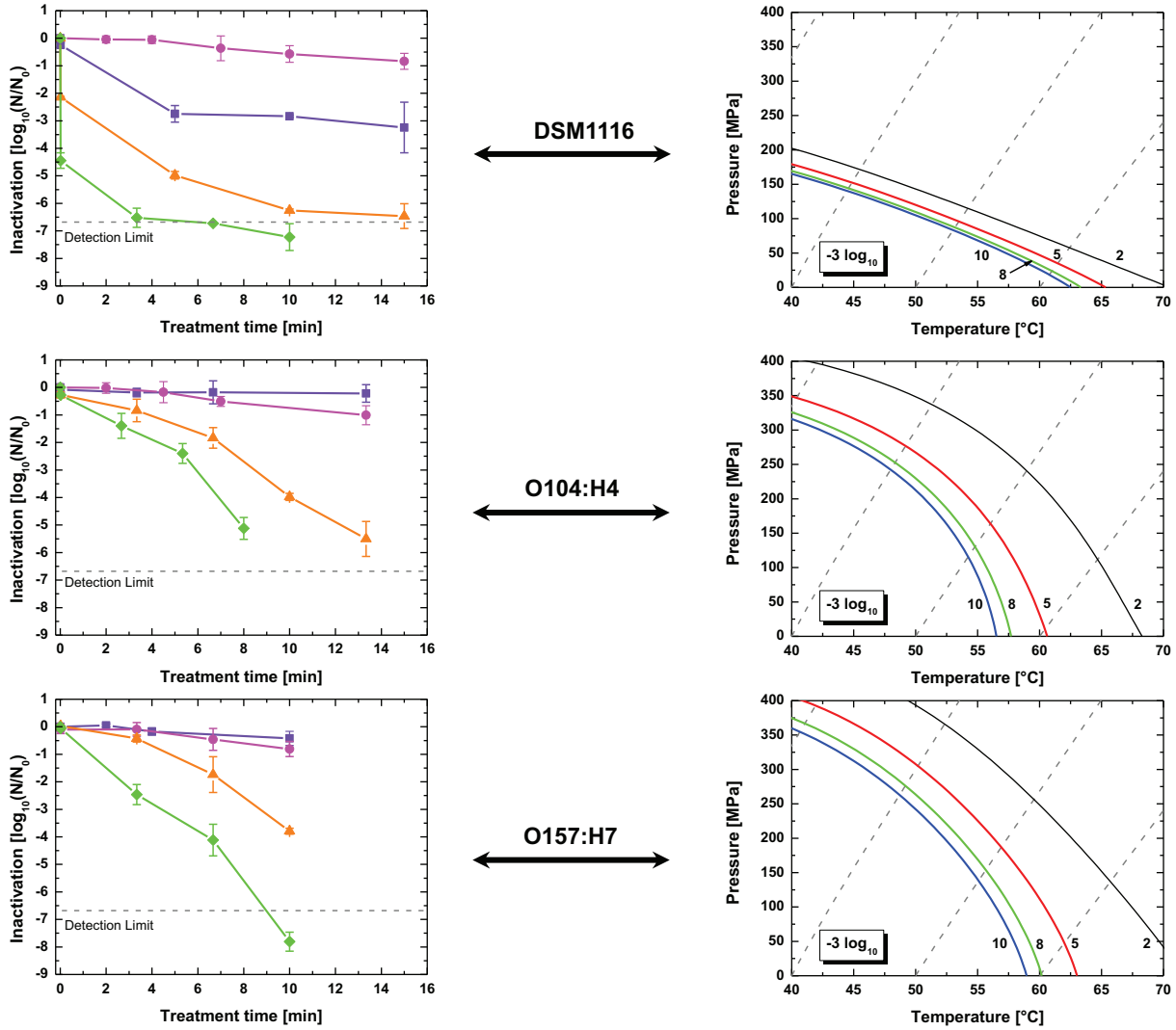

Fig. 1. A; inactivation kinetics at 50 °C and 0.1 MPa (■), 100 MPa (●), 200 MPa (▲) and 300 MPa (◆) for the STEC strains O157:H7 and O104:H4 and the *E. coli* DSM1116. B; corresponding isorate lines for a 3 $\log_{10}$ inactivation in 2 min, 5 min, 8 min and 10 min in the pressure–temperature landscape. Isorate lines for the STEC strains O157:H7 and O104:H4, represent the inactivation under isothermal and isobaric conditions during pressure dwell time. Isorate lines for the *E. coli* DSM1116 strain include the inactivation during compression and decompression. Gray dashed lines represent the adiabatic heat of compression.

Hence, matrix effects and the difference in pressure resistance of the two strains might contribute to this. However, the direct comparison of the inactivation behavior of the *E. coli* DSM1116 strain reveal that extrinsic factors could have an influence, but that at least some physiological changes in the cell population contribute to the higher resistance at lower pH-value.

Table 1
Average shape parameter n_{av}, adjusted coefficient of determination (Adj. R^2) and the residual sum of squares for the linear regression of experimental inactivation data versus calculated values from the respective model used.

Strain	Matrix	pH	n_{av}	Adj. R^2 of residuals	Residual sum of squares
O157:H7	ACES buffer	7	1.13	0.87	35.4
O104:H4	ACES buffer	7	0.91	0.89	33.5
O104:H4	Carrot juice	5.1	0.96	0.82	20.2
DSM1116	ACES buffer	7	0.23	0.91	29.7
DSM1116	ACES buffer	5	0.41	0.96	12.2

3.3. Loss of membrane potential

To identify cellular structures which might be responsible for the different pressure and temperature resistance of the tested *E. coli* strains at different pH values flow cytometry was used. The impact of high pressure and moderate or high temperatures on the cell membrane was used as an indicator of cell damage or cell death. The flow cytometric results of the $DiOC_2(3)$ staining, an indicator for the membrane potential of the analyzed cells, are shown in Fig. 3.

The data represent a full loss of membrane potential for the *E. coli* strain DSM1116 (≥90%), which can be directly compared with a 1 $\log_{10}$ (90%) inactivation after 2 min (dotted line). The modeling of the isorate lines was done as described above and the parameters for the goodness of fit of all models based on flow cytometric results are listed in Table 2. Again the residuals of the different models were nearly randomly distributed and hence, it is assumed that no heteroscedastic error is present in each model. Further, all average shape parameter n_{av} for the different staining methods are nearly equal and <1 for both pH values, denoting a similar and upward concave shape of the fluorescence intensity kinetics.

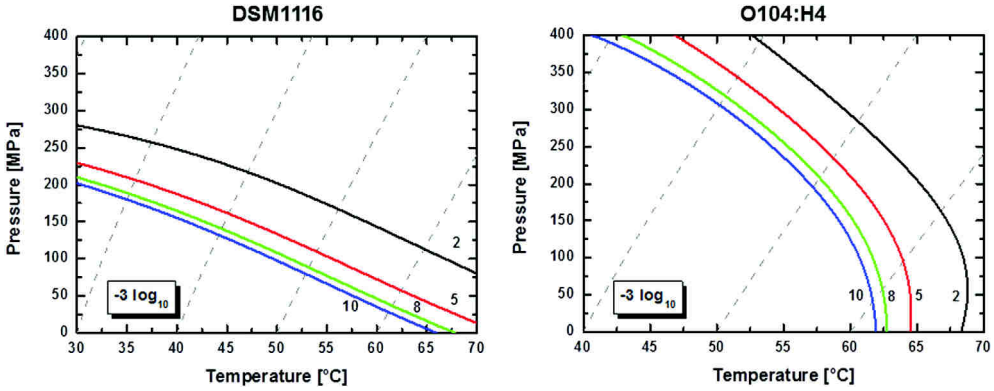

Fig. 2. Isorate lines for a 3 $\log_{10}$ inactivation in 2 min, 5 min, 8 min and 10 min in the pressure-temperature landscape for the STEC strain O104:H4 suspended in carrot juice (pH 5.1) and for the *E. coli* strain DSM1116 suspended in ACES buffer (pH 5). Isorate lines for the STEC strain O104:H4, represent the inactivation under isothermal and isobaric conditions during pressure dwell time. Isorate lines for the *E. coli* DSM1116 strain include the inactivation during compression and decompression. Gray dashed lines represent the adiabatic heat of compression.

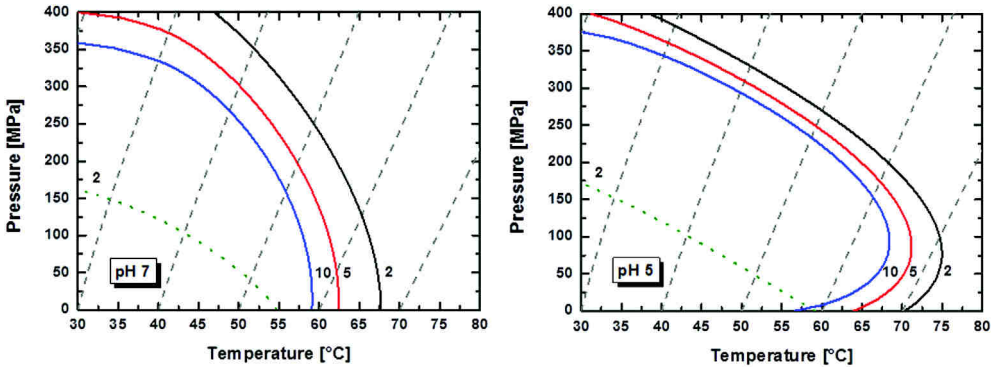

Fig. 3. Isorate lines for a full loss of membrane potential ($DiOC_2(3)$ red/green fluorescence intensity ratio = 0.1) in 2 min, 5 min and 10 min and a 1 $\log_{10}$ inactivation (dotted line) in 2 min in the pressure–temperature landscape for the *E. coli* strain DSM1116 suspended in ACES buffer (pH 7 and pH 5). Isorate lines include the impact of compression and decompression on the membrane potential. Gray dashed lines represent the adiabatic heat of compression.

The isorate lines have an elliptic shape and show a stabilization zone at 100 MPa for cells suspended in ACES buffer with pH 5. At atmospheric pressure (0.1 MPa) a rapid loss of membrane potential occurred at 60 °C in both buffers (pH 7 ratio 0.1 after 2 min and pH 5 ratio 0.07 after 2 min). At 50 °C and 0.1 MPa cells cultivated and treated at pH 5 showed a rapid loss of the membrane potential (ratio 0.27 after 2 min and full loss after 10 min), whereas cells cultivated and treated at pH 7 showed a much higher resistance (ratio 0.9 after 2 min and 0.58 after 10 min). However, under high pressure the behavior was vice versa (Fig. 3). At pressures below 200 MPa *E. coli* cells cultivated and treated at pH 5 showed a much higher stability, which converged for high pressures (>350 MPa) and moderate temperatures to the inactivation level achieved at pH 7. The direct comparison of the loss of membrane potential (Fig. 3) and the reduction of viable cells dotted line and Figs. 1 and 2 for the DSM1116 strain clearly depict, that most of the cells first lose their culturability and later on their membrane potential.

Table 2
Average shape parameter n_{av}, adjusted coefficient of determination (Adj. R^2) and the residual sum of squares for the linear regression of experimental flow cytometric data versus calculated values from the respective model used.

Method	Fluorescent signal used for modeling	ACES buffer pH	n_{av}	Adj. R^2 of residuals	Residual sum of squares
Membrane potential	$DiOC_2(3)$ red/green fluorescence intensity ratio	7	0.34	0.82	1.92
		5	0.20	0.75	2.17
Esterase activity	cF-fluorescence intensity	7	0.40	0.82	6.17
		5	0.39	0.77	5.24
Membrane permeabilization	PI-fluorescence intensity	7	0.47	0.81	3.38
		5	0.51	0.91	0.62

3.4. Loss of membrane integrity

A double staining with the fluorescence dyes TO and PI was used, to assess the membrane integrity of pressure treated *E. coli* DSM1116 cells suspended in ACES buffer with a pH of 5 or 7. Figs. 4 and 5 visualized the results for an isothermal treatment at 50 °C respectively the isorate lines for a 25% membrane permeabilization in dependence of pressure and temperature. As statistical data the goodness of fit is shown in Table 2.

Cells treated in buffer with pH 5 and 7 showed nearly a constant amount of cells without a staining (Fig. 4), or a slight decrease for longer treatment times (data not shown), but a variability between individual cultivation batches. A treatment at atmospheric pressure (0.1 MPa) and 50 °C in both buffers (pH 5 and 7) first reduced the amount of cells with intact cell membranes (TO-fluorescence signal), which stabilized for longer treatment times (Fig. 4A and B). An increase of temperature to 60 or 70 °C intensely reduced the cell population with intact cell membranes, resulting in an increased amount of cells with PI-fluorescence emission (data not shown). At a pressure level of 100 MPa a continuous decrease of *E. coli* cells with intact membranes (treated in buffer with pH 5) was measured,

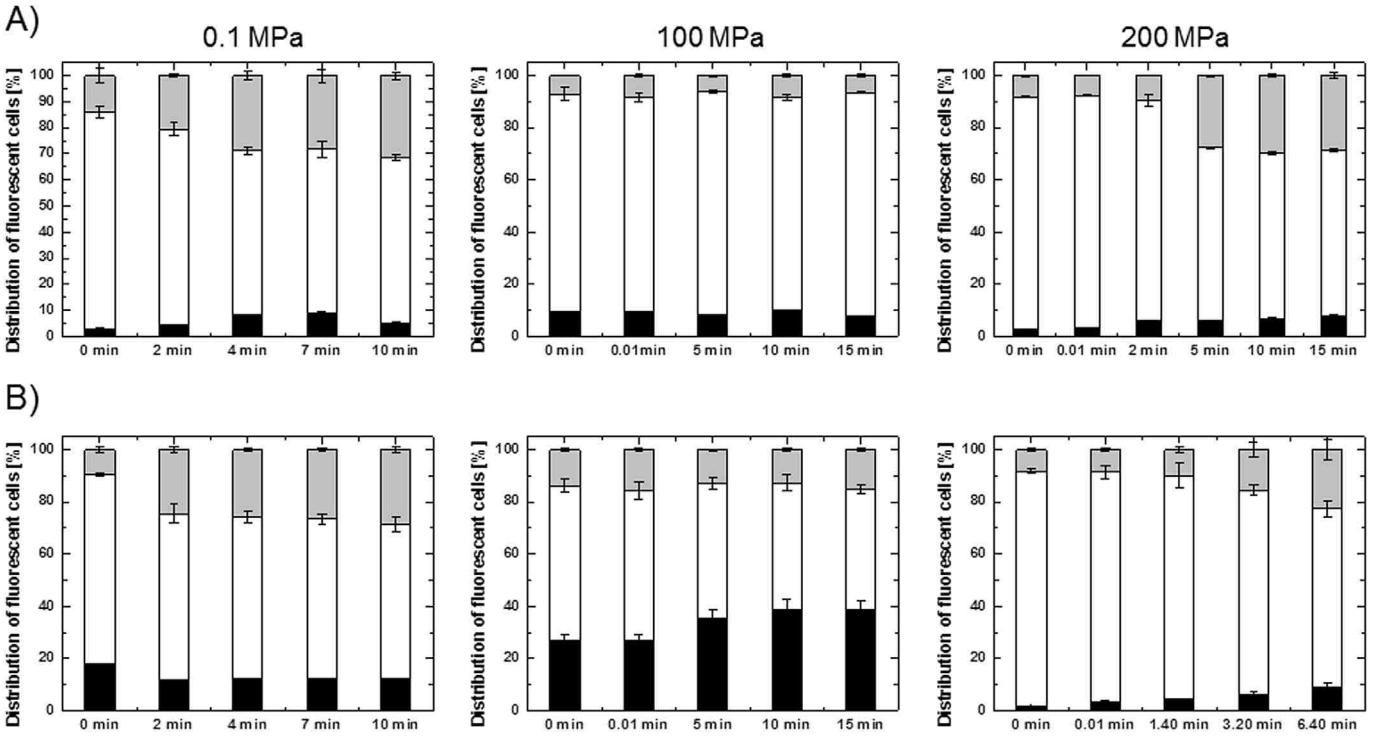

Fig. 4. Membrane integrity of *E. coli* DSM1116 cells suspended in ACES buffer with pH 7 (A) or pH 5 (B) for an isothermal treatment at 50 °C at different pressure levels. Black bars indicate cells without fluorescence emission, white bars indicate cells with TO-fluorescence emission (cells with membrane integrity) and gray bars indicate PI-fluorescence emission (cells with permeabilized membranes).

while a strong stabilization with no increase of cells with a positive PI staining was found for cells treated at pH 7 (Fig. 5). This stabilization phenomenon was dominant at 50 and 60 °C and decreased at 70 °C. For higher pressures (≥200 MPa) and moderate process temperatures (≤50 °C) cells cultivated and treated in media with pH 5 showed a slightly higher resistance.

A short pressure pulse (1 s pressure dwell time) had nearly no impact on the distribution of TO and PI stained cells compared to the untreated samples (Fig. 4).

High membrane permeabilization rates were only achieved during thermal treatments at atmospheric pressure and 70 °C with a maximum cell permeabilization of 93.2 ± 4.2% (pH 7) or 88.1 ± 6.8% (pH5) after 10 min treatment. Under high pressure, a maximum of 50% membrane permeabilization was detected for long pressure dwell times, but in most cases the permeabilization was less than 40%.

3.5. Esterase inactivation in pressure-treated cells

Non-fluorescent cFDA can penetrate the cell membrane and can be hydrolyzed by unspecific intracellular esterases into fluorescent cF. Hence, the decrease of cF-fluorescence intensity might indicate that intracellular enzymes are effected by high pressure combined with moderate or high temperatures. The isorate lines for a 25% reduction of cF-fluorescence signal/esterase activity for *E. coli* DSM1116 treated in ACES buffer with pH 7 and pH 5 is shown in Fig. 6, the statistic parameters for the goodness of fit in Table 2.

The isorate lines for both pH-values show again an elliptical shape and a stronger decrease of esterase activity at pH 7 compared to pH 5 for the same pressure–temperature–time combination. Furthermore, the reduction of esterase activity show a stabilization zone at 100 MPa for *E. coli* cells inactivated at pH 7, which was not measured for cells treated in buffer with pH 5. After a 10 min

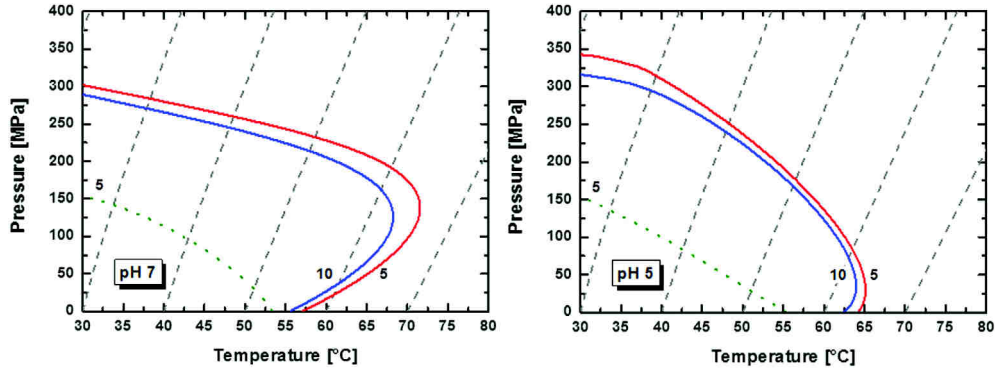

Fig. 5. Isorate lines for a 25% increase of PI-fluorescence emission (cells with permeabilized membranes) in 5 min and 10 min and a 1 $\log_{10}$ inactivation (dotted line) in 5 min in the pressure–temperature landscape for the *E. coli* strain DSM1116 suspended in ACES buffer (pH 7 and pH 5). Isorate lines include pressure effects on the membranes during compression and decompression. Gray dashed lines represent the adiabatic heat of compression.

treatment at 50 °C in ACES buffer with pH 5 respectively pH 7 a reduction of the esterase activity was measured (17.3 ± 6.7% at 0.1 MPa, 27.1 ± 2.5% at 100 MPa, and 36.3 ± 1.8% at 200 MPa [pH 5] resp. 33.1 ± 4.2% at 0.1 MPa, 25.9 ± 3.6% at 100 MPa and 37.9 ± 2.8% at 200 MPa [pH 7]). For longer pressure dwell times (>5 min) and moderate treatment temperatures the measured reduction of the easterase activity is nearly equal in both buffers, which is also shown by the convergence of the 10 min isorate lines at pH 7 and pH 5 (Fig. 6).

A short pressure pulse (1 s) up to moderate pressure (≤200 MPa) and higher temperatures (≥50 °C) or higher pressures (≥300 MPa) and moderate temperatures (≤40 °C) resulted in a reduction of the esterase activity of 6–13% (data not shown). To achieve a 75% or higher reduction of the esterase activity, treatment temperatures of ≥60 °C (in buffer with pH 7) or ≥ 70 °C (in buffer with pH 5) at atmospheric pressure were needed. Under high pressure a maximum reduction of <70% (in buffer with pH 7) or < 50°% (in buffer with pH 5) was achieved under the tested conditions.

4. Discussion

Within this study a large difference in pressure-temperature resistance between the three tested *E. coli* strains as well as tested media was found. Especially the two pathogenic STEC strains of the serotypes O157:H7 and 0104:H4 showed a much higher resistance than the non-pathogenic DSM1116 *E. coli* strain. A large variation of pressure and temperature resistance, particularly for different *E. coli* strains, is often reported in literature. Klotz et al. (2010), Buckow and Heinz (2008), and Van Opstal et al. (2005) reported a rapid inactivation of non-pathogenic *E. coli* strains in buffer with a comparable pressure-temperature-resistance to the tested DSM1116 strain. Further, some pathogenic O157:H7 strains behave similar, showing a rapid inactivation in PBS buffer (pH 7) at 500 MPa and ambient temperature (Benito et al., 1999), whereas other O157:H7 strains where extremely pressure resistant with less than 1 $\log_{10}$ reduction after a 30 min treatment under the same conditions (Benito et al., 1999). Many authors further report a high resistance of various O157:H7 strains in high (Linton et al., 1999; Teo et al., 2001) and low acid foods (Linton et al., 2001; Patterson et al., 2012; Teo et al., 2001; Van Opstal et al., 2005). Teo et al. (2001) investigated the destructive effect of a 2 min high pressure (615 MPa) exposure combined with low temperatures (15 °C) on a O157:H7 strain mix in different juices. For carrot juice they reported a maximum inactivation of 6.4 $\log_{10}$, which is much higher than for both pathogenic strains used in this study. A comparable inactivation in carrot juice (pH 6) was reported by Patterson et al. (2012). The authors tested the resistance of a mix of four Shiga toxin-producing *E. coli* at 500 MPa at ambient temperature for 1 min and achieved 1.82 $\log_{10}$, which is slightly higher than the inactivation of the O104:H4 strain (0.96 ± 0.11 $\log_{10}$) in carrot juice (pH 5.1, this study), but far more resistant than achieved inactivation in ACES-buffer with pH 7 (3.30 ± 0.42 $\log_{10}$ for the O157:H7 strain and 4.51 ± 0.826$\log_{10}$ for the O104:H4 strain) under comparable conditions. Similar findings were also reported by Van Opstal et al. (2005) for a non-pathogenic *E. coli* MG1155, were the inactivation in Hepes-KOH buffer (pH 7) was always more extensive than in carrot juice (pH 6.6) under all tested conditions (150–600 MPa and 5–45 °C). Consequently, these data denote that the inactivation behavior of pathogenic *E. coli* strains and non-pathogenic strains are not comparable. Further, the pH-value as well as the treatment matrix could have a tremendous impact on the inactivation rate and hence, kinetic data from buffer solutions cannot be directly compared with a real food matrix.

To reduce the impact of matrix interactions and investigate the influence of the pH-value exclusively, the *E. coli* strain DSM1116 was treated in ACES buffer with pH 5 and pH 7. The results confirm the effects found for the strain O104:H4 treated in ACES buffer (pH 7) and carrot juice (pH 5.1). Again a higher pressure-temperature resistance was found for the inactivation at lower pH-values.

For sake of clarity and to enable a comparison of the inactivation behavior of all tested strains in the different matrices, inactivation data were calculated as a functional relationship of pressure, temperature and time (Figs. 1 and 2). All of the calculated isorate lines are more or less elliptical, which is often found for the unfolding of proteins (Heremans, 2002), the inactivation of microorganisms (Knorr et al., 2011) or gelatinization of e.g. starches (Buckow and Heinz, 2008). For kinetic modeling the Weibullian power-law, based on the Weibull model was used. This model is very robust and a universal tool to fit both, linear and non-linear inactivation kinetics (Van Boekel, 2009). The progress of inactivation under isothermal isobaric conditions for the *E. coli* strains O157:H7 and O104:H4 was nearly linear, which is also seen in the shape parameters (n_{av}) in Table 1 (close to 1). The kinetic models for the strain DSM1116 have shape parameters <1 (Table 1 and Table 2) and cover the impact of the entire pressure cycle, including compression and decompression. This denotes that the decreasing kinetic curve becomes even more flat with prolonged treatment times. This denotes that *E. coli* cells that have not been killed respectively cellular compounds that remained intact are more resistant to the treatment than the ones that have been inactivated or altered. The sources of such heterogeneities in microbial population can be: i)

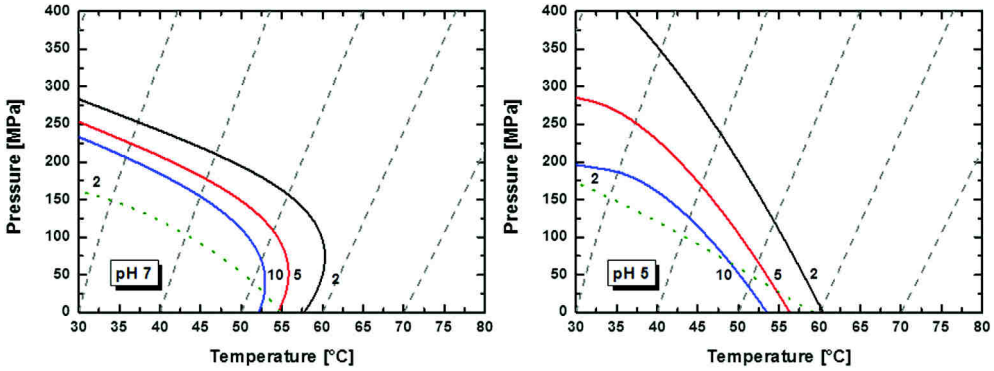

Fig. 6. Isorate lines for a 25% reduction of cF-fluorescence signal in 2 min, 5 min and 10 min and a 1 $\log_{10}$ inactivation (dotted line) in 2 min in the pressure–temperature landscape for the *E. coli* strain DSM1116 suspended in ACES buffer (pH 7 and pH 5). Isorate lines include pressure effects on esterase activity during compression and decompression. Gray dashed lines represent the adiabatic heat of compression.

genotypic because of mutations; ii) phenotypic via progression though the cell cycle, and iii) phenotypic due to changes in the exact local environment.

By the use of cells from a frozen stock culture, the daily preparation of a new cell suspension and pressure–treating of *E. coli* cells in the stationary phase, the first two factors for cell heterogeneity were minimized in this study. However, an important difference between the *E. coli* cell populations used for the inactivation at pH 7 and pH 5 was the modification of the pre-culture cultivation. Cells inoculated in a buffer with pH 7 were cultivated in nutrient broth with pH 7 whereas cells inoculated in carrot juice or ACES buffer with pH 5 were cultivated in nutrient broth with the corresponding pH-value, in order to reduce the pH-shock in the cells immediately after the inoculation. This modification in the cultivation most likely influenced the composition of the cell membrane (Gianotti et al., 2009; Yuk and Marshall, 2004) and other cellular compounds in the *E. coli* cells and hence, might give an insight into the mechanism contributing to the higher pressure resistance at pH 5.

A powerful tool to investigate the impact of different sublethal or lethal stressors on the cellular compounds is flow cytometry (Froehling, 2011; Shapiro, 1988), which can enable general insights into the underlying inactivation mechanisms of *E. coli* under pressure. Therefore, we tested three different staining methods to estimate the impact on the cell membrane potential [$DiOC_2(3)$-staining], cell membrane permeabilization (TO + Pi staining) and intercellular esterase activity (cFDA staining).

The results for a full loss of cell membrane potential are shown in Fig. 3. The isorate lines have an elliptical shape, according to the isorate lines for the inactivation, but interestingly point to a strong stabilization for pressures <200 MPa at pH5. The membrane potential is an essential part of bacterial physiology and caused by concentration gradients of Na^+, K^+, and Cl^- ions across the cell membrane and due to operation of proton pumps (Shapiro, 2000). It is involved in the generation of ATP, bacterial autolysis, glucose transport, chemotaxis, and survival at low pH.

Akopyan and Trchounian (2006) reported, that the membrane proton conductance depends on the pH of the growth medium and is also influenced by changes in the growth conditions. Further, Yuk and Marshall (2004) stated, that *E. coli* cells cultivated at low pH have a reduced membrane fluidity, which may be associated with changes in the proton flux. Cells adapted to a lower pH do not allow protons to flow into the cell as easily as non-adapted cells. Hence, these changes in proton flow through the cell membrane of acid adapted cells, might contribute to the higher pressure–temperature resistance of the *E. coli* DSM1116 strain cultivated and treated at pH 5.

A comparison of the data for the reduction of membrane potential (Fig. 3, 90% decrease) and cell permeabilization (Fig. 4, 25% permeabilization) might indicate that the cell membrane first loses its ability to maintain the membrane potential and is permeabilized in a following step. Some authors have investigated cell membrane permeabilization under high pressure by using PI-fluorescent dyes (Ananta et al., 2005; Klotz et al., 2010; Reineke et al., 2013b). Klotz et al. (2010) reported a transient loss of membrane integrity between approximately 200 and 600 MPa for the pressure resistant *E. coli* strain J1 treated in PBS buffer. Furthermore, they tested the PI uptake under pressure and after decompression for a pressure sensitive (NCTC 8164) and pressure resistant (J1) *E. coli* strain, showing that *E. coli* NCTC 8164 can reseal its membranes up to 125 MPa and *E. coli* J1 up to 200 MPa. From these findings they concluded that the difference of the pressure resistance of the two strains is due to a difference in the resilience of the cytoplasmic membranes towards high pressure stress. Interestingly, this is exactly the pressure range were the isorate lines for the *E. coli* strain DSM1116 tested in this study differ (Fig. 4). Pressures higher than 200 MPa lead to a rapid PI uptake at pH 7 and pH 5. However, cells incubated and treated at pH 7 showed high resistances of their membranes towards a pressure induced permeabilization at 125 MPa.

Gianotti et al. (2009) and Yuk and Marshall (2004) reported that *E. coli* cells growth at pH 5 have a significant lower amount of unsaturated fatty acids and hence, a reduced membrane fluidity, which may increase the acid resistance. However, a reduced amount of unsaturated fatty acid in a cell membrane, decreases its high pressure resistance according to Abe (2013) and Winter and Jeworrek (2009). Therefore, this might be an explanation for the higher resistance of the tested *E. coli* strain at low pressures (<200 MPa) in buffer with pH 7 (Fig. 5). After a phase transition of the membrane from liquid-crystalline to a gel phase, the uptake of PI and hence, the progress of membrane permeabilization nearly equals at both pH-values (Fig. 5).

Enzyme activity is another essential factor in cellular viability and thus, esterase activity measured by cF-fluorescence intensity is a useful indicator to estimate the impact of high pressure on intracellular enzyme activity (Ananta et al., 2005; Ananta and Knorr, 2009). The isorate lines for a 25% reduction of esterase activity (Fig. 6) show that *E. coli* cells treated at pH 5 or 7 had nearly the same heat resistance (at 0.1 MPa). Further the temperature needed to induce a 25% esterase enzyme inactivation was lower compared to the temperature needed to induce a 25% membrane permeabilization (Fig. 5). This difference in heat resistance was even more pronounced for higher treatment temperatures. Hence, thermal inactivation of esterase enzymes is possible without a membrane permeabilization. In combination with high pressure, a different response of the cells was observed, indicating that a different pathway of inactivation for *E. coli* is present. A double staining of *E. coli* DSM1116 with cFDA and PI showed, that there is no correlation between the absence of esterase activity and the loss of culturability, which was also reported by Ananta and Knorr (2009) for *Lactobacillus rhamnosus.*

A 5 min treatment at 400 MPa and 40 °C caused a >5.5 log inactivation at both tested pH-values, but only 37.4% (at pH 7) respectively 41.8% (at pH 5) reduction of the esterase activity. Contrary, Bang and Chung (2010) reported a full inactivation of esterase and esterase lipase in *E. coli* (ATCC11229) suspended in trypticase soy broth after a 30 s treatment at 23 °C and 448 MPa. The difference in the esterase inactivation mechanism under pressure is also visible at different pH-values (Fig. 6), were a much higher pressure-temperature resistance was found at pH 7.

Klotz et al. (2010) showed that *E. coli* cells have the ability to reseal its membrane after decompression. Hence, it might be possible that due to the pore formation under pressure a drop of the intracellular pH occurs, if the cells are suspended in a buffer with low a pH-value, which would decrease the resistance of the intracellular esterase.

5. Conclusion

The presented work deals with the inactivation of pathogenic and non-pathogenic *E. coli* strains in buffer solution and carrot juice. The two EHEC strains O157:H7 and O104:H4, isolated from HUS-patients, showed a much higher pressure-temperature resistance in ACES buffer at pH 7, than the non-pathogenic *E. coli* strain DSM1116. The resistance of the O104:H4 strain further increased, after pre-cultivation in nutrient broth at pH 5 and inactivation in carrot juice (pH 5.1). These data denote that the inactivation of pathogenic *E. coli* strains and non-pathogenic surrogates are not comparable and that an adaptation of the cells to the treatment matrix is essential. For sake of clarity and to enable a comparison of

the inactivation behavior of all tested strains in the different matrices, inactivation data were calculated as a functional relationship of pressure, temperature and time and presented by means of pressure–temperature diagrams. These iso-response pressure-temperature-graphs were also calculated for the results from flow cytometry. Flow cytometric analysis used in this study, to get an insight into the inactivation mechanisms of *E. coli* under pressure, enabled a rapid estimation of the high pressure impact on the membrane potential, membrane permeabilization and the inactivation of intracellular esterase for *E. coli* DSM1116 cells treated in ACES buffer at pH 5 and 7. Again significant differences were found for *E. coli* cells pre-cultivated and treated at a lower pH-value. These cells showed a stronger ability to retain its membrane potential, but a higher rate of membrane permeabilization at lower pressures (<200 MPa). For pressures higher than 200 MPa the isorate lines for membrane permeabilization at pH 7 and 5 converged, possibly denoting that differences in the fatty acid composition and membrane fluidity might be responsible for stabilization phenomenon at around 125 MPa. A comparison of the data for cell inactivation, with the reduction of the membrane potential (Fig. 3) and cell permeabilization (Fig. 4) indicate, that under high pressure a loss of culturability did not correlate with the absence of esterase activity. However, a thermal (≤60 °C) induced esterase inactivation was possible with (≥100 MPa) and without (0.1 MPa) membrane permeabilization, depicting a different pathway of cell inactivation.

Reproductively viable cells that are able to grow under laboratory conditions are still the benchmark method for the determination of successful pasteurization or sterilization in the food industry. However, inactivation pathways of microorganisms are complex, so flow cytometry could be a useful tool for a rapid detection in order to discriminate between damaged cells and dead cells.

Acknowledgment

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Impact of different water activities (a_w) adjusted by solutes on high pressure high temperature inactivation of *Bacillus amyloliquefaciens* spores

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Much research has been conducted to comprehend the mechanisms of high pressure (HP) inactivation of spores in aqueous systems but for food model systems these information are scarce. In these systems spores can interact with ingredients which then could possibly lead to retarded or reduced inactivation, which can cause a problem for the sterilization process. The protective mechanism of a reduced a_w-value is still unclear. HP processing might prove valuable to overcome protective effects of solutes and achieve shorter process times for sterilization under HP. To gain insight into the underlying mechanisms five a_w-values (0.9, 0.92, 0.94, 0.96, 1) were adjusted with two different solutes (NaCl, sucrose). Solutions were inoculated with spores of *Bacillus amyloliquefaciens* and treated at 105, 110, and 115°C at 600 MPa. Further a thermal inactivation was conducted at the same temperatures for a comparison with the HP data. Afterward, the influence of HP high temperature treatment on the inactivation, the dipicolinic acid (DPA)-release and membrane constitution was assessed by plate count, HPLC and flow cytometry (FCM). The results show that during HP treatments sucrose and salt both have a protective effect, in which the influence of sucrose on the retarded inactivation is higher. The threshold water activities ($a_{w)}$, which is 0.94, here salt and sucrose have a significant influence on the inactivation. The comparison of thermal (105–115°C) and HP and high temperature (600 MPa, 105–115°C) treated samples showed that the time needed to achieve a 4–5 $\log_{10}$ inactivation is reduced from 45 ($a_w = 1$) to 75 ($a_w = 0.9$) min at 105°C to 3 ($a_w = 1$) to 15 ($a_w = 0.9$) minutes at 600 MPa and 105°C. The release of DPA is the rate limiting step of the inactivation and therefore monitoring the release is of great interest. The DPA-release is slowed down in high concentrated solutions (e.g., sucrose, salt) in comparison to a_w 1. Since there is a difference in the way the solutes protect the spore it could be seen as an inner spore membrane effect. Maybe as shown for vegetative microorganism the solutes can interact with membranes, e.g., the inner spore membrane. Flow cytometry (FCM) measurement data show a similar trend.

Keywords: High pressure high temperature, reduced water activity, baroprotective effect, *Bacillus amyloliquefaciens*, spore inactivation

Introduction

One of the main aims of the food industry is the product safety but in the recent years due to consumer demand the product quality of the food has also gained more importance (Ramirez et al., 2009; Olsen et al., 2010; Belletti et al., 2013; Reineke et al., 2013a). The combination of elevated temperatures (90–121°C) and high pressure (HP) processing (up to 600 MPa) show a high technological potential to preserve foods in a more gentle and efficient way then only by heat processes. In the recent years a lot of research has been conducted to understand the influence of the so called high pressure thermal sterilization (HPTS) and its impact on; spores, food processing contaminants, the working principle of the technology and synergism of temperature and pressure (Margosch et al., 2006; Reineke et al., 2013a; Georget et al., 2015; Olivier et al., 2015; Sevenich et al., 2015). The mechanisms of spore inactivation under these servere conditions will not be discussed in detail since they are well described in literature elsewhere (Wuytack et al., 1998; Setlow, 2003; Margosch et al., 2006; Black et al., 2007; Barbosa-Canovas and Juliano, 2008; Reineke et al., 2012, 2013b). Although, it should be mentioned, since it is of importance for this work, that the release of dipicolinic acid (DPA), which makes up 5–15 % of the dry matter content of the spores, is thought to be the rate limiting step of the inactivation (Reineke et al., 2013b). At pressures above or equal 400 MPa an opening of the Ca^{2+} – DPA – channels occurs: (i) DPA is released from the spore core; (ii) the spore core gets dehydrated; and (iii) therefore, it becomes thermo- and pressure sensitive and can be inactivated (Reineke et al., 2012). If the treatment temperature is moderate ($\leq$50°C) the spore will be triggered to germinate. For a rapid and sudden inactivation of spores under pressure it is important to apply pressures $\geq$600 MPa and temperatures above 60°C to ensure the loss of heat resistance (Reineke et al., 2013b).

One possible explanation of the non-implementation of HPTS could be the fact that certain ingredients of foods such as fats, sugars, salts and the resultant water activities could lead to a retarded or reduced inactivation under these conditions. The so called baroprotective effect is not well studied yet for spores in combination with HP and high temperatures. Water activity (a_w) is a major factor in preventing or limiting growth (bacteria causing food borne diseases will not grow under a_w of 0.85) but further it can also lead to an increased resistance of microorganisms and spores. The influence of salts or sugars on the a_w of foods or suspension media does not explain the marked baroprotective effects of these solutes and it has been suggested that specific interactions between solutes and biological macromolecules contribute to their baroprotective effects (Fujii et al., 1996; Molina-Höppner et al., 2004). These observations were already made by researchers in the 1950–1980, which tried to understand the protective effect of solutes (sodium chloride, sucrose, glucose, fats, acids etc.) and the precipitated change in a_w during the thermal inactivation of microorganisms (Anderson et al., 1949; Secrist and Stumbo, 1958; Murrell and Scott, 1966; Molin and Snygg, 1967; Cook and Gilbert, 1968; Beuchat, 1974; Gould, 1977; Härnulv et al., 1977; Senhaji and Loncin, 1977). These researchers indicated that a protective effect of solutes/a_w-value exists and can lead to an increase of the *D*-value for the tested microorganisms. Some further stated that the protective effect is depending on the concentration of solute, the solute itself (ionic e.g., salt or non-ionic e.g., sugar), the selected microorganism and the temperature. However, the protective mechanism of a reduced a_w-value is still unclear. Molin and Snygg (1967) reported in their studies that fats/oils increased the heat resistance of *Bacillus cereus* and *B. subtilis* spores. They stated that the protective effect is not only due to the low heat conductivity or the water free environment. A more apparent possibility for that protective effect might be that the free fatty acids present in some oils could have a stabilizing effect on spores.

The use of an emerging technology such as HP processing might prove valuable to overcome the protective effect of the solutes. And by doing so, achieve shorter process times. Some interesting studies concerning the influence of HP on baroprotecive solutes exist. Oxen and Knorr (1993) showed that HP inactivation of *Rhodotorula rubra* in different a_w solutions was more effective than by heat alone. Molina-Höppner et al. (2004) stated that microorganisms (*Lactococcus lactis, Escherichia coli*) due to the osmotic up-shock accumulate solutes (NaCl and sucrose) which then can interact with biomolecules, as per the example of phospoholipid bilayers. Sucrose preserved the metabolic activity and membrane integrity of the cells during the high-pressure treatment, whereas salt preserved the membrane integrity but not the metabolic activity. Due to the accumulation, the membrane stays in a more fluid state during the treatment and therefore shows a higher HP resistance. However, the baroprotection of salt (ionic solutes) requires higher concentrations than the same baroportection by disaccharides.

