Lehrstuhl für Technische Mikrobiologie

# Behaviour of bacterial endospores and toxins as safety determinants in low acid pressurized food

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# Behaviour of bacterial endospores and toxins as safety determinants in low acid pressurized food

Dirk Margosch

Doctoral thesis

Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt

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**Vorwort**

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**Abbreviations**

| | |
|---|---|
| ATCC | American type culture collection, Manassas, Virginia, USA |
| ATP | adenosintriphosphate |
| *B.* | *Bacillus* |
| bp | base pair |
| *C.* | *Clostridium* |
| CFU | colony forming units |
| CIP | Institute Pasteur, Paris, France |
| Da | dalton |
| DNA | desoxyribonucleic acid |
| dNTP | desoxynucleotid phosphate |
| DPA | pyridine-2,6-dicarboxylic acid, dipicolinic acid |
| DSM | DSMZ, Braunschweig, Germany |
| DSMZ | Deutsche Sammlung für Mikroorganismen und Zellkulturen |
| *E.* | *Escherichia* |
| EDTA | ethylenediaminetetraacetic acid |
| Fig. | Figure |
| g | gram |
| h | hour |
| HPLC | high pressure liquid chromatography |
| IFO | Institute for fermentation, Osaka, Japan |
| LMG | Universiteit Gent, Laboratorium voor Microbiologie, Belgium |

| | |
|---|---|
| M | Mega ($10^6$), molar |
| m | Milli ($10^{-3}$), meter |
| min | minutes |
| N | viable counts |
| $N_0$ | initial viable counts |
| NCBI | National center for Biotechnology Information |
| OD | optical density |
| p | pressure [Pa] |
| PCR | poly chain reaction |
| rcf | relative centrifugal field |
| rRNA | ribosomal ribonucleic acid |
| SASP | small, acid soluble protein |
| SDS | sodium n-dodecylsulfate |
| s | second |
| T | type strain |
| *T.* | *Thermoanaerobacterium* |
| Tab. | Table |
| *Taq* | *Thermus aquaticus* |
| $T_M$ | transition temperature |
| TMW | Technische Mikrobiologie Weihenstephan |
| TRIS | tris (hydroxymethyl) aminomethan |
| v/v | volume / volume |

| | |
|---|---|
| vol. | volume |
| w/v | mass / volume |
| $\Delta V$ | reaction volume |
| $\mu$ | Micro ($10^{-6}$) |

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# 1 Introduction

Alternative food processing and preservation technologies attract special interest of the food industry. They are being developed to a large extent in reaction to consumers' requirements for food that are more natural and therefore less heavily preserved (e. g. less acid, salt, sugar) and processed (e. g. mildly heated), less reliant on additive preservatives (e. g. sulfite, nitrite, benzoate, sorbate), fresher (e. g. chill-stored) and more convenient in use (e. g easier to store and prepare) than previously (Gould, 2001). Wherever scientific proof is mostly lacking, such foods are believed to be nutritionally healthier. Among non thermal techniques (pulse-electric field pasteurization, high intensity pulsed lights, high intensity pulsed magnetic field, ozone treatment), high hydrostatic pressure is one technology in food preservation that offers the potential to inactivate microorganisms and enzymes while altering the flavor and nutrient content of food to a lesser extent than conventional heat treatments (Cheftel and Culioli, 1997; San Martin et al., 2002). Furthermore, pressure treatment as preservation method offers the possibility to reduce the energy requirement for food processing. High pressure processing is also gaining in popularity with food processors because of its potential to achieve interesting functional effects (Tewari et al., 1999). First attempts to use pressurization for food preservation date back to 1899 (as cited by San Martin et al., 2002), when Hite observed that shelf life of milk and other food products could be increased by pressure treatment. In recent years, pressure treatment has been extensively commercialized in Japan and a variety of food products like jams and fruit-juices have been processed (Cheftel, 1995). Examples of commercial pressurized products in Europe or US are: orange juice by UltiFruit®, Pernod Richard Company, France; acidified avocado puree (guacamole) by Avomex Company in US; and sliced ham (both cured-cooked and raw-cooked) by Espuna Company, Spain (Tewari et al., 1999). Furthermore, the European Parliament and the Council authorized the Danone Group with a commission decision (2001/424/EC) from 23 May 2001 to place pasteurized fruit-based preparations produced using high pressure pasteurization on the market.

## 1.1 Current criteria and standards for microbial safety

Generally, the adequacy of food processing should be established by scientific studies. As thermal preservation technologies, high pressure processes should be designed to ensure an appropriate reduction in the numbers of pathogens of public health concern. The decimal reduction time, termed D-value, is the time required to kill 90% or 1 log cycle of the spores or vegetative cells of a given microorganism at a specific temperature in a specific medium

(Price and Tom, 1997). In the following, the current criteria and standards for microbial safety of juices, pasteurized fish and low acid canned foods are described.

### 1.1.1 Juices

In 2001, a juice performance standard based on the best available scientific data and information was developed, after consideration of public comments on the microbial safety of juices. Unfortunately, there were no data available on the levels of the microorganism of concern, *Escherichia coli* O157:H7. In contrast, nonpathogenic *E. coli* can be isolated occasionally at low levels (< 10 cfu/mL) from apple juice. Thus, as the worst-case scenario, it was assumed that a level of 10 cfu/mL of the pathogenic strains represent a highly contaminated juice. Based on these data, a target concentration of *E. coli* O157:H7 of less than one cell per 100 mL was defined, as this volume was considered as a normal serving. An additionally safety factor of 100 was adopted, which resulted in a final target concentration of less than 1 cfu/10000 mL of juice. Thus, a process capable of achieving a minimum 5-D reduction would be required to assure a microbiologically safe product (Anonymous, 2003).

### 1.1.2 Pasteurized fish and fishery products

The purpose of heat treatment, performed after the product is placed in the hermetically sealed product container, is to make the product safe for an extended refrigerated shelf life, which, in most cases, involves the elimination of the spores of *Clostridium botulinum* type E and nonproteolytic B and F. Generally a reduction of six orders of magnitude is assumed to be suitable, which is called a 6-D process. Lower degrees of destruction are also believed to be acceptable if supported by a scientific study of normal inoculums in the food. Otherwise, if there is an especially high normal inoculums, higher levels of destruction may be necessary in some foods (FDA, 2001).

### 1.1.3 Low acid canned foods

In the early 1900s, the science behind the technology to produce canned foods was in its infancy, and thermal processes were often based on experience rather than experimental data. Limiting product spoilage was the primary focus, which was initially perceived as a greater problem than product safety. The facts that *C. botulinum* was widespread in the environment, that it was an anaerobe, and that boiling temperatures were insufficient to eliminate this microorganism, were not known until the early 1900s (Anonymous, 1998). Likewise, little was known about the illness and antitoxin was not available. Outbreaks of botulism in 1919 and 1920, which were liked to commercial canned California ripe olives, contributed both to

changes in regulation and to research. In New York alone, six members of a family of eight died from eating seemly "good" olives (Anonymous, 2003).

*C. botulinum* has an ubiquitous occurrence in the ground or in sediments of lakes and forms seven types of neurotoxins differing in their serological specificities. Strains of *C. botulinum* are classified in four groups according to physiological differences, and to the type of toxin which is formed. Strains of group I (proteolytic strains forming heat resistant spores) and group II (nonproteolytic, psychrotrophic strains forming spores with a much lower heat resistance) and toxins of the types A, B, E, and F are involved in human botulism (Doyle et al., 2001). In low acid food (pH > 4.5), spores of *C. botulinum* can germinate and produce neurotoxin. Until the early 1960s nearly all outbreaks of botulism in which toxin types were determined were caused by type A or B toxins usually associated with ingestion of home-canned vegetables, fruits, and meat products. Since 1980, infant botulism has been the most common from of botulism reported in the United States. In contrast to food borne botulism, no ingestion of preformed toxin in contaminated foods, but colonization of the intestine by spores of *C. botulinum*, with subsequent *in vivo* toxin production, causes botulism (Anonymous, 1998).

Botulinum toxin, generally regarded as the most poisonous of all poisons, acts on peripheral cholinergic nerve endings to block acetyl-cholin release. Its toxicity is dependent on its ability to penetrate cellular and intracellular membranes. Thus, toxin that is inhaled or ingested can bind to epithelial cells and be transported to the general circulation. Toxin that reaches peripheral nerve endings binds to the cell surface, then penetrates the plasma membrane by receptor-mediated endocytosis and the endosome membrane by pH-induced translocation. Internalized toxin acts in the cytosol, where it exerts its neuroparalytic effects, as a zinc-dependent endoprotease to cleave polypeptides that are essential for exocytosis. Blockade of transmitter release accounts for the flaccid and autonomic dysfunction that are characteristic of the disease botulism (Simpson, 2004).

Although botulism is rare, the mortality rate is high; the 962 recorded botulism outbreaks in the United States from 1899 to 1990 involved 2320 cases and 1036 deaths (Price and Tom, 1997). Thus, *C. botulinum* is recognized as the most heat-resistant microorganism of public health significance. The accepted minimum process to ensure safety of commercially sterile foods is one that achieves a 12-D reduction in the number of *C. botulinum* spores. With an estimated 1 spore per can of this clostridia, this process results in a product for which the probability of this microorganism surviving is 1 in $10^{12}$ cans. The initial published work

which was used to establish thermal processes in low acid canned foods was that of Esty and Meyer (1922). They described the heat resistance of suspensions of 109 strains of *C. botulinum* spores in phosphate buffer at temperatures above boiling, and developed a thermal destruction curve for spore suspensions of the three most heat resistant strains. These data were later used to calculate that a thermal process at 121°C for 2.45 min would eliminate a population of 1 x $10^{12}$ spores, which was the origin of the 12 D concept. Although the inactivation of $10^{12}$ spores of *C. botulinum* have never been demonstrated and the basic assumption that thermal inactivation of spores is linear has been challenged, the D-value concept is widely used to calculate thermal processes, as its application has a long history of safe use (Anonymous, 2003).

## 1.2 General principles of high pressure

Different physical principles underlie the effect of pressure treatment. The Microscopic Ordering Principle implies that at constant temperatures, an increase in pressure increases the degree of ordering of the molecules of a substance. Secondly, the principle of Le Chatelier, according to which any phenomenon in equilibrium (chemical reaction, phase transition, change in molecular configuration), accompanied by a decrease in volume, can be enhanced by pressure. Therefore, pressurization affects any phenomenon in food systems where a volume change is involved and favors phenomena which result in a volume decrease. In biological systems the volume decrease reactions are most important include the phase changes in lipids (and, therefore in cell membranes), gelation, denaturation of proteins, hydrophobic reactions and increases in the ionization of dissociable molecules due to 'electrostriction'. For the same reason the ionic dissociation of water and therefore the pH is enhanced under pressure. Some typical values for the volume effects connected with biochemical reactions involving the various interactions are given in Table 1.1.