No such detailed research was conducted at this point for spores but it is plausible that also spores could accumulate solutes by diffusion in the inner compartments, e.g., inner spore membrane, where these could interact with biomolecules. Some prior works already indicated that such a phenomenon could exist in food systems (cacao mass, fish systems, baby food puree, food concentrates) for HP high temperature processes as well (Ananta et al., 2001; Sevenich et al., 2013, 2014, 2015; Georget et al., 2015). Gerhardt and Black (1961) and Black and Gerhardt (1962) stated, that the central core of a spore is kept relatively dry but that the rest of the compartments are free to extensive water and solute permeation. Other authors mentioned that the predominant compartment that plays an important role in the uptake of solutes is the spore cortex. The cortex possesses a negative net charge. The low degree of cross-linking supposes that the spore cortex is able to change volume in response to changes as a result of balancing the electrostatic interaction (Kazakov, 2008; Kazakov et al., 2008). These induced changes in the different spore compartments could have an impact on the inactivation mechanism under HP. Another possible postulated impact of the baroprotective of solutes could be the influence on the rate limiting step of the spore inactivation under pressure, which is the release of DPA out of the spore core and the followed rehydration of the core by water. Lower a_w means that most of the outside water is bound to solutes and cannot

freely move. Therefore, one could assume that the exchange of water and DPA is slowed down due to changes in the osmotic pressure or the solubility of DPA in high concentrated solutions might differ, which both could result in retarded and delayed inactivation. In addition, a third postulated impact could be that high concentrations of solutes close to the oversaturation point can inhibit the pressure transmission of water to some degree and therefore generate an insufficient and in-homogenous pressure distribution within the system (Eder and Delgado, 2007; Min et al., 2010). The spores might, if the threshold pressure (500–600 MPa) is not reached, not undergo the postulated pathways of germination/inactivation.

The aim of this investigation was to obtain insights into mechanisms of baroprotection by NaCl and sucrose on *B. amyloliquefaciens* spores (TMW FAD82), which are very HP high temperature resistant (Margosch et al., 2006; Sevenich et al., 2013), under HP thermal sterilization conditions (600 MPa; 105, 110, and 115°C). Therefore, solutions with different a_w were prepared with two different solutes one was sodium chloride (1.2–2.7 mol/L) and the other sucrose (0.83–1.7 mol/L). The influence of the two different solutes and their resultant a_w values on inactivation was monitored by plate count, the DPA-release by HPLC and changes in the membrane barrier properties by flow cytometry (FCM).

Materials and Methods

Sporulation, Spore Preparation, and a_w-Solution Preparation

Using a method described elsewhere (Paidhungat et al., 2002), sporulation of *B. amyloliquefaciens* (Technische Mikrobiologie Weihenstephan, 2.479, Fad 82) was induced at 37°C on solid 2 × SG medium agar plates without antibiotics. The harvest was carried out when 90% of the spores were phase bright under the light microscope, which took 2–3 days. The spore suspension was cleaned by repeated centrifugation (threefold at 5000 *g*), washed with cold distilled water (4°C), and was treated with sonication for 1 min. The cleaned spore suspensions contained ≥95% phase bright spores and nearly no spore agglomerates, as was verified by a particle analysis system (FPIA 3000, Malvern Instruments, Worcestershire, UK). The spore suspensions were stored in the dark at 4°C.

Five different a_w-values were used for each solute (NaCl and sucrose); the range was selected from 1 to 0.9 (1, 0.96, 0.94, 0.92, 0.90), since this represents the a_w of many relevant food systems. To adjust the a_w with NaCl and sucrose a table published by the Food Safety Bulletin was used (Food-Saftey, 2010). The amounts of water (aqua dest.) and solute for the corresponding a_w were mixed in 500 ml flasks (Schott Ag, Mainz, Germany) and afterward autoclaved to gain a homogenous solution. After cooling down, the flasks were stored at 4°C.

High Pressure High Temperature Treatment

Twenty-four hours prior to the each HP trial *B. amyloliquefaciens* spores were suspended into the a_w-value solution to give the spores time to adapt to the new surroundings. The total cell count of the solution was between 10^7 and 10^8spores/ml. From these spore suspension 3*300 µl (one for the inactivation kinetics, one for DPA analyses and one for FCM analyses) were filled in shrinking tubes (Schrumpfschlauch 3/1, DSG-Canusa, Meckenheim, Germany, inner diameter 3 mm, outer diameter 3.6 mm) and were hermitically sealed with a soldering iron. All three shrinking tubes were put in 2 ml containers (2 mL, CryoTube Vials, Nunc Brand Products, Roskilde, Denmark) filled with same solution. For the pressure trials the U111 Monovessel unit (Unipress, Warsaw, Poland), with a 3.7 mL vessel volume and a compression rate of 25 MPa/s was used. In this pressure equipment, Di-2-ethyl-hexyl-sebacate served as the pressure-transmitting medium. To reach the designated treatment temperature, the pressure vessel was immersed in a thermostatic bath (cc2, Huber GmbH) filled with silicon oil (M40.165.10, Huber GmbH). To monitor the temperature during the treatment the temperature was measured in the geometrical center of container by a thermocouple. The temperatures selected for the treatment were 105, 110, and 115°C at 600 MPa with isothermal dwell times between 0.0166 and 15 min. The oil bath was set on the selected process temperature and the start temperatures for each food system were obtained in a pretrial. Before and after the trials the samples were stored on ice. After the treatments one of the shrinking tubes was used for the appropriate dilutions of each sample for surface-plated on nutrient agar (CM 003, Oxoid Ltd., Hampshire, England). The dishes were incubated at 37°C for 2 days, and the colony-forming units (CFUs) were counted. All trials were conducted in duplicates. The samples for the DPA or FCM analyses were put in 1 ml reaction cups (1 mL, CryoTube Vials, Nunc Brand Products, Roskilde, Denmark) and stored at −80°C until further analysis.

Thermal Inactivation of Spores Suspended in the Different Water Activities

The thermal sensitivity of *B. amyloliquefaciens* spores (10^7 CFU/ml) in the different a_w-values was conducted at ambient pressure in the temperature range of 105–115°C. Static temperature treatments of the spores were performed in a temperature-controlled oil bath using glass capillaries (Hirschmann Labogeräte, inner diameter 1 mm and outer diameter 1.3 mm, Germany). This was done to ensure instantaneous heating and cooling of the solutions. After the treatment, samples were immediately transferred to an ice bath to prevent further inactivation. The samples were diluted and incubated as described under section "High Pressure High Temperature Treatment."

DPA-Analyses by HPLC

Prior to the DPA analyses the samples were filtered through 0.2 µm Nylon filters (Rotilabo® Spritzenfilter; Carl Roth GmbH & Co KG, D-76185 Karlsruhe) to remove spores and other particles which could interfere with the analyses. A Dionex Ultimate 3000 system was used (Dionex Corporation, Sunnyvale, CA, USA), with a reversed phase separating column (RP 18—5 µm LiChroCART 124-4; Merck, KGaA, Darmstadt, DE) that was protected with a guard column (LichroCART 4-4; Merck KGaA, Darmstadt, DE). The DPA detection limit of this HPLC setup

was 1 μM. To determine the total amount of DPA in the spore suspensions, 1 mL of each individual batch was thermally treated at 121°C for 20 min (Reineke et al., 2013b) and then analyzed. To identify the peaks and to determine the DPA amount released standard solutions of known DPA concentrations were used to calculate a calibration curve. Pressure-induced DPA release was calculated relative to the total DPA content of each individual spore batch, and all of the data are represented as the mean of at least two independent experiments.

Flow Cytometry

For the FCM analyses a method described by Mathys et al. (2007) was used. For the sample preparation a double staining was used, involving SYTO16 (Invitrogen, Carlsbad, CA, USA) and propidium iodide (PI; Invitrogen, Carlsbad, CA, USA). Both fluorescent dyes are able to stain DNA, but the membrane permeant SYTO16 acts as an indicator for spore germination, whereas the membrane impermeant PI indicates membrane damage. The treated spore suspensions were diluted with *N*-(2-Acetamido)-2-aminoethanesulfonic acid (ACES) buffer solution (0.05 M, pH 7) to achieve a flow rate of about 1000 events/s, as well as a constant ratio of spores for the staining. The concentrations of the fluorescent dyes for staining were 15 μM PI and 0.5 μM SYTO16 in the diluted spore suspension. Afterward, the samples were stored in the dark at ambient temperature for 15 min. The analyses were carried out using a CyFlow ML flow cytometer (Sysmex Partec, GmbH, Münster, Germany) equipped with Partec FlowMax Operating and Analysis Software for Partec Flow Cytometry Particle Analyzing systems Version 3.0 (b5) (January 12 2009) was used as operation and acquisition software. Excitation was set to 488 nm and the fluorescence of Syto 16 was measured with the photomultiplier FL1 and a band pass filter with 536 ± 20 nm cut off. For the fluorescent of PI the photomultiplier FL3 and a short pass filter with 620 nm cut off ±15 nm (fluorescence intensity is recorded between 615 and 620 nm), was used. The parameters were collected as logarithmic signals and the obtained data was analyzed using Software FCS Express Version 4 for Flow and Image Cytometry Analysis (De Novo Software, Los Angeles, CA, USA). All analyses were performed in triplicates. Only certain samples (a minimum, intermediate, maximum a_w (1, 0.94, 0.90) for sucrose and NaCl in a temperature range of 105–115°C) were analyzed to get an overview of the influence of solutes on the physiological state of spores.

Calculation of Isokineticity Lines

For the calculation of the isokinetic lines the Weibullian approach (Equation 1) was used. This approach is a vitalistic approach and is suitable to describe inactivation kinetics of HP processing (van Boekel, 2002; Juliano et al., 2009; Reineke et al., 2013a).

$$\log\left(\frac{N}{N_0}\right) = -\left(\frac{t}{\Delta}\right)^b \quad (1)$$

With Δ as the scale parameter, b as the shape parameter. The shape parameter, b, determines the curve progression of the inactivation curve, and simultaneously gives information about the dying behavior of the respective microorganisms. For values of b smaller than 1 the decreasing curve becomes increasingly flatter. This implies that the microorganisms, not yet killed, are more resistant to the treatment than the microorganisms that have already been killed. If b equals 1 the graph is linear and therefore corresponds to a first order inactivation. For values of b greater than 1 the inactivation curve decreases progressively it can be deduced that the remaining cells exhibit increasing sensitivity against the applied treatment (van Boekel, 2002).

The parameter b and Δ were obtained by using the Weibull fit of the analytical software "Geeraerd and Van Impe Inactivation model Fitting Tool" (GinaFit Version 1.6 March 2012, Katholieke Universiteit Leuven). The mean shape parameter b for all inactivation kinetics was calculated and all inactivation-kinetics were refitted with the mean b to obtain Δ. To get a functional dependency of Δ(T), Δ and T were fitted with all equation set of TableCurve3D (SPSS Inc., Chicago, IL, USA). The equation with the minimal sum of square errors for Δ(T) was then used in (1) for Δ. The isorate lines were calculated with MathCAD 2001i professional (Mathsoft Engineering & Education, Inc., USA).

Statistical analyses: the statistical analysis of the data was performed using Statgraphics (Version 4.0, StatPoint Technologies, Warrenton VA, USA) Multiple range test was used to analyze the significance of the tested data. Significance for all statistical analysis was defined as $p < 0.05$.

Results

High Pressure High Temperature in Comparison to Thermal only Inactivation of *Bacillus amyloliquefaciens* in Different Water Activities

Spores of *B. amyloliquefaciens* inoculated in different a_w, adjusted by NaCl and sucrose, were used to investigate the influence of the baroprotective effect of these solutes on the HP high temperature and thermal only inactivation. In **Figures 1A,B** selected inactivation kinetics of the tested kinetics are shown to give an overview of the inactivation behavior (105°C 600 MPa and 115°C 600 MPa). **Figure 1A** shows the inactivation kinetics at 600 MPa, 105°C for the a_w-values 1, 0.94, and 0.90 for NaCl and sucrose. It becomes obvious that the higher the concentration of the solute, respectively the lower the a_w, the slower the inactivation becomes. Sugar has a more severe impact on the retardation of the inactivation as NaCl. If one looks at the inactivation kinetics $a_w = 0.9$ of NaCl it takes 8 min to achieve a 4.2 $\log_{10}$ inactivation whereas a similar inactivation for sucrose with the same a_w takes 15 min. Interesting to see is that an $a_w = 1$ and $a_w = 0.94$ adjusted with salt shows equal inactivation behavior. This underlines that not only the a_w needs to be taken into account but the solute as well. The other inactivation data which are not shown show similar trends. If the temperature is increased by 10°C up to 115°C (**Figure 1B**) the treatment times in comparison to 105°C, 600 MPa (**Figure 1A**) to achieve inactivation of 4–5 $\log_{10}$ are much shorter. Further the graphs converge closer together, which can be seen as an indication

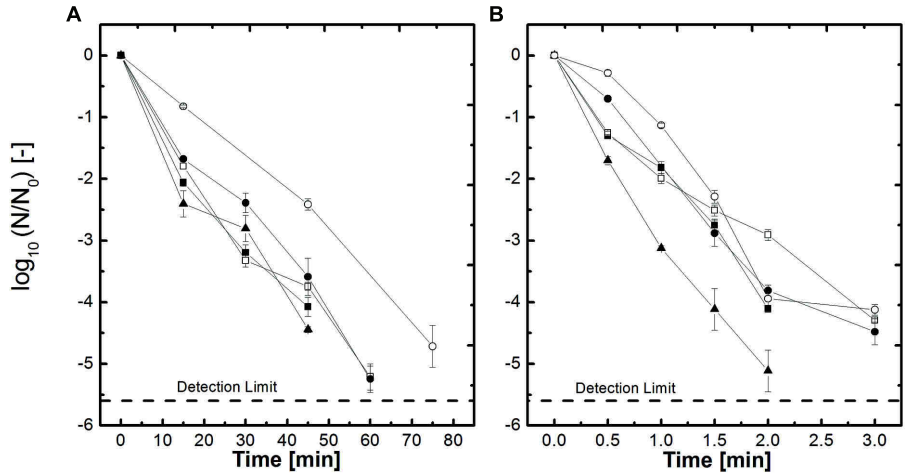

FIGURE 1 | Effect of pressure and heat on the inactivation of *Bacillus amyloliquefaciens* spores in selected water activities adjusted by NaCl and sucrose (A) 600 MPa, 105°C and (B) 600 MPa, 115°C. $a_w = 1$ (▲), $a_w = 0.94$ NaCl (■), $a_w = 0.90$ NaCl (□), $a_w = 0.94$ sucrose (●) and $a_w = 0.90$ sucrose (○). Initial spore count ~10^7 CFU/ml.

that the applied temperature is able to overcome the protective effect.

The inactivation results for the thermal treatment at 105–115°C of *B. amyloliquefaciens* spores in the different a_w-solutions (here only $a_w = 1$, 0.94, and 0.90 are shown) adjusted by NaCl and sucrose are depicted in **Figure 2**. In comparison to the HP treated samples the time needed to achieve a 4 log inactivation at 105°C only is between 45 and 65 min (**Figure 2A**) whereas at 600 MPa. 105°C (**Figure 1A**) the dwell time is between 2 and 14 min. At 115°C (**Figure 2B**) the inactivation rates are higher but still the HP treated samples at the same temperature (**Figure 1B**) are inactivated slightly quicker. The behavior of the spores in the different a_w-solutions for the thermal only inactivation is the same as described for the HP treated samples.

To gain a better understanding of the T, t dependencies at 600 MPa a modeling for the spore inactivation in the different a_w (1, 0.96, 0.94, 0.92, 0.90) adjusted with NaCl and sucrose was conducted based on the obtained inactivation kinetics for a 3 $\log_{10}$ and 5 $\log_{10}$ inactivation of *B. amyloliquefaciens* (**Figures 3A,B**). The inactivation that was achieved during the pressure build up (kinetic point of 1 s) was subtracted from the other kinetic points of each temperature, to have a valid model for isothermal and isobaric conditions. **Figure 3** shows the most relevant domain of a_w for the food industry and the influence on the inactivation of spores depending on the solute. A 3 and 5 $\log_{10}$ inactivation of *B. amyloliquefaciens* is possible for all tested a_w and solutes in a time range of 5–10 min and temperatures between 94 and 114°C at 600 MPa. The isokinetic lines also depict

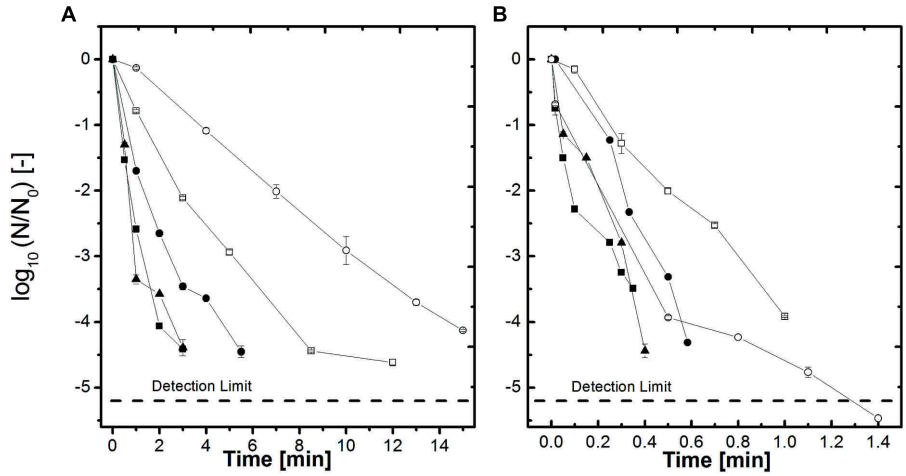

FIGURE 2 | Effect of heat on the inactivation of *B. amyloliquefaciens* spores in selected water activities adjusted by NaCl and sucrose (A) 105°C and (B) 115°C. $a_w = 1$ (▲), $a_w = 0.94$ NaCl (■), $a_w = 0.90$ NaCl (□), $a_w = 0.94$ sucrose (●) and $a_w = 0.90$ sucrose (○). Initial spore count ~10^6 CFU/ml.

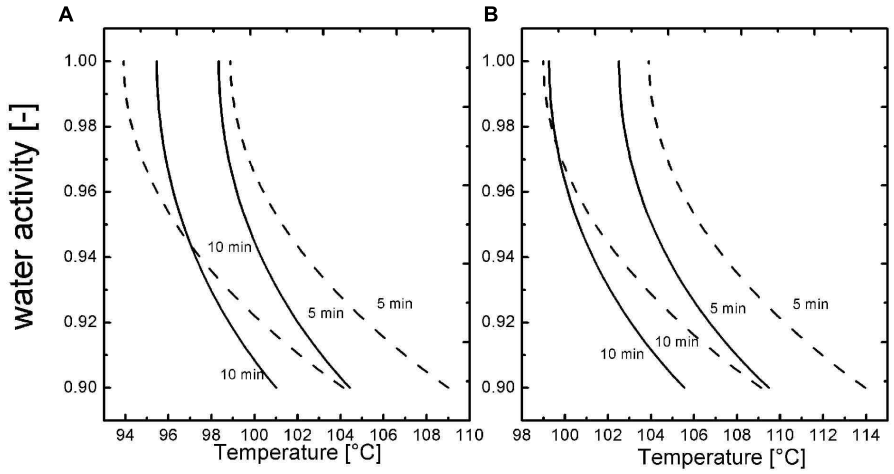

FIGURE 3 | Isokientic lines of weibull approach for NaCl (solid line; $b = 0.57$) and sucrose (dashed line $b = 0.72$) (A) -3 $\log_{10}$ and (B) -5 $\log_{10}$ inactivation at 600 MPa.

that sucrose has a more pronounced baroprotective effect then NaCl and therefore higher temperatures are needed to achieve the same kind of inactivation.

Quantitative Analyses of DPA-Release in the Different Water Activities by HPLC

Release of DPA during the HP-induced spore inactivation was monitored by HPLC to draw inferences from the released DPA about the physiological state of the cells, respectively, the inactivation in the presence of solutes (sucrose and NaCl in a_w range 0.90–1). Here, the ability of the spores to retain DPA can be seen as an indicator of the pressure and heat resistance of spores (Margosch et al., 2004). Furthermore, Reineke et al. (2013b) found that the release of DPA appears to be the rate-determining step of spore inactivation for a combined pressure and heat treatment. To determine the relative DPA-release in the tested spore population the maximum amount of available DPA was quantified by batch sterilization in an autoclave for 20 min at 121°C. The measured amount of released DPA was used as the maximum detectable DPA content and was set in relation to the treated samples. For the released DPA only the endpoints of the corresponding treatment and a_w were used. An overview of the relative DPA-release and the corresponding treatment time for the different a_w is given for a temperature range of 105–115°C in **Tables 1** and **2**. In general one can state for the a_w adjusted with NaCl and sucrose (**Tables 1** and **2**) that the DPA-release is depending on the a_w ($\leq$0.94). For NaCl the analyses showed that with increasing temperature the time to release the same amount of DPA is getting shorter and the released amounts increase to 84–92 % at 115°C in comparison to 72–92% at 105°C (**Table 1**). For higher temperatures the protective effect diminishes and is only more or less present for the a_w 0.92 and 0.90. In comparison to NaCl, the DPA release into the sucrose solutions (**Table 2**) shows the same tendencies described for NaCl. However, the temperature increase to 110°C, 600 MPa (**Table 2**) does not have such a huge impact on the DPA release and time; as for NaCl (**Table 1**). At 115°C, 600 MPa (**Table 2**) the impact of temperature becomes dominant but not as intense as for NaCl.

Flow Cytometry Analyses to Identify Possible Changes in the Membrane Constitutions by Solutes

The method uses a double staining approach, the membrane permeable Syto16, is as an indicator for germination, since staining with this dye is not possible until the degradation of the spore cortex was initiated (Black et al., 2005). The membrane impermeable PI, is an indicator for the spore inactivation since the rupture of the inner spore membrane is necessary for its detection. For both fluorescent colorants there have been no documented interactions with sucrose or NaCl. This at least can be disapproved by the following results. The staining of the spores in the different a_w adjusted by solutes was quite difficult. Since for higher solute concentrations the viscose and concentrated solutions seemed to keep the dye away from the spores and staining might be insufficient. This is depicted in **Figure 4** where the mean PI fluorescence intensity is shown over the treatment time and temperature for the corresponding a_w. The mean PI fluorescence intensity describes the average intensity of PI of the spores over the entire set of detection channels and therefore can be used as an indicator for the influence of HP high temperature on the inner spore membrane. The PI concentration increases with increasing temperature but decreases with a_w. For **Figures 4A–C** this trend is obvious and indicates the influence of NaCl on the inner spore membrane with increasing a_w-value. The interpretation of the results of sucrose (**Figures 4D,E**) is quite difficult. The influence of the solute, as described for NaCl, cannot be verified by the obtained results. Tendencies are present for a_w 0.94 (**Figure 4D**) but a_w 0.9 does not follow a clear trend. Although, the results of the DPA-release and the inactivation in sucrose solutions seemed to indicate this kind of influence. Maybe the

TABLE 1 | Relative DPA-release in % at maximal treatment time and corresponding temperature of *Bacillus amyloliquefaciens* in different water activities adjusted by NaCl.

	Water activity [–]	Max. DPA-release [%]	SD Maximum DPA-release [%]	Maximum treatment time [min]
NaCl, 105°C, 600 MPa	1	82.3	0.276	3
	0.96	92.64	2.75	2
	0.94	84.84	2.17	3
	0.92	81.23	0.196	5
	0.9	72.76	2.58	12
NaCl, 110°C, 600 MPa	1	95.15	0.245	1
	0.96	94.39	1.352	2
	0.94	89.06	0.44	3.5
	0.92	90.53	0.801	3
	0.9	89.81	0.129	3
NaCl, 115°C, 600 MPa	1	92.65	3.26	0.3
	0.96	91.44	0.832	0.5
	0.94	85.53	5.56	0.3
	0.92	87.66	1.87	0.7
	0.9	84.1	0.461	1

TABLE 2 | Relative DPA-release in % at maximal treatment time and corresponding temperature of *B. amyloliquefaciens* in different water activities adjusted by sucrose.

	Water activity [–]	Maximum DPA-release [%]	SD Maximum DPA-release [%]	Maximum treatment time [min]
Sucrose, 105°C, 600 MPa	1	82.3	0.276	3
	0.96	82.82	0.538	4
	0.94	84.61	0.454	5.5
	0.92	89.22	0.186	13
	0.9	49.71	0.7588	15
Sucrose, 110°C, 600 MPa	1	95.15	0.245	3
	0.96	89	0.448	2.5
	0.94	87	3.52	3.5
	0.92	85.54	2.69	6
	0.9	86.36	0.767	11
Sucrose, 115°C, 600 MPa	1	92.65	3.26	0.3
	0.96	85	2.49	0.25
	0.94	86	2.33	0.85
	0.92	88.57	4.18	1.25
	0.9	88.19	2.21	2

solute concentration/dye ratio has an influence on the staining properties of PI.

Another way to illustrate the results of the FCM-analyses is by showing the histograms of the measurement. These results are depicted in dependencies of a_w, temperature and time in **Figures 5A–C**. At 105°C, 600 MPa (**Figure 5A**) one can see that the PI Fluorescence Intensity (PIFI) is depending on the solute concentration, the a_w and the dwell time. The PIFI moves from high intensities at $a_w = 1$ down to lower overall PIFI for $a_w = 0.9$, although longer treatment times were applied for lower a_w.

Discussion

In the present paper the influence of two solutes (NaCl and sucrose) and their corresponding a_w-values and their influence on inactivation of *B. amyloliquefaciens*, amount of DPA released and changes on the structural spore properties under HPTS conditions were studied.

High sucrose and high NaCl concentrations and corresponding a_w of $\leq$0.94 have a servere impact on the inactivation. As many researches have postulated and which was just recently proven by Olivier et al. (2015) that HP and high temperatures have synergistic effect on the spore inactivation. This effect could be used to treat spores already at lower temperatures and shorter dwell times to achieve similar inactivation as under thermal only conditions. Literature data on the influence of HP on spores suspended in different a_w solutions are rare. Nevertheless, these findings are in accordance to results obtained by Raso et al. (1998), who reported that the inactivation of *B. cereus* by HHP decreased when the a_w decreased with the addition of sucrose (non-ionic). These findings in this work and the findings of Raso et al. (1998) are in contradiction to Sale et al. (1970), who concluded that only ionic (NaCl etc.) solutes could protect the spores from being inactivated. The results here indicate that both solutes (NaCl and sucrose) both have a protective effect and the one of sucrose is enhanced. The results of Sale et al. (1970) were obtained in the same range of a_w as the ones described here but the applied kinetics was 100 MPa, 30 min at 65°C. Maybe in these temperature-pressure domain sucrose acts differently than at the conditions tested here or by Raso et al. (1998). Raso et al. (1998) tested also in the same a_w range but at pressures between 250 and 690 MPa at 40°C, so as mentioned before the differences could be caused by sucrose acting differently at higher pressures. Although, more research will be needed to prove this assumption. Further different sporulation conditions used in the papers could also have been the cause for the contradictory results.

The influence of the a_w adjusted by NaCl and sucrose on the DPA-release is given for $a_w \leq 0.94$. If one looks exemplary on the behavior of the DPA-release in $a_w = 0.92$ by NaCl and sucrose, where 80–95% of DPA are released, in comparison to $a_w = 1$ in the temperature range 105–115°C one can see the influence quite nicely. At 105°C the time needed for 80–95% of DPA to be released is double (5 min for $a_w = 0.92$ NaCl) respectively 4 times (13 min for $a_w = 0.92$ sucrose) in comparison to $a_w = 1$ (3 min). This ratio does not change for an increase to 110°C but the times needed to achieve the same release at this temperature are of course lower. At 115°C only the sucrose keeps the ratio in comparison to the other samples.

With increasing temperature the baroprotective effects of the solutes are more and more suppressed. This leads at 115°C, 600 MPa (**Tables 1** and **2**) to similar released relative DPA amounts and affirms the assumption made under section "High Pressure High Temperature in Comparison to Thermal only Inactivation of *Bacillus amyloliquefaciens* in Different Water Activities" that the baroprotective effects of the solutes decreases with increasing temperature (Reineke et al., 2013b; Nagler et al., 2014). Therefore, the temperature plays a dominant role for

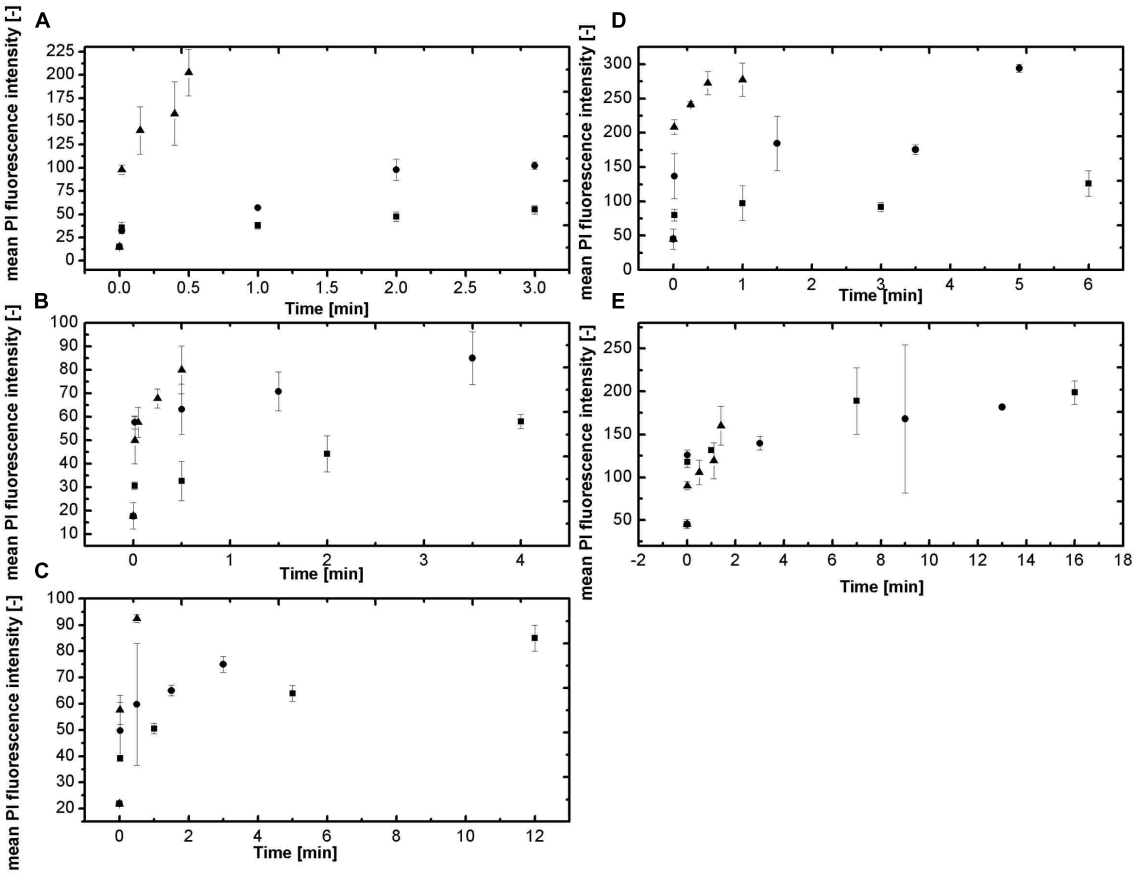

FIGURE 4 | Mean PI fluorescence intensity for the tested water activities at 600 MPa and a temperature range of 105–115°C (■ 105°C, ● 110°C, and ▲ 115°C). (A) $a_w = 1$; **(B)** 0.94 NaCl; **(C)** $a_w = 0.90$ NaCl; **(D)** $a_w = 0.94$ sucrose and **(E)** $a_w = 0.90$ sucrose.

the inactivation in the tested a_w as discussed by Reineke et al. (2013a,b) for buffer systems. Further, if one compares NaCl and sucrose at the same a_w (**Figure 4**) it shows that the ratio of relative DPA-release and maximal treatment time is lower for sucrose. This further indicates that the baroprotecive effect of sucrose on the HP high temperature inactivation of *B. amyloliquefaciens* is more pronounced than the one of NaCl. The proven and shown baroprotective effect of solutes on spores under HP high temperature conditions reduces and delays the release of DPA out of the spores if the a_w is $\leq$0.94 and therefore also the inactivation. For high NaCl concentrations (2.4–3.6 M) Nagler et al. (2014) stated that these concentrations can decelerate germination and decrease the overall germination efficiency under pressure. This might be due to an increase of the osmotic pressure in the outer and inner spore membrane and therefore lead to no water diffusion into the spore (Reineke, 2012; Nagler et al., 2014). This implies that a certain concentrations of sucrose and NaCl might be able to reinforce the ability of the spores to retain the DPA, but more research is needed to understand this mechanism of baroprotection completely. If this is due to the not completely functional DPA-protein channels, which might be in a dehydrated state; other spore compartments that are not correctly functional or their inner spore membrane is altered by the extrinsic factors. And/or DPA release is hindered due to the shortage of free water outside of the spore, which cannot be enlightened by HPLC alone. This hypothesis could be underlined by trials conducted at the Department of Food biotechnology and Process engineering of the Technische Universität Berlin (data not shown) with different water/oil concentrations. For 100 and 75% oil (olive and sunflower-oil) concentrations almost no inactivation occurred at 105°C 600 MPa (1 $\log_{10}$ inactivation took roundabout 30 min). With increasing water concentration 50 and 75% the inactivation rate increased drastically (-5.5 $\log_{10}$ inactivation in 3 respectively 7 min). These results support the hypothesis that free water must be available to guarantee a rapid inactivation and DPA-release.

The FCM-analysis is a sophisticated way to gain information on the physiological state of the spore and therefore gain more knowledge on the impact of HP high temperature processes on the inactivation. The method used by Mathys et al. (2007) is able to detected different kind of spore subpopulations (germinated, unknown state, inactivated) which stand for different membrane damage intensity and this can be used to identify possible influences of solutes on the spore membrane respectively inactivation under HP and high temperature conditions. This means that NaCl does have some

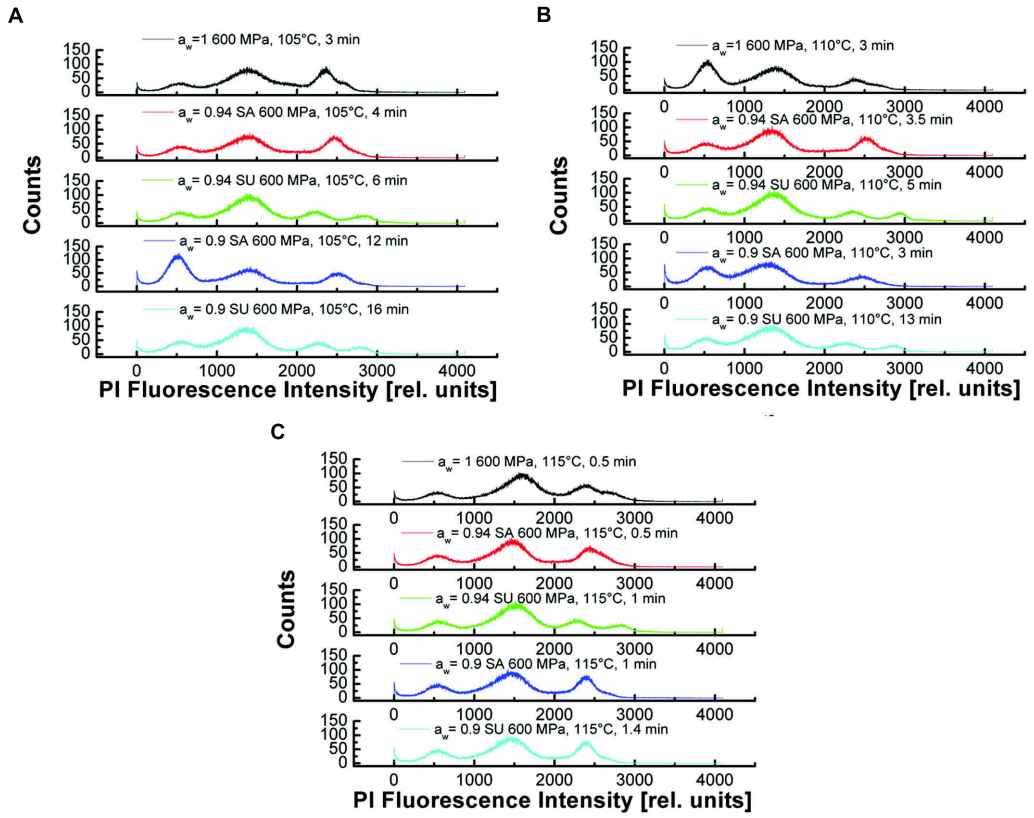

FIGURE 5 | Propidium iodide fluorescence intensity histogramms for different a_w – values (SA = NaCl and SU = sucrose), temperatures and times. **(A)** 105°C, 600 MPa with a_w = 1, 0.94, 0.90 for NaCl and sucrose **(B)** 110°C, 600 MPa with a_w = 1, 0.94, 0.90 for NaCl and sucrose **(C)** 115°C, 600 MPa with a_w = 1, 0.94, 0.90 for NaCl and sucrose.

kind of influence on the inner spore membrane. Although as shown for the inactivation data and the DPA-release the baroprotective effect is lost/is reduced at temperatures ≥115°C. For sucrose it seems like sucrose could have an influence on the dye since the concentrations of PI intensity are quite high, the values vary and the SD are high (**Figures 4D,E**). One is able to see similar tendencies as for NaCl but the influence of sucrose is not as distinct and dominant as seen for the inactivation kinetics and the DPA-release. As mentioned this could cohere with sucrose concentrations, which might interfere with the dye and therefore lead to an inconsistent staining.

This indicates that the baroprotective effect of the solutes is present for certain solute concentrations and that there is an influence of the solutes on the inner spore membrane. For the future the FCM analyses needs to be optimized to give consistent results in high concentrated solutions. Since the results obtained, at least for sucrose, leave a lot of room for interpretation. Further the a_w = 0.94 seems to be, as already described for the inactivation and the DPA-release, a threshold a_w where the baroprotective effect becomes pronounced. A similar trend is seen in **Figure 5B**, although here only a_w = 0.9 adjusted with sucrose shows a lower PIFI then the other samples. As mentioned, for the DPA-release and the inactivation, at 115°C, 600 MPa the PIFI shows no differences for the tested a_w-values and further the dwell times applied are equal. At 115°C, 600 MPa the baroprotective effect of the solutes used to adjust the a_w is lost and the temperature plays the dominant role (**Figure 5C**).

Conclusion

The aim of this work was to investigate the baroprotective influence of solutes (NaCl and sucrose) and the corresponding a_w (1-0.9) on the inactivation mechanisms of *B. amyloliquefaciens* in a temperature range of 105–115°C at 600 MPa. This work showed that for certain solute concentrations (corresponding to an $a_w \leq 0.94$ and temperature ≤110°C) a baroprotective effect is present but a more rapid inactivation is possible if pressure and heat are applied together then only by heat. As other researchers already indicated the a_w respectively the substances responsible for the a_w can have an impact on the inactivation under HP high temperature conditions (Sevenich et al., 2013, 2014, 2015; Georget et al., 2015). Sucrose has a higher protective effect then NaCl but the effect is minimized when temperature ≥115°C at 600 MPa are used. The calculations of the isorate lines could be used to optimize HPTS processes in food systems where NaCl

and sucrose are the major solutes/ingredients. Based on this a 12 D-concept could be established as an orientation for more complex foods with similar a_w values, such as liquid foods.

The DPA-release is slowed down by lower a_w values which might be due to interactions of the solutes with the inner membrane as the results of the FCM analyses indicate. More research is needed in the future to fully understand these effects. This work was able to point out that solutes have an impact on the spore inactivation under HP. Although the influence at 600 MPa on a retarded inactivation is depending on the concentration, the solute and the temperature applied. As described for spore inactivation in aqueous solutions (a_w 1) the release of DPA is crucial for spore inactivation under pressure and the inner spore membrane is the presumably the target structure affected by HPs, high temperatures and by solutes, as shown in this study. For the future, to further look into the physiological changes in the spores due to solute uptake, possible tools could be the use of transmission electron microscopy (TEM) analyses.

Furthermore, the mechanisms of individual solutes and food matrices need to be fully understood in order to optimize the process design of HPTS. Therefore it is of great importance to analyze at what time and at what pressure/temperature combinations the baroprotective effect of typical food ingredients occur and in what way do these affect spore components. HP processing proved valuable to overcome protective effects of solutes and achieve shorter process times for sterilization under HP. The gained data could be used as a basis for the inactivation behavior of spores in real food systems under the same a_w-value conditions and lead to case by case optimized treatment times for foods containing mainly sucrose or salt.

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This work is dedicated to my daughter Lotta and my parents.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00689

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5. Effect of high pressure thermal sterilization on the formation of food processing contaminants

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Effect of high pressure thermal sterilization on the formation of food processing contaminants

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ABSTRACT

The use of high pressure thermal sterilization (HPTS) as an emerging technology for the sterilization of foods could be a big turning point in the food industry. HPTS can result in a better overall food quality, lower thermal load applied to the product and less unwanted food processing contaminants (FPCs) as e.g. furan and monochloropropanediol/-esters.

Hence, within the EU FP7 founded Prometheus-project HPTS treatments were performed for selected food systems. Therefore, two spore strains were tested, the *Geobacillus stearothermophilus* and the *Bacillus amyloliquefaciens*, in the temperature range from 90 to 121 °C at 600 MPa. The treatments were carried out in different fish system and ACES-buffer. The treatment at 90 and 105 °C showed that the *G. stearothermophilus* is more pressure sensitive than the *B. amyloliquefaciens*. The formation of FPCs was monitored during the sterilization process and compared to the amounts found in retorted samples of the same food systems. Depending on the food system the amounts of furan could be reduced between 71 and 97% for the tested temperature pressure combination even at sterilization conditions of F_0-value 7 min.

Industrial relevance: The high pressure thermal sterilization (HPTS) process is an emerging technology to produce high quality low acid food products, which are shelf-stable at ambient temperature. In addition the consumer today demands foods which are minimally processed and are healthy and safe. However, an industrial scale process has not yet been implemented.

The work in this paper shows different temperature combinations (90 to 121 °C) at 600 MPa and their influence on the endospore inactivation and also on the formation of unwanted food process contaminants (FPCs), such as furan and monochloropropanediol/-esters, in comparison to retorting. The use of HPTS could lead to shorter process times and a means by which a better quality of the foods could be achieved.

1. Introduction

The concept of high pressure processing (HPP) as a tool for sterilization has been around for several years. Until now there has not been an implementation of high pressure thermal sterilization (HPTS) (Heinz & Knorr, 2005; Hjelmqwist, 2005; Matser, Krebbers, van den Berg, & Bartels, 2004; Moezelaar, Matser, & van den Berg, 2003), whereas high pressure processing in terms of food pasteurization was introduced on to the Japanese market during the 1994's and on to the US market soon thereafter (Cheftel, 1995; Hogan & Kelly, 2005; Patterson, 2005; Ramaswamy, 2011).