Covalent bonds are hardly affected by pressure whereas some non-covalent bonds are very sensitive to pressure. This means that low molecular weight food components (responsible for nutritional and sensory characteristics) are not affected, and high molecular weight components (whose tertiary structure is important for functionally) are sensitive (Tewari et al., 1999). Thirdly, the Isostatic principle, which implies that the transmittance of pressure is uniform and instantaneous (independent of size and geometry of food), however, transmittance is not instantaneous when gas is present. Therefore, and in contrast to conventional heat treatment, the processing time is independent of the sample volume. Furthermore, another interesting rule concerns the small energy needed to compress a solid or

liquid compared to heating, because compressibility is small (Cheftel, 1995; Gould, 2001; Tewari et al., 1999).

**Table 1.1.** Reaction volumes associated with selected biochemically important reactions at 25°C (Gross and Jaenicke, 1994). ΔV (mL/mol).

| **Reaction** | **Example** | **ΔV** |
|---|---|---|
| Protonation/ion-pair formation | $H^+ + OH^- \rightarrow H_2O$ | + 21.3 |
| | Imidazole + $H^+ \rightarrow$ Imidazole • $H^+$ | - 1.1 |
| | TRIS + $H^+ \rightarrow$ TRIS • $H^+$ | - 1.1 |
| | $HPO_4^{-2} + H^+ \rightarrow H_2PO_4^-$ | + 24.0 |
| | $CO_3^{-2} + 2H^+ \rightarrow HCO_3^- + H^+ \rightarrow H_2CO_3$ | + 25.5[a] |
| | Protein-$COO^- + H^+ \rightarrow$ protein-COOH | + 10.0 |
| | Protein-$NH_3^+ + OH^- \rightarrow$ protein-NH2 + $H_2O$ | + 20.0 |
| Hydrogen-bond formation | Poly (L-lysine) (helix formation) | - 1.1 |
| | Poly (A + C) (helix formation) | + 1.1[b] |
| Hydrophobic hydration | $C_6H_6 \rightarrow (C_6H_6)_{water}$ | - 6.2 |
| | $(CH_4)_{hexane} \rightarrow (CH_4)_{water}$ | - 22.7 |
| Hydration of polar groups | n-propanol $\rightarrow$ $(\text{n-propanol})_{water}$ | - 4.5 |
| Protein dissociation / association | Lactate dehydrogenase (M4 → 4M) apoenzyme | - 500 |
| | Holoenzyme (satured with NADH) | - 390 |
| | Microtubule formation (tubulin propagation; ΔV per subunit) | + 90 |
| | Ribosome association (*E. coli* 70S) | ≥ 200[c] |
| Protein denaturation | Myoglobin (pH 5, 20°C) | -98 |

[a] V for each ionization step
[b] for DNA denaturation: 0-3 ml/mol base pair
[c] 200-850 mL/mol, depending on pressure and state of charging

Since microbial inactivation by pressurization is improved at higher temperature levels, the benefits of high pressure can be maximized when the adiabatic heat of compression which occurs during the pressure build-up is considered. Adiabatic heating is the uniform temperature rise within the product, which is solely caused by pressurization (Matser et al., 2004). All compressible materials change temperature during physical compression, depending on their compressibility and specific heat (Ting et al., 2002). Thus, the adiabatic temperature increase of food may vary from 3 to 9°C / 100 MPa, depending on the initial temperature, on the rate of compression, and on the nature of the product (Table 1.2 according

to De Heij et al., 2003). Pressure release leads to a decrease in temperature of the same order of magnitude. As a result, the temperature of a product may rise 20-40°C during pressurization, but the metal pressure vessel that surrounds the product is not subjected to significant compression heating. Therefore, that part of the product near the vessel wall cools down and does not reach the same temperature as that part of the product in the center of the vessel (Ting et al., 2002).

**Table 1.2.** Temperature changes of selected substances due to compression heating according to De Heij et al. (2003).

| **Substance** | **Initial temperature (°C)** | **Temperature change (°C/100 MPa)** |
|---|---|---|
| Water | 20 | 2.8 |
| | 60 | 3.8 |
| | 80 | 4.4 |
| Steel | 20 | ≈ 0 |
| Chicken | 20 | 2.9 |
| Gouda | 20 | 3.4 |
| Milk fat | 29 | 8.5 |

## 1.3 Effect of pressure treatment on microorganisms

Although the use of pressurization has proven to be effective in preserving nutritional and sensory attributes in many products, the main concern regarding its spreading is still related to food safety issues. The effect of pressurization on microorganisms in food are determined by the effect of pressure on water, temperature during pressure treatment, the food constituents and the properties and the physiological state of the microorganisms (Smelt, 1998). Most bacteria are able to grow at pressures around 20 – 30 MPa. Piezophiles have optimal growth rates at pressures above atmospheric pressure and piezotolerant bacteria are capable of growth at high pressure, as well as at atmospheric pressure. Pressure treatment at ambient temperature in the range of 200 to 800 MPa is effective in eliminating vegetative bacteria (San Martin et al., 2002; Smelt et al., 2002). The pressure resistance of ascospores of yeast and moulds is comparable to that of vegetative cells (Butz et al., 1996). However, bacterial spores are not inactivated by pressure treatment at ambient temperature (Sale et al., 1970). Endospores of the

genus *Bacillus* and *Clostridium* tolerate at 25°C a pressure over 1000 MPa (San Martin et al., 2002).

Since the latter half of the 19$^{th}$ century bacterial endospores have been recognized as the hardiest known form of life on Earth, and considerable effort has been invested in understanding the molecular mechanisms responsible for the almost unbelievable resistance of spores to environments which exist at (and beyond) the physical extremes which can support terrestrial life (Nicholson et al., 2000). Spore forming bacteria are rather widespread within the low G+C subdivision of the gram-positive bacteria and represent inhabitants of diverse habitats, such as aerobic heterotrophs (*Bacillus* and *Sporosarcina* spp.), halophiles (*Sporosarcina halophila* and the gram-negative *Sporohalobacter* spp.), microaerophilic lactate fermenters (*Sporolactobacillus* spp.), anaerobes (*Clostridium* and *Anaerobacter* spp.), sulfate reducers (*Desulfotomaculum* spp.), and even phototrophs (*Heliobacterium* and *Heliophilum* spp.) (Nicholson et al., 2000).

Most commonly, bacteria multiply by symmetric division of single organisms into two daughter cells with identical morphological and genetic characteristics during their vegetative form. The changes in morphology and gene expression induced by sporulation are regulated by a complex regulatory network involving more than 125 genes (Stragier and Losick, 1996). Starvation is the main stimulus for the induction of sporulation (Errington, 2003). Other environmental and physiological signals arise from cell density, the Krebs cycle, DNA synthesis, and DNA damage (Stragier and Losick, 1996). As no single nutritional effect acts as the trigger, the cell has an extremely complex apparatus, which monitors a huge range of internal and external signals. The information is channeled through several regulatory systems, of which the most prominent component is a crucially important transcriptional regulator called Spo0A. The other key positive regulator of sporulation is a sigma factor, $\sigma^H$, which interacts with core RNA polymerase. Having made the decision to embark on sporulation, the endospore is formed by an unusual mechanism involving asymmetric cell division, followed by engulfment of the smaller cell (prespore or forespore) by its larger sibling (mother cell or sporangium) (Errington, 2003). In subsequent stages the forespore matures into a spore through a series of complex biosynthetic and morphogenic processes that take place in the mother cell, in the forespore, and in the space between the forespore membrane and the mother-cell membrane that surrounds the forespore. Finally, after about 6-8 hours of development, when maturation is complete, the fully ripened spore is liberated by lysis of the mother cell (Stragier and Losick, 1996). The structure of the mature spore of *B.*

*subtilis* from the outmost to the innermost layers are: Coats (composed of layers of protein), outer spore membrane, cortex (peptidoglycan structure), germ cell wall, inner spore membrane, and core (spore protoplast) (Setlow, 2003; Driks, 1999). Spores are metabolically dormant, can survive treatments that rapidly and efficiently kill other bacterial forms and can remain dormant for immense periods of time, perhaps millions of years (Cano and Borucki, 1995; Vreeland et al., 2000).

Despite such extreme dormancy, spores maintain an alert sensory mechanism, which enables them to respond to specific nutrients and a variety of non-nutrient agents including high pressure, $Ca^{2+}$-DPA, lysozyme, salts and cationic surfactants such as dodecylamine. Spore germination is divided into two stages. Stage I consists of $H^+$, monovalent cation and $Zn^{2+}$ release (release of $H^+$ elevates the core pH from ~ 6.5 to 7.7, a change essential for spore metabolism once spore core hydration levels are high enough for enzyme action), $Ca^{2+}$-DPA release, partial core hydration causing some decrease in spore wet-heat resistance (although this initial increase in core hydration is not sufficient for protein mobility or enzyme action in the spore core). Cortex hydrolysis, further core hydration, core expansion, more loss of resistance, and loss of dormancy is characterized as stage II. This leads to the latest event, the outgrowth (metabolism, small, acid soluble protein (SASP) degradation, macromolecular synthesis, and escape from spore coats) and to the return of vegetative growth (Setlow, 2003; Atrih and Foster, 2002).

The resistance of bacterial endospores to chemical and physical methods of preservation is mediated by a number of factors. These factors include the genetic makeup of the sporulating strain, the precise sporulating conditions, the temperature, the spore coats, the relative impermeability of the spore core, the low water content of the core, the high level of minerals in the spore core, the presence of DNA with α/β-type small, acid soluble proteins, and repair of damage to macromolecules during spore germination and outgrowth. In contrast, the role of the spore core's large depot of DPA with which much of the spore's divalent cations are likely chelated, in spore resistance is less clear. Correlations have been noted between spore wet heat resistance and DPA content, but there are a number of observations indicating that DPA need not be essential for spore heat resistance (Paidhungat et al., 2000). After removing DPA and associated divalent cations from the mature spores, spores of *Bacillus stearothermophilus* with <1% of untreated spore DPA levels appeared to have more highly hydrated core regions than untreated spores yet still retained high wet heat resistant (Beaman et al., 1988). Furthermore, a *B. subtilis* mutant that produces DPA-less spores that retain heat

resistance has also been isolated (Nicholson et al., 2000). However, several studies have found that the loss of the ability to synthesize DPA results in the production of wet heat sensitive spores (Balassa et al., 1979; Paidhungat et al., 2000; Daniel and Errington, 1993; Errington et al., 1988; Wise et al., 1967). It has been shown that in *B. subtilis* spores lacking DPA due to a specific mutation in the spoVFA (dpaA) or spoVFB (dpaB) locus, which encode the two subunits of DPA synthetase, have significantly increased core water and decreased heat resistance (Nicholson et al., 2000; Daniel and Errington, 1993; Balassa et al., 1979). Therefore it is not clear, if the loss of heat resistance is only due to a change in spore hydration or also to the reduction in core mineralization which accompanies the loss of DPA from spores. Since spore core mineralization also plays a role in wet heat resistance (Marquis and Bender, 1985), it is possible that changes in both core hydration and mineral levels contribute to the loss of wet heat resistance of DPA-less spores. It could be also possible that DPA accumulation during sporulation is required for the attainment of some state that is essential for full spore wet heat resistance (Paidhungat et al., 2000).

**Table 3.1.** Microbial safety criteria for some heat- and pressure treated foods according to Garcia et al. (2002).

| **Product group** | **Target pathogen** | **Proposed reduction value** | **Required heat treatment** | **Proposed pressure treatment** |
|---|---|---|---|---|
| Products currently marked without heat treatment | None, inactivation of spoilage organisms | 3D to 5D | none | <500 MPa |
| Acid products (pH<4.5), refrigerated storage | Low infective dose pathogens e. g. *E. coli*, Salmonella | 5 D | <70°C, 2min | <600 MPa, quarantine time |
| Non-acid products, refrigerated storage < 10d | Listeria monocytogenes | 6 D | 70°C, 2 min | <600 MPa, >50°C |
| Non-acid products, refrigerated storage > 10d | *Clostridium botulinum* type E | 6 D | 90°C, 10 min | <600 MPa, >55°C |
| Non-acid products, long term storage at ambient temperature | *Clostridium botulinum* type A | 12 D | 121°C, 5 min | >600 MPa, >75°C |

Inactivation of bacterial endospores requires the combination of pressure with moderate heat (Mallidis and Drizou, 1991) and the efficacy of pressure treatment is enhanced by low pH (Stewart et al., 2000; Wuytack et al., 2001), in the presence of nisin (Roberts and Hoover, 1996; Stewart et al., 2000) or argon (Fujii et al., 2002), and by oscillatory compression procedures (Furukawa et al., 2003; Hayakawa et al., 1993, 1994a, 1994b). The resistance of bacterial spores to pressure treatment is also influenced by environmental factors prevailing during sporulation, e.g. the mineral content and the temperature of the sporulation medium (Igura et al., 2003). Currently available data indicate that endospores of *Bacillus* and *Clostridium* species are inactivated by treatments with pressures ranging from 500 – 800 MPa at temperatures ranging from 60 – 80°C. Microbial safety criteria were recently proposed based on the current knowledge of food preservation by pressure processes by Garcia and co-authors (Tab. 3.1).

Studies on pressure effects on vegetative cells of bacteria have demonstrated that the resistance to pressure strongly varies within strains of one species (Benito et al., 1999; Garcia-Graells et al., 2002). Likewise, the heat resistance of endospores of various strains of one species may exhibit strong variations (Sarker et al., 2000). The majority of studies on the pressure resistance of bacterial endospores were performed with a limited number of laboratory strains. Furthermore, these examinations were carried out with an almost exclusive focus on spores of *Bacillus* sp., namely *B. subtilis*, and *Clostridium sporogenes,* and only few reports on the pressure resistance of *C. botulinum* spores are available. Because the resistance of endospores to pressure does not correlate to their resistance to heat (Nakayama et al., 1996), those strains and species with a high resistance to pressure as target organisms for food processing remain to be identified. The sporulation conditions as well as the matrix in which the spores are suspended during pressurization further affect the pressure resistance of spores of *B. subtilis* (Ananta et al., 2001; Igura et al., 2003).

As already noticed above, to ensure the safety of low acid canned food, processes are employed that attain a 12-decimal reduction of the heat –resistant spores of *C. botulinum*. Reddy et al. (1999, 2003) evaluated the effects of pressure in combination with moderate heat on spores of four *C. botulinum* strains. Spores of heat-sensitive *C. botulinum* type E were less pressure resistant than spores of heat-resistant *C. botulinum* type A and spore counts of the latter were not reduced by more than three log following treatments with 827 MPa and 75°C. Sizer et al. (2002) emphasized the need for a suitable target organism with a pressure resistance higher than that of *C. botulinum*, which has not been identified to date.

Moderate pressures of 100 to 600 MPa induce the germination of endospores of *B. subtilis* (Wuytack et al., 1998, 2000, 2001) and these germinated spores are more sensitive to chemical and physical agents compared to dormant spores (Herdegen, 1998; Knorr, 1999). Therefore, pressure induced germination of spores enables a subsequent inactivation of germinated spores by mild heat or pressure. At ambient pressure, the release of DPA from the spores results from activation of (nutrient)-receptors and is one of the early steps in spore germination (Paidhungat et al., 2002). Wuytack and Michiels (1998) compared spores which were induced to germinate at 100 MPa or 600 MPa. Germination of spores induced by 100 MPa resulted in the loss of DPA from the spores, degradation of SASP's and rapid generation of ATP. DPA release was also observed in spores germinated under high pressure conditions, however, the degradation of SASP's and ATP generation were not observed. Treatment with 550 MPa induces spore germination independent of nutrient receptors by opening channels that allow the loss of DPA and lead to later steps in spore germination (Paidhungat et al., 2000; Wuytack et al., 2000).

## 1.4 Effect of pressurization on biomolecules

As 62% of the total biosphere is characterized by pressures greater than 0.1 MPa, the earth is predominantly a high pressure environment. Nevertheless, information on the effects of pressure treatment on biomolecules has been relatively rare until recently (Boonyaratanakornkit et al., 2002). The effects of pressure treatment on biological systems appear to constitute an interesting tool for their study. As a thermodynamic parameter, pressurization was known for many years to act on biological materials in a similar but not identical way as temperature (Lullien-Pellerin and Balny, 2002). Perhaps the most important argument to measure the effect of pressure on a wide variety of thermodynamic systems is that the effects of volume and thermal energy changes can be separated, which appears simultaneously in temperature experiments (Weber and Drickamer, 1983).

### 1.4.1 Effect of pressure treatment on water

Most systems which were used to study the effect of pressurization on biomolecules were aqueous solutions. The whole pressure-temperature phase diagram of water is rather complicated containing several ice phases. The most unusual property of water is the lower density of the corresponding ice formed at ambient pressure. As a consequence, pressure treatment of water below 200 MPa at temperatures slightly below 0°C stabilizes the liquid phase. The lowest temperature at which water can be held liquid is –22°C at 207 MPa. This phenomenon makes many applications possible. One of it are basic studies on pressure

assisted cold denaturation. The storage of unfrozen food under subzero temperature, in order to avoid the damaging effect of ice crystal formation, or the rapid freezing of pre-cooled food trough depressurizing, could be practical applications (Smeller, 2002).

### 1.4.2 Effect of pressure treatment on biomembranes

Biomembranes are mentioned as main target of high pressure (Hoover et al., 1989). They are composed of a bilayer of phospholipids with transmembrane and membrane bond enzymes. Lipid bilayers undergo phase transitions (from the liquid crystalline to the gel phase) under pressure. According to the microscopic ordering principle, the hydrocarbon chains are conformationally disordered in the liquid crystalline phase, gel phases correspond to relatively ordered and more extended hydrocarbon chains (San Martín et al., 2002). Depending on the composition of the membrane (acyl length, saturation, and phospholipid groups) and reaction temperature (the gel-fluid transition temperature increases with a rate of approx. 0.2°C/MPa), these phase state transitions occur far below 300 MPa (Winter, 1996). The gel phase is lost after decompression and the fluid-crystalline phase is reestablished. Thus and in contrast to proteins, membrane phase transistions are reversible (Heremans, 1992). Pressure application results in a wide coexistence of liquid-crystalline and gel formations. The thermodynamical behavior of pressurized biomembranes is further affected by the mixture of different phospholipids and the integration of enzymes. These strong interrelations between phospholipid bilayers and incorporated or membrane bound enzymes mainly determine functionality of the cell and its ability to survive. The decrease in biomembrane fluidity may result in breakage of the membrane and in denaturation of the membrane bound proteins causing a functional disorder of these proteins (San Martín et al., 2002).

### 1.4.3 Effect of pressure treatment on nucleic acids

Relative to the effect of pressure on other biomolecular systems, nucleic acids are hardly influenced as such: wherever their "*in vivo*" functionality may be strongly affected. The majority of the reports dealing with pressure treatment and nucleic acids have focused on its effect on the conformational stability of DNA. In general, the helical form of DNA or RNA is abolished at high temperature and stabilized by pressure (Macgregor, 1998). This 'melting' is an endothermic process. The most common method is detecting it by the increase in UV absorption above the melting or transition temperature ($T_m$), which is the disappearance of the hypochromic effect due to the breaking of the double helix (Smeller, 2002). The value of $T_m$ is determined by the sequence and base composition of the nucleic acid polymer as well as solvent parameters as the ionic strength (Macgregor, 1998). The values of the change in $T_m$

with pressure ($dT_m/dP$) has been studied for several types of DNA, double-stranded DNA with natural sequences, non-random sequence synthetic polymers, and oligonucleotides of different lengths. These molecules have been investigated in solution with different types of cations and under different salt concentrations (Macgregor Jr., 2002). The effect of pressure on the $T_m$ of *Clostridium perfringens* DNA was studied by Hawley and MacLeod (1974). The pressure-temperature diagram of DNA helix-coil formation show, that the double-stranded structure is stabilized by elevated pressure, and that $dT_m/dP$ becomes larger and varied linearly with NaCl concentration (Smeller, 2002). Thus, the volume of the system that includes the double-stranded polymer, water, and cations is less than the volume of the system containing the single-stranded species. Enhanced stacking of the hydrophobic bases with increasing pressure is responsible for the stabilization (Macgregor Jr., 2002). The conclusion from this study is that melting of DNA does not show an elliptic phase diagram.