The process currently used in the food industry to achieve a sterile product is the retort heating, where the holding times and high temperatures can have a negative impact on the nutritional value for the food (Matser et al., 2004). Due to the growing consumer demand of minimally processed low acid foods in the recent years, an alternative process is needed to produce foods which are healthy, have a long shelf life and are produced in an economically compatible manner (Toepfl, Mathys, Heinz, & Knorr, 2006). High pressure thermal sterilization (HPTS) may offer an alternative to retort processing, and a means by which high quality products are achieved. HPTS can combine the synergistic effect of elevated temperatures (90–121 °C) and pressures above or equal to 600 MPa for a better overall inactivation of spores and pathogenic microorganism as well as the retention of the food structure (Knoerzer, Juliano, Gladman, & Fryer, 2007; Matser et al., 2004; Sommerville, 2009). Therefore the product needs to be preheated to 70–90 °C and through internal compression heating, during pressure build-up, the process temperature can reach to 90–130 °C. It is important that preheating and pressure come-up time are fine-tuned to guarantee optimal treatment conditions (Juliano & Barbosa-Canovas, 2005). Hence, the temperature non-uniformity in the treatment chamber, which can

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vary for industrial units ~10 °C, is also a factor that needs to be taken into account to ensure the safety of the process (Grauwet et al., 2012; Knoerzer et al., 2007; Martínez-Monteagudo, Saldana, & Kennelly, 2012). However, the rapid heating during HPTS reduces the lack of temperatures uniformity that occurs in traditional thermal sterilization processes (Knoerzer, Buckow, & Versteeg, 2010).

In the last decade much research has been conducted to comprehend the underlying mechanisms in the high pressure inactivation of spores in simple aqueous systems (Knorr, Reineke, Mathys, Heinz, & Buckow, 2011; Mathys, Reineke, Heinz, & Knorr, 2009; Paidhungat et al., 2002; Reineke, 2012a; Reineke, Mathys, Heinz, & Knorr, 2013). The next step in research must be to test that inactivation in real food or food model systems to see if the stated mechanisms are applicable (Welti-Chanes, Palou, Lopez-Malo, & Bermudez, 2005). In these systems, spores and microorganisms can interact with certain ingredients (sugars, fats, salt, etc.) which then could possibly lead to a retarded or incomplete inactivation (Olivier et al., 2011).

HPTS has not yet been implemented as a process in the food industry for sterilization. This might be due to a lack of a suitable microbiological indicator, similar to the use of *Geobacillus stearothermophilus* (GBS) for the thermal sterilization. For the HPTS, some authors have mentioned that spores of *Bacillus amyloliquefaciens* (BA) and *Clostridium botulinum* are high pressure resistant (Buckow & Heinz, 2008; Bull, Olivier, van Diepenbeek, Kormelink, & Chapman, 2009; Margosch, Gänzle, Ehrmann, & Vogel, 2004; Margosch et al., 2006).

For a high pressure process control, it is also stated by some research groups to use other indicators for the HPP of foods such as the density of copper tablets or the activity of a certain test enzyme, after the treatment (Grauwet, van der Plancken, Vervoort, Hendrickx, & van Loey, 2010; Minerich & Labuza, 2003).

Since in HPTS and conventional thermal sterilization the main target is the inactivation of all pathogenic microorganisms and spores, elevated temperatures are needed, even for high pressure processing, to gain a commercial sterile product (Rajan, Pandrangi, Balasubramaniam, & Yousef, 2004; Wimalaratne & Farid, 2008).

Temperatures between 90 and 121 °C at 600 MPa are proposed by numerous authors to achieve economical holding ($\leq$10 min) times through HPTS (Balasubramaniam, 2009; Koutchma, Guo, & Patazca, 2005; Margosch et al., 2004; Mathys et al., 2009; Rajan et al., 2004).

It is crucial that the threshold pressure of 600 MPa or higher is reached during the process to assure the complete and rapid inactivation of the spores (Reineke, 2012b).

At this point the only certified process for a sterilization using high pressure is the so called pressure assisted thermal sterilization (PATS) using 121.1 °C at 600 MPa (U.S. Food and Drug Administration, 2009) (Institute of food safety and health Illinois, 2009).

The main advantages of PATS are the short heating times due to the compression heating, which can range between 3 and 9 °C/100 MPa depending on the food system and the reduction of the overall thermal load applied to the product (Juliano & Barbosa-Canovas, 2005; Knoerzer et al., 2010; Matser et al., 2004). All these attributes can lead to a better and healthier product, because unwanted reactions, like the Maillard reaction which are temperature and time dependent, might not occur or can be reduced by high pressure (De Vleeschouwer, Van der Plancken, Van Loey, & Hendrickx, 2010). Thereby the formation of unwanted and harmful substances could be limited, if its specific reaction volume is positive (Le Chatelier's principle).

In the recent years, attention has been given to known and suspected carcinogenic food processing contaminants (FPCs). By definition FPCs are substances present in food as a result of food processing/preparation that are considered to exert adverse physiological (toxicological) effects in humans, i.e., substances which create a potential or real risk to human health. Little data has been published supporting the possible formation or reduction of unwanted FPCs, such as furan or monochloropropanediols and their esters (MCPD/MCPD-esters), by high pressure processing or conventional heat processing in general.

The sources of furan in foods are manifold; one point of origin is the Maillard reaction (thermal degradation) with the precursors being sugars (glucose and fructose) and amino acids other reaction pathways start with poly-unsaturated fatty acids, ascorbic acid or carotenoids (Crews & Castle, 2007; Vranová & Ciesarová, 2009). Furan is generally more abundant in closed heated food systems like cans and glasses and is associated with the flavor of the food.

High amounts of furan have been found in canned soups, canned meat, canned vegetables and coffee (239–5050 μg kg^{-1}).

Furan contents between 20 and 70 μg kg^{-1} can be described as moderately high (Crews & Castle, 2007). The furan-induced toxicity is due to cytochrome P450 activity (liver enzyme CYP2E1) which converts furan into cis-2-butene-1,4-dial. The compound formed can bind to proteins and nucleosides.

This can lead to serve DNA damage and a deficit of function in the affected compounds (Stadler & Lineback, 2009). The acceptable daily intake (ADI) was set to 2 μg kg^{-1} body weight (Lachenmeier & Kuballa, 2010).

The formation of MCPD/MCPD-esters is mostly associated with fat-containing food systems where refined fats or oils are used. These ingredients can already contain high amounts of MCPD/MCPD-esters due to prior steps during the refining of these fats/oils (Larsen, 2009). Precursors are glycerol, glycerides and chloride (Hamlet, Sadd, Crews, Velisek, & Baxter, 2002). 3-MCPD is known to be an animal carcinogen (Tomar, Feng, & Martha, 2010) but studies concerning the toxicity of MCPD-esters are limited at this point, but there is evidence of kidney and testicular damage similar to the effects of free 3-MCPD (Crews et al., 2013). Many internationally recognized authorities (Bundesinstitut für Risikoforschung (Bfr), European Food Safety Authority (EFSA), U.S. Food and Drug Administration (FDA)) see a demand for action to reduce amounts of these compounds in foods (BfR., 2004, 2012; Larsen, 2009; Tan, 2011). The amounts of MCPD-esters found in refined edible vegetable oils can range from 0.3 to 10 mg kg^{-1}, in other foods e.g. French fries (0.04–0.40 mg kg^{-1}) and jarred foods (0.011 mg kg^{-1}) are lower but still reasonably high (Crews, 2012).

Since both compounds are thought to be carcinogenic, it is uncertain to what amount the MCPD-esters are hydrolyzed within the digestive system and toxic MCPD is set free (Crews et al., 2013; Larsen, 2009; Stadler & Lineback, 2009). The ADI therefore is 2 μg of free MCPD per kg body weight. The need to reduce these FPCs exists because at this point not much is known about possible long-term accumulative effects of these compounds in the human body.

The aim of this study was to investigate the influence of the HPTS on the inactivation of spores of the strains *B. amyloliquefaciens* and *G. stearothermophilus* inoculated into commercially available food systems: tuna in brine (TB), tuna in sunflower oil (TO), sardines in olive oil (SO) and in an high pressure stable buffer solution (ACES-buffer). The formation of FPCs was monitored during the sterilization process and compared to the amounts found in retorted samples of the same food systems.

2. Materials and methods

2.1. Spore strains and spore preparation

Using a method described elsewhere for (Paidhungat et al., 2002) *B. amyloliquefaciens* (Technische Mikrobiologie Weihenstephan, 2.479, Fad 82), sporulation was induced at 37 °C on solid 2× SG medium agar plates without antibiotics.

The spore suspension was cleaned by repeated centrifugation (3-fold at 5000*g*), washed with cold distilled water (4 °C), and was treated occasionally with sonication for 1 min.

The cleaned spore suspensions contained $\geq$95% phase bright spores and nearly no spore agglomerates, as was verified by a particle analysis system (FPIA 3000, Malvern Instruments, Worcestershire, U.K.).

The spore suspensions were stored in the dark at 4 °C. The spores of *G. stearothermophilus* (Sterikon with bioindicator) were purchased from Merck (Darmstadt, Deutschland) with a certified $D_{121.1\ °C} = 1.5–2$ min and also stored at 4 °C in the dark.

2.2. Sample preparation

Untreated unretorted fish cans (tuna in brine, tuna in sunflower oil and sardines in olive oil) were obtained through Nouvelle Vague (Boulogne-sur-Mer, France). The tuna in sunflower oil and the sardines in olive oil in the cans had been precooked and only the tuna in brine was raw. Before the HP treatment the fish samples were minced in a blender and pressed through a sieve afterwards to gain a homogenous matrix.

As a model system ACES-buffer (N-(2-Acetamido)-2-aminoethanesulfonic acid) (Merck KGaA, Darmstadt, Germany, 0.05 mol, pH 7) a relatively pH stable HP buffer was used.

The pressurization media (fish matrices and buffer) were inoculated with spores of *B. amyloliquefaciens* or *G. stearothermophilus* to a spore count of 10^5 to 10^7 per g and mixed with a Stomacher (Seward Medical, London, UK).

For the analyses of furan and MCPD/MCPD-esters, portions of the mixed un-inoculated matrix (1.5 g) were placed in a container (Nunc Cryo Tubes Nr. 375299, Nunc A/S, Roskilde, DK) and put on ice. All the trials were conducted in duplicates.

2.3. HPTS-treatment

The high pressure unit U 111 (Unipress, Warsaw, PL), a lab scale system, was used to conduct the inactivation and formation of FPCs studies. With this unit pressures up to 700 MPa and temperatures up to 130 °C can be reached. The high pressure transmitting medium is silicone oil. The unit consists of five chambers, with a volume of 4 ml, immersed in an oil bath which can be separately used for the high pressure trials. Pressure build-up rate was 25 MPa/s. The temperatures selected for the treatment were 90, 105, 110, 115 and 121 °C at 600 MPa. The oil bath was set on the selected process temperature and the start temperatures for each food system were obtained in pretrials (Table 1).

To monitor the temperature during the treatment a control sample filled with the same fish as used for microbial analysis was put in one of the chambers and equipped with thermocouple (Unipress, Warsaw, PL) and the temperature was measured in the geometrical center of the sample (Fig. 1).

In the fish industry the F_0-value for conventional retorting of fish cans is 7 min, this represents the treatment conditions of 28 min at 115 °C. Therefore the formation of FPCs was monitored under HPTS conditions, equal to $F_0 = 7$ min, which were 115 °C, 28 min and 121 °C, 7 min at 600 MPa. For the temperatures 90 to 110 °C the holding times applied were not equal to $F_0 = 7$ min. Five to seven kinetic points were obtained during these holding times.

Since the inactivation of spores under thermal and high pressure conditions differs in mechanism and speed, the holding times for the HPTS were between 1 s and 30 min depending on the temperature. Five to seven kinetic points were obtained during these holding times.

Table 1
Temperature set up for the HPTS trials at 600 MPa.

Treatment T [°C]	Oil bath T [°C]	ACES T start [°C]	Tuna in brine T start [°C]	Tuna sunflower oil T start [°C]	Sardines in olive oil T start [°C]
90	95	50	50	45	45
105	110	65	65	60	60
110	115	70	70	75	75
115	120	75	75	80	80
121	125	80	80	85	85

After the treatment the samples were put on ice and the samples for the FPCs analysis were later on frozen at −80 °C.

2.4. Analysis of FPCs

Furan: The analysis of furan was conducted by automated headspace gas chromatography–mass spectrometry (HS-GC–MS). Since furan is very volatile (BPt 31 °C) the use of a headspace sampling is the obvious method for furan. The samples were incubated at low temperature (40 °C) to equilibrate furan into the headspace, which was sampled by an injection loop. Furan was quantified by comparison of the peak area of the furan response with that of deuterium labeled furan added at low level to the sample. The method used is described in detail elsewhere (Crews & Castle, 2007).

MCPD/MCPD-esters: The method to analyze free MCPD was described elsewhere (Hamlet et al., 2002; Zelinková et al., 2008). For the MCPD-esters determination two approaches are possible. One involves the conversion of the MCPD-esters to free MCPD by either a base-catalyzed transmethylation (bct), where a loss of MCPD can occur, or an acid-catalyzed transmethylation (act), where the formation of additional MCPD is possible; the other approach is the direct analysis as esters by LC–MS (liquid chromatography–mass spectrometry) or DART-MS (direct analysis in real time-mass spectrometry) described elsewhere (Cajka, Hajslova, & Mastovska, 2008; Hajslova, Cajka, & Vaclavik, 2011; Moravcova et al., 2012), which was used in this case.

2.5. Analysis of the spore inactivation and regression analyses

After the pressure treatment the portions (1.5 g) were mixed with 6 ml of Ringer-solutions and homogenized with glass beads. Afterwards a serial dilution with Ringer-solution down to 10^{-6} was performed and survivors after pressurization were measured by plate count using nutrient agar. Colonies of *B. amyloliquefaciens* were incubated at 37 °C and the *G. stearothermophilus* at 55 °C. Both were counted after 2 days.

In numerous papers describing the influence of high pressure on the inactivation of spores it is mentioned that the inactivation of spores is better described with an nth-order (Eq. (1)) than with a linear approach, since for longer holding times a tailing can occur, but no shouldering was observed (Ananta, Heinz, & Schlüter, 2001; Margosch et al., 2006; Mathys, 2008; Reineke, 2012b).

$$\text{for } n = 1 \quad \log_{10}\left(\frac{N}{N_0}\right) = \log_{10}\left(e^{-k*t}\right) \tag{1}$$

$$\text{for } n > 1 \quad \log_{10}\left(\frac{N}{N_0}\right) = \log_{10}\left(\left((1 + k * N_0^{n-1} * t * (n-1)\right)^{\frac{1}{1-n}}\right) \tag{2}$$

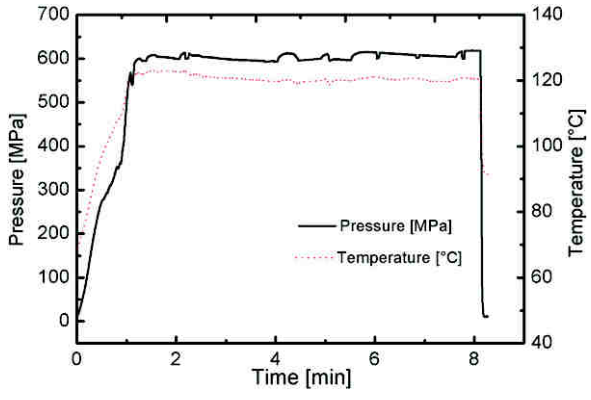

Fig. 1. Pressure temperature profile (121 °C, 600 MPa; PATS) measured inside the vessels of the U 111.

Two parameters can be derived from the model approach, which is unique for every inactivation kinetic and a means by which they can be compared: reaction order and the rate constant. The constant k, the inactivation rate constant, represents the spore-inactivating effect of each pT-combination. N is the shape factor, if n = 1 it is a first order reaction else if n > 1 is nth-order, the shape is up concave and represents the tailing.

For this study the data obtained from plate count was $\log_{10}\left(\frac{N}{N_0}\right)$ and plotted over the time. Isokinetic lines for the temperature–time diagrams, to obtain a 3, 5 and extrapolated 12 $\log_{10}$ inactivation, were derived from kinetic analysis of the experimental inactivation data. To obtain the reaction order all temperature–pressure kinetics were fit over a range of reaction orders (n = 1.0–1.7) (TableCurve2D SPSS Inc., Chicago, IL, USA). The minimal sum of the standard error identified the optimal reaction order. All inactivation-kinetics were refitted with the optimal n to obtain k. To get a functional dependency of k(T), k and T were fitted with all equation set of TableCurve2D (SPSS Inc., Chicago, IL, USA). The equation with the minimal sum of square errors for k(T) was then used in (1) for k. The isorate lines were calculated with MathCAD 2001i professional (Mathsoft Engineering & Education, Inc., USA).

3. Results and discussion

3.1. HPTS of the fish systems

Spores of *B. amyloliquefaciens* and *G. stearothermophilus* inoculated in different food systems were used to investigate the influence of HPTS. The trials were conducted at 600 MPa and temperatures ranging from 90 to 121 °C under isothermal and isobaric conditions (Fig. 1).

For the certified indicator of the thermal sterilization, *G. stearothermophilus*, a 4 log inactivation at 90 °C, 600 MPa could be achieved for all tested systems within 6 to 8 min (Fig. 2). In comparison to the *B. amyloliquefaciens* at 90 °C, 600 MPa much longer holding times were needed to achieve the same inactivation for *B. amyloliquefaciens* (Fig. 3A). Furthermore, the increase to a temperature of 105 °C at 600 MPa resulted in an inactivation of *G. stearothermophilus* (≥5 $\log_{10}$) within a few minutes (results not shown). This is the reason why for higher temperatures the experiments were only conducted with *B. amyloliquefaciens*. At this point, it can be stated that the tested *G. stearothermophilus* strain might be an indicator for the thermal sterilization but is very sensitive towards high pressure processing at elevated temperatures in the selected food systems.

At 600 MPa, 90 °C a 5 log inactivation was reached for ACES-buffer, tuna in brine and tuna in sunflower oil after 15 to 21 min. For sardine in olive oil only 3.3 logs could be inactivated after 28 min (Fig. 3A). In general the food systems at 600 °C, 90 MPa seemed to have an impact on the inactivation or a so called protective effect.

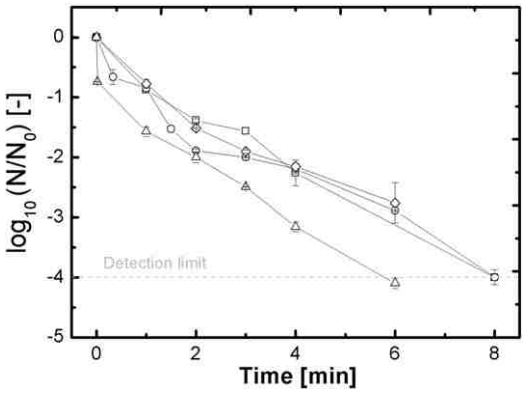

Fig. 2. Effect of pressure and heat on the inactivation of *Geobacillus stearothermophilus* spores in the tested food systems, 600 MPa, 90 °C. Sardines in sunflower oil (□), tuna in sunflower oil (○), tuna in brine (Δ) and ACES-buffer (◇). Initial spore count approximately 10^4 CFU/g.

The increase to 105 °C at 600 MPa (Fig. 3B) showed a shortening of the dwell time to reach an inactivation of 5 logs between 4 and 6 min as well as a slight loss in the protective effect of the food systems.

The inactivation of 5 logs of *B. amyloliquefaciens* spores in the food systems for 110 °C, 600 MPa (Fig. 4A) was possible within 2 to 3 min. The application of 115 °C, 600 MPa resulted in a very rapid and sudden total inactivation of spores in all food systems of approximately 1 min (Fig. 4B). For 121 °C, 600 MPa the inactivation of spores was difficult to show because the inactivation takes place within the first few seconds of the treatment.

To gain a better understanding of the T, t dependencies at 600 MPa a modeling for the spore inactivation in the fish systems and the ACES buffer was conducted for a 3 $\log_{10}$, 5 $\log_{10}$ and an extrapolated 12 $\log_{10}$ inactivation of *B. amyloliquefaciens* (Fig. 5A–C). The inactivation that was achieved during the pressure build up (kinetic point of 1 s) was subtracted from the other kinetic points of each temperature, to have a valid model for isothermal and isobaric conditions. Fig. 5 indicates that an inactivation of the tested spore strain is possible with HPTS even at relatively low temperatures, like 90 °C, although a long dwell times were necessary to achieve an inactivation: the higher the temperature the more rapid the inactivation. The reason for the long and alternating dwell times within the foods can likely be ascribed to the protective effect of certain food ingredients on the spores and the relatively low treatment temperature (Anderson & Esselen, 1949; Knorr & Oxen, 1993; Loncins & Senhaji, 1977a). In addition, sardines in olive oil and tuna in sunflower oil have a water activity which is quite low, between 0.91 and 0.93, which can lead to a retarded inactivation as well (Staack, Ahrné, Borch, & Knorr, 2008). The oils used, e.g., the olive oil with high amounts of polyunsaturated fatty acids (PUFA), can have a protective effect against heat as well (Ababouch, Grimit, Eddatry, & Busta, 1995; Loncins & Senhaji, 1977a, 1977b; Molin & Snygg, 1967). The spores inoculated in tuna in brine where the ones which were inactivated the quickest, even at lower temperatures like 90 °C. For higher temperatures (above 105 °C) the protective effect of the food systems seems to vanish (Figs. 3B & 5).

The dwell times are depending on the food system, whereas temperatures between 107.5 and 115 °C and dwell times between 9.90 and 0.755 min are needed to produce a calculated sterile product (Table 2). However, an indicator spore strain has to be found and established to implement this promising technology (Reineke et al., 2013). In this case, the composition and ingredients of the foods only play an important role for the delayed inactivation of spores at lower temperatures (90 to 105 °C). As stated by Reineke (2012b), if the threshold pressure of 600 MPa is reached, the driving force of the inactivation is the temperature.

3.2. FPCs formation in the high pressure sterilized foods

The comparison between an emerging technology such as high pressure processing and the common retorting in terms of formation of FPCs in real food systems is new at this point and literature concerning this is rare. All the retorted cans were treated at 115 °C for 28 min (total process time 55 min), which equals an F_0 = 7 min.

3.2.1. Formation of furan

The amount of furan in commercial available canned fish can vary from 1.5 to 8 μg kg^{-1} (Crews & Castle, 2007). The analyses of furan in fish samples showed that significant amounts could only be found in canned sardines in olive oil.

In all pressure treated and retorted samples of tuna in brine (0.23–1.5 μg kg^{-1}) and tuna in sunflower oil (0.37–1.1 μg kg^{-1}) furan was nominal and close to the detection limit of the analytical method.

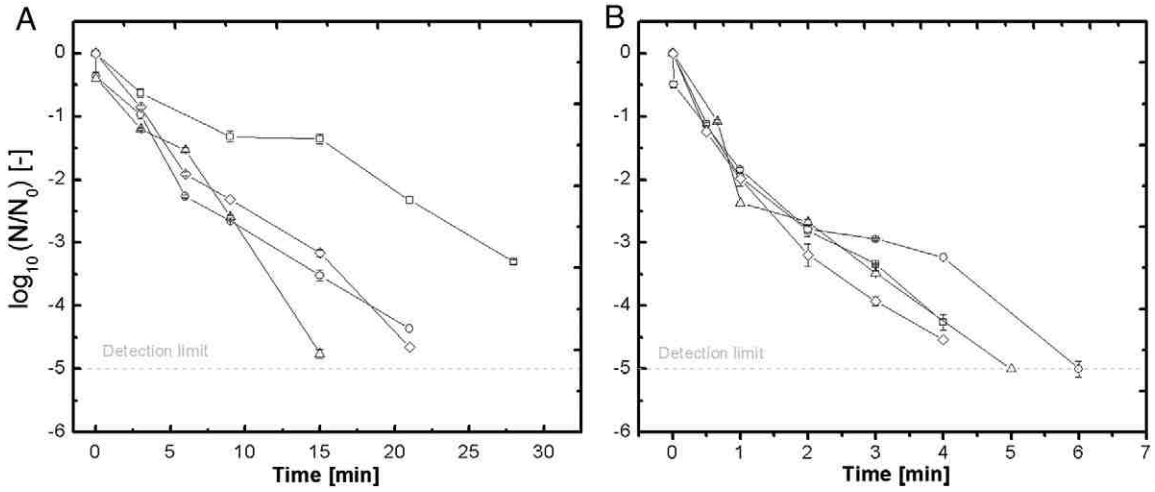

Fig. 3. Effect of pressure and heat on the inactivation of *Bacillus amyloliquefaciens* spores in the tested food systems A) 600 MPa, 90 °C and B) 600 MPa, 105 °C. Sardines in sunflower oil (□), tuna in sunflower oil (○), tuna in brine (Δ) and ACES-buffer (◊). Initial spore count approximately 10^6 CFU/g.

For this reason in Fig. 6A only the furan results for sardines in olive oil under high pressure conditions are shown. For the treatment with 90, 105 and 110 °C at 600 MPa no serious increase of furan over treatment time of 30 min was detectable. The increase of furan over the treatment time was obvious for 115 and 121 °C at 600 MPa. The formation of furan under high pressure conditions might be more time dependent than temperature dependent, since higher amounts are reached after 115 °C, 600 MPa, 28 min in comparison to 121 °C, 600 MPa, 7 min. More research is needed to validate these trends. The data for the formation of furan clearly show that one of the main sources of furan, is the olive oil, in which PUFAs are probably the precursor (Lachenmeier & Kuballa, 2010). As seen for tuna in sunflower oil and tuna in brine (Fig. 6A) the use of refined oil low in PUFA, or no oil leads to very low amounts of furan. The formation of furan is also dependent on the treated food system and the treatment conditions.

Also interesting is that an increase of 5 °C from 105 to 110 °C does not result in higher formation of furan (Fig. 6 A) but in a better overall inactivation of the spores (Fig. 4 A), therefore the temperature pressure combination seems very promising for processing food by HPTS treatment.

The reasons for the lower amounts of furan in the high pressure treated samples could be the shorter overall processing times which result in a reduction of the thermal load; and the Le Chatelier's principle, which states that under high pressure conditions only reactions are favored who have a negative reaction volume (Bravo et al., 2012; Cheftel, 1995; De Vleeschouwer et al., 2010; Stadler & Lineback, 2009). This factor might lead to less or no formation of furan in the tested food systems, if the reaction volume is positive. The comparison of the retorted and the high pressure treated sardines in olive oil (Fig. 6B) shows that depending on the treatment conditions, a reduction of furan in the high pressure treated samples is possible between 71 and 97%. Even at sterilization conditions of 121 °C, 600 MPa a reduction of 78% is possible.

3.2.2. Formation of MCPD/MCPD-esters

In preliminary trials treatment conditions with temperatures ranging from 90–121 °C and holding times of 7–30 min at 600 MPa (Fig. 7) were performed to comprehend which food system contained respectively produced the highest amount of MCPD/-esters. In all tested samples only really low amounts of free MCPD were found.

The results of the preliminary trials indicate that the main focus concerning the formation of MCPD-esters should be put on tuna in sunflower oil. Since here the highest amounts of MCPD-esters are formed respectively found. The quantities found in tuna in brine and in sardines in olive oil (0.8 to 19 μg kg^{-1}) were nominal in all HP treated samples (B–E), the untreated sample (A) and the retorted sample (F) as well. In addition, the untreated sample already contains quite high amounts of MCPD-esters (167 μg kg^{-1}) which are derived from the refined oil

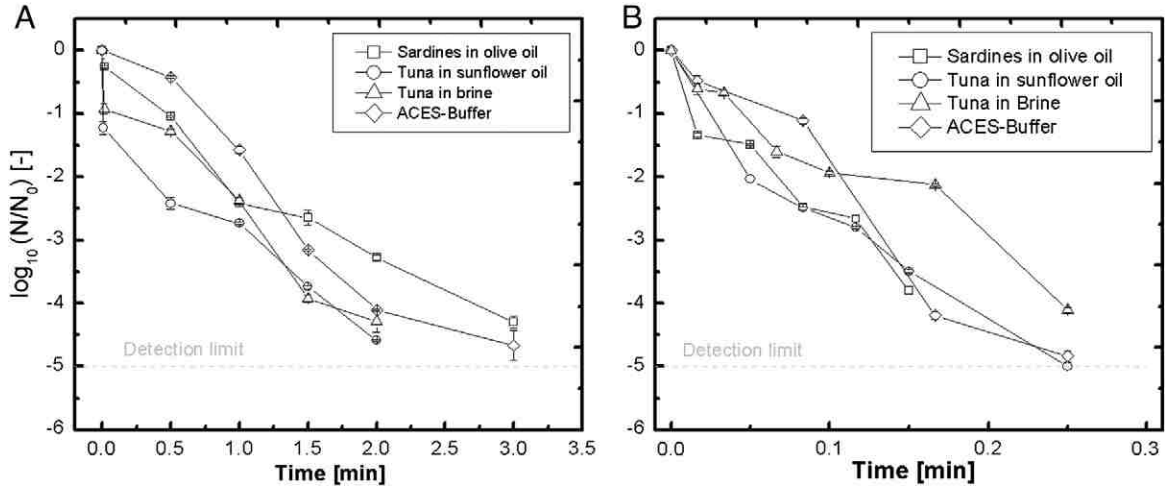

Fig. 4. Effect of pressure and heat on the inactivation of *Bacillus amyloliquefaciens* spores in the tested food systems A) 600 MPa, 110 °C and B) 600 MPa, 115 °C. Sardines in sunflower oil (□), tuna in sunflower oil (○), tuna in brine (Δ) and ACES-buffer (◊). Initial spore count approximately 10^6 CFU/g.

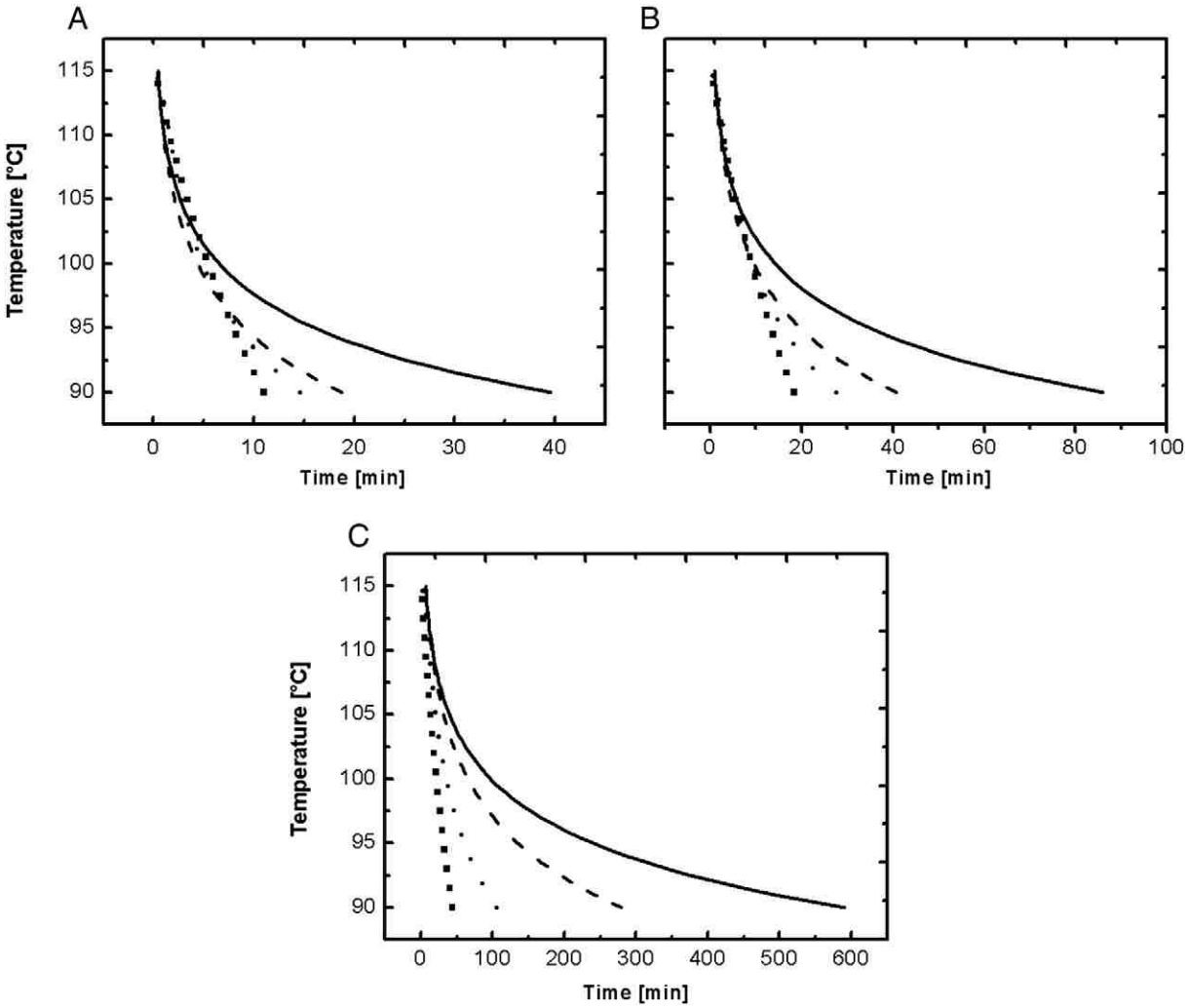

Fig. 5. Isorate lines for A) 3 $\log_{10}$, B) 5 $\log_{10}$ and C) extrapolated 12 $\log_{10}$ inactivation of *Bacillus amyloliquefaciens* in sardines in olive oil (solid line), tuna in sunflower oil (dashed line), tuna in brine (squares) and ACES-buffer (dotted line).

used (672 μg kg^{-1}). All treated samples contain lower (A–D) or equal (E) amounts in comparison to the retorted sample (F). Further analytic studies of the formation of MCPD-esters in tuna in sunflower oil over different time–temperature conditions at 600 MPa were conducted (Fig. 8).

The quantities found in tuna in sunflower oil for the different temperature time combinations at 600 MPa showed no clear trend for any combination. In the untreated sample, 167 μg kg^{-1} of MCPD-esters was found and the highest amount in the HP treated samples was found in 115 °C, 13 min, 600 MPa with 201 ± 60 μg kg^{-1}. In general, there was a slight increase of MCPD-esters in the samples over time and pressure. In comparison to the retorted samples with 180 ± 81 μg kg^{-1} of MCPD-esters equal amounts were formed but since the standard deviations of the samples were quite high a clear comparison is rather difficult.

The use of sunflower oil as an ingredient for foods can already lead to increased amounts in the untreated food systems. Otherwise the results (Fig. 7) indicate that the use of non-refined oils or no oil in the tested food systems results in no or a low formation of MCPD-esters.

The interpretation of the formation of MCPD-esters over time, temperature and pressure is quite difficult but it can be indicated that in comparison to the untreated samples no further increase can be detected during the HPTS (Fig. 8).

If one compares the retorted sample (180 ± 81 μg kg^{-1}) with the HPTS (max. value 201 ± 60 μg kg^{-1}) treated samples the choice of the process does not make a difference in terms of formation of MCPD-esters. Here the main goal should be to reduce the amounts of MCPD-esters in the food by changing the recipe towards non-refined oils.

Since for both compounds, furan and MCPD-esters, the bioavailability and possible long-term effects are still uncertain, the reduction or no increase during the processing of foods is an important matter. For the MCPD-esters, the percentage of hydrolysis into MCPD is unknown but experiments with rats showed that between 86 and 100% of the 3-MCPD was released (BfR., 2012). In the worst case scenario the ADI would be reached for the average person weighing 74.5 kg after eating 0.780 kg of canned fish or other heat treated enclosed foods containing equal amounts of MCPD-esters.

Table 2
Extrapolated temperature and time combinations to achieve a 12 $\log_{10}$ inactivation of *Bacillus amyloliquefaciens* in the tested food-/model systems at 600 MPa.

	T, t [°C, min] combination ≤10 min	t [min] at 115 °C, 600 MPa
Sardines in olive oil	113, 9.40	6.60
Tuna in sunflower oil	113, 9.90	7.4
Tuna in brine	107.5, 9.85	0.755
ACES-buffer	110, 9.40	1.15

4. Conclusion

The results of this study clearly show that a reduction of unwanted food processing contaminants is possible with HPTS compared to thermal retorting with an equal F_0 of 7 min. Furthermore, this technique

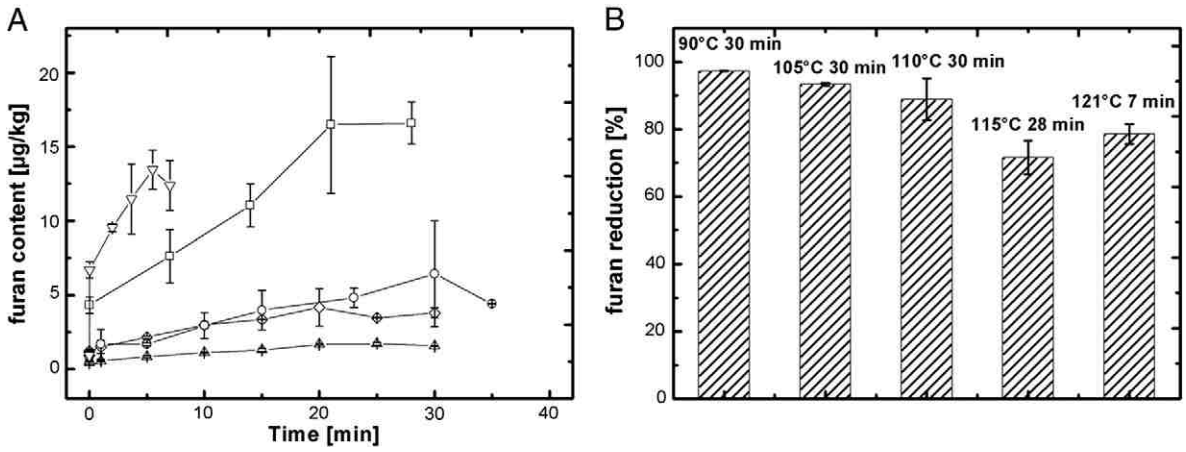

Fig. 6. A) Formation of furan under high pressure (600 MPa) conditions in sardines in olive oil. B) Reduction of furan in sardines in olive oil under high pressure (600 MPa) in comparison with the amount formed during retorting of 57.88 µg kg^{-1} (28 min, 115 °C). 90 °C, 600 MPa (Δ); 105 °C 600 MPa (◊); 110 °C 600 MPa (○); 115 °C 600 MPa (□) and 121 °C 600 MPa (▽).

might be a means by which a better overall quality can be achieved in foods. This is even the case for sterilization conditions at 121 °C and 600 MPa.

More importantly than a reduction of FPCs from a food technologist's point of view is the availability of a safe, spore and pathogenic microorganism free product with a long shelf life.

The results show that the *G. stearothermophilus* is unsuitable as an indicator for the HPTS since the *B. amyloliquefaciens* is more pressure–temperature resistant. The modeled inactivation kinetics also indicate that economical dwell times (≤10 min) could have been reached with the temperature–pressure combination of 107–115 °C, 600 MPa. Although more research must be conducted in the future to verify these findings.

At this point, the process most suitable for the sterilization of the tested food system with pressures being 600 MPa is the PATS (121.1 °C, 600 MPa). Since it is the only certified process and as the data sets depict, PATS can guarantee a safe and sterile product with dwell times of 7 min, which equals an F_0-value of 7 min, and also reduces the amounts of FPCs in comparison to the retorting. In comparison to the *G. stearothermophilus* and also compared to other tested spore strains (Margosch et al., 2004; Olivier et al., 2011) *B. amyloliquefaciens* might can become a suitable microorganism for the HPTS. This could lead to the implementation of this promising process within the food industry.

The advantages of PATS are the short heating times due to the additional compression heating, the isobaric and isothermal conditions during the holding time and the cooling effect after decompression due to the loss of the compression heating (Fig. 9).

All these factors mentioned cater for a reduced thermal load in comparison to the retorted samples which leads to less FPCs formed in the food and therefore to a better quality even for equal F_0-values. It could be possible to create individual treatment conditions to obtain the best results possible for each food system. The data clearly indicate that HPTS can result in lower processing times needed and thus in an overall higher product quality and reduced formation of FPCs such as furan and MCPD-esters.

For the future, more research needs to be carried out with other food systems under the same conditions. The construction of a system that can cope with high pressures and high temperatures needs to be driven on to a pilot scale and an industrial scale level to ensure homogenous temperature distribution in vessel and product.

Acknowledgments

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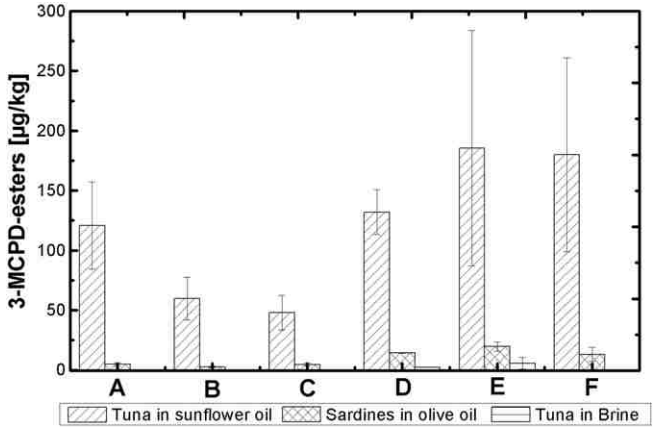

Fig. 7. Formation of MCPD-esters in the different treated food systems, A) untreated, B) 600 MPa, 90 °C, 30 min, C) 600 MPa, 105 °C, 20 min, D) 600 MPa, 115 °C, 28 min (F(0) = 7), E) 600 MPa, 121 °C, 7 min (F(0) = 7), F) retorted samples 115 °C, 28 min.

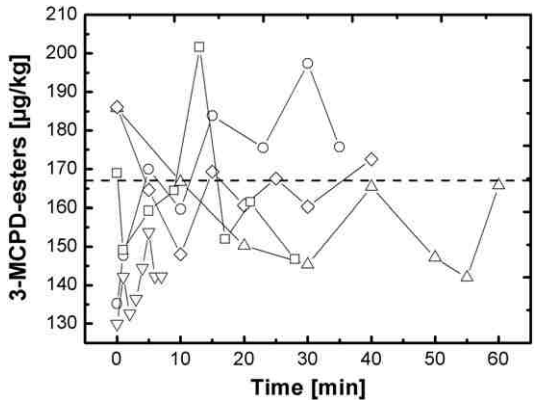

Fig. 8. Formation of 3-MCPD-esters under high pressure conditions (600 MPa) in tuna in sunflower oil. Standard deviations are not shown, range between ±40–59 µg kg^{-1}. 90°C, 600 MPa (Δ); 105 °C, 600 MPa (◊); 110 °C, 600 MPa (○); 115 °C, 600 MPa (□) and 121 °C 600 MPa (▽) and (- - -) untreated.