The most important new result concerning the effect of combined pressure temperature treatment on the stability of double-stranded nucleic acid polymers were published by Dubins et al. (2001). All previous studies explored the region were $dT_m/dP$ is greater than zero and the region where this parameter is negative was not observed. The prediction of their analysis was borne out by studying the effect of pressurization on the helix-coil transition of polymers with $T_m$ values below ~50°C (Macgregor Jr., 2002). On the basis of their calculated stability diagram, which is consistent with the earlier measurements in the low pressure range from Hawley and MacLeod (1974), a destabilizing effect of pressurization below 200 MPa (melting temperature lowered by pressure) if the melting point was below 50°C (at atmospheric pressure), and a stabilizing effect if the melting point was above 50°C could be anticipated. This trend reversed above 200 MPa, and at even higher pressures (~1 GPa) the melting temperature was 50°C, independent of the $T_m$ at atmospheric pressure (Smeller, 2002). In other words, it was shown that value of $dT_m/dP$ could be positive or negative (Macgregor Jr., 2002).

### 1.4.4 Effect of pressure treatment on proteins

It has been known for a long time that both pressure and temperature have significant effects on proteins including enzymes. There are four structural levels in the conformation of a protein. Secondary (coiling of peptide chains joined with hydrogen bonding), tertiary (arrangements of chains into globular shape by non-covalent bonding), and quaternary structures (present when a protein consists of more than one polypeptide chain) can be significantly affected by pressurization (Tewari et al., 1999). As the tertiary structure is

important in determining protein functionality, pressure treatment can result in novel functional properties. Pressure induced gel formation by proteins, resulting in gels with properties different from gels obtained by heat, was observed as early as 1914 on the coagulation of albumen (as cited by San Martin et al., 2002). The required pressure level depends upon the type and concentration of protein, pH and ionic strength of treated solutions (Tewari et al., 1999). Surimi gels obtained by pressurization were less opaque than traditionally heat set gels. Only after addition of microbial transglutaminase, pastes of turkey formed gels by pressure treatment, but the strength of these gels was lower than heat formed gels. High whiteness, high brightness, and fresh cream like flavor were highlighted for pressure-induced gels from freeze-concentrated milk and its use for high-quality cold dessert manufacture was suggested. Pressure treated albumen results in a microbial stable product, conserve its functional properties, and increases its digestibility (San Martín et al., 2002).

Likewise, the response of enzymes subjected to pressure treatment is varied. Orange juice was subjected to pressure treatment to investigate the stability of cloud, because the enzyme pectinesterase can cause the loss of its stability. Pressure treatments at 500 MPa for 10 min or 700 MPa for 1 min yielded microbiologically and cloud stable products. In contrast, polyphenoloxidase (PPO) and α- and β-amylases from malt barley was reported to increase its activity after pressurization in food matrices. That pressure treatment of purified PPO did not cause an enhancement in activity may be due to changes in interactions between extract constituents or from the release of membrane-bound enzymes (San Martín et al., 2002).

Proteins unfold at high temperature, as well as at high pressure and cold denaturation was also predicted, but only measured for a relative small number of proteins (Heremanns and Smeller, 1998; Smeller, 2002). In contrast to pressure, temperature denaturation involves changes in both the thermal energy and the volume. Pressure denaturation corresponds to the incorporation of water molecules into the protein, resulting in disruption of the structure, whereas heat denaturation corresponds to the transfer of nonpolar groups into water (Hummer et al., 1998). Pressurization tends to denature proteins because the protein-solvent system for the denatured state occupied a smaller volume than that for the native state. Similarly, pressure treatment causes oligomeric proteins to dissociate because this results in a decrease in the net volume of the system. A combination of factors have been made responsible for these effects. The oligomer interface, or the presence of cavities within the folded protein structure, favors unfolding or dissociation. Disruption of electrostatic interactions results in a large decrease in volume due to the electrostriction of water molecules around the unpaired

charged residues. Similarly, solvation of polar and hydrophobic leads to a decrease in volume of the water molecules. These effects balance the increase in volume as the crystalline-like state of the protein interior is disrupted and exposed to solvent on unfolding. (Perrett and Zhou, 2002).

Pressure / temperature diagrams of several proteins are published and their elliptic shape shows, that there is an optimum temperature at which proteins are most resistant to pressure (Smeller, 2002). The phase diagram of proteins (Fig. 1.1) indicate that proteins are stable in their native state inside the ellipse and pressure stabilizes against temperature mediated inactivation. The consistent thermodynamic description of the phase boundary of protein unfolding was developed by Hawley (1971). The experimental data on chymotrysinogen and ribonuclease A conformed his theory (Brandts et al., 1970; Hawley, 1971). These diagrams could be used to select biotechnological treatments for modulating the structure and stability of proteins and therefore to generate new food textures (Lullien-Pellerin and Balny, 2002). It is noteworthy that these phase diagrams are simplified as the hole theory is based on the assumption that there are only two possible states of the protein, i.e. folded and unfolded, a process that is known to sometimes involve a number of intermediates in the folding pathway, and also a reversible unfolding was implied. The role of water in protein unfolding is proven by the fact that the elliptic diagram can only be observed for protein solutions as proteins in the dry state are very pressure stable. Furthermore, the denaturation pressure and temperature do usually decrease at extreme pH values (Smeller, 2002).

While the dissociation of oligomeric proteins is supported by moderate pressures ( < 150 MPa), pressurization above 150-200 MPa induces unfolding of proteins. Beyond 200 MPa, significant tertiary structures changes are observed and at higher pressures (above 300-700 MPa) secondary structure changes take place, leading to non-reversible denaturation (Lullien and Balny, 2002). However, the secondary structure of Green fluorescent protein (27 kDa) is not influenced by pressures even up to 1300 MPa (Scheyhing et al., 2002), indicating that protein structures may be very pressure resistant. Although the effect of pressure treatment on food relevant enzymes as peroxidase and polyphenol oxidase are available (Hernández and Cano, 1998; Présramo et al., 2001), the effect of high pressure on bacterial toxins has not been studied.

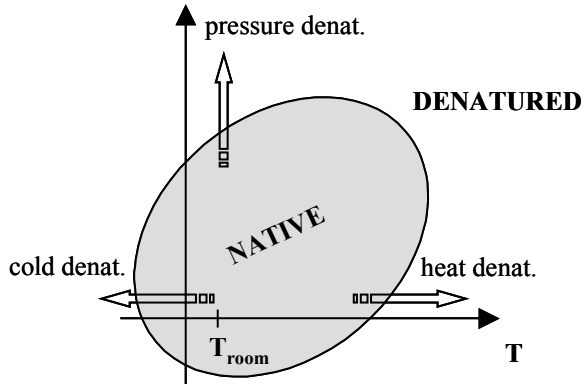


**Figure 1.1.** Schematic representation of the elliptic phase diagram of proteins according to Smeller (2002). The arrows show the specific denaturation ways known as pressure, heat, and cold denaturation.

### 1.4.5 Bacterial toxins

Enterotoxins of *Staphylococcus aureus*, *Bacillus cereus*, *Vibrio cholerae* and pathovars of *Escherichia coli* are an important cause of a variety of diseases. Thermal stability of these proteins was used to classify them as heat labile or heat stabile.

For more than 40 years, *B. cereus* has been recognized as a causative agent of food poisoning (Ghelardi et al., 2002). It is known to cause two different types of food poisoning (Jay, 1992). The vomiting type of intoxication is caused by an emetic toxin produced by growing cells in the food (Granum and Lund, 1997). The emetic toxin, named cereulide, has a molecular mass of 1.2 kDa and remains active after heat treatment at 121 °C for 90 min, and stable at pH 2-11 (Doyle et al., 2001). The diarrheal type is caused by various heat labile enterotoxins. Treatment at 56°C for 10 minutes leads to a complete loss of their biological activities (Glatz et al., 1974; Spira and Goepfert, 1975). They are produced during vegetative growth of *B. cereus* in the small intestine (Granum and Lund, 1997; Jay, 1992). The most extensively studied enterotoxin is Haemolysin BL (HBL), containing the protein components B (37.5 kDa), $L_1$ (38.2 kDa) and $L_2$ (43.5 kDa).

One of the leading causes of food poisoning in North America is the ingestion of staphylococcal enterotoxins (SEs) produced by certain strains of *S. aureus* (Park et al., 1994). Various different SEs are recognized by the use of serological methods: A, B, C1, C2, C3, D, E, G, H, I and J (Doyle et al., 2001). Heat stability is one of the most important properties of SEs in terms of food safety (Balaban and Rasooly, 2000). Normal cooking times and temperatures are unlikely to completely inactivate the toxins SEA, SEB, and SEC. At 120°C,

the three toxins are completely inactivated in 20 to 30 min (Tibana et al., 1987). Staphylococcal food borne diseases are characterized by a short incubation period (2 to 6h) after ingestion of preformed toxins, followed by nausea, vomiting, abdominal pain, and diarrhea (Balaban and Rasooly, 2000). SEs are single polypeptides of approximately 25 to 28 kDa , have an overall ellipsoidal shape, and are folded into two unequal domains containing a mixture of α and β structures (Dinges et al., 2000).

*E. coli* causes no food poisoning and is a common commensal organism of the normal microflora in the intestinal tract of humans and warm –blooded animals. Most strains are non-pathogenic; however, some isolates, which were categorized by mechanisms of pathogenicity virulence properties, clinical syndromes, and distinct O:H serotypes cause diarrhea. Enterotoxingenic *E. coli* (ETEC) strains colonize the surface of the small bowel mucosa and cause diarrhea through the action of two types of enterotoxins, heat stable enterotoxins (STs), and heat labile enterotoxins (LTs), whereby only ST, only LT, or both LT and ST may be expressed. STs are small, monomeric toxins that contain multiple cysteine residues, whose disulfide bonds account for the heat stability of these toxins. While STa is an 18 or 19-amino-acid peptide with a molecular mass of ca. 2 kDa, the LTs of *E. coli* are oligomeric toxins of ca. 86 kDa, composed of one 28 kDa A subunit and five identical 11.5 kDa B subunits, that are closely related in structure and function to the cholera enterotoxin (CT) expressed by *V. cholerae* (Nataro and Kaper, 1998) and therefore have similar antigenic structures (Spira and Goepfert, 1975). CT is the toxin responsible for severe, cholera like disease in epidemic and sporadic forms. It is produced after vibrios have colonized the epithelium of the small intestine (Doyle et al., 2001). The clinical disease is characterized by the passage of voluminous stools of rice water character that rapidly lead to dehydration (Kaper et al., 1995).