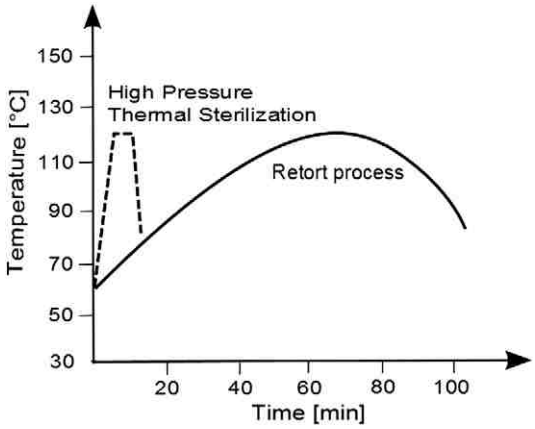

Fig. 9. Comparison of the HPTS and retort process. Adapted from Matser et al. (2004).

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6. High-Pressure Thermal Sterilization: Food Safety and Food Quality of Baby Food Puree

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High-Pressure Thermal Sterilization: Food Safety and Food Quality of Baby Food Puree

Robert Sevenich, Elke Kleinstueck, Colin Crews, Warwick Anderson, Celine Pye, Katerina Riddellova, Jaromir Hradecky, Eliska Moravcova, Kai Reineke, and Dietrich Knorr

Abstract: The benefits that high-pressure thermal sterilization offers as an emerging technology could be used to produce a better overall food quality. Due to shorter dwell times and lower thermal load applied to the product in comparison to the thermal retorting, lower numbers and quantities of unwanted food processing contaminants (FPCs), for example, furan, acrylamide, HMF, and MCPD-esters could be formed. Two spore strains were used to test the technique; *Geobacillus stearothermophilus* and *Bacillus amyloliquefaciens,* over the temperature range 90 to 121 °C at 600 MPa. The treatments were carried out in baby food puree and ACES-buffer. The treatments at 90 and 105 °C showed that *G. stearothermophilus* is more pressure-sensitive than *B. amyloliquefaciens*. The formation of FPCs was monitored during the sterilization process and compared to the amounts found in retorted samples of the same food. The amounts of furan could be reduced between 81% to 96% in comparison to retorting for the tested temperature pressure combination even at sterilization conditions of F_0-value in 7 min.

Keywords: baby food puree, *Bacillus amyloliquefaciens*, bacterial endospores, food processing contaminants, high-pressure thermal sterilization

Practical Application: High-pressure thermal sterilization (HPTS) is an emerging technology to produce high-quality low-acid foods, which are shelf-stable at ambient temperature. However, an industrial scale process has not yet been implemented. The conducted work shows different temperature combinations (90 to 121°C) at 600 MPa and their influence on the endospore inactivation and on the formation of unwanted food process contaminants (FPCs), such as furan, compared to retorting. Use of HPTS could lead to shorter process times compared to retorting. Since the consumer groups are babies and infants, the reduction of harmful substances in their daily diet is an extra benefit.

Introduction

The use of high-pressure processing (HPP) in the food industry was developed as an alternative to common thermal processes, such as pasteurization and sterilization, to produce a microbiologically safe food while avoiding and reducing undesirable changes in sensory, physiochemical, and nutritional properties of foods (Smelt 1998; Hogan and Kelly 2005; Welti-Chanes and others 2005; Oey and others 2008). The use of high pressure at high temperatures as a tool for sterilization (high-pressure thermal sterilization [HPTS]) may lead to benefits in terms of food safety and food quality when compared to conventional retorting. This promising technology is not yet available at industrial scale level; however, recent research may trigger the use of this application for certain foods (Matser and others 2004; Heinz and Knorr 2005; Knoerzer and others 2007; Juliano and others 2009; Reineke and others 2013a). As shown by Sevenich and others (2013) for canned fish products (tuna in brine, tuna in sunflower oil, and sardine in olive oil), shorter dwell times in comparison to the retorting as well as a reduction of unwanted and carcinogenic food processing contaminants (FPCs), such as furan, can be achieved depending on the food matrix. The consumer groups for this product are infants and babies, who are sensitive and potentially vulnerable. Therefore, it is important to minimize the risk of their exposure to harmful food constituents, and the outcome of a safe product must be a priority in food processing (Escobedo-Avellaneda and others 2011; Bravo and others 2012).

HPTS combines the synergistic effects of elevated temperatures (90 to 121 °C) and pressures above or equal to 600 MPa to realize a quick and sufficient inactivation of endospores (Matser and others 2004; Knoerzer and others 2007; Reineke and others 2013a). An additional benefit is the compression heating, which is caused by the compression work against intermolecular forces if pressure is applied and results in a temperature increase. Depending on the food system, this temperature increase can range from 3 to 9 °C per 100 MPa and helps additionally to heat up the product to the required temperatures; whereas the thermal load applied to the product can be reduced (Matser and others 2004; Barbosa-Canovas and Juliano 2008; Knoerzer and others 2010). However, heat losses to the equipment and environment must be taken into account. For HPTS treatment, the product needs to be preheated to 70 to 90 °C and through compression heating, during the pressure buildup, the process temperature can reach 90 to 130 °C. For scale-up applications of HPTS, it would be important that preheating, for example, in a water bath and pressure

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buildup time are fine-tuned to guarantee optimal and quick process (Heinz and Knorr 2005; Barbosa-Canovas and Juliano 2008). The temperature nonuniformity in the treatment chamber can vary for industrial units approximately 10 °C, and is also a factor that needs to be taken into account to ensure the safety of the process (Knoerzer and others 2007; Grauwet and others 2012; Martinez-Monteagudo and others 2012).

A substantial amount of research has been put into understanding the underlying mechanisms of the high-pressure inactivation of spores in aqueous solutions (mostly ACES-buffer) over the last decade but until today not all is totally understood (Wuytack and others 1998; Paidhungat and others 2002; Ahn and Balasubramaniam 2007; Black and others 2007; Mathys 2008). The dissociation equilibrium (for example, pH-shift) under high pressure can play an important role for sensitive reactions, for example, inactivation of microorganisms (Mathys and others 2008). Therefore, it is important that for the understanding of the underlying mechanisms of the high-pressure inactivation to use a stable buffer system (ACES, pH = 7, 0.05 M). In real food systems, this pH-shift also may be of great importance for a quick inactivation.

The inactivation of spores under high-pressure conditions is at least a 2-step mechanism (Wuytack and others 1998; Margosch and others 2004a; Mathys and others 2007; Reineke and others 2012; Reineke and others 2013a). At pressures above or equal 600 MPa, an opening of the Ca^{2+}—Dipicolinicacid (DPA)—channels occurs: (i) DPA is released from the spore core; (ii) the spore core gets dehydrated; (iii) the spore starts to germinate; and (iv) therefore, it becomes thermo- and pressure-sensitive and can be inactivated (Setlow 2003; Reineke and others 2013b). For a rapid and sudden inactivation of spores under pressure, it is important to apply pressures $\geq$ 600 MPa and temperatures above 60 °C to ensure the loss of heat resistance (Reineke and others 2013a). Since for HPTS and conventional thermal sterilization of low-acid foods, the main target is the inactivation of pathogenic microorganisms and spores, elevated temperatures are needed to gain a commercially sterile product, even for HPP (Rajan and others 2006; Wimalaratne and Farid 2008). Temperatures between 90 and 121 °C at 600 MPa have been proposed by numerous authors to achieve economical holding times through HPTS (Margosch and others 2004b; Koutchma and others 2005; Rajan and others 2006; Balasubramaniam 2009; Mathys and others 2009).

The lack of implementation of HPTS in the food industry might be due to the lack of an indicator strain to demonstrate an acceptable inactivation of pathogenic and spoilage bacteria spores. Clostridium spores (*Clostridium botulinum*, *Clostridium sporogenes, and Clostridium perfringens*) and Bacillus spores (*Bacillus amyloliquefaciens, Geobacillus stearothermophilus*) are mentioned by numerous research groups as being very highly pressure and temperature resistant (Ahn and Balasubramaniam 2007; Wimalaratne and Farid 2008; Juliano and others 2009; Ramirez and others 2009; Reineke and others 2012; Reineke and others 2013a).Although no commercial units are currently operating, pilot scale systems are available, and have been used to demonstrate that HPTS can work as a surrogate for the thermal sterilization (Barbosa-Canovas and Juliano 2008).

The food system itself can have a protective effect on to the spores because certain ingredients such as fats, sugars, salts, and the resultant water activity of the food can lead to retarded inactivation (Anderson and others 1949; Härnulv and others 1977; Senhaji and Loncin 1977; Oxen and Knorr 1993; Ababouch and others 1995; Sevenich and others 2013). This is why the application of HPTS needs to be tested in real food systems to ensure the safety of this process, the only certified process to this point that uses high pressure for sterilization purposes is the so-called pressure-assisted thermal sterilization (PATS), with 600 MPa and 121.1 °C (U.S. Food and Drug Administration, 2009 [NCFST 2009]). These conditions, as shown by Sevenich and others (2013), could work in terms of food safety (12 D-concept) and also reduction of FPCs. Although depending on the food system, lower temperatures are possible.

FPCs, in general, are compounds that are formed during the processing of a food with a negative impact and a risk on human health. Many FPCs arise from the Maillard reaction with precursors being sugars (glucose and fructose) and amino acids; other reaction pathways can involve poly-unsaturated fatty acids, ascorbic acid, or carotenoids (Crews and Castle 2007; Vranova and Ciesarova 2009; Lachenmeier and Kuballa 2010).

Little is known about the long-term cumulative effects of FPCs in the human body. Due to this, the acceptable daily intake (ADI) for most of these compounds (MCPD, acrylamide, and furan) is set to 2 μg per kg body weight (Jestoi and others 2009; Lachenmeier and Kuballa 2010). Its low ADI makes furan a threat for the aforementioned consumer groups (babies and infants) since given their body weight around 8 to 10 kg and with a daily diet of 1 kg of food, 18 μg/kg of furan would be critical.

Furan is generally more abundant in closed heated food systems like cans and jars and is associated with the flavor of the food (Vranova and Ciesarova 2009). The furan-induced toxicity is due to cytochrome P450 activity (liver enzyme CYP2E1) which converts furan into cis-2-butene-1,4-dial. The compound formed can bind with proteins and nucleosides, which causes severe DNA damage and a deficit of function in the affected compounds (Bolger and others 2009). Levels of furan found in baby food can vary depending on the food's ingredients. The mean concentrations of furan in baby food products reported by Jestoi and others (2009) were 9.2, 37.0, and 49.6 μg/kg for fruit-, vegetables-, and meat-containing baby foods, respectively.

The benefits which HPTS offers—compression heating and decompression cooling and a quick spore inactivation—could lead to a valuable and safe product because unwanted reactions, such as the temperature and time-dependent Maillard reaction, might not occur or might be reduced by high pressure (Ramirez and others 2009; De Vleeschouwer and others 2010; Sevenich and others 2013). The formation of unwanted and harmful substances could be limited, if their specific reaction volume is positive (Le Chatelier's principle) (Ramirez and others 2009; Escobedo-Avellaneda and others 2011; Bravo and others 2012). The Le Chatelier's principle states that any phenomenon (chemical reaction, phase transition, and so on) accompanied by decrease in reaction volume is enhanced by pressure. Therefore, pressure shifts the system to that of the lowest volume (Cheftel and Culioli 1997; Black and others 2007).

Moreover, the pressure-induced pH-shift during the HPTS could also be a rate limiting factor (Escobedo-Avellaneda and others 2011). The aim of this study was to investigate the influence of the HPTS on the spore inactivation of the strains *B. amyloliquefaciens* and *G. stearothermophilus* inoculated into an vegetable-based baby food puree and in a high-pressure stable buffer solution (ACES-buffer, pH = 7) (Mathys and others 2008). The formation of FPCs (furan) was monitored during the sterilization process and compared to the amounts found in retorted samples of the same food system.

Materials and Methods

Spore preparation

Using a method described elsewhere for *B. amyloliquefaciens* (Technische Mikrobilogie Weihenstephan, 2.479, Fad 82) (Paidhungat and others 2002), sporulation was induced at 37 °C on solid 2× SG medium agar plates without antibiotics. The harvest was carried out when 90% of the spores were phase bright under the light microscope, which took 2 to 3 d. The spore suspension was cleaned by repeated centrifugation (3-fold at 5000×*g*), washed with cold distilled water (4 °C), and was treated with sonication for 1 min. The cleaned spore suspensions contained ≥95% phase bright spores and nearly no spore agglomerates, as was verified by a particle analysis system (FPIA 3000, Malvern Instruments, Worcestershire, U.K.). The spore suspensions were stored in the dark at 4 °C.

The spores of *G. stearothermophilus* (Sterikon with bioindicator) were purchased from Merck (Darmstadt, Germany) with a certified $D_{121.1\,°C} = 1.5$ in 2 min and also stored at 4 °C in the dark.

Preparation of samples

For the baby food puree, frozen vegetables were mixed in accordance with common baby food puree recipes, the following composition was used as a model recipe for a vegetable-based baby food puree: carrots 40%, peas 20%, zucchini 15%, water 24.9%, and 0.1% salt. The mixture was heated up to 85 °C and then pureed with a blender. For a better homogenization, the puree was pressed through a sieve (normal kitchen sieve). The a_w-value of the puree was 0.96, the pH 6.47, and dry matter content 8%. As a model system ACES-buffer (Merck KGaA, 0.05 mol, pH 7), a relatively pH stable HP (high pressure) buffer was used. The pressurization media (baby food puree and buffer) were inoculated with spores of *B. amyloliquefaciens* or *G. stearothermophilus* to a spore count of 10^5 to 10^7 and mixed with a Stomacher (Seward Medical, London, U.K.). Portions (1.5 g) of the mixed matrix were placed in a container (Nunc Cryo Tubes Nr. 375299, Nunc A/S, Roskilde, Denmark) and pressure treated under isothermal isobaric conditions during the pressure dwell time.

The thermal inactivation of the tested spores strains in phosphate buffered saline (PBS) buffer and baby food puree was carried out in thin glass capillaries ($V = 60\ \mu L$). These were hermetically sealed to prevent evaporation according to a method described elsewhere (Mathys 2008).

High pressure and thermal treatment

The high-pressure unit U 111 (Unipress, Warsaw, Poland) was used to conduct the inactivation and formation of FPCs studies. With this unit, pressures up to 700 MPa and temperatures up to 130 °C can be reached. The high-pressure transmitting medium was silicone oil. The unit consists of 5 chambers, with a volume of 4 mL, immersed in an oil bath. Pressure buildup rate was 25 MPa/s.

To monitor the temperature during the treatment, a control sample filled with baby food puree was put in one of the chambers equipped with a thermocouple (Unipress) placed in the geometrical center of the sample (Figure 1). The temperatures selected for the treatment were 90, 105, 110, 115, and 121 °C at 600 MPa. The oil bath of the U111 was set on the selected process temperature and the start temperatures for each food system were obtained in pretrials (Table 1).

Table 1–Temperature setup for the high-pressure thermal sterilization trials at 600 MPa.

Target temperature [°C]	Temperature of oil bath [°C]	Matrix	Start temperature [°C] of pressure buildup
90	95	Baby food puree	38
		ACES-buffer	35
105	110	Baby food puree	63
		ACES-buffer	65
110	115	Baby food puree	68
		ACES-buffer	67
115	120	Baby food puree	70
		ACES-buffer	73
121	126	Baby food puree	77
		ACES-buffer	80

The thermal treatment in glass capillaries was conducted with the same temperatures at 0.1 MPa. A thermostatic bath (cc2, Huber GmbH, Offenburg, Germany) filled with silicon oil (M40.165.10, Huber GmbH) was used for the treatment. Following thermal treatment, the samples were immediately transferred into an ice bath.

After the high pressure and thermal treatment, a serial dilution with Ringer solution down to 10^{-6} was performed and survivors after the 2 treatments were measured by plate count in 2 replicates using nutrient agar. Colonies of *B. amyloliquefaciens* were incubated at 37 °C and the *G. stearothermophilus* at 55 °C. Both were counted after 2 d.

In the food industry, the F_0 at 121 °C for conventional retorting of food is most often 7 min. However, a comparison with the high-pressure sterilization in terms of spore inactivation is difficult, because at high temperature and 600 MPa, the inactivation can be very sudden and rapid. Therefore, the holding times for the spore inactivation at 600 MPa from 90 to 115 °C are not equal $F_0 = 7$ min. For the analysis of FPCs, the longest holding time at 115 °C, 600 MPa and 121 °C, 600 MPa was equal to $F_0 = 7$ min to achieve a better comparability to the retorted samples that were treated at an equivalent temperature to 115 °C for 28 min.

The trials for the analysis of furan were completed in duplicate. Portions of the mixed matrix (1.5 g) were placed in a container (Nunc Cryo Tubes Nr. 375299, Nunc A/S) and put on ice. After

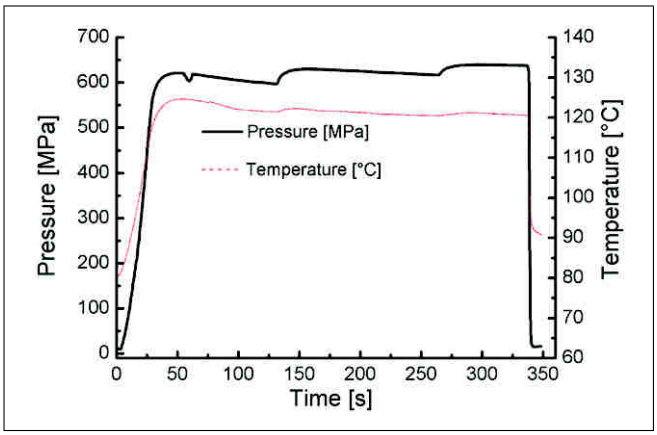

Figure 1–Pressure temperature profile measured in the geometrical center of a baby food puree sample (121 °C, 600 MPa; pressure-assisted thermal sterilization) inside the vessels of the Unipress Multivessel U 111.

the treatment, the samples were put on ice to reduce further FPC formation and soon after frozen at −80 °C.

Analyses of FPCs

The amount of furan in the samples was measured by 2 different approaches. The analysis of furan was conducted by automated headspace gas chromatography-mass spectrometry (HS-GC-MS), which is the most common approach (hereinafter referred to as method A) carried out by the Food Environmental Research Agency (FERA) (York, England). Because furan is very volatile (boiling point temperature 31 °C), the use of a headspace sampling is the obvious method for furan. The samples were incubated at low temperature (40 °C) to equilibrate furan into the headspace, which was sampled by an injection loop. Furan was quantified by comparison of the peak area of the furan response with that of deuterium-labeled furan added at low level to the sample. The used method is described in detail elsewhere (Crews and Castle 2007).

Another approach to measure the volatiles (furan) was done with a solid-phase microextraction (HS-SPME) and then run through gas chromatography-mass spectrometry (GC-MS) (hereinafter referred to as method B) carried out by the Inst. of Chemical Technology (Prague, Czech Republic). The SPME offers the possibility to analyze even really low levels of furan, however, at 5 μg/kg with a 29% of uncertainty (Jestoi and others 2009).

Regression analysis

Most of the inactivation kinetics for emerging technologies such as high pressure, pulsed electric fields, or plasma do not follow first-order kinetics. The reason for this can be manifold: protective effect of media, preservation factor, or the experimental procedure (Bermudez-Aguirre and Barbosa-Canovas 2011). For this study, spore inactivation was explained by a mathematical model based on the *n*th-order kinetics. In numerous papers describing the influence of high pressure on the inactivation of spores, it is noted that the inactivation of spores is better described with an *n*th order (Eq. 1 and 2) than with a linear approach, since for longer holding times, a tailing can occur (Ananta and others 2001; Margosch and others 2006; Mathys 2008; Bermudez-Aguirre and Barbosa-Canovas 2011; Reineke and others 2012).

$$\text{for } n = 1, \quad \log_{10}\left(\frac{N}{N_0}\right) = \log_{10}(e^{-k*t}) \tag{1}$$

$$\text{for } n > 1, \quad \log_{10}\left(\frac{N}{N_0}\right) = \log_{10}\left(1 + k * N_0^{n-1} * t * (n-1)^{\frac{1}{1-n}}\right) \tag{2}$$

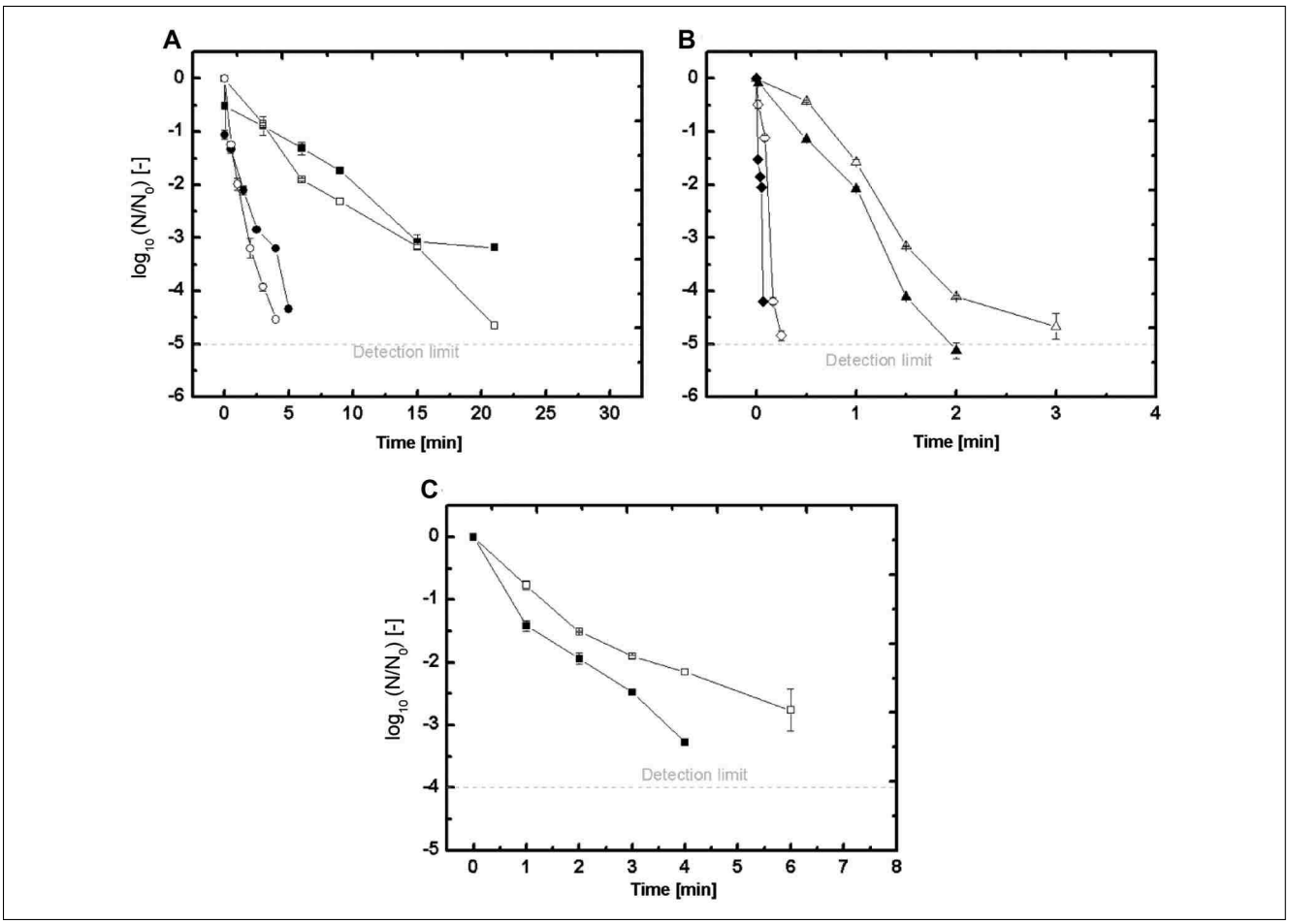

Figure 2–Effect of pressure (600 MPa) and heat on the inactivation of (A) *B. amyloliquefaciens* in baby food puree and ACES-buffer for *T* final being 90 °C (■,□) and 105 °C (●,○), (B) *B. amyloliquefaciens* in baby food puree and ACES-buffer for *T* final being 110 °C (▲,Δ) and 115 °C (♦,◇), and (C) *G. stearothermophilus* spores in baby food puree and ACES-buffer for *T* final being 90 °C (■,□). Initial spore count approximately 107 CFU/g for (A) and (B) and 105 CFU/g for (C).

Two parameters can be derived from the model approach 1 and 2), which is unique for every inactivation kinetic and a means by which they can be compared: n reaction order and k the rate constant. The constant k, the inactivation rate constant, represents the spore-inactivating effect of each pT-combination. n is the shape factor, if $n = 1$, it is a first-order reaction, while if $n > 1$ is nth-order, the shape is up concave and represents the tailing.

Isokinetic lines for the temperature–time diagrams were derived from kinetic analysis of the experimental inactivation data. To obtain the reaction order, all temperature–pressure kinetics were fit over a range of reaction orders ($n = 1.0$ to 1.7) (*TableCurve2D* SPSS Inc., Chicago, Ill., U.S.A.). The minimal sum of the standard error identified the optimal reaction order. All inactivation kinetics were refitted with the optimal n to obtain k. To get a functional dependency of $k(T)$, k and T were fitted with all equation set of *TableCurve2D* (SPSS Inc.). The equation for $k(T)$ was then used in (1) for k. The isorate lines were calculated with MathCAD 2001i professional (Mathsoft Engineering & Education, Inc., Cambridge, Mass., U.S.A.).

Results and Discussion

HPTS of the baby food puree

Spores of *B. amyloliquefaciens* and *G. stearothermophilus* inoculated in baby food puree and in ACES-buffer were used to investigate the HPTS impact on the inactivation of the spores. The pressure being 600 MPa and temperatures applied were 90 to 121 °C under isothermal and isobaric conditions.

The inactivation kinetics displayed in Figure 2 show that for a 3 $\log_{10}$ inactivation at 600 MPa and 90 °C for the certified indicator of the thermal sterilization, *G. steraothermophlius* (Figure 2C), 3 to 4 min of holding time were needed in the baby food puree. The spores in the ACES-buffer were more stable; 6 min were needed for a 3 $\log_{10}$ inactivation (Figure 2C). To achieve the same inactivation of *B. amyloliquefaciens* (Figure 2A) at 600 MPa, 90 °C, 15 min were needed. The increase to 105 °C resulted in a complete inactivation of the inoculated *G. sterarothermophlius* within a minute. Hence, for higher temperatures, the experiments were only conducted with *B. amyloliquefaciens*.

The increase of temperature leads to a 3 $\log_{10}$ inactivation at 105 °C, 600 MPa within 4 min (Figure 2A). For the p,T-combination of 115 °C, 600 MPa, the inactivation of the spores was so sudden and rapid that after 4 s, 4 $\log_{10}$ were inactivated (Figure 2B). The treatment at 121 °C, 600 MPa resulted in an inactivation so severe that no spores were detectable on the plate count after a treatment time of 1 s.

Regression analysis of the inactivation kinetics

To gain a better understanding of the T, t dependencies at 600 MPa, a modeling of the spore inactivation in the baby food puree and the ACES-buffer was conducted for a 3 $\log_{10}$, 5 $\log_{10}$, and an extrapolated 12 $\log_{10}$ inactivation of *B. amlyoliquefaciens*. The inactivation that was achieved during the pressure buildup (kinetic point of 1 s) was subtracted from the other kinetic points of each temperature, to have a valid model for isothermal and isobaric

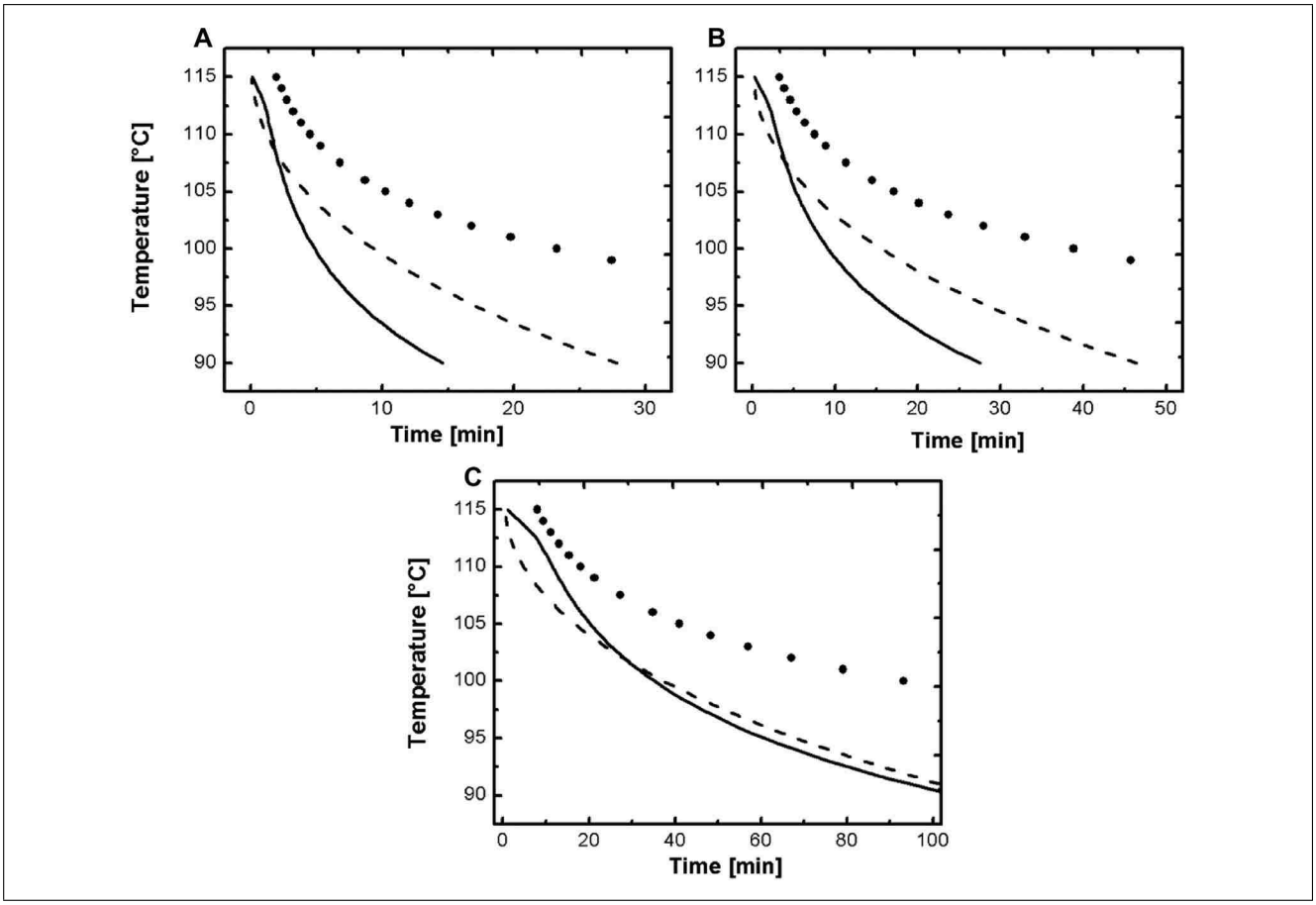

Figure 3–Isorate lines for (A) 3 $\log_{10}$, (B) 5 $\log_{10}$, and (C) extrapolated 12 $\log_{10}$ inactivation of *B. amyloliquefaciens* in ACES-buffer and baby food puree under HPTS conditions. Baby food puree (dotted line), ACES-buffer (black line), and thermal inactivation in the baby food puree (black dots).

Table 2–Extrapolated *T*,*t*-combination for a 12 $\log_{10}$ inactivation of *B. amyloliquefaciens* @ 600 MPa (Figure 3C).

	T [°C]	*t* [min]
Baby food puree	107.5	9.80
Baby food puree	115	0.45
ACES-buffer	111.5	9.42
ACES-buffer	115	1.15

conditions. The model that described the inactivation of the spores in the baby food puree the best was an *n*th-order approach with $n = 1$ and for the ACES-buffer with $n = 1.05$. Figure 3 indicates that an inactivation of the tested spore strain is possible with HPTS even at relatively low temperatures, like 90 °C, although a long holding time is necessary to achieve the desired inactivation: the higher the temperature, the more rapid the inactivation. The food system offered a protective effect for the spores, and therefore the dwell times were longer in comparison to the model buffer system (Figure 3A, B, and C). Although at 105 °C, the 2 isorate lines cross each other and the ACES-buffer seems more stable. Here, the time difference is so little that it cannot be seen as significantly different. All isorate lines of the HPTS-treated samples showed that the inactivation in comparison to the thermal inactivation was more rapid (black dotted line). In Table 2, the extrapolated dwell times to achieve a sterile product are shown. To reach economical dwell times under HPTS conditions (≤10 min) (Tonello Samson C, 2012, Hiperbaric, Spain, personal communication), temperatures of 107.5 °C and 9.80 min are needed. If the temperature is increased to 115 °C, less than half a minute is needed for the baby food puree to inactivate 12 $\log_{10}$.

FPC formation in the high-pressure sterilized baby food puree

The comparison between an alternative technology such as HPTS and common retorting in terms of formation of FPCs is new and literature concerning this matter is scarce. The quantities of furan formed in retorted baby food puree were obtained by autoclaving baby food puree in a container (Nunc Cryo Tubes Nr. 375299, Nunc A/S) at an $F_0 = 7$ min (115 °C, 28 min). Amounts of furan found were 30.11±1.6 μg/kg. These values fit the amounts of furan found in commercial available vegetable baby food purees by Jestoi and others (2009) with mean concentration of 37 μg/kg. The untreated samples in both cases contained 0.32 μg/kg of furan. The formation of furan in the baby food puree under HPTS is shown in Figure 4(A) and (B). The levels analyzed by both method A and method B are much lower than the levels common in retorted vegetable baby food puree (usually around 40 μg/kg and more). Both figures (Figure 4A and B) show slightly higher levels at 115 °C in comparison to a treatment at 121 °C.

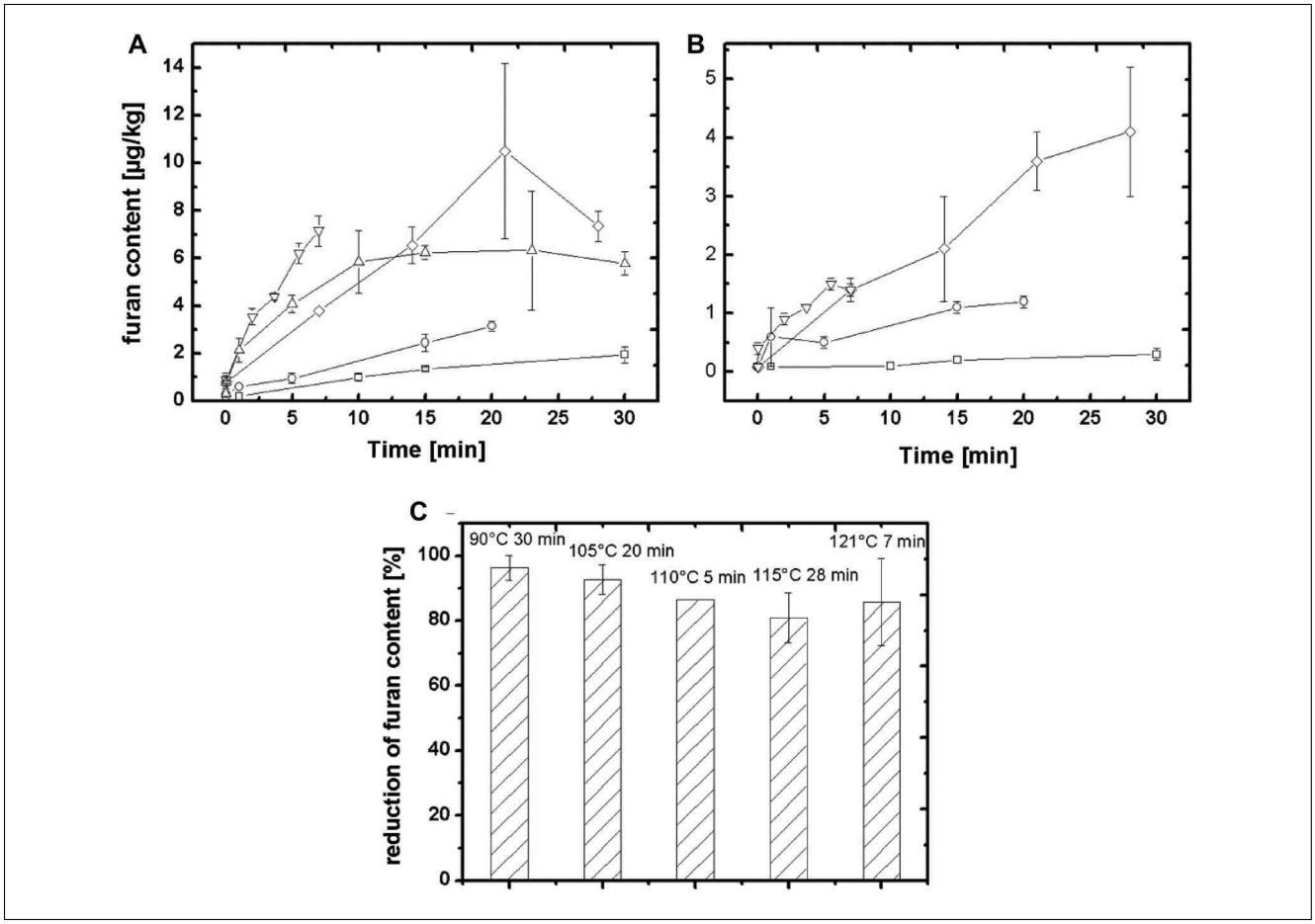

Figure 4–(A) Formation of furan under high-pressure thermal sterilization conditions (600 MPa) analyzed by automated headspace gas chromatography-mass spectrometry (method A). (B) Formation of furan under high-pressure thermal sterilization conditions (600 MPa) analyzed by solid-phase microextraction-gas chromatography-mass spectrometry (method B). (C) Reduction of furan in the baby food puree by high-pressure thermal sterilization (600 MPa) in comparison to retorting. 90 °C, 600 MPa (□); 105 °C, 600 MPa (○); 110 °C, 600 MPa (Δ); 115 °C, 600 MPa (◇); 121 °C, 600 MPa (∇).

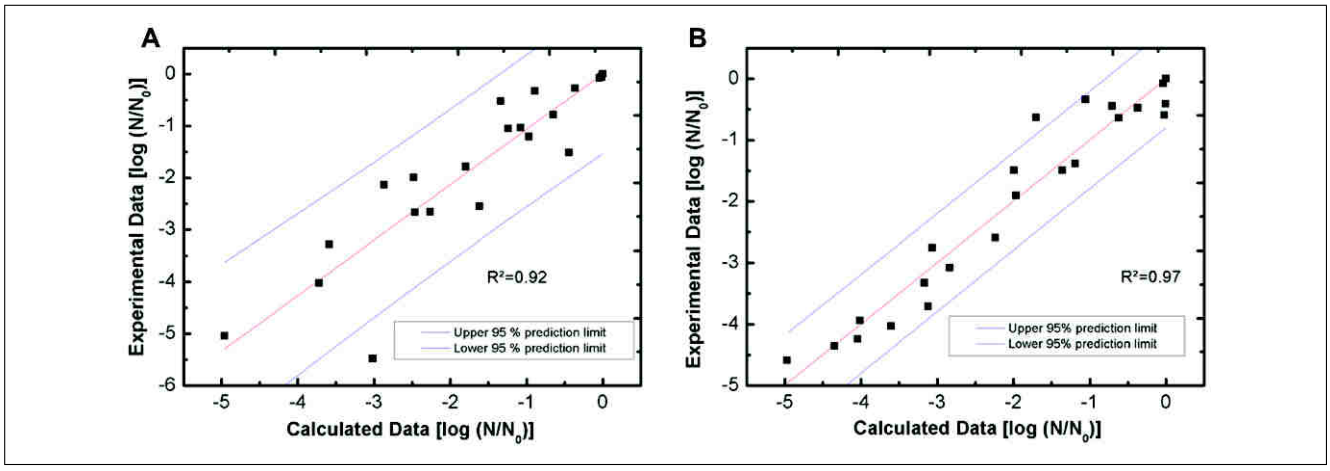

Figure 5–Comparison among calculated and experimental data with 95 % prediction bands for (A) baby food puree and (B) ACES-buffer.

But this could be just a possible tendency; the difference is not significant considering the low levels of furan. The amounts for 90 and 105 °C at 600 MPa are nominal. The highest amounts of furan were detected for 115 °C, 28 min with 7.35 ± 0.6 μg/kg (Figure 4A), respectively, 4.1±0.1 μg/kg (Figure 4B).

The results of method A and method B are in most cases very comparable, within the uncertainty of the methods (at such low levels ≤5 μg/kg, 29% is considered uncertainty), although the amounts analyzed by method A are a little bit higher than the ones analyzed by method B. The main reasons for this can be 2-fold: the use of a different method to collect the volatiles and the samples that were sent to the 2 labs were not from the same batch, and therefore variations/differences in the ingredients of the food system cannot be excluded. To summarize, the results are very comparable, since both show the same tendency. A reduction of furan is possible under HPTS conditions in comparison to the retorting, depending on the temperature and time, the reduction was within a range of 81% to 96% (Figure 4C). Even under sterilization conditions $F_0 = 7$ min (115 °C, 28 min; 121 °C, 7 min) at 600 MPa, a reduction of 81% to 86% is possible depending on the applied T, t-combination.

The modeling approach (Figure 3) for the inactivation in baby food puree and ACES-buffer is well suitable for the description of the inactivation of *B. amyloliquefaciens* spores under HPTS conditions. It describes the impact of T, t as well as the protective effect of the food systems on the inactivation at $T \leq 105$ °C. In Figure 5, the calculated and the experimental data obtained are compared. An R^2 of 0.82 for the baby food puree and 0.97 for the ACES-buffer indicate that the chosen model fits quite well to describe the inactivation under the HPTS conditions for the selected systems. Another parameter that gives helpful information about the goodness of a fit is the root mean squared error (fit standard error) with 0.69 for the baby food puree and 0.39 for the ACES-buffer. In both cases, the model fits quite well although in the case of the buffer system, the real data are better described by the model in comparison to the model of the baby food system (Figure 5).

The protective effect is present until a temperature above 105 °C is reached. Above this temperature, the driving force of the inactivation is the temperature (Reineke and others 2013a). The buffer system and the tested food system gave quite similar results. A 12 $\log_{10}$ inactivation is theoretically possible, the modeling predicts an economical T,t-combination (≤10 min) (Tonello Samson C. 2012. Hiperbaric, Spain, personal communication) of 107.5 °C, 9.80 min or 115 °C, 0.45 min at 600 MPa for the baby food puree (Table 2). These process parameters would shorten the overall process time in comparison to retorting.