## 1.5 Objectives of the work

It was the aim of this thesis to investigate the behaviour of bacterial endospores and toxins as safety determinants in low acid pressurized food. To obtain a shelf stable and save product having a pH well above 4.5, endospores must be inactivated by food processing, as spores are able to germinate during storage. Therefore, the pressure resistance of spores of toxigenic *C. botulinum* and *Bacillus cereus* as well as that of the food spoilers *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, *B. smithii* and *Thermoanaerobacterium thermosaccharolyticum* should be determined. Furthermore, a non-pathogenic, non-toxinogenic strain which forms spores with a higher resistance to pressure than *C. botulinum* or *B. cereus* should be identified for use as a target strain for process development. Various sporulation conditions, non-proteolytic and proteolytic strains of *C. botulinum* should be employed in order to determine the variation in pressure resistance within this species. The pressure resistance of selected strains should further be compared to their heat resistance. An extended spectrum of combined pressure (600 to 1400 MPa) / temperature (70 to 120°C) treatment with isothermal holding times should be used to determine the behaviour of the most resistant spores concerning spoilage and food safety, respectively.

The role of DPA-release during pressure inactivation and germination of representative strains as a possible reason for the varying pressure resistances should be examined as well as the role of DPA in spore pressure resistance by using a mutant strain of *B. subtilis*.

As a food model system mashed carrots should be used. They have a pH well above 4.5. Strains concerning food spoilage should be isolated from the carrot habitat (e.g. mashed carrots obtained out of a commercial process just before heat treatment) to get “wild strains” with pratical relevance. Since the survival of bacterial spores during and after high pressure treatment depends highly on the matrix of food, most investigations concerning their inactivation should be made in carrot porridge.

Furthermore, the effect of combined pressure / temperature treatment of enterotoxins from *S. aureus*, *B. cereus*, *V. cholerae* and *E. coli* (STa) on their reactivity in enzyme immunoassays (EIA’s) should be determined. These toxins, which differ in heat resistance, should be used as models, as some of the toxins are only formed in humans. Cytotoxicity of the pressure treated supernatant of toxigenic *B. cereus* DSM 4384 should be investigated in order to compare its toxicity with the results obtained by the immunoassay.

# 2 Material and Methods

## 2.1 Analysis of the carrot flora

To identify target organisms, typically associated with carrots, the flora of carrots from the local supermarket, from the local market place, and of bio carrots was investigated. The two specified first were mashed (10g carrots with 90g of ¼-strenght Ringer's solution (Merck, Darmstadt, Germany)) and aliquots were treated at 0.1 MPa / 80°C or 600 MPa / 20°C for 10 min, respectively. The aerobic total account number was determined using ST1 agar (per L: 15.0 g peptones, 3.0 g yeast extract, 6.0 g sodium chloride, 1.0 g D(+) glucose, and 12.0 g agar agar; adjusted to pH 7.5) at 30°C, and the anaerobic total account number was determined using DRCM agar (per L: 5.0 g peptone from casein, 5.0 g peptone from meat, 8.0 g meat extract, 1.0 g yeast extract, 1.0 g starch, 1.0 g D(+) glucose, 0.5 g L-cysteinium chloride, 0.5 g sodium acetate, 0.5 g sodium disulfite, 0.5g ammonium iron (III) citrate, 0.002 resazurin sodium, and 12.5 g agar agar; adjusted to pH 7.1) at 37°C and 60°C. The colony forming units of lactic acid bacteria was determined using MRS agar (per L: 10.0 g peptone from casein, 8.0 g meat extract, 4.0 g yeast extract, 20.0 g D(+) glucose, 2.0 g di-potassium hydrogen phophate, 1.0 g Tween® 80, 2.0 g di-ammonium hydrogen citrate, 5.0 sodium acetate, 0.2 g magnesium sulfate, 0.04 manganese sulfate, and 12.5 g agar agar; adjusted to pH 5.7) at 37°C, of coliforms using ENDO-S agar (per L: 8.0 g peptones, 10.0 g lactose, 0.2 g pararosaniline (fuchsin), 2.0 g di-potassium hydrogen phosphate, 3.0 g sodium chloride, 2.5 g sodium sulfite, and 12.0 g agar agar; adjusted to pH 7.5) at 37°C and of yeasts using YGC agar (per L: 5 g yeast extract, 20 g D(+) glucose, 0.1 g chloramphenicol, and 14.9 g agar agar; adjusted to pH 6.6) at 30°C was only determined for the flora of the carrots from the local supermarket. Bio carrots were also mashed and heat treated as described above, diluted, plated on ST1, and incubated aerobically at 30°C and 60°C to isolate aerobic both meso- and thermophilic spore formers.

Industrially produced mashed carrots, which were obtained out of a commercial process just before heat treatment at 121°C, were further analyzed. Process-related, the glasses had already been treated for 30 min at 80°C, as this is a part of the normal manufacturing method before filling. Therefore, determination of cell counts were carried out just after sampling and also after incubation of the whole glasses at 30°C or 60°C, respectively. The aerobic and anaerobic total account number was determined as described above.

Identification of the isolates was made PCR-supported by finger printing (RAPD) and 16S rRNA amplification and sequencing.

## 2.2 DNA Isolation, RAPD-PCR, and electrophoresis conditions

The method of Lewington et al. (1987) for DNA preparation was modified. Cells of an overnight culture (2 mL) were centrifuged (10000 rcf for 5 min at room temperature), washed twice, and resuspended in 200 µL Tris-EDTA-saline-buffer (TES, 50 mM Tris/HCl, 50 mM NaCl, 10 mM EDTA, adjusted to pH 8.0). 30 µL of lysozyme solution (20 mg/mL lysozyme (Boeringer, Mannheim, Germany) in TES buffer) was added and the sample was incubated for 30 min at 37°C. Subsequently, 20 µL of SDS solution (25% (w/v) sodium n-dodecyl sulfat) and 10 µL of proteinase K solution (10 mg/mL proteinase K (Boeringer, Mannheim)) was added. The sample was incubated for 20 min at 60°C. 60 µL of ice cold NaCl solution (5 M NaCl) was added after putting the samples on ice. One vol. of phenol/cholorform was added, mixed, centrifuged (10000 rcf for 5 min at room temperature). The upper aqueous phase was removed to a clean tube. One vol. of chlorform was added, mixed, centrifuged (10000 rcf for 5 min at room temperature) and the upper aqueous phase was again removed to a clean tube. For the precipitaion of the DNA, 1/9 vol. sodium acetate EDTA solution (3M sodium acetat, 0.01 M EDTA, adjusted to pH 7.0) and 0.56 vol. cold isopropanol was added. After centrifugation for 5 min at 14000 rcf, the harvested DNA was washed with 1 mL of cold 70 % ethanol, dried in a vacuum oven and then redissolved in 100 µL of Tris-EDTA-buffer (TE, 10 mM Tris/HCl, 1 mM EDTA, adjusted to pH 8.0).

RAPD-PCR was carried out with the oligonucleotide primer M13V (5'-GTT TTC CCA GTC ACG AC-3'), obtained from MWG-Biotech (Ebersberg, Germany). The reaction mixture (50 µl) contained 100 pmol of primer M13V, 0.2 mM each deoxyribonucleotide triphosphate, 3.5 mM $MgCl_2$, reaction buffer, 1.5 U of *Taq* polymerase, and 1 µl of DNA solution. Oligonucleotides, *Taq* polymerase, and reaction buffer for use in PCRs were obtained from Amersham Pharmacia, Uppsala, Sweden. All PCR reactions were carried out on a Gradient Master Thermocycler (Eppendorff, Hamburg, Germany). The cycling program was (96°C/3 min; 35°C/5 min; 75°C/5 min) 3 cycles; (96°C/1 min; 55°C/2 min; 75°C/3 min) 32 cycles. Each set of reactions included a negative control. Amplification products were separated on 1% agarose gels, stained with ethidium bromide, and visualized by UV transillumination.

## 2.3 Cluster analysis

The patterns were evaluated by the Gel Compar 4.1 package (Applied Maths, Kortriijk, Belgium). A clustering algorithm, the Unweighted Pair Group Method using arithmetic averages (UPGMA), was applied. The similarity between the RAPD patterns was expressed by the Pearson product moment correlation coefficient (r x 100).

## 2.4 16S rDNA amplification and sequencing

16S rDNA was amplified with the primer 616VII (5'- AGA GTT TGA TYM TGG CTC AG -3'; 3' terminus of the primer was located at position 7; *Escherichia coli* numbering convention; Brosius et al., 1978) and 630R (5'- CAK AAA GGA GGT GAT CC -3'; terminus of the primer was located at position 1528; *E. coli* numbering convention). The reaction mixture (50 µl) contained 25-pmol amounts of each primer, 0.2 mM concentrations of each deoxyribonucleotide triphosphate, reaction buffer, 20 mM tetramethylammonium chloride, 2.5 U of r*Taq* polymerase (Amersham Pharmacia Biotech), and 1 µl of DNA solution. The PCR program used was: 94°C for 2 min; 35 cycles of 94°C for 45 s, 54°C for 1 min, and 72°C for 30 s; and finally 72°C for 4 min. PCR products were purified using the QIA-quick PCR purification kit (Qiagen, Hilden, Germany) according to the instructions of the supplier and sequenced by SequiServe (Vaterstetten, Germany). Oligonucleotides used for sequencing were: 616VII (sequence see above), 630R (sequence see above), 97K (5'- CTGCTGCCTCCCGTA –3'; terminus of the primer was located at position 342; *E. coli* numbering convention), 607 (5'- ACGTGTGTAGCCC –3'; terminus of the primer was located at position 1220; *E. coli* numbering convention), 609 (5'- GGACTACCTGGTATCTAATCC –3'; terminus of the primer was located at position 784; *E. coli* numbering convention), and 612RII (5'- GTAAGGTTYTNCGCGT –3'; terminus of the primer was located at position 968; *E. coli* numbering convention). Sequence symbols are A (Adenin), C (Cytosin), G (Guanosin), and T (Thymin). Wobble IUPAC-IUB symbols used in this study were: R (A or G), Y (C or T), M (A or C), K (G or T), S(G or C), W (A or T), and N (G or A or T or C). To determine the closest relatives of the partial 16S rDNA sequences, a search of the GenBank DNA database was conducted by using the BLAST algorithm (Altschul et al., 1990). A similarity of >99% to 16S rDNA sequences of type strains was used as the criterion for identification.

## 2.5 Bacterial strains, growth conditions and preparation of spore suspensions

The bacterial strains used in this study and their origin are shown in Table 2.1. All bacilli were grown aerobically in ST1 broth (composition as ST1 agar, just without agar agar) at 60°C (*B. smithii* TMW 2.487) or 30°C (other bacilli). *T. thermosaccharolyticum* was grown anaerobically in *C. thermohydrosulfuricum* broth (per L: 10.0 g tryptone, 10.0 g sucrose, 2.0 g yeast extract, 0.2 g ferrous sulfate heptahydrate, 0.2 g sodium sulfite, 0.08 g sodium thiosulfate pentahydrate, 1 mg resazurine; adjusted to pH 7.4) at 60°C and strains of *C. botulinum* were grown anaerobically in RCM broth (per L: 10.0 g meat extract, 10.0 g peptone from casein, 3.0 g yeast extract, 5.0 g D(+) glucose, 1.0g starch, 5.0 g sodium chloride, 3.0 g sodium acetate, 0.5 g L- cysteinium chloride, adjusted to pH 6.8) at 30°C. Spores were prepared by plating aliquots of 0.1 mL from fresh overnight cultures on agar plates at the temperatures as noted above.

**Table 2.1.** Strains used and their origin.

| Organism | Strain designation and origin |
|---|---|
| *Bacillus licheniformis* | TMW 2.492, pasteurized carrots[1)] |
| *Bacillus licheniformis* | TMW 2.534, carrot surface[1)] |
| *Bacillus licheniformis* | TMW 2.545, carrot surface[1)] |
| *Bacillus licheniformis* | TMW 2.551, carrot surface[1)] |
| *Bacillus subtilis* | TMW 2.484, pasteurized carrots[1)] |
| *Bacillus subtilis* | TMW 2.485, pasteurized carrots[1)] |
| *Bacillus subtilis* | TMW 2.469, chocolate cracker[1)] |
| *Bacillus subtilis* | TMW 2.533, pasteurized carrots[1)] |
| *Bacillus subtilis* | DSM 347 |
| *Bacillus subtilis* | DSM 6405 |
| *Bacillus subtilis* | DSM 618 |
| *Bacillus subtilis* | DSM $10^T$ |
| *Bacillus subtilis* | TMW 2.476, Fad 110, ropy bread[2)] |
| *Bacillus subtilis* | TMW 2.483, Fad 109, ropy bread[2)] |
| *Bacillus sp.* | TMW 2.480, Fad 94, ropy bread[3)] |
| *Bacillus subtilis* | CIP 76.26[4)] |
| *Bacillus smithii* | TMW 2.487, pasteurized carrot juice[1)] |
| *Bacillus simplex* | TMW 2.535, carrot surface[1)] |

| | |
|---|---|
| *Bacillus amyloliquefaciens* | DSM 7[T] |
| *Bacillus amyloliquefaciens* | TMW 2.474, Fad 99, ropy bread[5)] |
| *Bacillus amyloliquefaciens* | TMW 2.475 Fad We, ropy bread[5)] |
| *Bacillus amyloliquefaciens* | TMW 2.477, Fad 108, ropy bread[5)] |
| *Bacillus amyloliquefaciens* | TMW 2.478, Fad 77, ropy bread[5)] |
| *Bacillus amyloliquefaciens* | TMW 2.479, Fad 82, ropy bread[5)] |
| *Bacillus amyloliquefaciens* | TMW 2.481, Fad 97, ropy bread[5)] |
| *Bacillus amyloliquefaciens* | TMW 2.482, Fad 11/2, ropy bread[5)] |
| *Bacillus cereus* | DSM 4384 |
| *Bacillus cereus* | TMW 2.383, soil[6)] |
| *Bacillus macroides* | TMW 2.544, carrot surface[1)] |
| *Bacillus mycoides* | TMW 2.550, carrot surface[1)] |
| *Bacillus gelatini* | TMW 2.552, pasteurized carrots[1)] |
| *Bacillus psychrodurans* | TMW 2.549, carrot surface[1)] |
| *Bacillus sp.* | TMW 2.532, pasteurized carrots[1)] |
| *Bacillus sp.* | TMW 2.540, carrot surface[1)] |
| *Bacillus sp.* | TMW 2.543, carrot surface[1)] |
| *Bacillus sp.* | TMW 2.546, carrot surface[1)] |
| *Bacillus sp.* | TMW 2.547, carrot surface[1)] |
| *Paenibacillus lautus* | TMW 2.539, carrot surface[1)] |
| *Geobacillus sp.* | TMW 2.531, pasteurized carrots[1)] |
| *Geobacillus thermodenitfrificans* | TMW 2.536, carrot surface[1)] |
| *Geobacillus stearothermophilus* | TMW 2.537, pasteurized carrots[1)] |
| *Geobacillus stearothermophilus* | TMW 2.538, pasteurized carrots[1)] |
| *Thermoanaerobacterium thermosaccharolyticum* | TMW 2.299, dung[1)] |
| *Clostridium botulinum* type A, proteolytic | TMW 2.356, REB 1750[7)] |
| *Clostridium botulinum* type B, proteolytic | TMW 2.357, REB 89[7)] |
| *Clostridium botulinum* type F, proteolytic | TMW 2.358, REB 1072[7)] |
| *Clostridium botulinum* type B, proteolytic | TMW 2.359[8)] |
| *Clostridium botulinum* type A, proteolytic | ATCC 19397, Nr. 83[9)] |
| *Clostridium botulinum* type B, nonproteolytic | ATCC 25765, Nr. 156[9)] |
| *Clostridium botulinum* type B, nonproteolytic | TMW 2.518, Nr. 160, ham[9)] |
| *Staphylococcus warneri* | TMW 2.541, carrot surface[1)] |

| | |
|---|---|
| *Staphylococcus epidermidis* | TMW 2.542, carrot surface[1)] |
| *Staphylococcus haemolyticus* | TMW 2.548, carrot surface[1)] |

[1)] TMW = Strain collection Technische Mikrobiologie Weihenstephan; classified based on the sequence of the 16S rRNA (see appendix).
[2)] Röcken and Spicher, 1993
[3)] Röcken and Spicher, 1993; strain re-classified as *Bacillus sp.* based on the sequence of the 16S rRNA (see appendix).
[4)] Balassa et al., 1979
[5)] Röcken and Spicher, 1993; strain re-classified as *B. amyloliquefaciens* based on the sequence of the 16S rRNA and RAPD patterns (see Figure 2.1 and appendix).
[6)] Previously isolated from Christian Dotzauer, Lehrstuhl für Technische Mikrobiologie, Freising and classified as *B. cereus* based on the sequence of the 16S rRNA (see appendix).
[7)] Institut für medizinische Mikrobiologie und Infektionsepidemiologie, Leipzig
[8)] Fraunhofer-Institut für Verfahrenstechnik und Verpackung, Freising
[9)] Lehrstuhl für Hygiene und Technologie der Lebensmittel tierischen Ursprungs, München

All *Bacillus* strains were grown aerobically on ST1 agar additionally supplemented with 10 mg $L^{-1}$ $MnSO_4$ x $H_2O$ unless otherwise stated. Strain CIP 76.26 (Balassa et al., 1979) was grown on ST1 or ST1 additionally containing DPA (50 µg $mL^{-1}$) to control the DPA content of these spores. To investigate the effect of minerals on pressure resistance, 5 mM of $CaSO_4$ x $2H_2O$, $MnSO_4$ x $H_2O$ or $ZnSO_4$ x 7 $H_2O$ were added to ST1 agar. To determine differences between spores obtained from agar plates or broth, spores from strain TMW 2.485 were obtained from ST1 broth with 10 mg $L^{-1}$ $MnSO_4$. *T. thermosaccharolyticum* was grown anaerobically on *Caldicellulosiruptor* agar (per L: 0.9 g ammonium chloride, 0.9 g sodium chloride, 0.4 g magnesium chloride heptahydrate, 0.75 g potassium dihydrogen phosphate, 1.5 g di-potassium hydrogen phosphate, 2.0 g trypticase, 1.0 g yeast extract, 1 mL trace element solution, 2.5 mg ferrous (III) cloride hexahydrate, 1.0 g cellobiose, 0.75 g L- cysteinium chloride, 0.5 mg resazurine, and 12.5 g agar agar, adjusted to pH 7.2; the composition of the trace element solution was: 10 mL 7.7M hydrochloric acid, 1.5 g ferrous (II) chloride tetrahydrate, 70 mg zinc chloride, 0.1 g manganese chloride tetrahydrate, 6 mg boric acid, 190 mg carbonyl chloride hexahydrate, 2 mg copper (II) chloride dihydrate, 24 mg nickel chloride hexahydrate, 36 mg sodium molybdate dihydrate, and 990 mL aqua dest.) and strains of *C. botulinum* were grown anaerobically on ST1, RCM (composition as RCM broth, but with 12.5 g/L agar agar), egg meat (BD Difco™, Heidelberg, Germany) or WSH agar. WSH agar contained the following per liter: To 1 L of soil extract prepared according to Gams et al. (1998) were added 20 g of meat extract, 3 g of yeast extract, 0,5 g of cysteine x HCl, 5 g of $CaCO_3$ and egg white of 1.5 fresh eggs, and the pH was adjusted to 7.0. The agar plates or broth were incubated 5 days for the bacilli and 10 days for the other bacteria. Preparations

showed between 90 and 99% phase bright spores as examined by phase-contrast microscopy. Spores were collected from the plates by flooding the surface of the culture with 10 ml aliquots of cold sterile distilled water. After harvesting the spore suspensions were washed four times by centrifugation at 5000 rpm for 15 min at 5°C, resuspended in sterile distilled water and stored at –80°C until use. Between the second and third wash cycle the suspensions were pasteurized at 80°C for 10 min to kill all the vegetative forms. Spores of strain CIP 76.26 were prepared after 7 days of growth and the heating step was omitted. It was verified by microscopic observation that more than 99% of the cells had sporulated.