At this point, it can be stated that the tested *G. stearothermophilus* strain might be an indicator for the thermal sterilization but it is very sensitive toward HPP at elevated temperatures in the selected food system, as already mentioned. However, other studies for a different *G. stearothermophilus* strain (Mathys and others 2009) showed a much higher resistance, which highlights, for example, the tremendous impact of the sporulation conditions (Reineke and others 2013a). The results of the spore inactivation in the selected food system are very promising but for an implementation of this alternative technology, an indicator spore strain needs to be found (Reineke and others 2013a; Sevenich and others 2013).

The formation of furan under HPTS conditions in the baby food puree show similar results as already mentioned by Sevenich and others (2013) for certain fish-containing foods. A reduction of furan is possible in a range of 81% to 96%. The analyses also showed that depending on the method used, the amounts of furan found can vary slightly. Although the tendency in terms of formation of furan over the T,t-combinations is similar. The lower formation of furan in comparison to the retorting might be ascribed to the following explanations: HPTS results in a shorter process time ergo in a lower thermal load applied to the product; and the Le Chatelier principle that states that under high-pressure conditions, only reactions are favored which have a negative reaction volume and if the reaction volume of furan is positive, the reaction might be limited (Cheftel and Culioli 1997; Ramirez and others 2009; De Vleeschouwer and others 2010; Escobedo-Avellaneda and others 2011).

Conclusion

HPTS could guarantee a safe product, by applying short dwell times and temperatures between 107 and 121 °C. The reduction of FPCs was shown to be possible not only for the baby food puree but also for other food systems as well (Sevenich and others 2013). Furan was reduced in the baby food by HPTS in comparison to retorting by 81% to 96%, even at conditions equal to the thermal sterilization ($F_0 = 7$ min). The consumer groups of this food are infants and babies. Reducing harmful substances, such as furan, in their daily diet is clearly an advantage given by HPTS over conventional thermal processing. The combination of a low ADI,

body weight, and the amounts of furan found in the retorted samples is quite critical. A scaling up is needed to validate these findings and help to implement this promising technology in the food industry.

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This work is dedicated to Rainer Selle.

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7. The impact of high pressure thermal sterilization on the microbiological stability and formation of food processing contaminants in selected fish systems and baby food puree at pilot scale

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The impact of high pressure thermal sterilization on the microbiological stability and formation of food processing contaminants in selected fish systems and baby food puree at pilot scale

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ABSTRACT

To this day there is no implementation of the high pressure thermal sterilization (HPTS) in the food industry. HPTS could result in better food quality, lower thermal load applied to the product and less unwanted food processing contaminants (FPCs) such as furan.

Based on findings for selected foods at lab-scale extrapolated temperature-time combinations for a 12 $\log_{10}$ inactivation of *Bacillus amyloliquefaciens* were chosen for a scale-up with a 55 L vessel (HPHT system Hiperbaric). Temperature-time-combinations at 600 MPa were between 100 and 115 °C and 0.45–28min. The scale-up resulted in a reduction of furan, depending on the food system, between 41 and 98% to retorting. Results at pilot scale were similar to lab-scale experiments. The performed storage trials (standardized method NF V 08-408) showed that only for the baby food puree two selected treatment conditions (107.5 °C, 9.8 min and 115 °C, 0.45 min at 600 MPa) resulted in an unstable product. Overall the results of the scale-up process support the idea that the HPTS could be feasible for the implementation in the food industry.

1. Introduction

The food industry is looking for new ways to produce safe, healthy and shelf-stable foods. One possible way to meet this goal is applying high pressure to foods, up to 600 MPa. A processing at 600 MPa and ambient temperatures is referred to in the literature as cold pasteurization (Knoerzer, Juliano, Gladman, Versteeg, & Fryer, 2007; Matser, Krebbers, Van Den Berg, & Bartels, 2004; Mújica-Paz, Valdez-Fragoso, Samson, Welti-Chanes, & Torres, 2011). The use of high pressure as a technology for pasteurization of different kinds of foods, such as juices, ham, sauces and seafood is a growing trend sector in the food industry since the 1990's (Hogan & Kelly, 2005). In 2014 over 252 high pressure systems produced over 500.000 metric tonnes of high pressure treated foods, which were put on the market worldwide. The numbers are increasing each year (Samson, 2014). Furthermore, there is a high acceptance of pressurized foods by consumers (Olsen, Grunert, & Sonne, 2010). High pressure can also be used in combination with elevated temperatures of 90–121 °C as a combined process to inactivate spores. This technique is called high pressure thermal sterilization (HPTS), which is to this point is not implemented in the food industry but could be seen as an alternative process for the conventional thermal sterilization as a mean to produce shelf-stable low acid foods. The only process within the HPTS that was certified by the U.S. FDA in 2009 is the so called pressure assisted thermal sterilization (PATS), where pressure is used for a rapid homogenous heat-up to the designated sterilization temperature (NCFST, 2009). In the last decade, much research was conducted to

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understand the underlying mechanisms of spore inactivation under high pressure conditions (Barbosa-Canovas & Juliano, 2008; Black et al. 2007; Margosch et al. 2006; Reineke et al. 2012; Reineke, Mathys, Heinz & Knorr, 2013; Setlow, 2003; Wuytack, Boven, & Michiels, 1998). The inactivation of spores under these severe conditions is based on a non-physiological germination (Reineke, Mathys, et al. 2013; Setlow, 2003). This results in a destruction of the inner spore membrane and or an opening of the dipicolinic acid (DPA)-channels. DPA is released from the spore core, a rehydration of the spore core occurs, the spore becomes pressure-sensitive and thermo-sensitive and can be inactivated (Reineke, Mathys, et al. 2013). At these conditions (600 MPa), the driving force of the inactivation is the applied temperature (Reineke, Schlumbach, Baier, Mathys, & Knorr, 2013; Sevenich et al. 2013, 2014). Reasons for the non-implementation of HPTS to this point are threefold: i) There is no existing sterilization indicator microorganism to demonstrate a sufficient inactivation of pathogenic and spoilage bacteria spores. Clostridium spores (*Clostridium botulinum*, *Clostridium sporogenes and Clostridium perfringens*) and Bacillus spores (*Bacillus amyloliquefaciens, Geobacillus stearothermophilus*) are mentioned by numerous research groups as being very highly pressure and temperature resistant (Ahn & Balasubramaniam, 2007; Georget et al. 2014; Juliano, Knoerzer, Fryer, & Versteeg, 2009; Margosch et al. 2006; Mathys, Reineke, Heinz, & Knorr, 2009; Reineke, Mathys, & Knorr, 2011; Reineke, Schlumbach, et al. 2013; Wimalaratne & Farid, 2008; Wuytack et al., 1998). ii) Certain ingredients (sugar, fats, salts etc.) and the corresponding water activity can cause a baroprotective effect, which can lead to retarded or incomplete inactivation of spores and microorganisms (Härnulv, Johansson, & Snygg, 1977; Molina-Höppner, Doster, Vogel, & Gänzle, 2004; Oxen & Knorr, 1993; Senhaji & Loncin, 1977; Sevenich et al. 2013; Van Opstal, Vanmuysen, & Michiels, 2003). iii) For the production of pressure sterilized foods a homogenous distribution of temperature during processing is mandatory and key for the inactivation of microorganism and enzymes. In recent years a lot of research was conducted to understand the temperature distribution in high pressure vessels, since a inhomogeneous temperature distribution could be a limitation, during high pressure processing (Grauwet et al. 2012; Knoerzer et al. 2007). For high pressure processing the only variable that could lead to non-uniformity during the process is the temperature. Pressure due to the isostatic principle is assumed uniform and the treatment time is fixed. The temperature uniformity has been described by many research groups and can be explained by differences in adiabatic heating of the product, pressure medium and the heat transfer from the vessel wall (Grauwet et al. 2012; Juliano et al. 2009). Hence, the temperature nonuniformity in the treatment chamber, which can vary for industrial units ~10 °C between the bottom and the top of a horizontal industrial scale high pressure system, is a factor that needs to be taken into account to ensure the safety of the process (Grauwet et al. 2012; Knoerzer et al. 2007; Martinez-Monteagudo, Saldana, Torres, & Kennelly, 2012). However, the rapid heating during HPTS reduces the lack of temperatures uniformity that occurs in traditional thermal sterilization processes (Knoerzer, Buckow & Versteeg, 2010). The mapping of the temperature inhomogeneity can be made visible by a quantitative analysis of uniformity of temperature using three-dimensional numerical simulations (Rauh, Baars, & Delgado, 2009) or by a so called pressure temperature time indicator (pTTi) (Grauwet, Van der Plancken, Vervoort, Hendrickx, & Loey, 2009). The pTTi is a protein based indicator that can be placed on several different positions within the vessel and the read-out can be conducted after the treatment. The question here is: are the used proteins the ones which are most high pressure high temperature resistant or depending on the treatment are there case by case pTTi and further will the protein/enzyme behave the same in a cell in comparison to the solution they are in (adiabatic heating). More promising seems to be the development of an inline tool to measure the temperature directly in the vessel and the product, such as the "Thermo-egg" developed by CISRO Australia (Knoerzer, Buckow & Versteeg, 2010; Knoerzer, Smith, et al. 2010) or an wireless measurement device developed by Hiperbaric (Samson, 2014, NC Hyperbaric, Spain, personal communication). Research for the development of this kind of equipment is still ongoing to this day.

Also since pilot scale and small industrial systems are available these need to be optimized to guarantee an economical process for the food industry. This signifies that the process line needs to be fine-tuned in terms of output, the heat up time of the vessel needs to be shortened, optimized intensifiers for quicker pressure build up and tools need to be developed to guarantee safe and constant temperature-pressure contribution in the packed food. Due to these mentioned effects the next step in research must go toward treatment of spores inoculated in complex food systems to evaluate the influence of the food matrix on the inactivation mechanism (Georget et al. 2014). This is of importance since the baroprotective effect on spores could be a possible limitation of the use of HPTS in the food industry for certain foods. Sevenich (2013, 2014) showed that for *B. amyloliquefaciens* inoculated in selected food systems (sardine in olive oil, tuna in brine, tuna in sunflower oil and baby food puree on the basis of vegetables) depending on the food composition different temperature time combinations (107.5–115 °C and 0.45–10 min) at 600 MPa are suitable for a extrapolated 12 $\log_{10}$ inactivation at lab scale. Nevertheless, none of these combinations are certified and the *p*, *T*-resistance could vary tremendously if the sporulation conditions are changed. Furthermore, the HPTS seemed to have a positive impact on a lower formation of the so called food processing contaminants (FPCs), such as furan or monochlorpropandiol-esters (MCPD-esters), in the tested foods. This is in comparison to the thermal sterilization and might be due to shorter process times and lower temperatures (Palmers, Grauwet, Kebede, Hendrickx, & Van Loey, 2014; Sevenich et al. 2013; Sevenich et al. 2014; Vervoort et al. 2012). The formation of unwanted and harmful substances under pressure could also be limited, if the specific reaction volume is positive (Le Chatelier's principle) (Bravo et al. 2012; Escobedo-Avellaneda et al. 2011; Ramirez, Saraiva, Perez Lamela, & Torres, 2009). The Le Chatelier's principle states that any phenomenon (chemical reaction, phase transition etc.) accompanied by decrease in reaction volume is enhanced by pressure. Therefore, pressure shifts the system to that of lowest volume (Black et al. 2007; Cheftel & Culioli, 1997). FPCs, in general, are compounds that are formed during the processing of a food with a negative impact and a risk on human health. Many FPCs arise from the Maillard reaction with precursors being sugars and amino acids; other reaction pathways can involve poly-unsaturated fatty acids (PUFAs; linoleic acid), ascorbic acid, sugars (glucose, fructose) or carotenoids (Crews & Castle, 2007; Lachenmeier & Kuballa, 2010; Vranova & Ciesarova, 2009). Furan is thought to be carcinogenic, since it can bind to proteins and nucleosides (Bakhiya & Appel, 2010; Bolger, Shirley, & Dinovi, 2009). This is why the ALARA (as low as reasonably achievable) principle is applied to foods for furan (Crews & Castle, 2007). Furan is very volatile (boiling point temperature 32 °C), so one could assume that during the normal heating practices that precede consumption, furan would evaporate out of the food. As Hasnip, Crews, and Castle (2006) have shown for fruit purees, baby food and other food systems that is not the case. Furan rather seems to have accumulated well into the matrix if the food was sterilized in cans or jars prior to the preheating. A reduction of furan or other FPCs in general would be a huge benefit of the HPTS since this would lead to the reduction of the toxicological potential of harmful substances,

which results in a better overall product quality. An additional benefit of HPTS is the compression heating, which is caused by the compression work against intermolecular forces if pressure is applied and results in a temperature increase of the pressure transmitting medium and the treated product. Depending on the food system this temperature increase by "volumetric heating" can range from 3 to 9 °C per 100 MPa and helps additionally to rapidly heat up the product to the required temperatures; whereas the thermal load applied to the product can be reduced (Barbosa-Canovas & Juliano, 2008; Knoerzer, Buckow and Versteeg 2010; Matser et al. 2004). Furthermore, during decompression (≤4 s), due to the adiabatic loss of the compression heating, there is an additional rapid volumetric cooling effect. However heat losses to the equipment and environment must be taken into account. For HPTS treatment the product needs to be preheated to 70–90 °C and through compression heating during the pressure build-up, the process temperature can reach 90–130 °C. For scale up applications of HPTS it would be important that both preheating, such as in a water bath, and pressure build-up time are fine-tuned to guarantee optimal and quick process (Barbosa-Canovas & Juliano, 2008; Heinz & Knorr, 2005). The calculated costs for an high pressure sterilization process are between 0.16 (300 L) and 0.50 (50L) €/kg depending on the size of vessel (Mújica-Paz et al. 2011) and this might make an industrial implementation feasible for some selected food products only. Although pilot scale high-pressure systems as well as high-pressure-high-temperature stable packaging are available on the market for HPTS (Koutchma, Guo, Patazca, & Parisi, 2005), up to now there is to our knowledge no HPTS treated product on the market. There has not yet been an approach to go from lab scale based modeled inactivation kinetic data of a high pressure high temperature resistant spore forming bacteria into an pilot scale system with economical *T*, *t* combination ($t \leq 10$ min) in combination with storage trials in the public domain. Based on inactivation data (5 $\log_{10}$) derived in a 3.5 ml vessel, by Sevenich (2013, 2014), under isothermal, isobaric conditions during pressure dwell-time a scaling up of the HPTS with tuna in brine, tuna in sunflower oil, sardine in olive oil and a baby food puree was conducted with the 55 L vessel HT from Hiperbaric at the AZTI-Tecnalia (Derio, Spain) to verify the findings in a large scale under non-uniform temperature. The foods were not inoculated with spores they were only treated at the calculated conditions for a 12 $\log_{10}$ inactivation of *B. amyloliquefaciens*, which is regarded to be one of the most *p*, *T* resistant spore strains (Olivier, Bull, & Chapman, 2012; Olivier et al. 2011; Margosch et al. 2006; Margosch, Ehrmann, Ganzle, & Vogel, 2004; Margosch, Ganzle, Ehrmann & Vogel, 2004; Rajan, Pandrangi, Balasubramaniam, & Yousef, 2006). Later on, the samples were stored at 37 °C and room temperature for 21 days, to see if the calculated *T*, *t*-combinations at 600 MPa lead to a shelf stable product. The aim of this paper was 1) to validate extrapolated temperature time combinations obtained at a lab scale at a pilot scale; 2) Evaluate the formation of FPCs in comparison to lab scale; 3) Examine the technical feasibility of HPTS as a pilot scale process.

2. Material and methods

2.1. Sample preparation

For the preparation of the baby food puree frozen vegetables were used and mixed following a recipe for a vegetable based baby food puree (BF): Carrots 40%, Peas 20%, Zucchini 15%, Water 24.9%, 0.1% Salt. The mixture was heated up to 85 °C and then pureed with a blender (Thermomix, Vorwerk, Wuppertal, Germany). For a better homogenization the puree was pressed through a sieve (normal kitchen sieve). The a_w-value of the puree was 0.96, the pH 6.47 and dry matter content 8%. Frozen unprocessed fish cans were obtained through Nouvelle Vague (Boulogne-sur-Mer, France). The tuna in sunflower oil (TO) (a_w-value 0.91) and the sardines in olive oil (SO) (a_w-value 0.92) in the cans had been precooked and only the tuna in brine (TB) (a_w-value 0.94) was raw. Before the HP treatment the fish samples were minced in a blender and then pressed through a sieve to obtain a homogenous sample. Two hundred grams of each food were then filled in high-pressure, high-temperature stable Meals-Ready-to-Eat (MRE)-pouches (Smurfit-Kappa, Dublin, Ireland) which are made of three layers: 48 ga. (12 μm) Polyethylene/adhesive/; 0.0005″ (13 μm) Aluminum foil/adhesive/and 4 mL polyolefin. This layer set up has shown to be very high pressure high temperature resistant (Brown, Meyer, Cardone, & Pocchiari, 2003; Koutchma et al. 2005). The filled pouches were sealed with hand sealing machine (Hawo, Obrigheim, Germany). Prior to the high pressure treatment the pouches were put in a 85 °C hot water bath for 10 min, as a preheating step to reach 85 °C.

2.2. Sterilization

2.2.1. High pressure thermal sterilization

High Pressure Thermal Sterilization treatments were carried out in a Wave 6000/55HT equipment (Hiperbaric, Burgos, Spain) situated at the Pilot Plant facilities of AZTI-Tecnalia (Derio, Spain). This machinery is specially designed to develop HPTS at industrial level, capable of combining high pressure (up to 620 MPa) and high temperature (up to 117 °C) processing. Its main differential characteristic, apart from its unique volume of treatment for an HPTS equipment (55 L) and the horizontal design of the vessel (a cylinder of 20 cm in inner diameter), is that it also includes an accurate control of the vessel wall, plugs and inlet water temperatures in order to avoid heat loss and consequently assure a nearly constant temperature during the treatment (Fig. 1). For treatments, inlet water was heated to the corresponding initial temperature, between 85 and 90 °C, taking into account the adiabatic heating during the treatment. Vessel wall and plugs were heated to the final temperature of each treatment (temperature under pressure). The samples were placed in baskets designed for the high pressure systems. In the middle of the baskets a bar was affixed, on which the pouches were placed horizontally, to ensure the temperature homogeneity for all pouches (8–10 in one basket) at least inside the basket. Since there is literature data available that showed that the temperature within (top to bottom) the basket can differ up to 10 K. The temperature of the water (pressure transmitting medium) during the processing was measured by thermocouples, but not

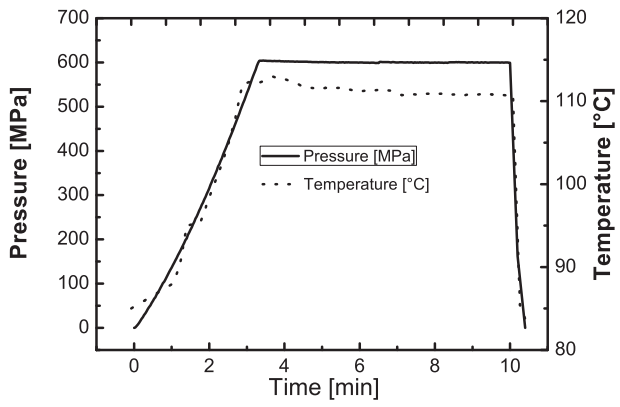

Fig. 1. Pressure-temperature profile of the Wave 6000/55HT equipment measured in the center of the chamber. $T = 110$ °C and holding time 6.53 min at 600 MPa.

directly inside the pouches. The temperature was a mean of the temperature measured by two thermocouples, which protrudes into the vessel from the center of each plug. One would have to assume that with adiabatic heating, the high water content of the foods etc. the temperature inside the pouch is the same as the one of the water. The rate of pressure increase was about 170 MPa/min and the pressure release was immediate (within seconds) at the end of the time of treatment. The treatment time and temperature combination were based on the findings by Sevenich et al. (2013, 2014) of the nth-order modeling approach for a 5 $\log_{10}$ inactivation at lab scale for *B. amyloliquefaciens* (Technische Mikrobilogie Weihenstephan, 2.479, Fad 82) which was extrapolated to a 12 $\log_{10}$ inactivation in the selected food systems. Considered were only those *T*, *t*-combinations for each food system that led to a 12 $\log_{10}$ inactivation $\leq$10 min (Table 1). A treatment time of 10 min is considered to be economical for high pressure processing (Samson, 2014, Hiperbaric, Spain, personal communication). For a comparison with normal retorting regime with an F_0-value of 7, samples of each food were treated at 115 °C, 28 min at 600 MPa. Also some conditions were chosen which represent an underprocessing of the system with *T* being 100 °C and *t* being 10 min at 600 MPa. Furthermore untreated samples were prepared to see to what extent a formation of FPCs occurred in comparison to treated samples and also to have a reference sample for the storage trials.

2.2.2. Conventional thermal retorting

To have a comparison with the retorting, four pouches of each product were autoclaved in Varioklav Steam Sterilizer 500E (V = 156 L), (H + P Labortechnik GmbH, Oberschleiβheim/Germany) at 115 °C for 28 min and 121 °C for 7 min.

All trials were done in duplicates. Table 1 shows the treatment conditions for the selected food systems. Four pouches of each food system were used for the storage trials, two pouches of each food system were used for the furan analytics and for the TO two extra pouches were used for the analyses for the MCPD-esters. The treatment conditions at 100 °C, 10 min at 600 MPa were only used for the storage trials (four pouches). All food systems that had the same treatment conditions were processed together otherwise separate treatments were conducted. After all the treatments, the samples were cooled down in an ice-cold water bath.

Table 1
Treatment conditions of the Sardine in olive oil (SO), Tuna in sunflower oil (TO), Tuna in Brine (TB) and Baby food puree (BF).

Matrix	Treatment	*F*-value [min]
SO	Untreated	–
SO	100 °C, 10 min undertreated	0.08
SO	115 °C, 28 min @ 600 MPa	7
SO	115 °C, 6.56 min @ 600 MPa	1.64
SO	113 °C, 9.40 min @ 600 MPa	1.49
TO	Untreated	–
TO	100 °C, 10 min undertreated	0.08
TO	115 C, 28 min @ 600 MPa	7
TO	115 °C, 7.41 min @ 600 MPa	1.86
TO	113 °C, 9.9 min @ 600 MPa	1.56
TB	Untreated	–
TB	100 °C, 10 min undertreated	0.08
TB	115 °C, 28 min @ 600 MPa	7
TB	115 °C, 0.75 min @ 600 MPa	0.18
TB	110 °C, 6.53 min @ 600 MPa	0.52
TB	107.5 °C, 9.80 min @ 600 MPa	0.43
BF	Untreated	–
BF	100 °C, 10 min undertreated	0.08
BF	115 C, 28 min @ 600 MPa	7
BF	115 °C, 0.45 min @ 600 MPa	0.11
BF	110 °C, 4.84 min @ 600 MPa	0.38
BF	107.5 °C, 9.80 min @ 600 MPa	0.43

2.3. Storage trials

The standardized method "Microbiology of food and animal feeding stuffs. Control of stability of preserved and assimilated products, Routine method" NF V 08-408 (AFNOR, 1997) was used to evaluate if the treatment conditions were suitable to produce a shelf-stable low acid product by HPTS. Therefore 2 samples of each treatment-condition were stored for 21 days at room temperature and 37 °C. The norm also includes one storage test at 55 °C for 7 days but instead it was chosen to store the samples for 21 days although the norm only requires 7 days. After the storage time had ended, the following chart was used to determine if the product is stable or not (Fig. 2). This includes a pH-measurement of the samples stored at room temperature (reference) and 37 °C, depending on the pH difference between the same samples differently stored further investigations are necessary. After the storage period microbiological analyses on nutrient agar were also conducted to evaluate if barro/thermo microorganisms survived the treatment in the samples. To sum up this means that a product is considered to be stable if the samples show: No deformation of the container, no modification of the appearance and smell, no pH difference >0.5 unit, between the samples and no specific microflora in the different samples under the microscope. All trials were carried out in duplicates.

2.4. Analyses of FPCs

Furan: The analysis of furan was conducted by automated headspace gas chromatography-mass spectrometry (HS-GC-MS). Since furan is very volatile (Boiling Point 31 °C) the use of a headspace sampling is the obvious method for furan analysis. The samples were incubated at low temperature (40 °C) to equilibrate furan into the headspace, which was sampled by an injection loop. Furan was quantified by comparison of the peak area of the furan response with that of deuterium labeled furan added at low level to the sample. The method used is described in detail elsewhere (Crews & Castle, 2007).

Monochlorpropandiol (MCPD)-esters: For the MCPD-esters determination the DART-MS direct analysis in real time-mass spectrometry described elsewhere (Moravcova et al. 2012) was used in this case.

2.5. Statistical analysis

The statistical analysis of the data was performed using Statgraphics (Version 4.0, StatPoint Technologies, Warrenton VA, USA). Multiple range test was used to analyze the significance of the tested data. Significance for all statistical analysis was defined as $p < 0.05$.

3. Results/discussion

3.1. Formation of MCPD-esters and furan

As shown by Sevenich et al. (2013, 2014) at lab scale, HPTS offers the ability to reduce the amounts of furan in certain foods (baby food puree and sardine in olive oil) by 71–98% in comparison to the retorting, due to shorter holding times, to lower thermal load applied to the product, and the possibility that the specific chemical reaction volume is positive (Le Chatelier-Principle). No significant amounts of additional MCPD-esters were formed by either processing with HPTS or retorting; the reason for MCPD-esters in the foods was the use of refined oils for the production of these foods. Interesting to see will be if a possible furan formation can be reduced and MCPD-ester formation only occur nominal for a

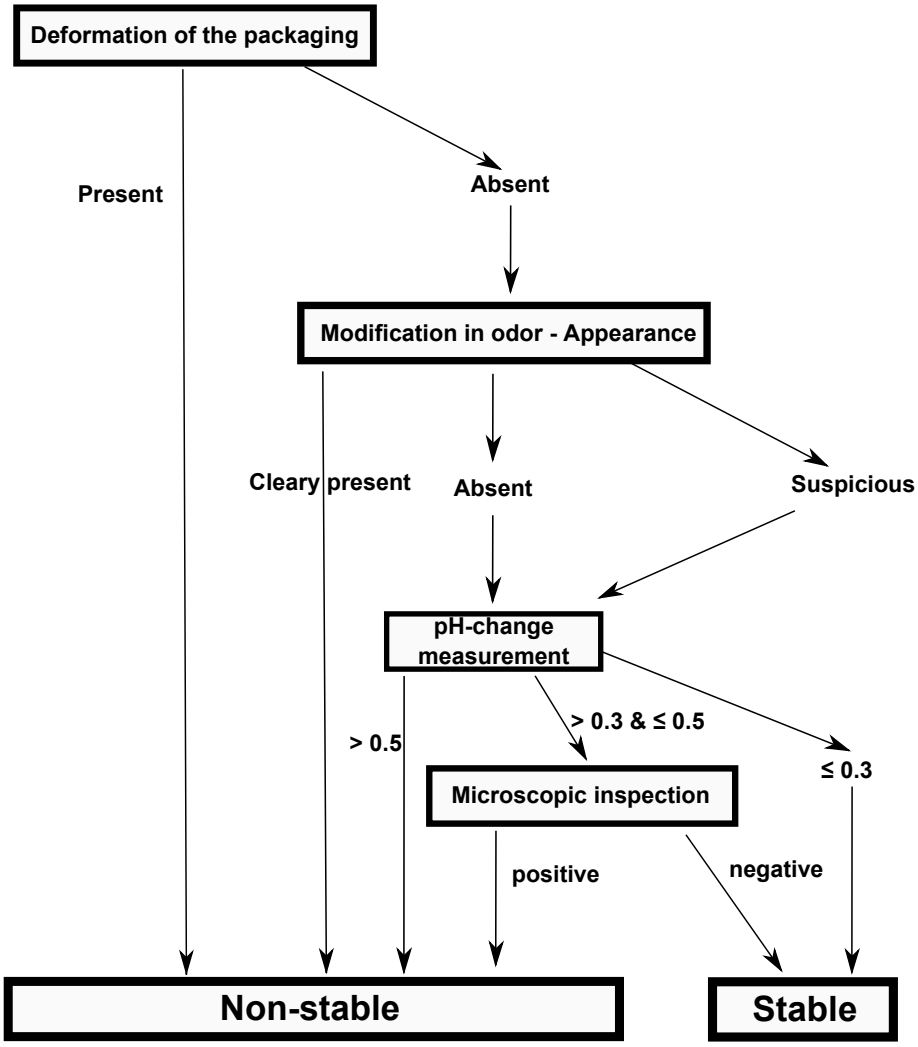

Fig. 2. Chart to interpret the results of the storage trials conducted at room temperature and 37 °C. Adapted from Standard NF V 08 408.

process run at pilot scale level, where longer pressure build up times, different packaging and food volumes are used.

Due to the findings at lab scale, only tuna in sunflower oil was investigated for the presence of MCPD-esters (Fig. 3). The results at pilot scale level confirm the results at lab scale. The amounts of MCPD-esters between (84–160 μg kg^{-1}) found in the differently treated samples; untreated samples (3A), thermal sterilized samples (3 B) and under pressure with 600 MPa (3 C-D) were not significantly different. The higher amounts of MCPD-esters in the sample treated at pilot scale level with 113 °C, 9.9 min, 600 MPa (3E), containing 293 ± 69 μg kg^{-1} of MCPD-esters, could be attributed to the fact that in this case probably relatively more sunflower oil then fish was filled in the pouches (higher oil then fish ratio). An analysis of the amount of MCPD-esters present in pure oil showed that an amount of 672 μg kg^{-1} was detected. This could be an explanation for the higher contents in comparison to the other samples. Nevertheless, the tendency of MCPD-esters present in the samples is the same for a treatment at pilot and lab scale level. No significant formation could be detected for these two approaches and the retorting, since it is mainly due to the use of the refined oil. In general one could say that the amounts found in tuna in sunflower oil are very low. As low amounts are defined as values between 500 and 1500 μg kg^{-1} (Larsen, 2009).

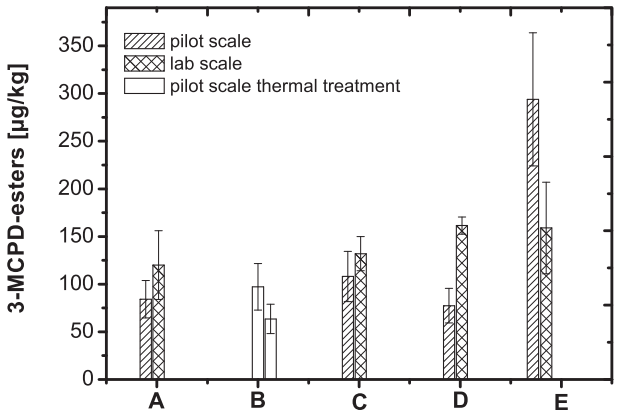

Fig. 3. Amount of MCPD-esters with standard deviations in tuna in sunflower oil at the *p*, *T*-conditions for a calculated 12 $\log_{10}$ inactivation in comparison to the lab scale results. A) untreated; B) retorting 115 °C, 28 min & 121 °C, 7 min; C) 115 °C, 28 min, 600 MPa; D) 115 °C, 7.41 min, 600 MPa; E) 113 °C, 9.9 min, 600 MPa & 110 °C, 10 min, 600 MPa.

No amounts of furan resp. only amounts of furan close to the detection limit (≤0.1 μg kg^{-1}) of the method were found in tuna in brine and tuna in sunflower oil for the retorted and high pressure sterilized samples. Which is in accordance to previous findings at lab scale by Sevenich et al. (2013) and due to the absence of possible precursors such as poly unsaturated fatty acids. This is why the data is not shown here. The amounts of furan detected in sardine in olive oil and baby food puree are shown in Fig. 4. Furan in the unprocessed samples of sardine in olive oil and the baby food puree were 4.0 ± 0.2 μg kg^{-1} resp. ≤0.1 μg kg^{-1}. The amounts of furan within sardine in olive oil show (Fig. 4a) that similar amounts of furan were obtained in the retorted samples at lab scale and pilot scale. The difference between lab scale 115 °C, 28 min with 57.0 ± 4.0 μg kg^{-1} and at pilot scale 115 °C, 28 min with 41.0 ± 9.0 μg kg^{-1} (Fig. 4a A) can be attributed to minor differences within the composition of the foods. The results also indicate the same trends as seen for the lab scale experiments that for the same F_0-value (7) lower amounts of furan are measured under HPTS conditions as for normal retorting (Fig 4a B). At sterilization conditions under pressure (600 MPa) the amounts of furan were 17.0 ± 2.0 μg kg^{-1} (lab scale 115 °C, 28 min, 600 MPa) and 28.0 ± 1.0 μg kg^{-1} (pilot scale 115 °C, 28 min, 600 MPa). In the case of the HPTS treated samples, this means that more than 2 times lower amounts of furan were present in the samples under sterilization conditions equal to F_0-value of 7. For the treatment conditions of 115 °C, 6.56 min, 600 MPa (Fig 4a C) 15.0 ± 1.0 μg kg^{-1} and for 113 °C, 9.4 min, 600 MPa (Fig. 4a D) 10.0 ± 2.0 μg kg^{-1} of furan were detected. Here the reduction of furan in comparison to the retorted samples at pilot scale, is 3–4-fold. Overall, the trials at pilot scale validated the trials conducted at lab scale. A reduction of furan in sardine in olive oil in comparison to the retorting was also found in the scaled-up process. At lab scale the reduction of furan was between 71 and 96 % depending on the process conditions (Sevenich et al. 2013). For the pilot scale trials the results are similar and reductions of furan between 42% (115 °C, 28 min, 600 MPa) and 77% (113 °C, 9.4 min, 600 MPa) were achieved in comparison to the retorting. The higher reduction at lab scale in comparison to the pilot scale can have many reasons. One possible explanation could be the preheating step of the pouches in the water bath (85 °C 10 min) – the longer pressure build up time (3–3.5 min for the pilot scale system to 1 min at lab scale), the slower cooling in the water bath due to bigger sample volume and at lab scale temperature as low as 90 °C were used. All these mentioned steps contributed to a higher thermal load applied to the product in comparison to the trials conducted at lab scale, where less intermediate steps and time was needed to heat and cool the samples. Furthermore, the difference within the formation of furan between lab scale and pilot scale could be due to the fact that for the lab scale experiments the temperature control during the dwell time was controlled via in-line measurement of the temperature and to variations in food and oils composition. This is valid for all the samples treated at lab scale in comparison to the pilot scale. Therefore, it could be possible that the temperature within the samples was not quite constant (either higher or lower due to different adiabatic heat of compression in comparison to water, which was the pressure transmitting fluid, with 3 °C/100 MPa) for the pilot scale trials although the temperature of the water in the high pressure chamber was constant over the dwell times. The presence of these low and high temperature zones during HPTS was reported by Grauwet et al. (2012). Nevertheless, a reduction of furan in the samples is still possible for the selected temperature-time combinations at 600 MPa in comparison to the retorting. The amounts of furan found in the baby food puree are similar to the formation of furan in sardine in olive oil. Although, in general lower amounts of furan, between 30.1 and 0.4 μg kg^{-1}, were detected in comparison to sardine in olive oil. The lower amounts can be partly explained for Fig. 4b C, D, E by shorter holding times and lower temperatures applied. If one compares the amounts found in baby food puree at 115 °C, 28 min and at 600 MPa (27.0 ± 12.0 μg kg^{-1}; 2.5 ± 0.4 μg kg^{-1}) with sardine in olive oil at 115 °C, 28 min and at 600 MPa (41.0 ± 9.0 μg kg^{-1};17.0 ± 2.0 μg kg^{-1}) this brings up the question: Do the different precursors of furan, PUFAs (linoleic acid) in sardine in olive oil and carotenoids plus sugars (glucose, fructose) in baby food puree, differ in terms of reaction speed and reactivity to form furan? In the literature there are studies available on the efficiency (molar yield) of furan formed from different precursors. Märk, Pollien, Lindinger, Blank, and Märk (2006) reported that the potential to form furan goes after the following order: ascorbic acid > PUFAs > sugars (glucose, fructose). However, Crews and Castle (2007) mentioned that when ascorbic acid is present in real foods and model systems, less furan is produced, as if ascorbic acid is heated alone. Based on this it can be concluded that the formation of furan is not only dependent on the treatment conditions but also on the formation potential of the precursors in foods. High amounts of precursor do not automatically lead to high amounts of furan. This might explain the difference in furan

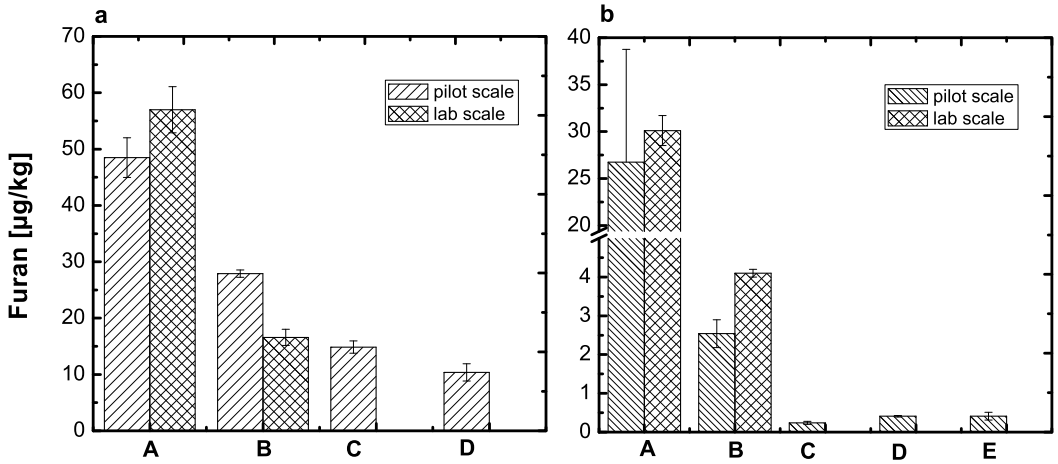

Fig. 4. (a) Amount of furan in sardine with standard deviations in olive oil under HPTS-conditions: A) retorting 115 °C, 28 min; B) 115 °C, 28 min, 600 MPa; C) 115 °C, 6.56 min, 600 MPa; D) 113 °C, 9.4 min, 600 MPa. (b) Amount of furan in baby food puree with standard deviations under HPTS-conditions: A) retorting 115 °C, 28 min; B) 115 °C, 28 min, 600 MPa; C) 115 °C, 0.45 min, 600 MPa; D) 110 °C, 4.84 min, 600 MPa; E) 107.5 °C, 9.8 min, 600 MPa.

observed for the two systems at equal treatment conditions. The reduction of furan in baby food puree is with 94–98% in comparison to retorting similar to the reductions obtained at lab scale, where the reduction for a broader temperature time range (*T*: 90–121 °C, *t*: 0–30 min) was between 81 and 96 % (Sevenich et al. 2014). The results of the formation of FPCs under pilot scale conditions overall agree with the results obtained at lab scale. In comparison to retorting, with an equivalent temperature/time combination, HPTS is a promising alternative preservation method which could give food products with considerably less undesirable components.

3.2. Extrapolated inactivation kinetics and storage trials

The temperature-time combinations at 600 MPa, for the processing of the different food samples at pilot scale, were based on calculated and extrapolated isokinetic lines of the inactivation kinetics of *B. amyloliquefaciens* obtained at lab scale. Fig. 5 shows the isokinetic lines in a temperature-time range of 90–115 °C and 0–40 min based on data from Sevenich et al. (2013, 2014). The dotted and dashed line above the ACES-buffer (straight) represent as follows: sardine in olive oil (dotted) and tuna in sunflower oil (dashed). The behaviors of the curves show that the oil within these systems has a protective effect on the spore inactivation. Whereas tuna in brine (dashed-dotted line) and the baby food puree (dot-dot-dashed line) are running beneath the ACES-Buffer and therefore represent food systems that have a neutral or enhancing influence on the spore inactivation in comparison with this buffer system. This clearly indicates that the composition of the food plays an important role in the inactivation of spores. Due to this independent optimized treatment conditions could be obtained for the different foods without having to deal with an over-processing of the foods. The results of the storage trials revealed that altogether three temperature-time combination at 600 MPa 107.5 °C, 9.8 min; 115 °C, 0.45 min for baby food puree and 107.5 °C, 9.8 min for tuna in brine resulted in a failure of the stability trials and therefore these conditions could not be applied to produce a stable product under HPTS-conditions in our tests (Table 2). As expected, all the undertreated samples (100 °C, 10 min) and untreated samples resulted in spoilage (Table 2). All other treatment conditions for the other tested foods could be suitable for the production of a high pressure sterilized products. Although more trails and research must be conducted in the future, with other spore formers, such as *C. botulinum*, to validate these findings. According to the used norm and the flowchart (Fig. 2), the pH-difference between the samples stored at room temperature and 37 °C for 21 days needs to be taken into account. In all cases, the difference of the samples was ≤0.11 and therefore these samples could be defined as stable (Fig. 2). To be sure and certain that no spore recovery occurred, all pouches were checked for revivable thermo-/baroresistant microorganism via plate count. Except for the ones, which were mentioned above, all other results were negative. This shows that a performed storage trial after the treatment is a powerful tool to validate the safety of the applied extrapolated *T*, *t* – combinations at 600 MPa. The process window that opens up in the temperature-time domain at 600 MPa for the different food systems is shown in Fig. 6. Here the assumption was made that if a sample at given *T*, *t* – combination would be stable it would also apparently be stable if the temperature would be held constant and the time would be extended to a time ≤10 min. The connected symbols (for *T*, *t*-combinations see Table 2) represent the treated and stable temperature time combination at 600 MPa for the different food systems. The process window that leads to a "theoretical" sterility in case of all the selected food products is situated between 113 and 115 °C, 7.4–10 min at 600 MPa. The samples treated at 115°, 28 min and

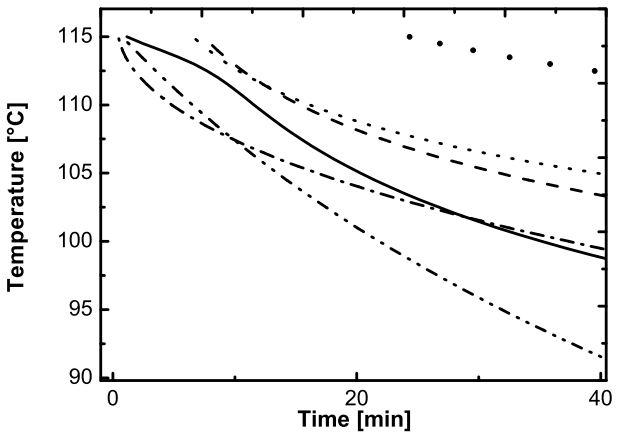

Fig. 5. Isokinetic lines of selected food systems for an extrapolated 12 $\log_{10}$ inactivation of *Bacillus amyloliquefaciens* (from a 5 $\log_{10}$ inactivation kinetics obtained at lab scale) at 600 MPa based on data from Sevenich et al. (2013, 2014) in a *T*, *t*-range of 90–115 °C and 0–40 min. Sardine in olive oil a_w 0.92 (small black dotted line); Tuna in sunflower oil a_w 0.91 (black dashed line); ACES-Buffer (solid black line); Baby food puree a_w 0.96 (black dash dotted line); Tuna in brine a_w 0.94 (dash dot dotted line); border of the thermal inactivation (big black dots).

600 MPa, which equals and $F_0 = 7$, have also shown to be stable, so one could also consider 121.1 °C, 7 min, 600 MPa (equal $F_0 = 7$) to obtain safe foods. In Table 2 the corresponding *F*-values for the different *T*, *t* combinations at 600 MPa are shown, which range from

Table 2
Results of the storage trials conducted after Norm: NF V 08-408 at room temperature and 37 °C for 21 days after the HPTS treatment for the selected food systems with corresponding *F*-value: Sardine in olive oil (SO); Tuna in sunflower oil (TO); Tuna in Brine (TB) and baby food puree (BF).

Matrix	Treatment	*F*-value [min]	Run 1	Run 2	Comment
SO	Untreated	–	Failed	Failed	Coccus but no thermo resistant
SO	100 °C, 10 min undertreated	0.08	Failed	Failed	Coccus but no thermo resistant
SO	115 °C, 28 min @ 600 MPa	7	Stable	Stable	
SO	115 °C, 6.56 min @ 600 MPa	1.64	Stable	Stable	
SO	113 °C, 9.40 min @ 600 MPa	1.49	Stable	Stable	
TO	Untreated	–	Failed	Failed	Coccus but no thermo resistant
TO	100 °C, 10 min undertreated	0.08	Stable	Failed	Coccus but no thermo resistant
TO	115 C, 28 min @ 600 MPa	7	Stable	Stable	
TO	115 °C, 7.41 min @ 600 MPa	1.86	Stable	Stable	
TO	113 °C, 9.9 min @ 600 MPa	1.56	Stable	Stable	
TB	Untreated	–	Failed	Failed	
TB	100 °C, 10 min undertreated	0.08	Failed	Failed	
TB	115 C, 28 min @ 600 MPa	7	Stable	Stable	
TB	115 °C, 0.75 min @ 600 MPa	0.18	Stable	Stable	
TB	110 °C, 6.53 min @ 600 MPa	0.52	Stable	Stable	
TB	107.5 °C, 9.80 min @ 600 MPa	0.43	Stable	Failed	Coccus but no thermo resistant
BF	Untreated	–	Failed	Failed	
BF	100 °C, 10 min undertreated	0.08			
BF	115 C, 28 min @ 600 MPa	7	Stable	Stable	
BF	115 °C, 0.45 min @ 600 MPa	0.11	Failed	Failed	Bacillus gram+ and spores
BF	110 °C, 4.84 min @ 600 MPa	0.38	Stable	Stable	
BF	107.5 °C, 9.80 min @ 600 MPa	0.43	Failed	Stable	Bacillus

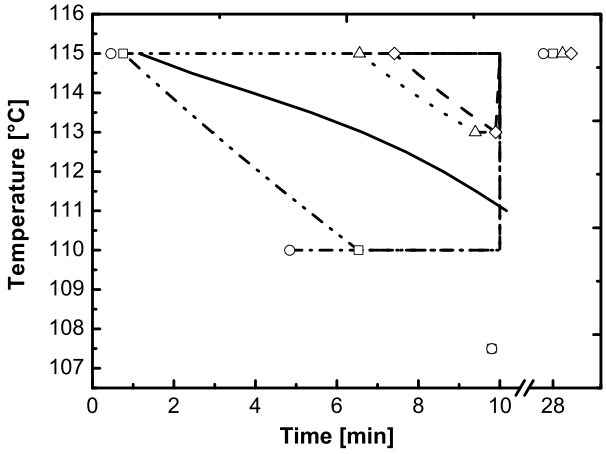

Fig. 6. Process windows for the selected food systems for a 12 $\log_{10}$ inactivation of *Bacillus amyloliquefaciens* that lead to stable product at 600 MPa in the temperature time range of 107.5–115 °C and 0.45–28 min. Sardine in olive oil (Δ and small black dotted line); Tuna in sunflower oil (◊ and black dashed line); ACES-Buffer (solid black line); Baby food puree (○ and black dash dotted line); Tuna in brine (□ and dash dot dotted line).

0.08 min–7 min. Although the results of this study showed that lower *F*-values for HPTS, due to the synergism of pressure and temperature, could be used in comparison to the conventional retorting. However, since the number of samples in this study was too small to make a general statement on the *T*, *t*-combinations used, one should consider that at least an *F*-value of 2.5 is applied. This again shows that more research is needed to understand the impact of temperature and pressure for future applications in the industry.

Furthermore holding times longer than 3–3.5 min need to be considered since the high pressure system needs this time for the pressure build up and therefore it would not be economical to use dwell times ≥3–3.5 min. The storage trials showed that for the selected food systems, case-by-case, at least a temperature between 110 °C and 115 °C and a dwell time of 4.84–10 min and a corresponding *F*-value of 2.5 min needs to be assured to gain a stable product. In all cases the temperature-time combinations under HPTS-conditions that would lead to a safe product in terms of microbiological safety and are lower in terms of time and temperature in comparison to the thermal retorting. Furthermore treatment conditions result in a lower formation of carcinogenic food processing contaminants.

4. Conclusion

The results obtained at pilot scale verified the results from lab scale. It was possible to go from lab scale based modeled inactivation kinetic data of a high pressure high temperature resistant spore strain into an pilot scale system with economical *T*, *t* combination ($t \leq 10$ min) in connection with storage trials for the selected food systems. The experiments showed that based on the calculation the storage trials were successful and that the temperature time combination at 600 MPa could be suitable for future research and application of this technology at pilot or industrial scale. Furthermore, an over-processing can be avoided if HPTS is used as the sterilization technique and also results in a benefit in terms of food quality, reduction of furan between 41 % and 98 % depending on the food and food safety. In the future, more research needs to be conducted with more food systems and other possible target microorganisms for the HPTS-process. Also since pilot scale and small industrial systems are available, these need to be optimized to guarantee an economical process for the food industry. This signifies that the process line needs to be fine-tuned in terms of output, the heat up time of the vessel needs to be shorten and tools need to be developed to guarantee safe and constant temperature-pressure contribution in the packed food. The HPTS-process could lead to a new principle of application for high pressure processing, where the benefits of this emerging technology merge to create healthier and high quality foods.

Industrial relevance

The high pressure thermal sterilization (HPTS) process is an emerging technology to produce high quality low acid food products, which are shelf-stable at ambient temperature. However, an industrial scale process has not yet been implemented. The work in this paper shows an approach to go from lab scale based modeled inactivation kinetic data of a high pressure high temperature resistant spore strain into an pilot scale system with economical *T*, *t* combination ($t \leq 10$ min) in combination with storage trials. Also a comparison between the results obtained at lab scale in comparison to the pilot scale results, in terms of the formation of food processing contaminants and food safety is made. The HPTS-process could lead to a new principle of application for high pressure processing, where the benefits of this emerging technology merge to create safer, healthier and high quality foods.

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This work is dedicated to Lina.

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8. Conclusion and perspective

The work conducted and the results obtained in this thesis show the potential of the high pressure technology at different temperatures and pressure conditions for the inactivation of foodborne pathogens, resistant spores, and the mitigation of food process contaminants (FPCs). Due to its variety of applications in the food industry high pressure processing has the potential to become a routine tool for the food industry in the coming years (Figure 8-1).

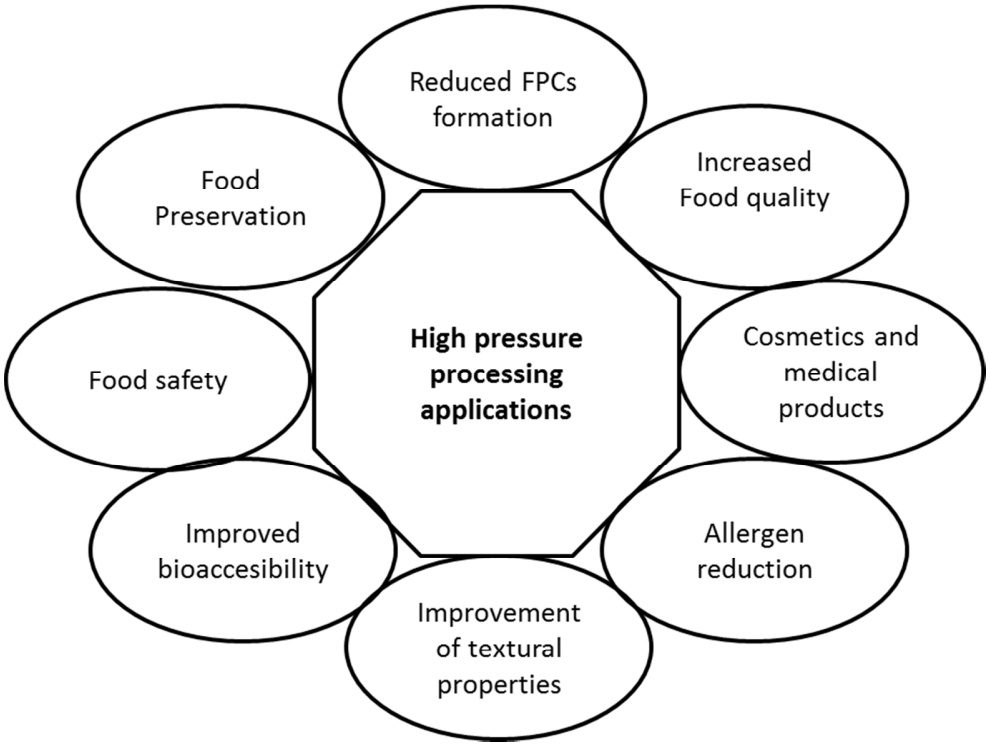

Figure 8-1: Current and possible applications of the high pressure technology.

One of the possible applications is the decontamination of products containing vegetative pathogens. The 2011 German EHEC-outbreak associated with fenugreek raised common concern; therefore, minimizing the risk of exposure to this pathogenic *E.coli* should be an aim of great importance. Thus, within this work the inactivation of EHEC-strains under different p,T (0.1-500 MPa at 20-70°C) conditions were tested. EHEC strains O157:H7 and O104:H4, isolated from HUS-patients, showed a much higher pressure-temperature resistance in ACES buffer at pH 7, than the non-pathogenic *E. coli* strain DSM1116. Resistance of the O104:H4 strain further increased, after pre-cultivation in nutrient broth (pH 5) and inactivation in carrot juice (pH 5.1). These data denote that the inactivation of pathogenic *E. coli* strains and non-

pathogenic *E.coli* surrogate (DSM 1116; DIN 58959-7) under high pressure are not comparable and that an adaptation of the cells to the treatment matrix is essential. For sake of clarity and to enable a comparison of the inactivation behavior of all tested strains in the different matrices, inactivation data was calculated as a functional relationship between pressure, temperature and time and was presented by means of pressure-temperature diagrams. Flow cytometric analysis was used in this study, to gain an insight into the inactivation mechanisms of *E. coli* under pressure and to enable a rapid estimation of the high pressure impact on the membrane potential, membrane permeabilization and the inactivation of intracellular esterase for *E. coli* DSM1116 cells treated in ACES buffer at pH 5 and 7. Significant differences were also found here for *E. coli* cells pre-cultivated and treated at a lower pH-value. These cells showed a stronger ability to retain its membrane potential, but a higher rate of membrane permeabilization at lower pressures (<200 MPa). For pressures higher than 200 MPa the isorate lines for membrane permeabilization at pH 5 and 7 converged, possibly denoting that differences in the fatty acid composition and membrane fluidity might be responsible for stabilization phenomenon at around 125 MPa. A comparison of the data for cell inactivation, with the reduction of the membrane potential and cell permeabilization indicated that under high pressure a loss of culturability did not correlate with the absence of esterase activity. However, a thermal (60°C) induced esterase inactivation was possible with (600 MPa) and without (0.1 MPa) membrane permeabilization, depicting a different pathway of cell inactivation. Reproductively viable cells that are able to grow under laboratory conditions are still the benchmark method for determining of successful pasteurization or sterilization in the food industry.

Even more resistant to all kinds of inactivation technologies (heat, chemicals, high pressure etc.) are spores. The possible exposure to spoiled canned foods by e.g. *Clostridium botulinum* is lethal. One of common approaches used in the industry today to sterilize canned and packed foods is by retort heating. This can often lead to an over-processing of the foods to fulfil the 12 D-concept. The huge thermal load applied favors the formation of carcinogenic compounds such as furan, 3-MCPD-esters and acrylamide. Alternative technologies and approaches for mitigation of these FPCs, without a loss of quality, are needed in the future, since EFSA and the U.S. FDA want to establish guidelines for allowed amounts of FPCs in foods. One alternative is the high pressure thermal sterilization, although it is not yet certified for temperatures < 121°C at 600 MPa and is still missing a sterilization indicator.

Therefore, more data and research in both model and real food systems are needed. Much research has been conducted to comprehend the mechanisms of high pressure (HP) inactivation of spores in aqueous systems. Nevertheless, prior to the treatment in model or real food system *Bacillus amyloliquefaciens* (TMW 2.479), *Clostridium sporogenes* (DSM 633) and *Geobacillus stearothermophilus* (DSM 5934) were treated in ACES-Buffer in a broad p,T-domain (200-700 MPA and 60-121°C) to find out which one of the selected is the most resistant one. The results obtained within this study for *Bacillus amyloliquefaciens* (TMW 2.479), *Clostridium sporogenes* (DSM 633) and *Geobacillus stearothermophilus* (DSM 5934) indicated that *Bacillus amyloliquefaciens* in the tested domain is the most resistant (Figure 8-2). Longer treatment times, higher pressures and high temperatures must be applied for *Bacillus amyloiquefaciens* (Figure 8-2 A) to obtain the same inactivation (here 5 $\log_{10}$ inactivation) as for *Geobacillus stearothermophilus* (Figure 8-2 B) and *Clostridium sporogenes* (Figure 8-2 C). The results in this thesis indicate that for the tested spore strains the sensitivity of *Clostridium sporogenes* and *Geobacillus stearothermophilus* spores towards high pressure and high temperature and therefore these seem not suitable as sterilization indicator strain under HPTS conditions. Although, results may differ for other sub-species or different sporulation conditions.

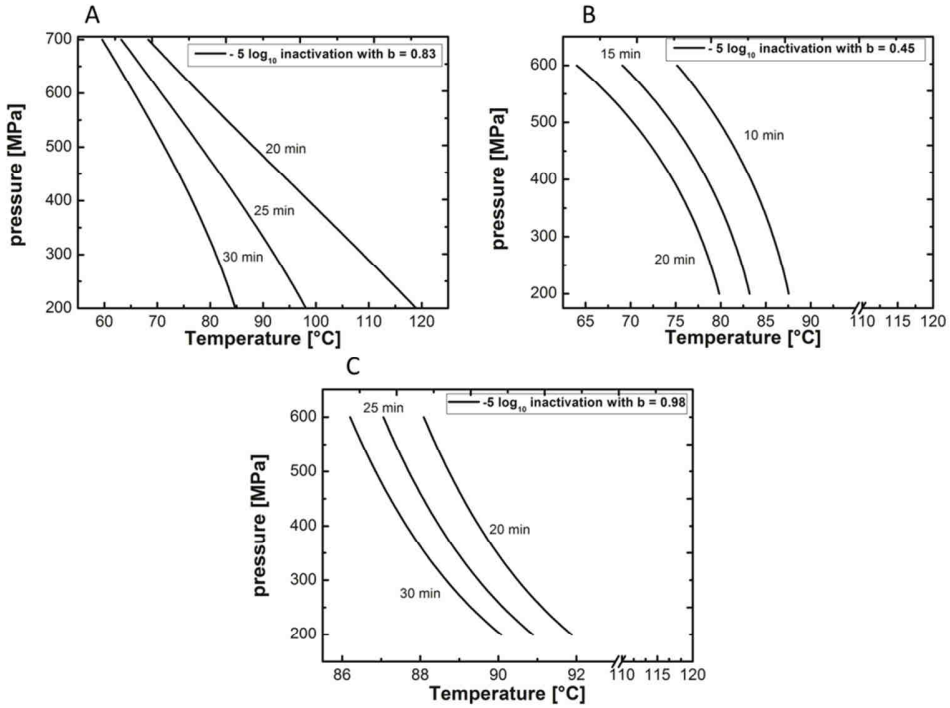

Figure 8-2: Isokinetic lines for different treatment times in the pressure temperature domain for a 5 $\log_{10}$ inactivation of A) *Bacillus amyloliquefaciens*, B) *Geobacillus stearothermophilus*, C) *Clostridium sporogenes* in ACES-Buffer

This trend becomes even more intense / obvious in the isorate lines for a 5 $\log_{10}$ inactivation at 600 MPa and elevated temperatures (Figure 8-3).

The only spore strain that is slightly more resistant is the *Clostridium botulinum* (TMW 2.375) based on inactivation data (600 MPa and 70-120°C) obtained from Margosch et al. (2006). In this case the data points were taken out of the graphs of the publications. Here, only maximal treatment time of 8 min was used. Therefore, longer treatment times and low temperatures (80-100°C) are not well described by the model as in comparison to the model used for the *Bacillus amyloliquefaciens*.

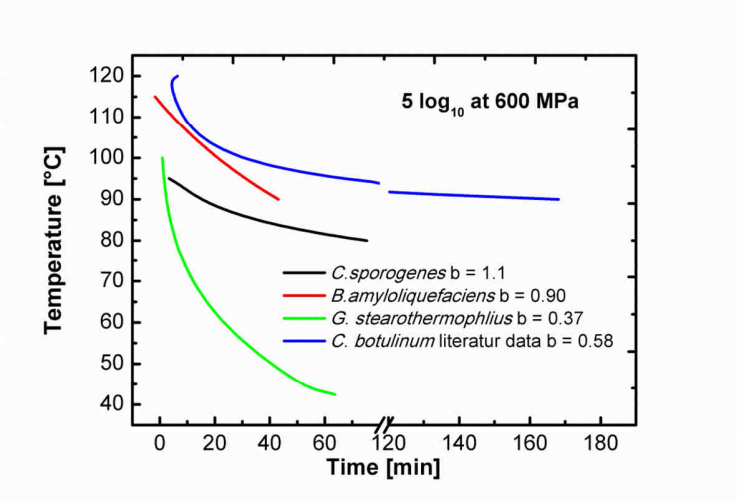

Figure 8-3: Isokinetic lines for a 5 $\log_{10}$ inactivation in ACES-Buffer in the high pressure high temperature domain for different spore strains.

In a study conducted by Lenz et al. (2015) another *Clostridium botulinum* strain (TMW 2.990; proteolytic Type E) was used where at 600 MPa and 75°C and a treatment time of 2.5 min lead to an inactivation of around 7 $\log_{10}$. Reddy et al. (2006) tested three different non-proteolytic *Clostridium botulinum* strains (Type B) in buffer and crabmeat at pressures of 875 MPa, temperatures between 40-75°C and dwell times between 10-30 min, which resulted in an inactivation between 0.4 to 7.0 $\log_{10}$. This again depicts the problem this technology is still facing that the search for indicator strain is ongoing. More research needs to be conducted to fully understand the inactivation mechanisms of spores under high temperature and high pressure conditions. Based on the results in ACES-buffer (Figure 8-2) for further studies the *Bacillus amyloliquefaciens* was chosen. The next step was to test that inactivation of *Bacillus amyloliquefaciens* within real-or food model systems to see if the stated mechanisms are applicable. In these systems spores can interact with ingredients which then could possibly lead to retarded or reduced inactivation.

Numerous researchers mentioned that the protective effect of solutes is dependent on the concentration of solute, microorganism and temperature. The protective mechanism of a reduced a_w-value is still unclear. HP processing might prove valuable to overcome protective effects of solutes and achieve shorter process times for sterilization under HP. Therefore, to gain insight into the underlying mechanisms 5 a_w-values, ranging between 0.9-1, were adjusted with 2 different solutes (NaCl, sucrose) and were inoculated with *Bacillus amyloliquefaciens*, which is thought to be one of the most pressure resistant spore strains (Margosch et al., 2004b; Rajan et al., 2006; Olivier et al., 2011; Reineke et al., 2013a). Solutions were inoculated with spores and treated at 105-115°C at 600 MPa. This work showed that for certain solute concentrations (corresponding to an $a_w \leq 0.94$ and temperature ≤ 110 °C) a baroprotective effect is present but a more rapid inactivation is possible if pressure and heat are applied together rather than only by heat. As other researchers already indicated the a_w and respectively the substances responsible for the a_w can have an impact on the inactivation under high pressure high temperature conditions (Sevenich et al., 2013; Sevenich et al., 2014; Georget et al., 2015; Sevenich et al., 2015). Sucrose has a higher protective effect then NaCl but the effect is minimized when temperature ≥ 115°C at 600 MPa are used. The calculations of the isorate lines could be used to optimize HPTS processes in food systems where NaCl and sucrose are the major solutes/ingredients. Based on this a 12 D-concept could be established as an orientation for more complex foods with similar a_w-values, such as liquid foods. Furthermore, the mechanisms of individual solutes and food matrices need to be fully understood in order to optimize the process design of HPTS. Therefore, it is of great importance to analyze at what time and at what pressure/temperature combinations the baroprotective effect of typical food ingredients occur and in what way these affect spore survival and components. This indicates the following research needs:

- Solutes interact with inner spore membrane indicated by FCM. More research is needed on how the solutes interact with the inner membrane
- DPA-release is influenced resp. slowed down by solutes and therefore the inactivation. HPLC-analyses in high concentrated solution needs to be optimize to gain further knowledge of the rate limiting step of the inactivation
- Inner spore membrane is presumably the target structure affected by high pressures, high temperatures and by solutes. For a further look into the physiological changes in the spore inner membrane due to solute uptake, the use of FTIR (Fourier transform infrared spectroscopy) analysis should be considered

- Mechanisms of individual solutes and food matrices need to be fully understood in order to optimize the process design of HPTS

Therefore, it is of great importance to analyze time and pressure/temperature combinations the baroprotective effect of typical food ingredients occur and in what way do these affect spore components.

Even more complex are real food systems and their influence on the inactivation of spores and possible chemical reactions during the processing that can lead to the formation of FPCs. Hence, lab scale trials (4 mL U111 Multivessel Unipress, Warsaw, Poland) with *Bacillus amyloliquefaciens*, with different foods (baby food puree and different fish foods in oil) at 600 MPa in a temperature range of 90-121°C were done.

According to Angsupanich and Ledward (1998), pressures above 400 MPa can accelerate lipid oxidation due to the release of free metal ions, while pressures of 600 MPa and 800 MPa markedly enhanced lipid oxidation, which is one of the intermediate steps of the furan formation pathway. This subject has been recently reviewed by Medina-Meza et al. (2014). Mesias et al. (2015) compared the fatty acid profile of different fish foods for a high pressure thermal sterilization process (600 MPa, 115°C, 28 min, $F_0 = 7$) and a retorting process (116°C, 60 min, includes heating and cooling time). The results showed that during the high pressure thermal sterilization significant differences were found in sardine canned in olive oil, where total PUFA content and the sum of EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) were lower in samples treated by HPTS ($p < 0.05$). These results suggest that combination of high pressure in combination with high temperature could promote lipid oxidation. This fact may be explained by the higher mineral content of canned sardine in comparison with canned tuna, which during HPTS treatment may promote lipid degradation. Whereas, tuna in brine and tuna in sunflower oil showed no changes regarding their fatty acid profile for either HPTS or retorting. Nevertheless, more research needs to be conducted on this matter but here the treatment conditions of HPTS were chosen to be comparable to the retorting. In reality lower temperature and dwell times for the HPTS process could be applied and therefore might lead to a lower PUFA oxidation. Furthermore, the furan formation, which is mainly due to PUFA oxidation, was lower in the HPTS process (16.6 µg kg^{-1}) compared to the retort (57.9 µg kg^{-1}). This fact could be explained by the Le-Chatelier-principle that maybe the PUFA oxidation is promoted by pressure and intermediates of furan are formed (see pathway of furan Figure 2-15) but not furan itself.

Therefore the pressure must have some kind of influence on the reaction pathway of furan since else lower formation of furan under pressure (600 MPa, 115 °C, 28 min with 16.6 µg kg^{-1}) at equal conditions as in retorting (115 °C, 28 min with 57.9 µg kg^{-1}) could not be explained by lower thermal load alone.

The results of this study clearly showed that a reduction of unwanted food processing contaminants is possible with HPTS in comparison to thermal retorting. The reduction of furan achieved at the maximal holding time for the baby food puree was depending on the temperature between 81-96 % to the initial content found in the retorted sample (30.1 µg kg^{-1}). Similar results were obtained for sardine in olive; here the reduction was 71-97 % to the initial content of the retorted sample (57.9 µg kg^{-1}). In Figure 8-4 the modeled formation of furan in baby food puree and sardine in olive oil is shown for the lab-scale trials.

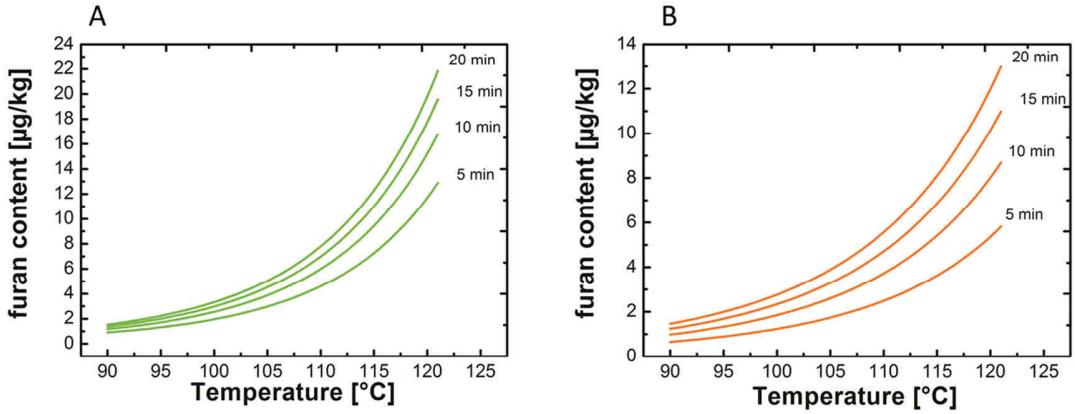

Figure 8-4: Formation of furan for different temperatures and processing times at 600 MPa in A) Sardine in olive and B) Baby food puree.

The key message here is that the formation of furan is dependent upon the precursor present in the food and also on the applied temperature-time combination at 600 MPa. In this case here the PUFAs in the fish food have a higher ability/tendency to form furan than the sugars and carotenoid in the baby food, as described by Märk et al. and Blank (2006; 2009). The formation tendencies can also be described while comparing the rate constants "k" obtained from the modeling of the formation kinetics via the Weibull approach ($y=k*X^n$). For example, the rate constant "k" for the treatment 110°C, 600 MPa, 5 min is for sardine in olive oil 2.5 and for the baby food puree a is 0.97, therefore the formation of furan under the same conditions in the sardine in olive was more than two times higher than in the baby food puree. To this day the literature on mitigation strategies of FPCs by innovative technologies is still scarce. Palmers et al.

(2014a) also showed the reduction of furan in different vegetables puree by HPTS (600 MPa, 117°C, 15 min) but here the initial furan concentrations after the heat treatment was low. The following is an approach to explain that the reduced amounts of furan found in the HPTS treated samples is not solely due to the lower thermal load applied but also that the pressure might interfere to some extend in the reaction pathway of the furan formation. This phenomenon appears at equal T,t conditions for thermal treatment and a high pressure treatment (See page 109).

To find out more about the influence of pressure on the formation pathway of furan one must examine the intermediate formation of furan formation pathway and check if the different intermediate reactions leading to furan are possible under high pressure high temperature conditions (Figure 8-5).

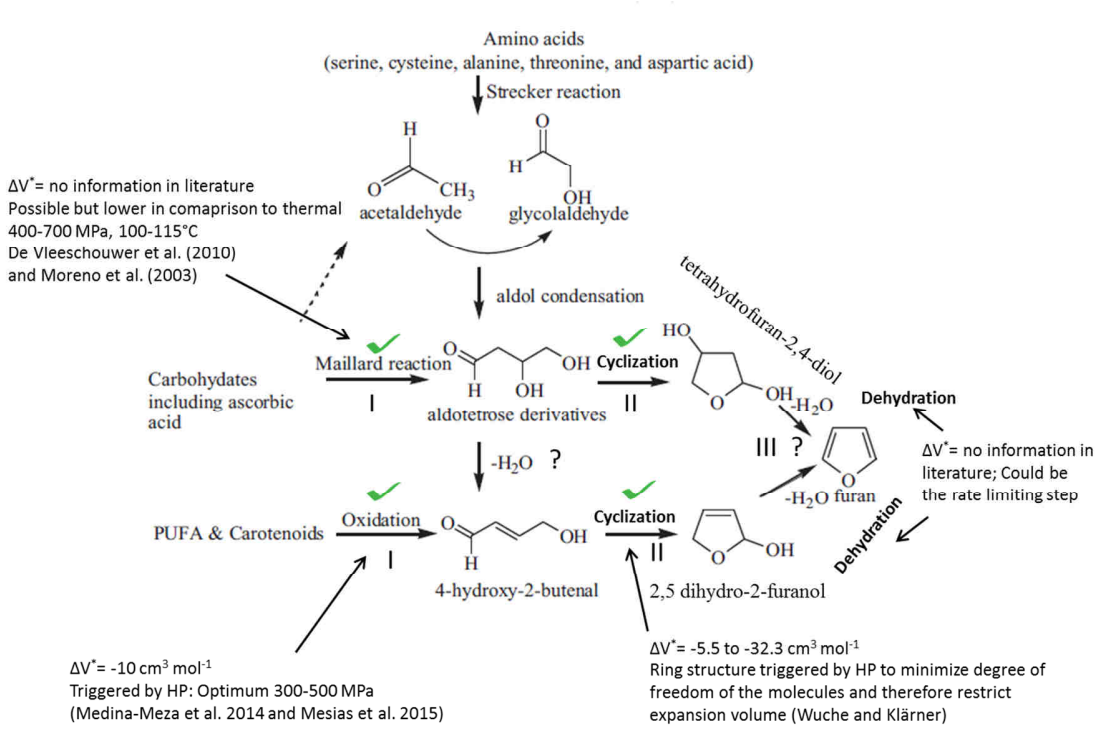

Figure 8-5: Pathway of furan formation under pressure adapted from Perez Locas and Yaylayan (2004)

Literature data on the reactions under pressure are available. Although not the entire pathway of furan is discussed but the single reactions found in the pathway are described. Mainly the data is described for reactions analyzed in model systems. The green arrows indicate that the reaction is possible under pressure. Oxidation reaction (I) from PUFA's and carotenoids and the Maillard reaction (I) from reducing sugars can occur under pressure (Isaacs and Coulson, 1996; Moreno et

al., 2003; De Vleeschouwer et al., 2010; Medina-Meza et al., 2014; Mesías et al., 2015). The cyclization (II) into the ring structure is also triggered by HP since this form lowers the degree of freedom in terms of expansion volume (Wurche and Klärner, 2002). The last intermediate reaction is a dehydration reaction (III) from 2,5-dihydro-2-furanol respectively tetrahydrofuran-2-4-diol to furan, for which no data in the literature is available. Therefore this could be the rate limiting step within the formation pathway. Question that arise from this pathway under pressure are: how toxic are possible intermediates and how stable are those?; does a negative ΔV automatically mean that the reaction will take place since it is only favored by pressure?; Does pressure have a retardation effect on the reaction? ; what happens to the intermediates after decompression and is a back reaction possible? The answers cannot be given here since no knowledge of the instable intermediates was gained within this study. For the future these intermediates must be detected and analyzed to totally understand the influence of pressure on the reaction pathway. The data obtained within this study shows that not only the lower thermal load under HPTS-conditions has an influence on the lower formation of furan but also that the pressure could interfere with the reaction pathway also mentioned by Palmers et al. (2014). Although, the formation of furan under pressure conditions is not 0 and furan is formed. The reaction surely occurs but not to the extend as under equal thermal conditions. The reaction pathway of furan is quite complex and therefore more research in the future needs to be conducted:

- Monitor possible oxidation and furan formation process during the storage period
- Use of modeling approaches in the future to predict the formation of FPCs and optimize processes to low formation rates of FPCs
- Analyze the intermediates of the furan reaction pathway to gain more knowledge of the influence of high pressure on this pathway

This work gives an insight where possible starting points are located. More research on this matter is needed but the potential of HPTS as a mitigation strategy could be shown. An end of new discoveries cannot be foreseen yet and one may assume that the sum of all these contaminants has a significant impact on life-style diseases such as cancer. Therefore in the future more risk assessments need to be performed also for the combined effect of toxins.

The inactivation kinetics of *Bacillus amyloliquefaciens* and *Clostridium sporogenes* revealed that the food system itself has a great influence on the inactivation (Figure 8-6). Further, as already

shown for the inactivation within the broad p-T-domain in ACES-Buffer (Figure 8-2 & Figure 8-3) *Bacillus amyloliquefaciens* is more resistant than the *Clostridium sporogenes* in real food systems as well. The order in which the inactivation was retarded goes as follows: tuna in brine ≤ baby food puree < tuna in sunflower-oil ≤ sardine in sunflower oil (Figure 8-5).

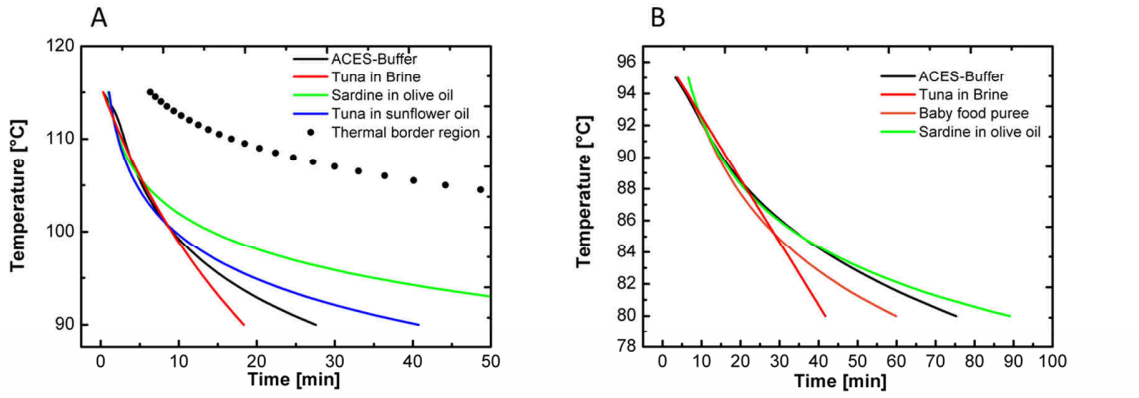

Figure 8-6: Isokinetic lines for a 5 $\log_{10}$ inactivation in the tested food systems and ACES-buffer of A) *Bacillus amyloliquefaciens*, B) *Clostridium sporogenes.*

The order also corresponds quite nicely to their resultant a_w-values. A 5 $\log_{10}$ inactivation was achieved at all temperatures but the inactivation time was shortened if the temperature was increased from 90 to 121°C. This indicates as proposed by Reineke et al. (2013) that at 600 MPa the driving force of the inactivation is the temperature. For the trials it was observed that at temperatures above 105 C the inactivation kinetics merge more and more and the protective effect was lost. Based on the modeled inactivation data (5 $\log_{10}$) under isothermal, isobaric conditions during pressure dwell-time at lab scale of *Bacillus amyloliquefaciens* an extrapolated 12 $\log_{10}$ inactivation for the tested food systems was calculated for economically feasible T, t combination (t ≤ 10 min). The trials were conducted utilizing the 55 L vessel HT from Hiperbaric at the AZTI-Tecnalia (Derio, Spain) to verify the findings in larger scale under non-uniform temperature distribution. These were not actual challenge studies, i.e., the foods were not inoculated with spores, they were only treated at the predicted conditions for a 12 $\log_{10}$ inactivation of *B. amyloliquefaciens*, which is regarded to be one of the most p,T resistant spore strains (Margosch et al., 2004a; Margosch et al., 2004b; Margosch et al., 2006; Rajan et al., 2006; Olivier et al., 2011; Olivier et al., 2012). The safety of the products was evaluated by storage trials (37°C for 21 days) after the norm NFV08408 (AFNOR, 1997). The results obtained at pilot scale showed that it could be possible to go from lab scale based modeled inactivation kinetic

data of a high pressure high temperature resistant spore strain into a pilot scale system with economically feasible T, t combination (t ≤ 10 min). The storage trials revealed that based on the calculation for 7 out of 9 calculated conditions were successful. This disapproves the predicted inactivation data although failure in the storage trials of the two tested conditions could also be attributed to temperature inhomogeneities inside the system. Nevertheless, future validation of these study trials foods inoculated with *Bacillus amyloliquefaciens* or other highly resistant spores need to be conducted. The temperature time combination at 600 MPa could be suitable for future research and application of this technology at pilot or industrial scale. For the tested foods different possible process windows could be obtained (Figure 8-7).

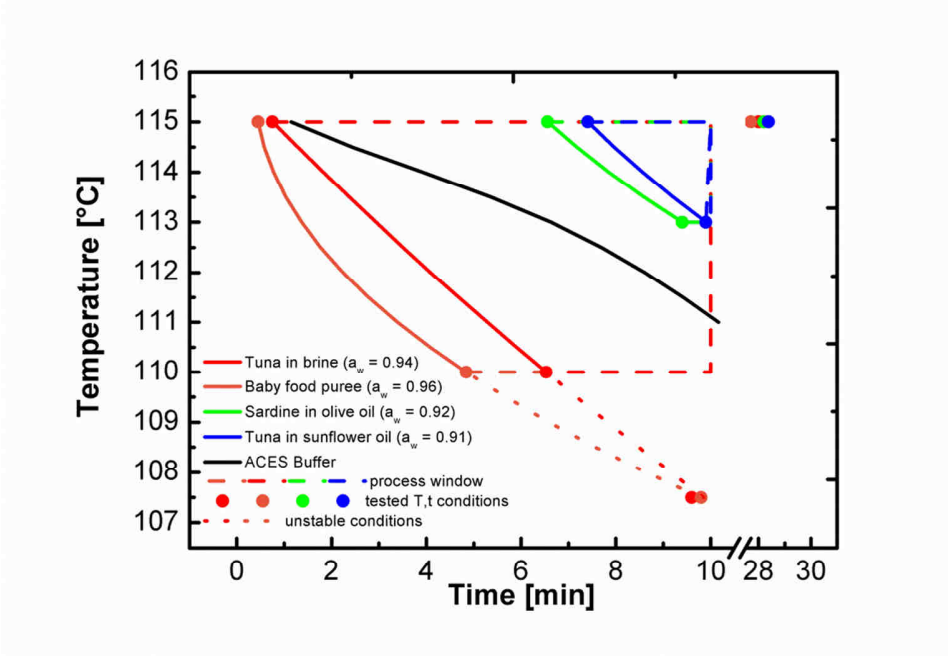

Figure 8-7: Process windows for the selected food systems for a 12 $\log_{10}$ inactivation of *Bacillus amyloliquefaciens* that lead to stable product at 600 MPa in the temperature time range of 107.5-115 °C and 0.45-28 min.

The connected symbols represent the treated and stable temperature time combination at 600 MPa for the different food systems. The process window that leads to a "theoretical" sterility in case of all the selected food products is situated between 113 and 115°C, 7.4-10 min at 600 MPa. The samples treated at 115°C, 28 min and 600 MPa, which equals an $F_0 = 7$, have also shown to be stable, so one could also consider 121.1°C, 7 min, 600 MPa (equal $F_0 = 7$) to obtain safe foods.

Furthermore, an over-processing can be avoided if HPTS is used as the sterilization technique and also results in a food quality benefit and reduction of furan between 41 % and 98 %

depending on the food and the applied T,t-conditions. If one seizes the idea mentioned earlier to use the modeling to predict/estimate the formation of FPCs the outcome from the lab scale based kinetic modeling for the formation of furan for the upscaling trials is depicted in table 8-1.

Table 8-1: Comparison of modeled data at lab scale and to actual obtained data for furan in up scaling trials

	T[°C],t [min] -conditions	Modeled Furan concentration based on lab scale trials [µg kg^{-1}]	Actual mean Furan concentration for upscaling trails [µg kg^{-1}]
Sardine in olive oil at 600 MPa	115°C, 28 min	13.9	27.90±0.7
	115°C, 6.56 min	8.1	14.9±1.1
	113°C, 9.4min	6.7	10.4±1.5
	100°C, 10 min	2.2	4±0.8
Baby food puree at 600 MPa	115°C, 28 min	9.8	2.5±0.4
	115°C, 0.45 min	0.9	0.2±0.04
	110°C, 4.84 min	2.4	0.4±0.02
	107.5°C, 9.8min	3.1	0.4±0.05
	100°C, 10 min	1.8	0.1±0

Table 8-1 shows that the modeled data based on the kinetics of the lab scale trials could only be used to estimate roughly the amounts of furan formed during the upscaling trials. The following factors might be responsible for the deviations' respectively higher actual amounts obtained during the upscaling trials: the preheating step for the 55 L vessel is with 10 min at 80-90°C much longer; longer pressure build up time (3-3.5 min) compared to lab scale (1 min) are applied; also no direct temperature control during dwell time was possible. Therefore it might be possible that higher temperature exist in the pouches as measured in the water inside the vessel; the amount of food was 100 times more in comparison to the lab scale trials and therefore cooling took longer. Lastly, since all of the ingredients are natural, deviations within the amounts of precursor for each batch can also not be neglected, although the higher formation at pilot scale is mainly due to the higher thermal load in comparison to the lab scale system.

In general these results indicate that one could use the kinetic data of the furan formation at lab scale to make rough estimates of the furan concentration after processing. For better comparison a kinetic study should be conducted at pilot scale level to factor in the aforementioned effect of the preheating of the sample and the cooling of the samples for the furan formation at pilot scale.