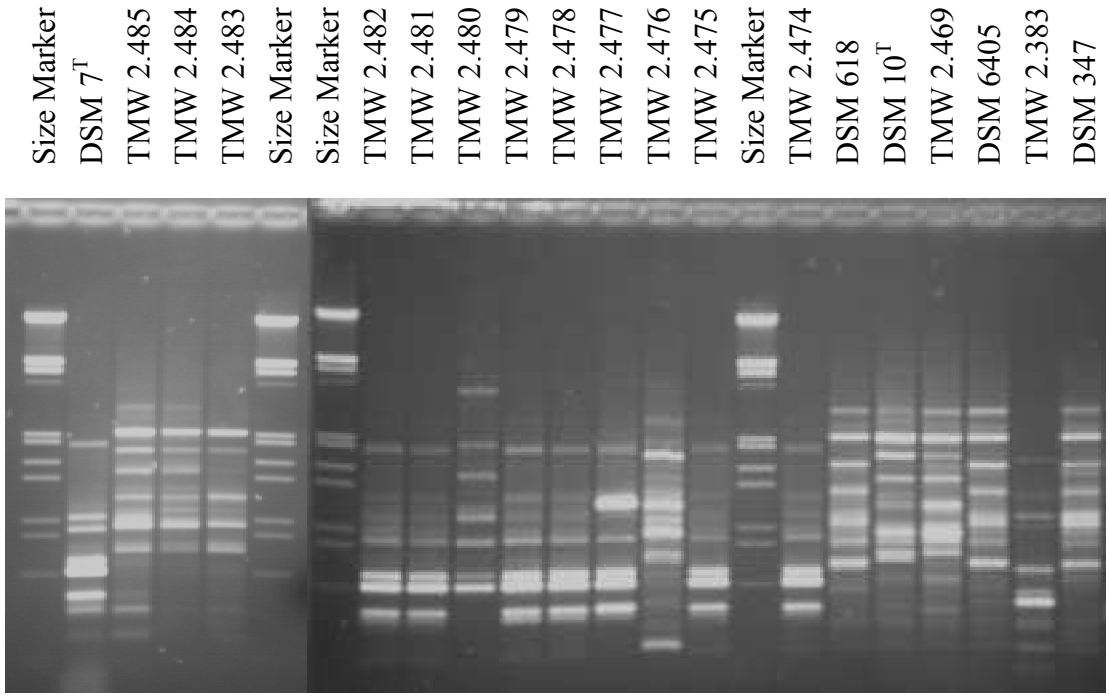


**Figure 2.1.** In combination with the sequence of the 16S rRNA of TMW 2.482 (see appendix), the RAPD patters were used for re-classification the strains TMW 2.474, TMW 2.475, TMW 2.477, TMW 2.478, TMW 2.479, TMW 2.481, and TMW 2.482 from the work of Röcken and Spicher (1993) as *B. amyloliquefaciens.*

## 2.6 Determination of cell counts

Cell counts of the *Bacillus* strains were determined on ST1 agar. Appropriate dilutions were plated using a spiral plater (IUL, Königswinter, Germany) and plates were incubated aerobically for 36 hours at 30°C or 60°C, respectively. Cell counts of *C. botulinum* and *T. thermosaccharolyticum* were determined in RCM agar and *C. thermohydrosulfuricum* -agar, respectively. Appropriate dilutions were pipetted into petri dishes, mixed with the respective agar, and plates were incubated anaerobically for 36 hours at 30°C or 60°C, respectively.

## 2.7 Pressure treatment of spores with non-isothermal holding times

Heat-sterilized, mashed carrots (pH 5.15) for use as pressurization medium were obtained in a local supermarket. Alternatively, Tris-His buffer (THB, 10 mM TRIS-HCl, 20 mM histidine-HCl) adjusted to pH 4.0, 5.15 or 6.0 was used. The pressurization media were inoculated with spores to a spore count of 2.0 x $10^6$ to 9.6 x $10^8$ spores $mL^{-1}$ and transferred to 2 mL Eppendorf reaction tubes, sealed with silicon stoppers avoiding enclosure of air, and stored on ice until treatment. Unless otherwise noted, the samples were pressurized at starting temperatures ranging from 60 to 80°C and pressures ranging from 0.1 to 800 MPa using a FoodMicroLab equipment (Stansted Fluid Power Inc., Stansted, UK) or high pressure autoclaves from Dunze Hochdrucktechnik (Hamburg, Germany) and ethanol:rhizinus oil (80:20) as pressure transmission fluid. The temperature of the pressure cell was maintained by thermostat jacket connected to a water bath (Haake GH, Karlsruhe, Germany) and the internal temperature was monitored by a thermocouple in the pressure vessel reaching into the sample container. The samples were preheated at the respective temperature for 10 min in the pressure vessel and the compression and decompression rates were 2 MPa $s^{-1}$ (standard conditions) or 6 MPa $s^{-1}$. In all figures, the pressure holding time, excluding the times required for compression and decompression are indicated. The samples were placed in the pressure vessel about 4 min prior to compression to equilibrate the sample temperature to the value indicated. Upon compression, the temperature in the samples rose by approximately 20°C or 36°C, respectively, and decreased to 80°C within 10 min (see chapter 3.2). After decompression, the sample tubes were stored on ice until determination of plate counts and measurement of DPA release, and stored at –20°C until investigation of lag times. For each combination of pressure and temperature, an inactivation kinetics with 6 –8 different pressure holding times were determined. For each experiment, an untreated sample was used as a control to determine the initial number of spores. Data are presented as means of at least two independent experiments and the standard deviation generally was 0.66 log units or less or as indicated with the error bars.

## 2.8 Pressure treatment of spores with isothermal holding times

If indicated, samples were pressurized, at temperatures ranging from 70 to 120°C and pressures ranging from 600 to 1400 MPa, using the high pressure equipment micro-system (Unipress, Warsaw, Poland) from the department of food biotechnology and food process engineering (TU Berlin). It consisted of one pressure vessel with a volume of approx. 150 µL, which was placed into a heating-cooling block. As the time for compression even up to 1400

MPa was less than 20s and as this block reproduced the increase of temperature caused through adiabatic heating, adiabatic conditions and isothermal holding times could be simulated. The initial temperature was calculated by a software written from Ardia (2004) on the basis of the adiabatic heating profiles of water, the requested processing pressure and temperature. Pressure release, which was as quick as the compression rate, was accompanied by a block mediated cooling. After decompression, the sample tubes were stored on ice until determination of plate counts. An untreated sample was used as a control to determine the initial number of spores. All these experiments were performed using THB at pH 5.15 as pressurization medium and di-2-ethyl-hexyl sebacate (Sigma, Munich, Germany) as pressure transmission fluid. The presentation of standard deviation through error bars indicates means of two independent experiments. Otherwise, experiments were not repeated.

### 2.9 Heat treatment of spores

Spore suspensions prepared as noted above were transferred to 2 mL Eppendorf reaction tubes and stored on ice until heating. The samples were put in boiling water for 5 (THB) or 10 min (mashed carrots), respectively, rapidly cooled and stored on ice until determination of plate counts. Determination of the inactivation kinetics at 100, 110 and 120°C was performed, using a oil bath. Therefore, spore suspensions were filled in glass capillaries (the internal and external diameter was 1.12 and 1.47 mm, respectively, the length was 10.8 mm), thermal incubated for up to 64 min, and further treated as noticed above.

### 2.10 Measurement of DPA release

The release of DPA from spores was determined by measurements of the DPA concentration in the supernatant of pressure treated and untreated spore suspensions. The DPA concentration of samples was analyzed by high performance liquid chromatography (HPLC) using a polyspher OAKC column (Merck, Darmstadt, Germany). The flow rate was 0.4 mL $min^{-1}$, the mobile phase was 5% acetonitrile in 5 mM $H_2SO_4$, and the temperature of the column was 70°C. A UV detector at 280 nm (Gynkotek, Germering, Germany) was used for the detection. The total DPA content of the spores was determined by quantification of DPA from spore suspensions after heat treatment at 121°C for 20 min to fully release the DPA from the spores (Janssen et al., 1958) and the release of DPA by pressure treatment was calculated relative to the total DPA content of the spores. Data are presented as means of at least two independent experiments and the standard deviation was generally less than 5% or as indicated with the error bars.

### 2.11 Detection of lag times

The determination of lag-times as a measure for the population heterogeneity in vegetative cells of bacteria was previously proposed (Baranyi and Pin, 1999). Pressure treated or untreated spore suspensions with known cell counts were diluted in ST1 broth to obtain 5, 2.5, and 1.25 spores per mL. For each of these three dilutions, 12 x 200 µl were transferred to microtiterplates and the growth kinetics were monitored by measuring absorption at 590 nm in a Spectraflour microtiter plate reader (Tecan, Grödig, Austria) at 30 min intervals for up to 120 hours at 30°C. It was assumed that the 200 µL cultures were inoculated with a single spore when 2 or more of the twelve cultures remained sterile. The experiment was repeated until observations for 96 or more individual spores from a given sample were obtained. The detection times were calculated as the time in hours that elapsed until the culture grew to an optical density (OD) of 0.02.

### 2.12 Detection of the L2 components of the HBL complex

*B. cereus* DSM 4384 was grown at 32°C in CYG medium (Beecher et al., 1994) containing 2% caseine, 0.6% yeast, 0.2% $(NH_4)_2SO_4$, 1.4% $K_2HPO_4$, 0.6% $KH_2PO_4$, 0.1% sodium citrate, and 0.2% $MgSO_4$, supplemented with 1% glucose for 6 h. EDTA (1 mM) was added at the time of harvesting. Cell-free supernatants, obtained by centrifugation (10.000 × rcf at 4°C for 20 min), followed by filtration through 0.2-µm-pore-size Millipore filters, were used in the enzyme immunoassay (EIA). For the determination of L2 component of the HBL in cell-free supernatants, the microtiter plates were coated with serial dilutions of the supernatants. The enzyme immunoassay, based on monoclonal antibodies, was performed according to (Dietrich et al., 1999). The antibodies 1A12 and 8B12 were specific for the $L_2$ component. Free protein binding sites of the plates were blocked with phosphate-buffered saline containing sodium caseinate (30 g/liter) for 30 min. Subsequently, 100 µl of the respective purified monoclonal antibody (2 µg/ml) were added, and the plates were developed as described in (Dietrich et al., 1999). Data are presented as means of three independent experiments and error bars indicate standard deviation. An untreated sample was used as a control, representing 100% reactivity, and a dilution of 1:320 gave absorbance values at 450 nm of 1.12, 0.96, and 0.94, respectively.

### 2.13 Determination of the cytotoxicity

Cytotoxicity of the cell-free supernatants was determined by measuring cell proliferation and cell viability using Vero cells (Dietrich et al., 1999). Growth medium and diluent consisted of

Eagle minimum essential medium (Biochrom KG, Berlin, Germany) with Earle salts supplemented with 1% calf serum and 2 mM glutamine. The activity was tested as serial dilutions in microtiter plates. Cell-free supernatant (0.1 ml) was added to 0.1 ml of the Vero cells, and the plates were incubated for 24 h at 37°C in a 5% $CO_2$ atmosphere. Cell Proliferation Reagent WST-1 (10 µl) (Roche Diagnostics, Mannheim, Germany) was added to 0.1 ml of the above suspension, and the plates were incubated for another hour under the same conditions. The absorbance was determined at 450 nm, and the 50% inhibitory concentration was calculated as described by Dietrich et al. (1999). Data are presented as means of three independent experiments and error bars indicate standard deviation. An untreated sample was used as a control, representing 100% cytotoxicity, and the dilution that gave a 50% reduction in the survival rate of the Vero cells was 1:348, 1:575, and 1:758, respectively.