In the future, additional research needs to be conducted with more food systems and other possible target microorganisms for the HPTS-process. Case-by-case optimized treatment

conditions could be obtained depending on the food system. Also since pilot scale and small industrial systems are available, these need to be optimized to guarantee an economical process for the food industry. This signifies a need for the process line to be fine-tuned in terms of output, the heat up time of the vessel surroundings need to be shorten and tools need to be developed to guarantee safe and constant temperature-pressure contribution in the packed food. Although, commercial reality is only ever going to happen if equipment manufacturers offer large scale HPTS units or other approaches are developed that allow HPTS treatment in another fashion. One possible approach, which would make the high pressure thermal sterilization more feasible for the industry would be the treatment of samples in insolated carriers, as shown by Knoerzer et al. (2007), filled with 80-90°C hot water and product. The insolated and pressure transmittable carriers could be placed in already available industrial scale systems used for pasteurization and treated at 600 MPa without any additional feature of the high pressure system needed. The HPTS process could lead to a new principle of application for high pressure processing, where the benefits of this emerging technology merge to create healthier and high quality foods. The trials and analyses within this thesis show that there are many factors that have an effect on the inactivation of spores in model or real food systems. An overview is given schematically in Figure 8-8.

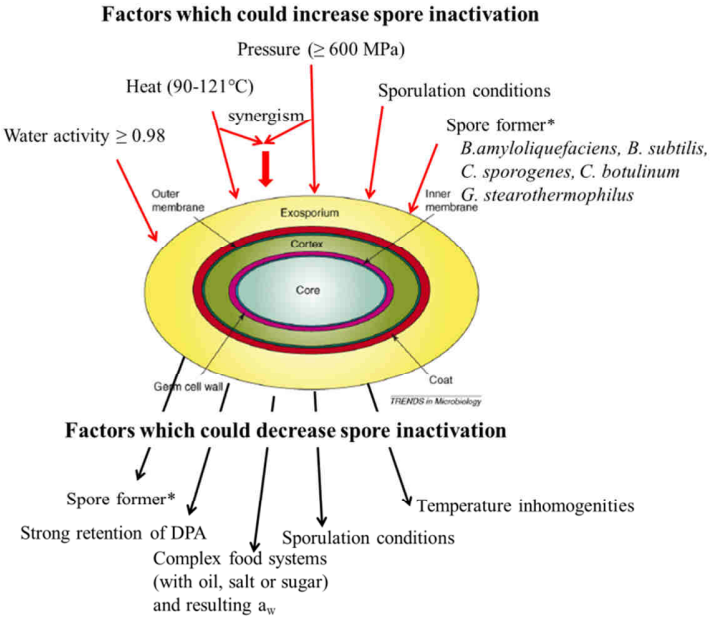

Figure 8-8: Non protective and protective factors of spore inactivation during high pressure processing.

All of these factors (Figure 8-8) need to be taken into account for the dimensioning of future processes conditions for the high pressure thermal sterilization. This work clearly showed that high pressure thermal sterilization is capable of producing safe and high quality foods with a lower toxicological risk for the consumer. But the question concerning the sterilization indicator strain needs to be solved before a commercial application of this technology is even remotely possible.

Over the last century there have been around 17,500 scientific publications on high pressure and its influence on spores, vegetative microorganisms etc. (Sciencedirect, 2015). Starting in 1899 until today but most significantly within the most recent decade, research has been focused more on the mechanisms of the inactivation of vegetative microorganisms or spores. Nevertheless still more research is needed to totally understand the mechanisms involved. It is interesting to note that within the last 5 years there has been an increase in number of the scientific papers dealing with the inactivation of *Bacillus amyloliquefaciens*: previously, it was primarily *Bacillus subtilis* (Annex 1). This seems like a small turning point since *Bacillus subtilis* was primarily used, since it is the best understood in terms of its molecular and cell biology, to understand the inactivation mechanisms of high pressure since it is well studied spore former and now the shift to finding a resistant spore strains for the process validation has begun. Therefore, the work conducted here is a step forward and demonstrates a new application of the high pressure technology. Further it could be shown within this study that *Bacillus amyloliquefaciens* is more resistant then the here tested *Clostridium sporogenes* and *Geobacillus stearothermophilus*.

Furthermore, high pressure can not only be used for the inactivation of spores or microorganism but also the decreased toxicological potential of a food is possible. Other applications might follow in the near future since high pressure has many hidden potentials, as e.g. the potential of high pressure to reduce of the allergenic potential of foods, such as rice and apples (Huang et al., 2014; Barba et al., 2015).

To get an idea how HPTS effects allergens, soy-milk was treated at 600 MPa in a temperature range from 90-121°C for 0.0166, 10 and 30 min. Treatment at 90°C and 110°C did not show any visual effects on the molecular weight distribution of soy proteins. However, at 121°C and after 7 min, β-conglycinin subunits were almost completely decomposed and glycinin subunits remained within 10 min (Figure 8-9). Glycinin “A” was partially affected, whereas glycinin “B” was more resistant.

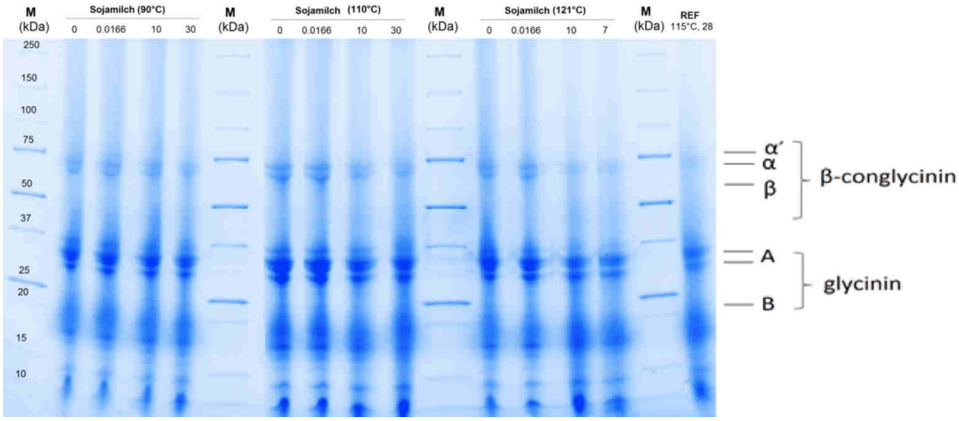

Figure 8-9: SDS-PAGE patterns of untreated soymilk, control (Ref) and HPP/heat-treated soymilk. M molecular weight standard indicated in kilo Dalton (kDa); SPI soy protein isolate; Ref reference of each protease (no enzyme addition); Electrophoresis was carried out with 4-20% polyacrylamide gradient gels. α'-, α- and β- subunits of β-conglycinin; A and B: acidic and basic subunit of glycinin.

This might be due to the fact that the basic group is located inside the glycinin complex and was therefore less affected by treatment conditions. In contrast, the acidic subunit is at the exterior of the complex, which means it is more susceptible for heat and HPP (Yin et al., 2008). The control sample (Ref, 115°C, 28 min) showed merely the same electrophoretic profile than HPP/heat-treated soymilk at 121°C for 7 and 10 min (Figure 8-9). More research on this matter is surly needed for further applications.

In the future the research of the high pressure thermal sterilization should be focused on finding a niche of products, where it can be applied usefully. As for example the production of foods which have a high quality, are microbiological safe, show low formation of unwanted substances during the processing and are minimal processed. The formation of furan and the corresponding extrapolated 12 $\log_{10}$ inactivation in sardine in olive oil and baby food puree is shown in Figure 8-10.

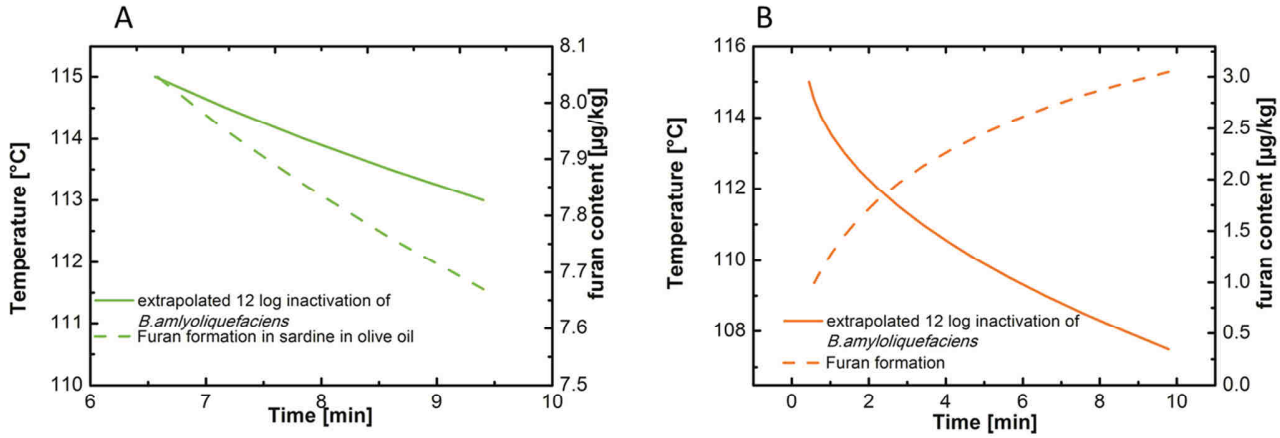

Figure 8-10: Correlation of 12 $\log_{10}$ *Bacillus amyloliquefaciens* spore inactivation and formation of furan under HPTS conditions in A) Sardine in olive oil, B) Baby food puree.

The use of HPTS leads to, as shown in T,t domain at 600 MPa in Figure 8-10, to low amounts of furan formation and to a microbiological safe product. The comparison between the designated precursor responsible for the formation in the sardine in olive oil (PUFAs) and in baby food puree (carotenoids) show a different behavior of the systems to form furan. It seems as the formation of furan via PUFA's is more temperature driven. Whereas the formation via carotenoids is more dependent on the time for the shown T,t conditions. The knowledge of the behaviors within the T,t domain of a microbiological safe product under HPTS (Figure 8-9 and Figure 8-6) conditions could be used to optimize future processes. If the precursor within the system and its tendencies to form FPCs are known this could lead to a better food quality without compromising the food safety.

This dual benefit shows an entirely new application of the high pressure technology in the food industry sector a future application will lead to the reduction of undesired processing byproducts of the food without affecting the food quality. The total energy consumption for an HPTS-process in a 55 L (600 MPa, 115°C, 28 min) would be 106 kWh/cycle which is two times lower as for a similar thermal process with 216 kWh/cycle. Although, the throughput and the investment costs need consideration.

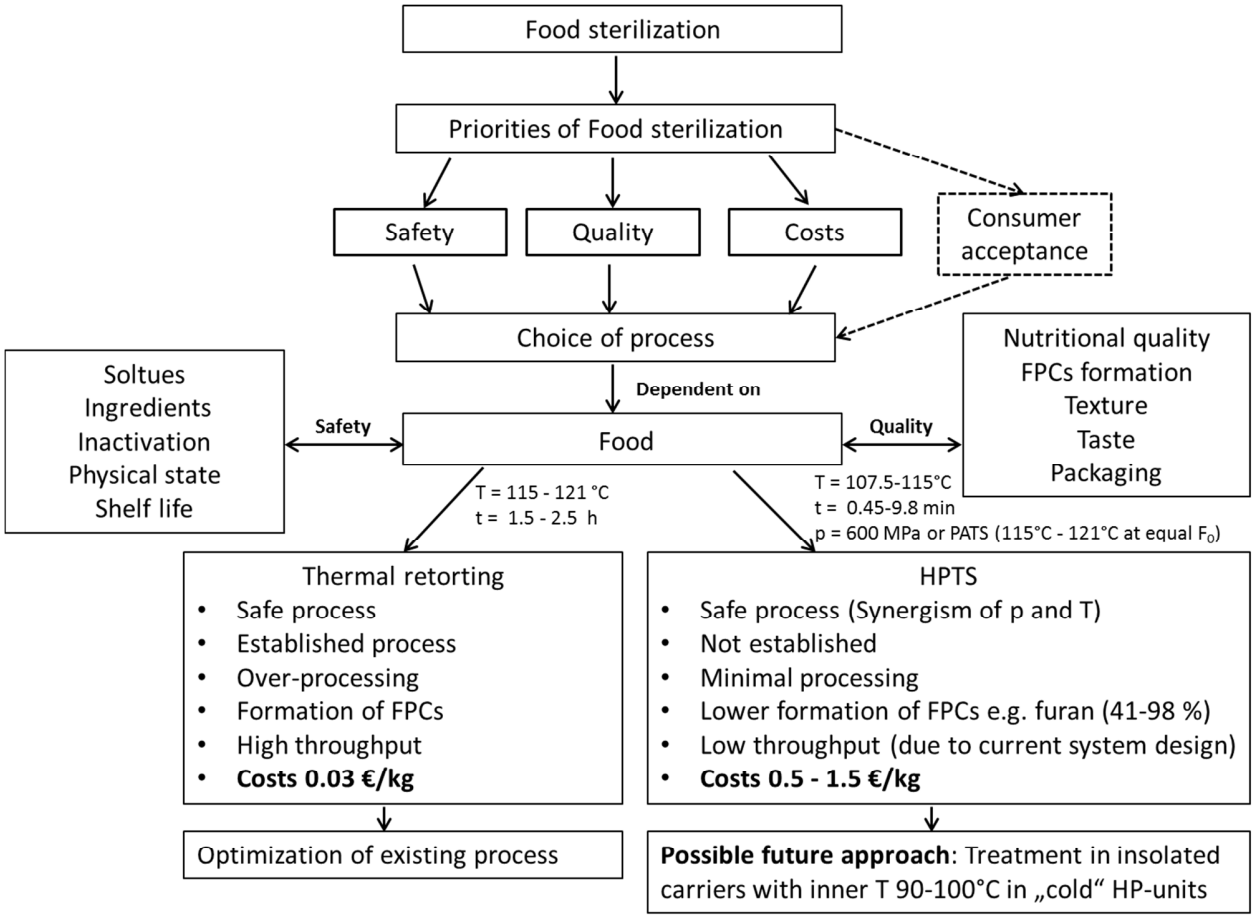

Figure 8-11: Flowchart of process relevant influential quantities for the selection of a sterilization process.

The choice which process to choose e.g. to sterilize foods is depending on many factors. An overview of the different influence quantities is given in the flowchart above (Figure 8-11). The most important factors are the safety and the costs of the process. The major concern of the food production is to keep the balance between safety and quality. Both thermal retorting and HPTS can keep the balance. UHT sterilization was not considered, although it is a safe process, because it is direct treatment of food and here only in package treatments were tested. HPTS would be in favor due to shorter processing times, lower thermal load applied and other advantages (Figure 8-10) but the current non-implementation of the technology at this point favors the conventional retorting due to lower processing costs and higher throughput.

If HPTS treated products would come onto the EU-market they would fall under the Novel Food regulation (EC NO. 258/97) and therefore would need a detailed study of potential for toxic, nutritional and allergic effects (EC, 1997).

Further, the Novel Food regulation includes a simplified procedure for marketing certain types of novel food or novel food ingredient in the EU if it is considered 'substantially equivalent' to an existing food or food ingredient that is already marketed within the EU (EC, 1997). In the case of the studied food systems within this thesis the substantially equivalent could be applied (safe product, low amount of undesired compounds) and therefore if labeled correctly the products treated by HPTS could be sold on the EU-market.

To date, very few pilot (> 15 L) and semi industrial scale (30-50 L) systems are available. In order to ensure economical processing once industrial scale systems become available, or other approaches are implemented that allow for high pressure thermal processing in existing industrial systems. It is essential that the general processing conditions required for certain food categories and target organisms are determined and optimized in the currently available smaller scale systems. This signifies that the process operation line is fine-tuned in terms of throughput; the heat up time of the vessel needs to be short and also the preheating time of the product to initial (elevated) temperatures needs to be reduced (e.g., through microwave heating rather than convection and conduction heating in a bath). Further, tools need to be developed to guarantee safe and constant temperature pressure distribution in the packaged food to develop safe temperature-time combinations other than the PATS-process. Since HPTS is considered very cost intensive due to only small pilot scale vessels being accessible to date, the current scope of the application of HPTS will not be the sole sterilization of products, due to the higher costs, 0.50-1.50 € kg^{-1} (Mújica-Paz et al., 2011), in comparison to the heat treatment with 0.03 € kg^{-1}. If an added value to the product by HPTS is possible such as, reduction of FPCs, retention of nutrients, reduction of allergens etc., the technology could be applied to high value and luxury products such as caviar in oil, medical solutions and cosmetics. Intensive research needs to be conducted for technologies that offer tailor-made safe and high quality foods for more susceptible consumer groups (e.g. immune suppressed patients, the elderly, infants and babies). HPTS combines the benefits of temperature and pressure to inactivate spores more rapidly and guarantee a safe product; also HPTS increases the quality of the food due to a lower thermal load on the product. Moreover, the toxicological potential of harmful substances could be reduced, so a further health risk for the aforementioned consumer group can be minimized.

Annex I

Annex Table 1: Bacterial spore inactivation and germination by high pressure: A literature overview [adapted from Heinz and Knorr (2002), Mathys (2008) and Reineke (2012) updated]

Reference	*Organism*		*Treatment*		*Type*	*Method*	*Medium*	*Comments*
	Bacillus	*Clostridium*	*Pressure [MPa]*	*Temperature [°C]*				
Chlopin & Tammann, 1903	ant		0.1-300	0-40	I	m	Nutrient broth	Spore formers are highly resistant; lethal pressure effect increases at higher T
Hite et al., 1914	sub		< 700	Room	I	m	Different foods e.g. fruits	Pressure tolerance of spore was inferred
Larson et al., 1918	sub		300-1200	Room	I	m	Infusorial earth	Still survivors after 14 h at 1200 MPa
Basset et al., 1932	sub		< 1750	Room	I	m		Rapid inactivation of vegetative form; survival of spores up to 1750 MPa and 45 min
Johnson & Zobell, 1948	sub		0.1-60	25-94	I	m	Buffer pH 7 Water	1 lg at 60 MPa/94°C/30 min; 4 lg at 0.1 MPa/94°C/30 min
Timson & Short, 1965	sub		0.1-800	-30-100	I	m	Milk	Spore can survive phase transitions of water, survivors after 700 MPa/100°C/30 min
Clouston & Wills, 1969	pum		0.1-170	25	I/G	m/b	Water Buffer pH 6/8	2 lg at 170 MPa/25°C/270 min; 4.5 lg with simultaneous irradiation
Clouston & Wills, 1970	pum		80-100	25	I/G	m	Buffer pH 6/8	Less than 1 lg at 80 MPa/25°C/100 min
Gould & Sale, 1970	sub, cer co , pum		25-100	20-80	G	m	Water	At 50 and 100 MPa, germination optimum at 50°C
Sale et al., 1970	co, cer, sub	sp	0.1-800	25-75	I/G	m/b	Buffer pH 8	6 lg after (70°C/30 min) + (300 MPa/75°C/120 min)
Murrel & Wills, 1977	sub, pum, cer		50-70	25-44	I/G	m	Buffer pH 6/8	1 lg at 63 MPa /41°C/40 min; germination optimum at 63 MPa/ 50°C
Bender & Marquis, 1982	meg		30-100	24-60	G	m	Buffer pH 6/8	>30 MPa/45°C can induce germination
Butz et al., 1990	st		200-300	40-60	I	m	Salt solution	2.5 lg at 60°C/300 MPa and 360 min
Mallidis & Drizou, 1991	st		1-30	117-128	I	m	Water	5 lg at 10 MPa/123°C/3 min
Taki et al., 1991	li		600	60	I	m	Buffer pH 7	Inactivation at 600 MPa/ 60°C/20 min

Continues

Annex Table 1: continued

Reference	*Organism*		*Treatment*		*Type*	*Method*	*Medium*	*Comments*
	Bacillus	*Clostridium*	*Pressure [MPa]*	*Temperature [°C]*				
Seyderhelm & Knorr, 1992	st		0.1-600	20-90	l	m	Water	6 lg at 350 MPa/80°C/30 min
Hayakawa et al., 1994a	st		0.1-800	20-70	l	m	Water	6 lg at 600 MPa/70°C/ 6 x 5 min oscillatory treatment
Hayakawa et al., 1994b	st		0.1-800	20-70	l	m	Water	6 lg at 600 MPa/70°C/6 x 5 min oscillatory treatment
Nishi et al., 1994	lic, sub, pol		50-200	25-60	l/G	m	Milk nutrient broth	6 lg after (200 MPa/60°C/10 min) + (incub. 37°C/60 min) + (65°C/30 min)
Okazaki et al., 1994	sub		0.1-500	25-111	l	m	Buffer pH 7	4 lg at 400 MPa/100°C/10 min
Sojka & Ludwig, 1994	sub		20-500	40-80	l/G	m	Germination Medium	>8 lg at 50°C/6 x 30 min/cycling between 150 and 500 MPa
Fornari et al., 1995	cer, li, co, st		200-900	20-70	l	m	PBS buffer pH 7	cer: 5 lg 20°C, 200 MPa/ 1 min+900 MPa/ 1 min li:complete inactivation 800 MPa/5 min /60°C st:complete inactivation 700 MPa /5 min /70°C co:4 lg at 900 MPa / 5 min / 70°C
Crawford et al., 1996		sp	410-820	80	l	m	Chicken	2 lg at 680 MPa/80°C/20 min and 6 lg with subsequent irradiation (3 kGy)
Gola et al., 1996	cer, li, st, co	sp	300-900		l	m	Buffer pH 7 Truffle cream	st: 5 lg at 700 MPa/70°C/5 min li: 6 lg at 700 MPa/70°C/3 min sp: 2 lg at 900 MPa/80°C/10 min
Ludwig at al., 1996	sub		0.1-500	4-70	l/G	m/b	Physio. NaCl	4.5 lg at 500 MPa/70°C/15 min; 8 lg by oscillation: 70°C/ 7x (1 min/500 MPa + 1 min/ 0.1 MPa)
Kakugawa et al., 1996	st		0.1-400	50-120	l	m	Buffer pH 7	5 lg at 150 MPa/90°C/30 min
Nakayama et al., 1996	sub, st, lic, meg, co		200-1000	5-10	l	m	Water	Less than 1 lg inactivation at 1000 MPa/10°C/40 min
Okazaki et al., 1996	sub, co	sp	0.1-400	35-110	l	m	Buffer pH 7	sub: 6 lg at 400 MPa/65°C/15 min; co: 6 lg at 400 MPa/110°C/18 min; sp: 6 lg at 400 MPa/110°C/18 min
Roberts & Hoover, 1996	co		400	25-70	l	m	Buffer pH 4-7	6 lg at 400 MPa/70°C/30 min/ pH 4/ + Nisin
Arroyo et al., 1997	cer, sub		100-400	10, 20	l	m	Tryptone soy broth	No significant reduction at 400 MPa
Heinz, 1997	sub		0.1-600	5-80	l/G	m/b	Ringer solution	6 lg at 150 MPa/70°C/30 min; depending on the p-T level germination is either initiated/ inhibited

continues…

Annex Table 1: continued

Reference	*Organism*		*Treatment*		*Type*	*Method*	*Medium*	*Comments*
	Bacillus	*Clostridium*	*Pressure [MPa]*	*Temperature [°C]*				
Hölters et al., 1997	sub/st		0.1-500	30-70	G	m/b	Physio. NaCl	Germination at 60 MPa/30°C or 30 MPa/ 50°C
Marquis, 1997	meg		100	25	l	m	Aqueous H_2O_2 solutions	0 lg at 100 MPa/25°C/3 h; 5.5 lg at 100 MPa/25°C/3 h in the presence of 1% H_2O_2
Sojka & Ludwig, 1997	sub		0.1-520	30-50	G	m/b	Physio. NaCl	> 50 MPa/ >30°C can induce germination
Wuytack et al., 1997	sub		100	40	G	m/b	Water	Rate of pressure-induced germination in mutant spores is decreased
Hayakawa et al., 1998	st		30-200	5-100	l	m	Buffer pH 7	6 lg at 200 MPa/95°C/60 min + rapid decompression (1.5 ms) 1 lg at 30 MPa/95°C/720 min + rapid decompression (1.5 ms)
Heinz & Knorr, 1998	sub		50-300	10-70	l/G	m/b	Water	6 lg at 150 MPa/70°C/30 min
Herdegen & Vogle, 1998	sub		0.1-700	40-80	l	m	Peptone solution pH 7	5 lg at (500 MPa/20°C/15 min) + (0.1 MPa/37°C/30 min) + (600 MPa/20°C/15 min)
Mills et al., 1998		sp	60-600	20-80	l	m	Water	2 lg at 0.1 MPa/80°C/10 min + 400 Ma/60°C/70 min
Raso et al., 1998a	cer		250-690	25-60	l/G	m	Buffer pH 7	8 lg at 690 MPa/60°C/1 min (sporulation at 37°C) 6 lg at 690 MPa/60°C/15 min (sporulation at 20°C)
Rovere et al., 1998	cer, li, st, co	bot, sp	700-900	50-70	l	m	Beff broth, Buffer pH 7	At 700 MPa/70°C/5 min: cer, 5 lg; li, 6 lg; st, 5 lg; co, 1 lg at 800 MPa/88°C/9 min: bot, 3 lg,
Wuytack et al., 1998	sub		100-600	40-55	l/G	m/b	Buffer pH 7	3 lg at (600 MPa/40°C/60 min) + (0.1 MPa/55°C/10 min)
Hölters et al., 1999		pas	0.1-500	30-60	l/G	m/b	Physio. NaCl	2 lg at 420 MPa/60°C/75 min 4 lg by oscillation: 60°C/6x(10 min/40 MPa + 5 min/0.1 MPa)
Reddy et al., 1999		bot	689-827	35-60	l	m	Buffer pH 7	5 lg at 827 MPa/40°C/10 min 5 lg at 227 MPa/50°C/5 min

continues

Annex Table 1: continued

Reference	*Organism*		*Treatment*		*Type*	*Method*	*Medium*	*Comments*
	Bacillus	*Clostridium*	*Pressure [MPa]*	*Temperature [°C]*				
Rovere et al., 1999		sp	600-1200	90-110	l	m	Meat broth	D(90°C) at 400/ 600/ 800/ 1200 MPa: 76.9/ 16.7/ 5.3/ 4.38 min D(110°C) at 0.1/ 400/ 600/ 800 MPa: 13.3/ 6.1/ 1.3/ 0.7 min
Wuytack et al., 1999	sub		0.1-600	20-80	l/G	m/b	Various aqueous media	6 lg 500 MPa/ 60°C/ 30 min, different ger-mechanisms at 100 MPa and 600 MPa use of fluorescent GFP-containing spores
Capellas et al., 2000	sub		60, 500	25, 40	l/G	m	Goat's milk cheese	5 lg 600 MPa/40°C/210min+500 MPa/40°C/15 min 2.7 lg with the same treatment at 25°C
Furukawa et al., 2000	sub		200-400	25-55	l/G	m	Water	>6 lg at 300 MPa/55°C/ 6 cycle a 5 min >4.5 lg at 400 MPa/55°C/1 cycle and 30 min
Meyer, 2000	cer	sp	2 x 690	90	l	m	macaroni cheese	Sterilization after two-pulse treatment 2 x 1 min
Meyer et al., 2000	cer, sub, st	sp	621, 690, 966	60, 90, 105	l	m	food pH > 4.5 fat<10%,a_w>0.8	2 or more cycles 621 MPa/ 105°C lead to sterility with 10 E+6/g spore load
Okazaki et al., 2000	sub, st, co	sp	0.1-400	35-120	l/G	m	PBS buffer(pH 7)	6 lg at 400 MPa/110°C/ B.co (17 min), C.sp(7 min) 4 lg at 400 MPa/113°C/ 10 min B.st with tailing
Shearer et al., 2000	sub, cer, co Acy	sp	392	45°C	l/G	m/b	milk, beef, apple tomato juice	Sucrose laurate(>1%)+392MPa 45°C,10-15 min lead to 3- 5.5 lg reduction laurate is inhibitory not lethal on spores
Stewart et al., 2000	sub	sp	404	25-90	l	m	Citrate buffer +Nisin +Sucr. laurate	sp at 25°C/30 min/ pH 4 (2.5 lg) pH 7(<0.5 lg) sub at pH 6-7/15 min/ 70°C (5 lg) 25°C(<0.5 lg) +/- synergism of HP/ pH/ Nisin/ sucr. laurate
Wilson & Baker, 2000	sub, st	sp	51-827	75-90	l	m	Meat emulsion	Treatment: 621 MPa/98°C/5 min >5 lg (sp), > 9 lg (sub), > 10 lg (st)
Wuytack et al., 2000	sub		100-600	40	G	m	Water	Different pathway for germination at 100 and 600 MPa
Ananta et al., 2001	st		50-600	60-120	l	m	Mashed broccoli Cocoa mass	6 lg at 600 MPa/80°C/60 min in mash. broccoli 6 lg 600 MPa/90°C/45min in cocoa mass
Furukawa et al., 2001	sub, li		200	25-65	l	m	NaCl solution	4 lg(li) at 65°C/180 min; 6 lg(sub) at 65°C/90 min 1 lg more after filtration and HP
Meyer, 2001	cer	sp	345-965	70-100	l	m	Different food	Two or more cycles of ultra-high pressure were combined with temperature treatment
Moerman et al., 2001	sub, st		50- 400	20- 80	l	m	Fried minc. pork Mash.potatoes	sp 5 lg at 400 MPa/80°C/ 60 min in pork sp 5 lg at 400 MPa/80°C/ 60 min in potatoes

continues…

Annex Table 1: continued

Reference	Organism		Treatment		Type	Method	Medium	Comments
	Bacillus	Clostridium	Pressure [MPa]	Temperature [°C]				
Wuytack and Michiels, 2001	sub		100, 600	40	I/G	m/b	Buffer pH 3 - 8	Higher inactivation if first pressure treated at neutral pH + then exposed to low pH for 1 h sensitive to heat inactivation at low pH
De Heij et al., 2002	st		700	121	I	m	Tryptone soy broth	6 lg after 2 x 700MPa/ 121°C/ 90 s
Fujii et al., 2002	cer		500-600	20-40	I	m	Water+argon	Pressure resistance of *B. cereus* spores was affected by a strong 'water ordering' effect
Furukawa et al., 2002	sub		100	45-75	I	m	Water	>6 lg after120 min with different initial concentrations; higher No=higher resistance
Lee et al., 2002	Acy		0.1-621	22,45,71,90	I	m	Apple juice	4 lg 41°C/207 MPa/10 min, 6 lg 71°C/207 MPa and 5 min, 6 lg at 90°C/414 MPa/1 min
Krebbers et al., 2002 spores	natural flora		700, 860	75, 85	I	m	Basil	Below detection line after 2 x 950 MPa/75°C/30 s or 2 x 700 MPa/85°C/30 s, initial 4 lg/g
Paidhungat et al., 2002	sub		100, 550	Room	G	m/b	KPO_4 (pH 7.4) Water	100 MPa activation of germinant receptors 550 MPa opens channels for DPA release
Balasubramiam et al., 2003	sub		827	50, 70	I	m	PBS, pH 7	Effect of pressure transmitting fluids, sodium benzoate highest dT/dp; 8 lg 827 MPa,70°C
De Heij et al., 2003	st, sub		300-800	84–122	I	m	Tryptone soy broth	6 lg 700 MPa/ 90°C/3 min in standard vessel 10 lg at 700 MPa/ 90°C/ 3 min in isolated
Farkas et al., accompanied 2003	sub		300, 600	Room	I/G	m/b	Nutrient broth	Germination of recombinant sub was by the emergence of bioluminescence
Furukawa et al., 2003	sub		200-500	25-55	I/G	m	Water	>6 lg at 300 MPa/55°C/6 cycle a 5 min >6 lg at 500 MPa/45°C/1 cycle and 30 min
Igura et al., 2003	sub		100-300	50	I	m	Distilled water	Sporulated at 30°C highest resistance, increased after demineralization
Kalchayanand et al., 2003		sp,la,per,te	345	60	I/G		Roast beef +Biopreservat.	la alone-extended shelf-life for 84 days at 4°C; with mixture of clostridial spores for 42 days
Krebbers et al., 2003	st		300,500,700	20, 80 90 initial T	I	m	in meat balls in tomato puree	6 lg at 700 MPa/ 20°C/ 2 min 6 lg pulse 2 x 700 MPa/ 80°C/ 30 s
Lopez-Pedemonte … 2003	cer		60-400				Cheese	2.4 lg after 60 MPa/30°C/210 min and 400 MPa/30°C/15 min+Nisin
März, 2003	sub	sp	600	60-90	I	m	Salt solution	1-2 lg of sp 600 MPa/ 60°C/ 300 min 6 lg of sp at 600 MPa/ 90°C/ 180 min
Oh and Moon, 2003	cer		0.1-600	20-60	I/G	m/b	Suspension	B. cer sporulated at pH 6 showed more resistance than at pH 7 or pH 8

continues…

Annex Table 1: continued

Reference	*Organism*		*Treatment*		*Type*	*Method*	*Medium*	*Comments*
	Bacillus	*Clostridium*	*Pressure [MPa]*	*Temperature [°C]*				
Reddy et al., 2003		bot	417-827	60-75	I	m	PBS pH 7.0 crabmeat blend	2-3 lg at 827 MPa/ 75°C/ 20 min in PBS same lg reduction in crab meat -->no protection
Watanabe et al., 2003	st, co, sub, cer li		0.1-200 + CO_2	35-95	I	b	Water	HP+CO_2 resulted in 5 lg at 30 MPa/95°C/120 min
Wilson and Baker, 2003	sub, st	sp	500-900	75-130	I	m, b	Phosphate Meat	Mixed spore culture (sub,st, sp) was inactivated 6 lg at 621 MPa/85°C/1 min
Ardia, 2004	st		200-1400	90-130	I	m	PBS, ACES Buffer pH7/ 6	8 lg in p,T diagram up to 1400 MPa/170°C for 3 different holding times(10, 30, 60 s) in PBS and 20 s holding time at pH 6 and 7 in ACES
Ardia et al., 2004a	Acy		0.1-700	initial 80-95	I	m	Orange juice	6 lg in p,T diagram for 5 holding times(10-30 min)
Clery-Barraud et al., 2004	ant		280-500	20-75	I	m		D(75°C) at 0.1/500 MPa: 348/4 min; D(20°C) at 500:160 min
Kalchayanand et al., 2004		te, per, la sp	138-483	25, 50	I/G	m/b	0.1% peptone pediocin AcH nisin A	483 MPa/ 50°C/ 5 min- te: 2.5 lg reduction per, la, sp: 0.1-0.2 lg reduction antimicrobial compound after HP
Margosch et al., 2004a	sub, cer, li, am		200-800	initial 60-80	I/G	m/b	Mashed carrots	>6 lg to no reduction, large resistance variation inact.=2 stage mechanism without germination *B. amyloliquefaciens* suggested target organism
Margosch et al., 2004b	sub, cer, li, am	bot	600-800	80-116	I	m/b	Mashed carrots	5.5 lg to no reduction at 600 MPa/ 80°C/ 1 s inact.=2 stage mechanism without germination *C. botulinum* TMW 2.357 most resistant *B. amyloliquefaciens* suggested target organism
Matser et al., 2004	naturally flora		900	initial 90°C	I/G	m	Vegetables	>3 lg; all under detection limit of ~1 lg, defined as sterility;2 pressure pulses
Rodriguez et al., 2004	st	bot	400-827	60-75; 92-110	I	m	PBS pH 7.0 (bot) dest.Water (st)	st- z(T) = 34.5°C, z(p)= 370 MPa; and transient process conditions
Van Opstal et al., 2004	cer		0-600	30-60	I/G	m	Milk	6lg-500 MPa/ 60°C/ 30 min or two-step treatment 200 MPa/ 45°C/ 30 min and then 60°C/10 min
Van Schepdael et al., 2004	st, sub		600-950	60-90	I	m	Tryptone soy	5.7 lg of B.st. at 60°C/950 MPa B. sub. at 90°C/700 MPa below detection limit
Aoyama et al., 2005a	sub, li, cer, co		60	40	I/G	m	PBS GAM broth	60 MPa/ 40°C/ 24 h sub -PBS: 1.6 lg; GAM broth: 5 lg li, cer, co- GAM broth, 1-3 lg
Aoyama et al., 2005b	sub		0.1-300	20-70	I/G	m	PBS glucose broth	Induction germination started at 10 MPa/ 40°C/1 h 2 lg at 20 MPa/ 60°C/ 1 h in broth 5 lg (PBS and broth) at 300 MPa/ 60°C/ 30 min

continues…

Annex Table 1: continued

Reference	*Organism*		*Treatment*		*Type*	*Method*	*Medium*	*Comments*
	Bacillus	*Clostridium*	*Pressure [MPa]*	*Temperature [°C]*				
Black et al., 2005	sub		150	initial 37°C	G	m/b	50 mM Tris-HCl pH 7.5	Detection of germinated spores with Syto16 and Flow Cytomerty;effects on pressure germination
De Heij et al., 2005	st, sub	bot, sp	400-800	70–110	I	m	Broth	st D(105°C) at 800 MPa = 20 s
Koutchma et al., z(p)~21°C 2005	st	sp	600-800;688 in 35 L vessel	91-121	I	m	PBS, Scrambled Egg patties	4.5 lg of C.sp at 800 MPa/108°C/3 min, in PBS, >6 lg at 688 MPa/121°C/3 min in patties >6 lg of B.st at 688 MPa/108°C/5 min in patties
Moerman, 2005	sub, st	sp, ty, sa	400	20, 50	I	m	Pork Marengo	20°C/30 min sub 1 lg, st 0.2 lg, sp ty sa 0.7 lg 50°C/30 min sub 4 lg, st 1.5 lg, sp ty sa ~3 lg
Farid, 2006	st		0.1 - 87	90 - 125	I	m	Water Milk	Heating a liquid food product to a temperature of 95°C will generate pressure of 700 bar 90 min leads to an inactivation of 4 lg
Gao et al., 2006a	st		432-768	63-97	I	m	Milk buffer	Optima at 86°C/625 MPa/ 14 min-->6 lg Response surface methodology (RSM)
Gao et al., 2006b	sub		323-668	63-97	I	m	Milk buffer	Optima at 87°C/576 MPa/ 13 min-->6 lg Response surface methodology
Islam et al., 2006	sub, coa, st		100	65-85	I	m	Potage, ketchup pH 4/ 7	4-8 lg dependent on pH (4 higher) higher inactivation in potage than ketchup
Lee et al., 2006	Acy		207,414,621	22,45,71,90	I/G	m	Apple juice 17, 35, 70°Brix	17.5° Brix 2 lg at 45°C, 5 lg at 71 and 90°C 30° Brix no effect at 45°C, 2 and 4lg at 71, 90°C 70° Brix, no inactivation
Margosch et al., 2006	am	bot	0.1-1400	70-120 const.	I	m	Tris-His buffer pH 4, 5.15, 6	p,T-diagram for 5 lg reduction of C.bot (pH 5.15) Isothermal conditions, tailing under pressure 4 lg reduction of B.am at 800 MPa/100°C
Patazca et al., 2006	st		500-700	92-111	I	m	Water	D(T,p) values 29.4 to 108.8 s at 92°C,17 - 76s at 100°C, 6.1 to 51 s at 111°C within 500-700 MPa
Rajan et al., 2006a	am		500-700	95-121	I	m	Egg patty mince	6.8 lg at 600 MPa/121°C/~1.7 min and 700 MPa at 110°C/ 3 min; z (700 MPa) = 28.6°C; z (12 1°C) = 332 MPa
Rajan et al., 2006b	st		400-700	105	I	m	Egg patty mince Water	4 lg at 700 MPa/ 105°C/ 5 min no inactivation difference in patties and water
Reddy et al., 2006		bot	551-827	40-100	I	m	Crabmeat blend PBS (pH 7)	>6 lg at 827 MPa/ 75°C/ 20 min in both media Crabmeat blend provided no protection
Sasagawa et al., 2006	sub		700	55°C	I/G	m	Orange juice PBS, Acetate	PEF+ HP, 7.1 lg in acetate buffer with pH 3.3 HP main lethal effect, HP 6 lg in orange juice

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Annex Table 1: continued

Reference	*Organism*		*Treatment*		*Type*	*Method*	*Medium*	*Comments*
	Bacillus	*Clostridium*	*Pressure [MPa]*	*Temperature [°C]*				
Scurrah et al., 2006	cer, co, li, pum		600	25, 75-95 initial T	I	m	Skim milk 9.5 % (w,w)	No inactivation to 6 lg at 600MPa/initial 72°C/1min high variability between species
Subramanian et al., 2006	am, sh	ty	700	121	I	m/b	Distilled water	Inactivation determined by FT-IR spectroscopy, adaquate comparison to plate count between
Black et al., 2006	sub		150, 500	50-76	G	m/b	Tris/HCl buffer pH 7.5	Germination detected by flow cytometry (Syto 16) 500 MPa no nutrient-germinantion, but DPA 0-8 lg inactivation after different dwell times
Ahn et al., 2007	am, sh	sp, ty	700	105, 121	I	m	Deionized water	sp, ty 6 lg at 700 MPa/ 105, 121°C(2, 0.2 min) am 6 lg at 700 MPa/ 105, 121°C(3, 0.2 min)
Mathys et al., 2007a	li		150, 600	10, 37, 77	I/G	m/b	Citrate buffer Nutrient broth	3 step mechanisms of inactivation detected by flow cytometry(PI,S16) with unknown population
Shigeta et al., 2007	sub, cer, pol		20-100	40	I/G	m	PBS glucose broth	60 MPa/ 40°C/ 30-60 min 5 lg germination with nutrients; 2-3 lg without nutrients
Subramanian et al., 2007	sh, am	ty	700	121	I	m	TSB, clostridial medium	Biochemical changes were studied by FT-IR 121°C no changes in the DPA content pressure leads to release of DPA
Vepachedu et al., 2007	sub		150, 500	Room	G	m/b	Buffer	SpoVAproteins involved in DPA release SpoVA proteins component of DPA channel
Mathys et al., 2008	st		500,600,900	80	I	m/b	PBS, ACES buffer (pH 5-8)	Different dissociation equilibrium shifts in buffer with effect on inactivation, up to 1.5 lg
Gao et al. 2008		bot	300-700	30-70	I	m	Milk	6 lg optimum (modelled) at 545 MPa/ 51 °C/ 13.3 min and 129 U/ml nisin
Suklim et al. 2008	cer		100,550	40	I/G	m	Water, Blue	Max. germination and inactivation in water at 550 MPa, crab meat in crab meat higher germination but retarded I Spores survived 4 °C storage
Robertson et al. 2008	6 species		600	75 start	I	m	Skimmed milk	p-transmitting media: silicon oil (T_{max} 90°C after 105 °C) higher inactivation compared to after cycling 103 °C)
Black et al. 2008	sub, cer		100, 500	40	I/G	m/b	Buffer, milk	B. sub germination 4 lg, 500 MPa and 2.5 lg I + 500U/ml nisin >5.7 lg / B. cer more resistant
Zhu et al. 100°C under 2008		sp	700-900	80-100	I	m	Ground beef	D-value: 15.8-7.0 min at 80°C and 1.5-0.63 at p; z-value: under pressure,
Ju et al. 2008	cer		400-600	60-80	I	m	Milk bufferRSM-model:	optimum 6lg at 540 MPa, 71 °C. 16.8 min
Bull et al. 2009	sp, bot		600	105	I	m	30% Bolognese, Rice water	$F_{105°C}$-value calculation, for some strains no protective effect of tested media, C. sp. no suitable surrogate for C. bot

continues…

Annex Table 1: continued

Reference	*Organism*		*Treatment*		*Type*	*Method*	*Medium*	*Comments*
	Bacillus	*Clostridium*	*Pressure [MPa]*	*Temperature [°C]*				
Wang et al. 2009	co		400-600	70-80	I	m	PBS buffer, Whole milk	Higher resistance in milk, tailing occurred which could be best fitted with Weibull-model
Wei et al. 2009	cer		150	40-50	I/G	m	Buffer	Treatment of wild-type and mutant strains that lack some nrGR, wild-type spore germinated slightly faster
Ratphitagsanti et al. 2010	am		700	35, 105	I/G	m	Organic acid	Full inactivation even without org. acids (3min, 700 MPa 105°C),
							Carrot puree	105 °C or HP at 35°C caused no inactivation,
Ramaswamy et al. 2010a		sp	700-900	80-100	I	m	Milk	Isothermal, isobaric treatment; no inactivation after 1s; at 900 MPa D-values of 9.1-0.73 min, synergism between p and T, z-value under pressure higher (16.5-18.2 min)
Ramaswamy et al. 2010b		sp	700-900	80-100	I	m	Salmon slurry	Synergism between p and T, z-value under p higher (14.5-15.5 min) compared to 0.1 MPa
Gao et al. 2010a	st		625	86	I	m	Ringer solution + /Sucrose /Soybean protein / Soybean oil	pH value variation and RSM-model, 6 lg I no effect of soybean oil; pH, soybean protein significantly effected spore inactivation
Wei et al. 2010	sub		150, 500	room	G	m	TRIS buffer	Superdormant spores germinate well after p
Reddy et al. 2010		bot	620	95, 105	I	m	TPGY-broth	Addition of lysozyme, L-alanine, L-aspartic acid, DPA, sodium bicarbonate and lactate; None of them improved spore recovery despite lysozyme (less than 1lg)
Balasubramanian et al. 2010	sub		690, 827	60-75	I	m	Citrate buffer (pH 3, 5,7) Tomato puree Minced crabmeat	Highest inactivation in citrate buffer (pH 3) 7lg, 825 MPa, 75 °C 5min
Gao et al. 2010b	cer		540	71	I	m	Ringer solution + /Sucrose /Soybean protein / Soybean oil	Soybean protein, pH and sucrose significantly effected spore inactivation; verification of quadratic predictive model to describe impact of food ingredients
De Lamo-Castellvi 2010	am		700	35, 105	I	m	Deionized water Mashed carrots	Addition of sucrose laurate (0.1-1%), 1% SL synergistic effect under pressure, no effect showed best at 105°C and 0.1 MPa or 35 °C and 700 MPa

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Annex Table 1: continued

Reference	*Organism*		*Treatment*		*Type*	*Method*	*Medium*	*Comments*
	Bacillus	*Clostridium*	*Pressure [MPa]*	*Temperature [°C]*				
Minh et al. 2010	sub		90-550	40	I/G	m	Distilled water	Spore germination more efficient at low p estimation of germination by 10min at 80 °C or 350 MPa, 40°C for 10 min, pressure resistant sp
Gao et al. 2011a	co		300-700	15-75	I	m	Milk buffer + nisin (0-200 U/mL)	Optimum for 6 lg inactivation: 550 MPa, 41 °C, 12.2 min, 120 U/mL nisin det. by RSM model;
Baier et al. 2011	sub		150-600	37-60	I/G	m/b	ACES buffer	Correlation of germination data detected by FCM (SYTO 16) and released amount of DPA, fluorescence Tb-DPA quantification suitable for screening p-T conditions for germination
Reineke et al. 2011b	sub		550-1000	37-80	I/G	m/b	ACES buffer	No inactivation at 550 MPa, 37 °C, 2h; above 600 MPa, 60 °C treatment temperature Dominant; mutant strains lacking one CLE showed only slight higher Inactivation (sleB)
Shao et al. 2011		sp	700-900	80-100	I	m	Milk (2% fat)	Pressure pulse inactivated 0.5 lg; D-value at 700 MPa from 13.6-2.4 min, at 900 MPa (16.5-20.3 °C) higher than at 0.1 MPa (9 °C)
Minh et al. 2011	sub		350	40	I/G	m/b	Distilled water	Effect of sporulation conditions on p-resistance spores produced at pH were most resistant
Marco et al. 2011	cer		200-500	20 start	I	m	BHI broth + 0-2.5% olive powder	Max 1.5 lg inactivation; 2.5% olive powder slightly decreased inactivation, highest 200MPa
Gao et al. 2011b		per	400-800	35-95	I	m	Milk + nisin	RSM-model calculated optimum parameter for 6 lg 654 MPa, 74 °C, 13.6 min & 328 U/mL nisin
Reineke et al. 2012a	sub		100-700	30-80	I/G	m/b	ACES buffer	Spore germination without nutrient receptors possible at 200 MPa, first step of inactivation is germination, multi-response kinetic modeling
Sokolowska et al. 2012	Acy		100-500	50	I	m	Apple juice	Highest inactivation at 200 MPa, stabilization at 500 MPa,200 MPa + 60 min at 0.1 MPa + 500 MPa resulted in 6.15 lg ,addition of 250 U/mL nisin enhanced inactivation, no effect of lysozyme
Vercammen et al. 2012	co, Acy		100-800	25-60	I/G	m	PBS buffer Tomato sauce	2lg germination of Acy at p< 300 MPa and co 800 MPa, pH 4 suppressed Acy germination but stimulated B. co germination, tomato sauce, 60 °C, pH 4.2. 3.5 lg Acy and 2.0 lg B. co I
Syed et al. 2012	sub		600	60-70	I/G	m/b	TRIS buffer Skimmed milk Orange juice	Compression and decompression rate effect spore inactivation, highest inactivation for slow compression and decompression

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Annex Table 1: continued

Reference	*Organism*		*Treatment*		*Type*	*Method*	*Medium*	*Comments*
	Bacillus	*Clostridium*	*Pressure [MPa]*	*Temperature [°C]*				
Aouadhi et al. 2012	spot		300-500	30-50	I	m	Distilled water Skimmed milk	Higher I in distilled water, RSM model – 5 lg optimum at: 477 MPa, 48 °C, 26 min (water), 495 MPa, 49 °C, 30 min (milk)
Olivier et al, 2012	am, co, spot		600	110	I	m	molten Nutrient agar	Impact of sporulation conditions on HP tested; high sporulation T decreased pressure resistance and effects for increasing minearl B. am spores sporulated at 37 or 30 °C: D-value 0.58 – 4.0 min under pressure
Reineke et al. 2012b	sub		100-700	30-80	I/G	m/b	ACES buffer	Structural analysis of internal structure of pressure treated spores (FIB-SEM), Mechanism proposed: p < 550 MPa physiological like germination – SASP degradation possible; p > 600 MPa, T > 60°C inner spore membrane most sensitive structure under pressure
Reineke et al. 2012c	sub		100-700	30-80	I/G	m/b	ACES buffer	Isorate-pressure-temperature-diagram for DPA release, differentiation between 3 different release mechanisms in dependence of p and T,
Zimmermann et al. 2013	co		300-600	50-60°C	I		tomato pulp	at 300 MPa 50-60°C -2-2.4 lg and at 600 MPa 50-60°C for 15 min - 3.1-5.7 lg inactivation
Sevenich et al. 2013	am,gbs		600 MPa	90-121°C	I		different fish foods	-4 lg of GBS at 90°C between 6-8 min; am I -4-5 lg for 90-115°C depending on food
Ramaswamy et al 2013	bot		800-900	90-100°C	I		phosphate buffer (0.1M)	1-3.5 lg inactivation at 90-100°C with time 5-15 min
Daryaei et al. 2013	co		600 MPa	75-105°c	I		tomato juice	holding times of 10-40s inactivated -7 lg
Hue Luu-Thi et al. 2014	cer		600 MPa	60-100°C	I		MES-buffer	-1-3 lg at 60-100°C for holding times 5-60 min
Georget et al. 2014	GBS		200 MPa	55°C	G		Laurdan	-3 lg at 200 MPa 55°C due to germination
Bekele et al. 2014	li		400-600 MPa	40-60°C	I		carrot juice	-3.3-4.9 lg depending on condition dwell time 3-40 min, pH 4.5-6.2 showed better I for low pH

continues…

Reference	*Organism*		*Treatment*		*Type*	*Method*	*Medium*	*Comments*
	Bacillus	*Clostridium*	*Pressure [MPa]*	*Temperature [°C]*				
Martinez-Monteagudo et al. 2014	am		600	121	I		milk	nisin (≥ 16 mg L^{-1}) to milk -7 lg inactivation after 5min
Sevenich et al. 2014	am,gbs		600	90-121	I		baby food puree, ACES	am 4-5 lg 0.2-20 min for T 90-115°C, gbs - 3.5 lg at 90°C between 4-6 min dwell time
Olivier et al. 2014	am, sp		600	100 and115	I		MPA broth	sp at 100-105°C with time 1-2 min 2.9-5.5 lg am at 115°C with time 1.5-3.5 min 4.4-7.2 lg
Devatkal et al. 2015	am		600	105	I		deionized water, beef broth	am 5 min at 105°C in water -2.5lg and beef broth 3 min at 105°C 2.1-3.58 lg
Sevenich et al. 2015	am		600	105-115	I		baby food puree, fish food systems	Based on lab scale trials T,t conditions for -12 lg were calculated and validated by storage trials
Ahn et al 2015	gbs		500 & 700	105	I		milk, mashed potato,beef	in milk and mashed potato at 700 MPa >-6 lg in beef -4.27 lg for all holding time = 3 min
Schnabel et al 2015	bot E		300-750	45-75	I		emulsion with 30-70 % fat	holding time = 10 min; depending on T,p and fat content inactivation between 1.29-3.23 lg
Lenz et. al 2015	bot E		200 & 800	40&80	I		IP-buffer	dwell time 10 min: 200 40 inativation ≤0.8 lg; 200 80 inactivation 2.6-3.3 lg 800 40 inactivation 3-4.5 lg 800 80 inactivation 6.5-7.3lg

Organism: sub, *B. subtilis*; st, *G. stearothermophilus*; cer, *B. cereus*; li, *B. licheniformis*; meg, *B. megaterium*; pum, *B. pumilis*; co, *B. coagulans*; pol, *B. polymyxa*; ant, *B. anthracis;* am, *B. amyloliquefaciens*; sh, *B. sphaericus*; *spot, Bacillus sporothermodurans*; Acy, *Alicylobacillus acidoterrestris*; sp, *C. sporogenes*; pas, *C. pasteurianum*; bot, *C. botulinum*; ty, *C. tyrobutyricum*; sa, *C. saccharolyticum*; la, *C. laramie*; per, *C. perfringens*; te, *C. tertium; gbs, Geobacillus stearothermophilus,*

Type (of experiment): I, inactivation; G, germination.

Methodology used: m, microbiological; b, biochemical.

Comments: lg = $\log_{10}$.

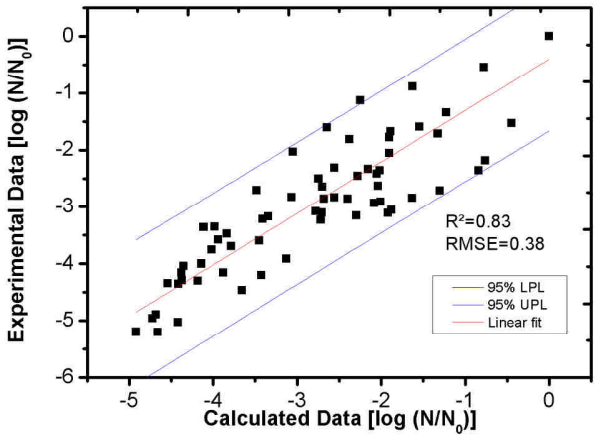
Annex II

Overview of modeling data for the different systems and spore strains

1. Selected spores in ACES-buffer system

Geobacillus stearothermophilus in ACES-buffer: Weibull fit data (Mafart et al., 2002) and comparison of experimental data and inactivation calculated data.

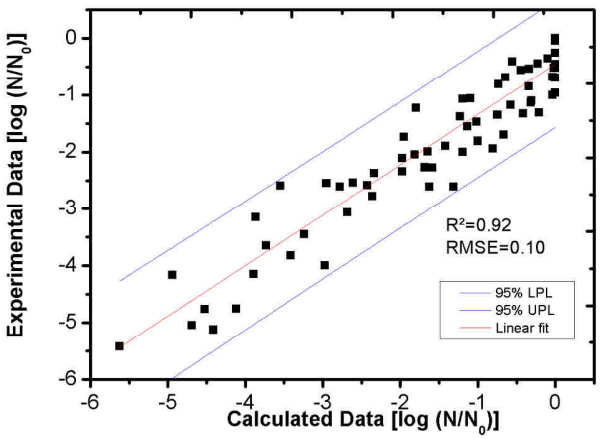
$\log (N/N_0) = -(t/k\,(p,T))^n$; n_{mean}= 0.45; t = 0-60 min



p [MPa]	T [°C]	k(p,T)
200	40	3.61
200	60	1.93
200	80	0.54
200	100	0.16
400	40	3.23
400	60	1.91
400	80	0.47
400	100	0.07
600	40	3.13
600	60	1.13
600	80	0.19
600	100	0.02

Clostridium sporogenes in ACES-buffer: Weibull fit data (Mafart et al., 2002) and comparison of experimental data and calculated inactivation data.

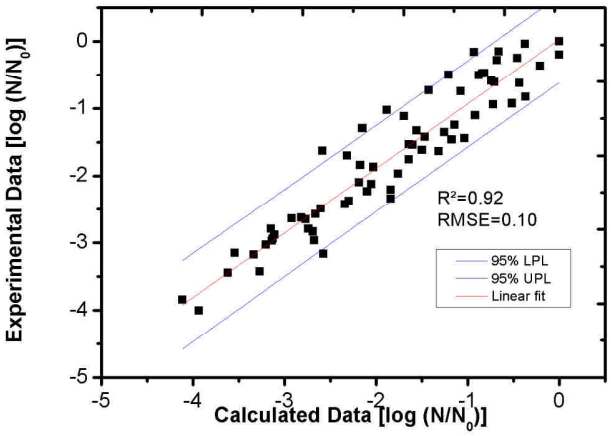
$\log (N/N_0) = -(t/k\,(p,T))^n$; n_{mean} = 0.98; t = 0-40 min



p [MPa]	T [°C]	k(p,T)
200	75	80.05
200	85	22.71
200	95	1.26
400	75	30.87
400	85	8.48
400	95	0.7
600	75	16.19
600	85	5.29
600	95	0.55

Bacillus amyloliquefaciens in ACES-buffer: Weibull fit data (Mafart et al., 2002) and comparison of experimental data and calculated inactivation data.

$\log (N/N_0) = -(t/k\,(p,T))^n$; $n_{mean} = 0.83$; t = 0-60 min

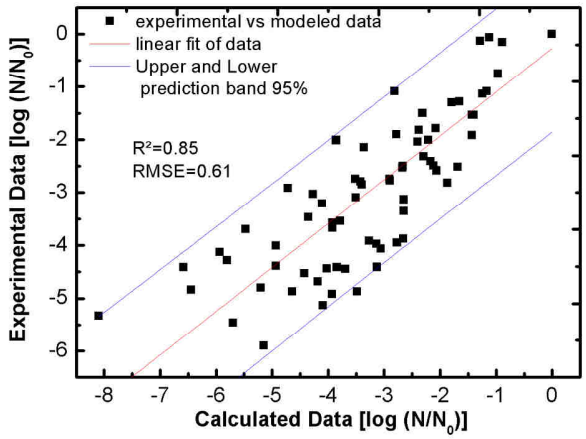


p [MPa]	T [°C]	k(p,T)
200	40	16.51
200	60	6.15
200	80	6.37
200	100	3.27
400	40	25.57
400	60	6.31
400	80	4.69
400	100	2.75
600	40	66.59
600	60	7.19
600	80	3.84
600	100	0.38

2. Bacillus amyloliquefaciens in different a_w-values adjusted by solutes (NaCl and sucrose)

Bacillus amyloliquefaciens in NaCl solution at 600 MPa : Weibull fit data (Mafart et al., 2002) and comparison of experimental data and calculated inactivation data.

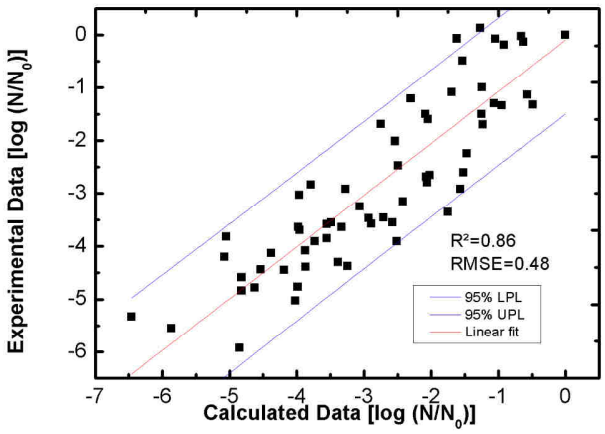
$\log (N/N_0) = -(t/k\,(a_w,T))^n$; $n_{mean} = 0.38$; p= 600 MPa; t = 0-15 min



a_w	T [°C]	k(a_w,T)
1	105	0.178
0.96	105	0.216
0.94	105	0.277
0.92	105	0.397
0.9	105	0.642
1	110	0.074
0.96	110	0.089
0.94	110	0.115
0.92	110	0.165
0.9	110	0.266
1	115	0.034
0.96	115	0.041
0.94	115	0.053
0.92	115	0.076
0.9	115	0.123

Bacillus amyloliquefaciens in sucrose solution at 600 MPa: Weibull fit data (Mafart et al., 2002) and comparison of experimental data and calculated inactivation data.

$\log (N/N_0) = -(t/k\ (a_w,T))^n$; $n_{mean} = 0.58$; p= 600 MPa; t = 0-15 min



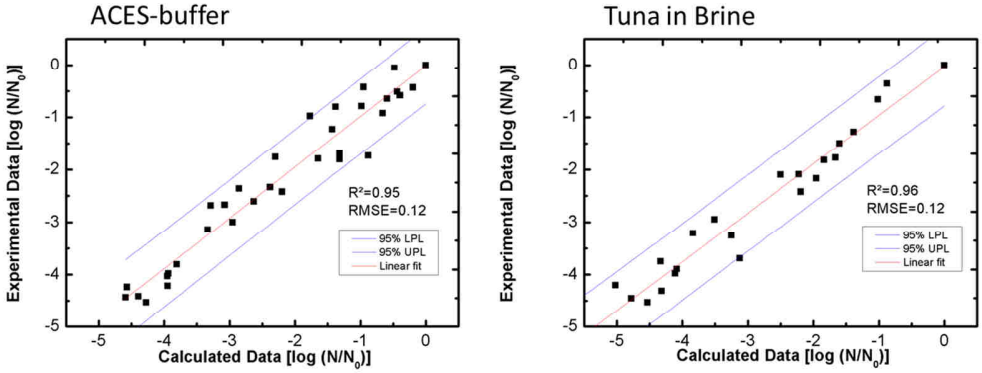
a_w	T [°C]	k (a_w,T)
1	105	0.458
0.96	105	0.586
0.94	105	0.752
0.92	105	1.125
0.9	105	1.924
1	110	0.225
0.96	110	0.279
0.94	110	0.369
0.92	110	0.552
0.9	110	0.943
1	115	0.11
0.96	115	0.136
0.94	115	0.18
0.92	115	0.269
0.9	115	0.46

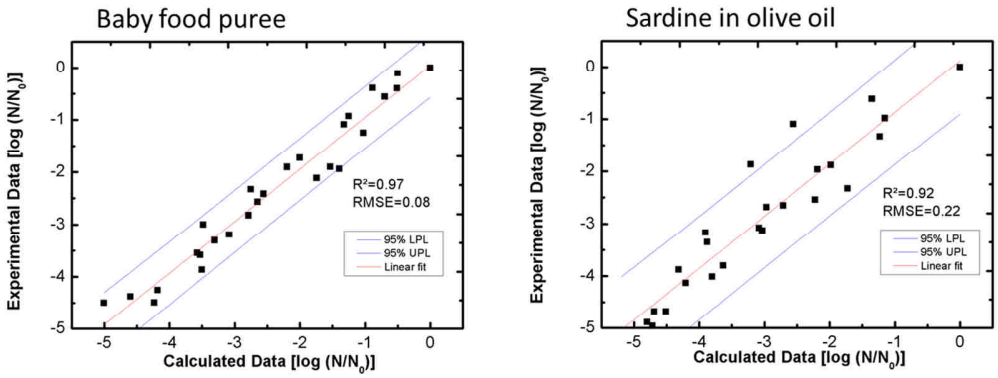
3. *Clostridium sporogenes* and *Bacillus amyloliquefaciens* in real food systems (tuna in brine, sardine in olive oil, tuna in sunflower oil and baby food puree

Clostridium sporogenes in real food systems at 600 MPa: n^{th}-order fit data and comparison of experimental data and calculated inactivation data.

$$log_{10}(\frac{N}{N_0}) = log_{10}(e^{-k(T)*t}) \text{ for n = 1}$$

$$log_{10}(\frac{N}{N_0}) = log_{10}(((1 + k(T) * N_0^{n-1} * t * (n-1))^{\frac{1}{1-n}}) \text{ for n > 1; t=0-80 min and T[°C]=80-95}$$



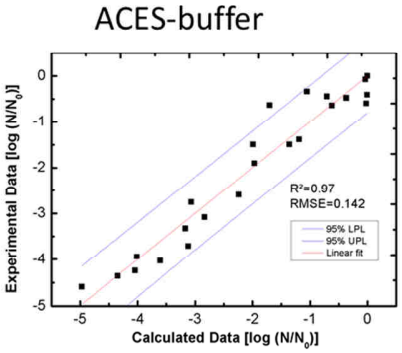


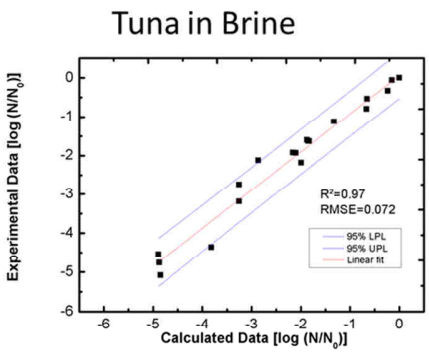
	p [MPa]	T [°C]	k(T)
ACES-Buffer; n=1.0	600	80	0.151
	600	85	0.337
	600	90	0.757
	600	95	3.287
Baby food puree; n=1.0	600	80	0.192
	600	85	0.392
	600	90	0.802
	600	95	3.047
Tuna in sunflower oil; n=1.1	600	80	0.518
	600	85	0.743
	600	90	1.314
	600	95	5.675
Sardine in olive oil; n=1.1	600	80	0.243
	600	85	0.608
	600	90	1.443
	600	95	3.269

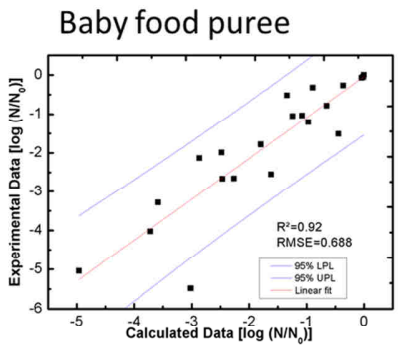
Bacillus amyloliquefaciens in real food systems: n[th]-order fit data and comparison of experimental data and calculated inactivation data.

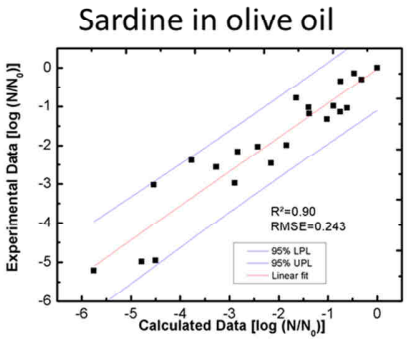
$log_{10}(\frac{N}{N_0}) = log_{10}(e^{-k(T)*t})$ for n = 1

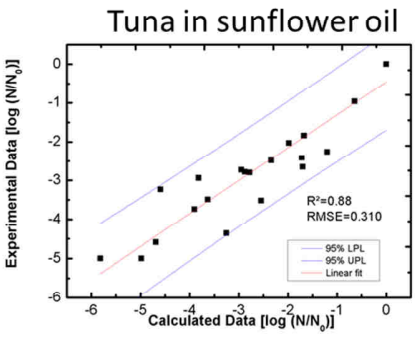
$log_{10}(\frac{N}{N_0}) = log_{10}(((1 + k(T) * N_0^{n-1} * t * (n-1))^{\frac{1}{1-n}})$ for n>1;t=0-30 min and T=90-121°C











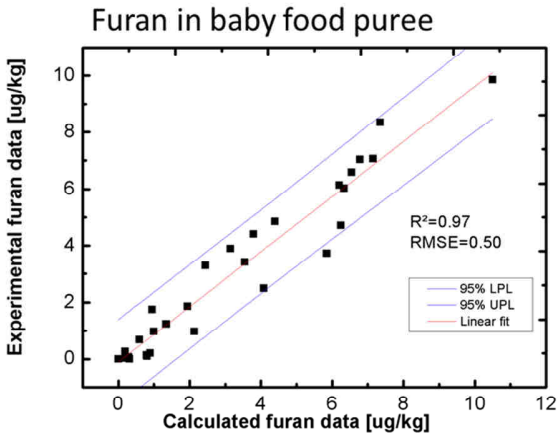
	p [MPa]	T [°C]	ln k (T)
ACES-Buffer; n=1.05	600	90	-0.58
	600	105	1.13
	600	110	1.60
	600	115	3.95
Baby food puree; n=1.0	600	90	-0.95
	600	105	0.37
	600	110	1.75
	600	115	4.12
Tuna in brine; n= 1.0	600	90	-0.51
	600	105	0.82
	600	110	1.37
	600	115	3.60
Tuna in sunflower oil; n = 1.1	600	90	-0.63
	600	105	1.55
	600	110	2.27
	600	115	3.00
Sardine in olive oil; n = 1.1	600	90	-1.38
	600	105	1.32
	600	110	2.22
	600	115	3.11

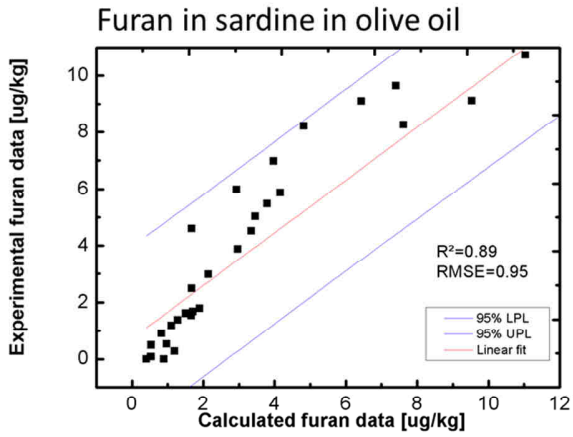
4. Formation of furan in sardine in olive oil and baby food puree

Furan formation data in the selected food systems: Weibull fit data and comparison of experimental data and formation calculated data.

$y=k*(X)^n$; n_{mean} = 0.38 (Sardine in olive oil); n_{mean} = 0.58 (baby food puree)

p= 600 MPa; t = 0-30 min





	p [MPa]	T [°C]	k (T)
Baby food puree	600	90	0.258
	600	105	0.684
	600	110	0.979
	600	115	1.425
	600	121	2.285
Sardine in olive oil	600	90	0.492
	600	105	1.613
	600	110	2.495
	600	115	3.938
	600	121	6.995

5. Corresponding F-values for the treatments at 600 MPa at lab scale for temperatures between 90-115°C and D-values at 600 MPa with their corresponding z-values

	z = 10 K	**F-value [min] for**				
Food	Temperature [°C]	shortest holding time [min]	longest holding time[min}	time [min] for extrapolated -12 $\log_{10}$	extrapolated -12 $\log_{10}$	actual holding time [min] to achieve F_0=7
Tuna in Brine	90	0.0024	0.022	54.3	0.043	8812
	105	0.012	0.1	33.65	0.845	278
	110	0.04	0.24	20.2	1.6	88.1
	115	0.012	0.1255	7.7	1.93	28
Baby food puree	90	0.0024	0.022	111.5	0.088	8812
	105	0.012	0.1	16.75	0.42	278
	110	0.04	0.24	4.85	0.38	88.1
	115	0.012	0.1255	0.45	0.11	28
Tuna in sunflower oil	90	0.0024	0.022	280	0.22	8812
	105	0.025	0.15	36	0.9	278
	110	0.04	0.24	18	1.43	88.1
	115	0.012	0.06	9	2.3	28
Sardine in olive oil	90	0.0024	0.022	600	0.48	8812
	105	0.012	0.1	40	1	278
	110	0.04	0.24	18	1.43	88.1
	115	0.012	0.06	9	2.3	28

	D-values [min] at 600 Mpa			
T [°C]	Tuna in brine	Baby food puree	Tuna in sunflower oil	Sardine in olive oil
90	3.4	4.8	5	9.3
105	1.1	1.05	1.3	1.4
110	0.48	0.35	0.6	0.8
115	0.04	0.04	0.04	0.04
Corresponding z-value [K]	7.2	5	4.9	3

6. **Treatment conditions of the Sardine in olive oil (SO), Tuna in sunflower oil (TO), Tuna in Brine (TB) and Baby food puree (BF) and corresponding F_0 at pilot scale (600 MPa, 100-115°C)**

Matrix	Treatment	F-value [min]
SO	untreated	-
SO	100°C, 10 min undertreated	0.08
SO	115°C,28 min @ 600 MPa	7
SO	115°C,6.56 min @ 600 MPa	1.64
SO	113°C, 9.40 min @ 600 MPa	1.49
TO	untreated	-
TO	100°C, 10 min undertreated	0.08
TO	115 C; 28 min @ 600 MPa	7
TO	115°C, 7.41 min @ 600 MPa	1.86
TO	113°C, 9.9 min @ 600 MPa	1.56
TB	untreated	-
TB	100°C, 10 min undertreated	0.08
TB	115 C; 28 min @ 600 MPa	7
TB	115°C, 0.75 min @ 600 MPa	0.18
TB	110°C, 6.53 min @ 600 MPa	0.52
TB	107.5°C, 9.80 min @ 600 MPa	0.43
BF	untreated	-
BF	100°C, 10 min undertreated	0.08
BF	115 C; 28 min @ 600 MPa	7
BF	115°C, 0.45 min @ 600 MPa	0.11
BF	110°C, 4.84 min @ 600 MPa	0.38
BF	107.5°C, 9.80 min @ 600 MPa	0.43

Curriculum vitae

Robert Severin Sevenich – Curriculum vitae

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Main Research fields

- Inactivation of bacterial spores under high pressures (600 MPa) and high temperatures (90-121°C)
- Understanding the influence of complex food systems on the inactivation of spores
- Formation of food processing contaminants in foods and mitigation strategies
- Optimization of the high pressure thermal sterilization process
- Mechanisms and influence of high pressure and mild temperatures on vegetative (pathogenic/non-pathogenic) microorganism
- Use of novel food technologies for the all-*trans*-lycopene isomerization into its more valuable 5-*cis*-form
- Influence of high pressure processing on the physical properties of food additives

Education

2011-2015 Technische Universität Berlin, Department of Food Biotechnology and Food Process Engineering

Ph.D. candidate

High pressure processing at ambient and high temperatures and its influence on food processing contaminants, food borne diseases and spores in model- and real-food systems

Supervision: Prof. Dr. Dietrich Knorr

Financial support by the European Commission (project PROMETHEUS, PROcess contaminants: Mitigation and Elimination Techniques for High food quality and their Evaluation Using Sensors & Simulation; FP7-KBBE-2010-4-265558)

2005-2011 Study of food technology at the Technische Universität Berlin

Diplom in food engineering (Dipl.-Ing.) July 2011 with grade average of 1.4

2009-2011 Student assistant in the Ultrasound working group (Ultraveg)

Languages

German native

English fluent

French basic skills

Research projects

- PROMETHEUS: Process contaminants: Mitigation and elimination techniques for high food quality and their evaluation using sensors and simulation. Subproject leader FP 7 EU Project.
- 2 Projects with WILD FLAVOR on High pressure pasteurization
- 1 Project with Analyze and Realize on Lycopene isomerization with alternative technologies
- 1 Project with Tate & Lyle on Influence of high pressure on different food additives

Awards

January 2016 1st place at IFTPS Charles Stumbo Graduate Student Paper Competition, San Antonio, TX, USA

July 2015 2nd place at the Aquatic Food Division IFT International Research Paper Competition" 2015 at IFT 15 Chicago, IL, US

January 2015 3rd place at the IFTPS Charles Stumbo Graduate Student Paper Competition, San Antonio, TX, USA

June 2014 Winner of the "George F. Stewart IFT International Research Paper Competition" Award 2014 at IFT 14 New Orleans, LA, US

October 2012 Supervisor of the team "Cruemel", 2nd place at the European Student Award for Food Innovation (ECOTROPHELIA 2012). SIAL 2012, Paris, France

April 2012 Supervisor of the team "Cruemel", winner of the German Student Award for Food Innovation (TROPHELIA 2012). FEI-meeting 2012, Bonn Germany

Research grants

July 2015 Travel Grant by the International Division of the IFT for traveling to IFT 15 in Chicago, IL, USA

Teaching activities

- Thermal technology of food processing (integrated seminar and practical courses)
- Innovative Technologies (integrated seminar)
- Supervisor of 6 Master/Diploma thesis and 8 bachelor/ scientific student thesis

Reviewer activities

- Innovative Food Science and Emerging Technologies
- Journal of Food Engineering

Membership in scientific organizations

- Institute of Food Technologists (IFT)
- Student representative of the International Division of the IFT
- International Association for Food Protection (IAFP)
- Member of the institute consul of the Institute of Food Technology and Food Chemistry

Internship experience

October-November 2010 Bakery DEWIBack, Berlin, Germany

Production and production control, optimization of process line

March 2009 BakeMark, Neu-Ulm, Germany

Production and production control, optimization of process line

April-June 2007 Versuchs und Lehrbrauerei Berlin

Brewing process, analytical control and other fermentation processes

List of publications

Peer reviewed publications

1) Gonzalez-Arenzana, L., Sevenich, R., Rauh, C., Lopez, R.,Knorr, D. and Lopez-Alfaro, I. "Inactivation of Brettanomyces bruxellensis by High Hydrostatic Pressure technology" Food Control (May 2015)
2) Sevenich, R., Hecht, P. ,Reineke, K., Fröhling, A., Schlüter, O., Rauh, C. and Knorr, D. "Impact of different water activities (aw) adjusted by solutes on high pressure high temperature inactivation of *Bacillus amyloliquefaciens* spores" Frontiers in Microbiology: Microbial decontamination by novel technologies - Analytic approaches and mechanistic insights (July 2015)
3) Mesias, M., Holgada, F., Sevenich, R., Briand, J.C., Marquez-Ruiz, G., Morales, F.J. "Fatty acids profile in canned tuna and sardine after retort sterilization and high pressure thermal sterilization treatment" Journal of Food and Nutrition Research (April 2015)
4) Sevenich, R., E. Kleinstueck, F. Bark, C. Crews, C. Pye, J. Hradecky, K. Reineke, M. Lavilla, I. Martinez de Maranon, J.C. Briand and D. Knorr "The impact of high pressure thermal sterilization on the microbiological stability and formation of food processing contaminants in selected fish systems and baby food puree at pilot scale" *Food Control* (April 2015)
5) E. Georget, R. Sevenich, K. Reineke, A. Mathys, V. Heinz, M. Callanan, C. Rauh and D. Knorr "Inactivation of microorganisms by high isostatic pressure processing in complex matrices: A review" *Innovative Food Science & Emerging Technologies* (January 2015)
6) K. Reineke, R. Sevenich, C. Hertwig, T. Janßen, A., Fröhling, D. Knorr, L. Wieler and O. Schlüter „Comparative study on the high pressure inactivation behavior of the Shiga toxin-producing E. coli O104:H4 and O157:H7 outbreak strains and a non-pathogenic surrogate." Food Microbiology (April 2014)
7) Sevenich, R., E. Kleinstueck, C. Crews, W. Anderson, C. Pye, K. Riddellova, J. Hradecky, E. Moravcova, K. Reineke, and D. Knorr. 2014. „High Pressure Thermal Sterilization: Food safety and food quality of baby food puree." Journal of Food Science (February 2014)
8) Sevenich, R., F. Bark, C. Crews, W. Anderson, C. Pye, K. Riddellova, J. Hradecky, E. Moravcova, K. Reineke, and D. Knorr. 2013. "Effect of High Pressure Thermal Sterilization on the Formation of Food Processing Contaminants." *Innovative Food Science & Emerging Technologies* (August 2013)
9) Reineke, K. , Sevenich, R., Mathys, A. and Knorr, D. Book Chapter "Mechanisms of spore germination and inactivation during high pressure processing" in the 2nd edition of High pressure processing of foods (2015)
10) Sevenich, R., Article on "High pressure thermal sterilization" in the Food Science Reference Module by Elsevier (September 2015)

Presentations at scientific meetings

Oral presentations

1. R.Sevenich, E.Kleinstück, F.Bark and D. Knorr „PROcess contaminants: Mitigation and Elimination Techniques For high food quality and their Evaluation Using Sensors & Simulation" at the European Committee, 10.4.2014 Brussles, Belgium.
2. R.Sevenich, E.Kleinstück und D.Knorr „High pressure thermal sterilization (HPTS): The way to increase microbial food safety and to mitigate food process contaminants in selected food systems" The 8th European Workshop on Food Engineering and Technology at the DIL, Quakenbrück, Germany 1-2.4.2014**.**
3. R. Sevenich, F.Bark, E. Kleinstück und D. Knorr „Hochdrucksterilisation ausgesuchter Lebensmittel: Vom Labormaßstab zur Pilotanlage" Dechema 2014 - Jahrestreffen der ProcessNet-Fachgruppe Lebensmittelverfahrenstechnik und Phytoextrakte; Freising, 26-28.2.2014
4. R. Sevenich und D. Knorr „Einsatz von neuen Technologien in der Lebensmittelprozessierung: Ein Überblick. Schwerpunkt Hochdruckbehandlung von Lebensmitteln" VZD Symposium, Erfurt, 3.12.2013
5. **"**High pressure thermal sterilization: The way forward to mitigate processing contaminants in fish canned products and baby food purees " Prometheus Industry Info day, Madrid, Spain. 29.11.2013
6. R. Sevenich, F.Bark, E. Kleinstück und D. Knorr "Résoudre le compromis entre la production de composes néoformés et la maîtrise du risque microbiologique à l'aide de technologies innovantes" INRA Symposium, Avignon, 14.11.2013
7. R. Sevenich, E. Thieme und D. Knorr "Baroprotective effect of solutes against the high pressure inactivation of *Bacillus amyloliquefaciens* spores" at the IFOOD 2013 in Hannover, 8-10.10.2013.
8. R. Sevenich, E.Kleinstück und D. Knorr "HIGH PRESSURE THERMAL STERILIZATION (HPTS) OF BABY FOOD PUREE (on the basis of vegetables) AND THE POSSIBLE REDUCTION OF FOOD PROCESSING CONTAMINANTS" at the IAFP Symposium in Marseille, 15-17.5.2013
9. Progress and major achievements of the HPTS in the review period". Review meeting with the EU - Paris – January 24th, 2013
10. R. Sevenich, F.Bark und D. Knorr "Reduction of furan and MCPD-esters during the High Pressure Sterilization (HPST) of fish " at the 50th EHPRG Meeting, Thessaloniki, Greece 16-21 September 2012

Poster presentations

1. Impact of different water activities (a_w) adjusted by solutes on high pressure high temperature inactivation of Bacillus amyloliquefaciens sporesRobert Sevenich[1]*, Kai Reineke, Philipp Hecht, Antje Fröhling, Cornelia Rauh, Oliver Schlüter and Dietrich Knorr at the IFT 2015 in Chicago, IL, USA 11-14.7.2015

2. HIGH PRESSURE THERMAL STERILIZATION (HPTS): ITS IMPACT ON THE FORMATION OF FOOD PROCESSING CONTAMINANTS AND SPORE INACTIVATION IN DIFFERENT FOODS CONATINING FISH AT LAB AND PILOT SCALE. R. Sevenich[1*], F. Bark[1] , E. Kleinstueck[1] , C. Crews[2], C. Pye[2] J.Hradecky [3], K. Reineke[4], M. Lavilla[5], I. Maranon[5], D. Knorr[1] at the IFT 2015 in Chicago, IL, USA 11-14.7.2015
3. High pressure thermal sterilization (HPTS): The way forward to mitigate food process contaminants in baby food purees. R. Sevenich[1*], F. Bark[1] , E. Kleinstueck[1] , C. Crews[2], C. Pye[2] J.Hradecky [3], K. Reineke[4], M. Lavilla[5], I. Maranon[5], D. Knorr[1] at the IFT 2014 in New Orleans, LA, USA 21-25.6.2014
4. Baroprotective effect of solutes against the high pressure inactivation of *B. amyloliquefaciens* spores. Robert Sevenich[1]*, Elena Thieme [1], Philipp Hecht[1] , Cornelia Rauh[1] and Dietrich Knorr[1]at the IFT 2014 in New Orleans, LA, USA 21-25.6.2014.
5. R. Sevenich, F.Bark und D. Knorr "High pressure thermal sterilization (HPTS): A possible tool to increase safety and quality of food products" at the IFT 2013 in Chicago, IL, USA 13-16.7.2013

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Eidesstattliche Erklärung

Hiermit versichere ich an Eides statt, dass ich die Dissertation selbständig verfasst habe. Alle benutzten Hilfsmittel und Quellen sind aufgeführt. Weiter erkläre ich, dass ich nicht schon anderweitig einmal die Promotionsabsicht angemeldet oder ein Promotionseröffnungsverfahren beantragt habe. Veröffentlichungen von irgendwelchen Teilen der vorliegenden Dissertation sind von mir, wie in der vorstehenden Publikationsliste aufgeführt, vorgenommen worden.

Berlin, 08.12.2016

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