### 2.14 Detection of the staphylococcal enterotoxins

RIDASCREEN® EIA kits, which utilize five monovalent capture antibodies against SEA to SEE, and a mixture of SET A, B, C, D, and E with a concentration of 2 ng $mL^{-1}$ for each toxin, were obtained from R-Biopharm GmbH, Darmstadt, Germany. The toxin mixture was diluted with phosphate buffered saline (per liter 0.55 g $NaH_2PO_4$ x $H_2O$, 2.85 g $Na_2HPO_4$ x 2 $H_2O$, 8.7 NaCl, and the pH was adjusted to7.4, PBS) to a concentration of 1.4 ng $mL^{-1}$, transferred to 0.5 mL Eppendorf reaction tubes, sealed with silicon stoppers avoiding enclosure of air, and stored on ice until pressure treatment. Afterwards 400 µL of each sample was diluted with 300 µL PBS. The enterotoxin assays were performed by the methods recommended by the manufacturers of the kits. Colored extracts resulting from the enzymatic reactions were measured by determining optical densities at 450 nm with a Spectraflour microtiter plate reader (Tecan, Grödig, Austria). Data are presented as means of two independent experiments and error bars indicate standard deviation. An untreated sample was used as a control, representing 100% reactivity, and gave absorbance values at 450 nm of 1.036 and 1.061 for SEC.

### 2.15 Detection of the heat-stable *E. coli* enterotoxin

Test kits for the detection of heat-stable *E. coli* enterotoxin by competitive enzyme immunoassay, and *E. coli* heat-stable enterotoxin STa were obtained from Oxoid GmbH, Wesel, Germany. Based on the competitive EIA format, the test uses a synthetic peptide toxin analogue and a monoclonal antibody to ensure specificity. 10 µg of the toxin was diluted with 50 mL TE buffer (10 mM TRIS-HCl, 1 mM EDTA, and the pH was adjusted to 8.0), transferred to 0.5 mL Eppendorf reaction tubes, sealed with silicon stoppers avoiding

enclosure of air, and stored on ice until pressure treatment. Enterotoxin assays were performed by the methods recommended by the manufacturers of the kits. An untreated sample was used as a control, representing 100% reactivity, and gave absorbance values at 485 nm of 0.56 ± 0.1. TE buffer without added toxin as negative control showed values of 1.38 ± 0.23. The $OD_{485}$ of the samples were subtracted from that of TE buffer without added toxin, multiplied with –1 and related to the untreated sample. Data are presented as means of two independent experiments and error bars indicate standard deviation.

### 2.16 Detection of *Vibrio cholerae* enterotoxin

Test kits for the detection of cholera toxin from *Vibrio cholerae* by reversed latex agglutination, which utilize polyvalent antibodies, and cholera toxin from *Vibrio cholerae* were obtained from Oxoid GmbH, Wesel, Germany. 500 µg of the toxin was diluted with 150 mL TE buffer, transferred to 2.0 mL Eppendorf reaction tubes, sealed with silicon stoppers avoiding enclosure of air, and stored on ice until pressure treatment. Enterotoxin assays were performed and classified by the methods recommended by the manufacturers of the kits.

### 2.17 Pressure treatment of toxins

The samples were pressurized at temperatures ranging from 5 to 80°C and pressures ranging from 0.1 to 800 MPa using the FoodMicroLab equipment (Stansted Fluid Power Inc., Stansted, UK). The compression and decompression rates were 2 MPa $s^{-1}$, the temperature of the pressure cell was maintained by a water bath and monitored by a thermocouple in the autoclave. The temperature in the samples rose by approximately 20°C due to adiabatic heating (see below) After decompression, the sample tubes were stored on ice until immunological or cytotoxical determination.

# 3 Results

## 3.1 Behavior of endospores concerning food spoilage in low acid pressurized food

### 3.1.1 Analysis of the carrot flora

Strains concerning food spoilage were isolated from the carrot habitat to obtain practice relevant "wild strains". Therefore, the flora of carrots from the local supermarket, from the local market place, of bio carrots and of industrial produced mashed carrots were investigated. The composition of the carrot-flora from the local supermarket was as follows: Aerobic total account number at 30°C: 1.62 * $10^6$; anaerobic total account number at 37°C: 2.3 * $10^5$ (at 60°C not detectable); lactic acid bacteria: 1.5* $10^5$; coliforms: 2.1* $10^5$; and yeasts: 7.4* $10^4$. After heat (80°C) or pressure treatment (600MPa / 20°C), colony forming units slipped under the detection limit. The composition of the untreated, the heat and pressure treated porridge of the carrots from the local market place was: Aerobic total account number at 30°C: 1.5 * $10^7$, 2.2* $10^3$, and 2.6* $10^3$; anaerobic total account number at 37°C: 3.4 * $10^3$, 2.0* $10^2$, and 1.2 * $10^3$. Anaerobic strains at 60°C were not detectable. Together with the strains obtained from the bio carrots, 153 pure cultures have been isolated from the heat treated carrot-surface. Analyses by finger printing (PCR-RAPD) showed a heterogeneous flora (Fig. 3.1.). Several times arising strains were classified by 16S rRNA amplification and partial sequencing. Thereby following 17 strains were identified: TMW 2.534 *Bacillus licheniformis*, TMW 2.535 *Bacillus simplex*, TMW 2.536 *Geobacillus thermodenitrificans*, TMW 2.539 *Paenibacillus lautus*, TMW 2.540 *Bacillus sp*, TMW 2.541 *Staphylococcus warneri*, TMW 2.542 *Staphylococcus epidermidis*, TMW 2.543 *Bacillus sp*., TMW 2.544 *Bacillus macroides*, TMW 2.545 *B. licheniformis*, TMW 2.546 *Bacillus sp*., TMW 2.547 *Bacillus sp*., TMW 2.548 *Staphylococcus haemolyticus*, TMW 2.549 *Bacillus psychrodurans*, TMW 2.492 *B. licheniformis*, TMW 2.550 *Bacillus mycoides*, and TMW 2.551 *B. licheniformis*. The respective sequences with indication of the sequenced base pairs and the mismatches to the closest related strain are listed in the appendix.

Furthermore, mashed carrots obtained out of a commercial process just before heat treatment at 121°C were investigated. This means the glasses have already been treated for 30 min at 80°C. The samples were derived from five different batches. From the first batch, the sample was taken at the mid of the processing time; from the others at the beginning, at the mid and at the end out of the process. Determination of cell counts were carried out just after sampling, but in all of the 13 glasses, microbial germs were absent or under the detection limit. After incubating the whole glasses at 30°C respective 60°C for several weeks, microbial

growth caused spoilage and 34 pure cultures could be isolated and finger printed as indicated above. The RAPD-pattern of these strains showed, that in every batch and at almost every point of processing time, a *B. subtilis* strain was detectable. The following 8 several times arising strains were identified: TMW 2.531 *Geobacillus sp*., TMW 2.532 *Bacillus sp*., TMW 2.533 *Bacillus subtilis*, TMW 2.357 *Geobacillus stearothermophilus*, TMW 2.538 *G. stearothermophilus*, TMW 2.484 *Bacillus subtilis*, TMW 2.485 *B. subtilis*, TMW 2.552 *Bacillus gelatini*. Likewise, the respective sequences are listed in the appendix.

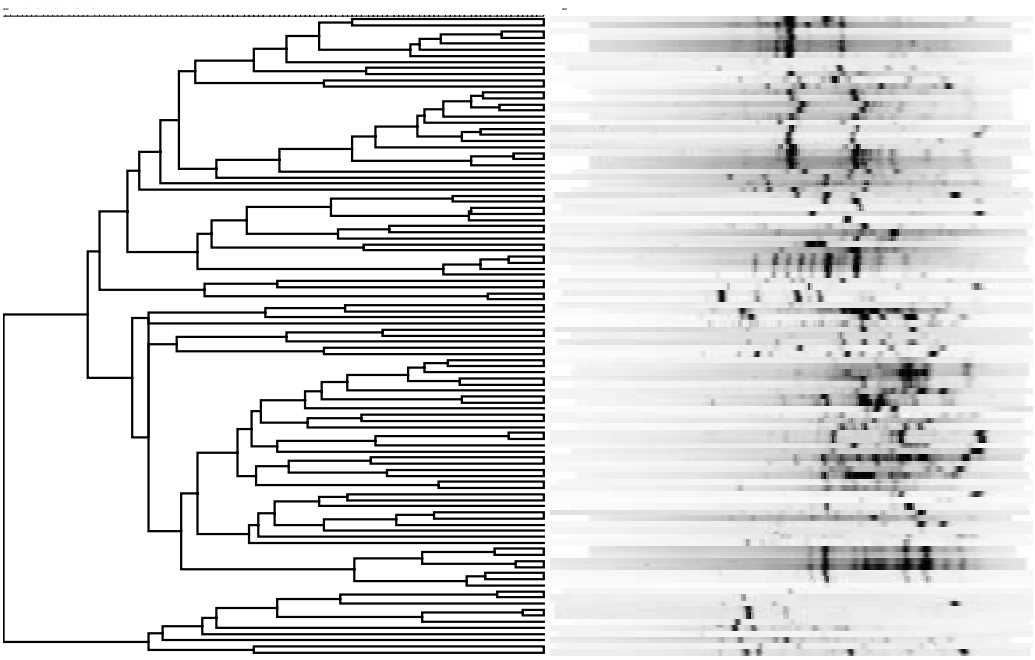

**Figure 3.1.** Representative part of the RAPD-PCR based fingerprinting of the 157 isolates from the carrot surface, which shows a heterogeneous flora.

Finally, commercial available pasteurized but spoiled carrot juice (pH < 4.5) was investigated and allowed the isolation of another thermophilic organism: *B. smithii* TMW 2.487. On the basis of the described results, the following species concerning food spoilage were used to determine the effect of pressure and temperature on the inactivation of bacterial endospores: Mesophilic and aerobic strains of *B. subtilis* and *B. licheniformis* as multible isolated, *B. smithii*, witch really caused industrial problems, as representative of thermophilic and aerobic strains, and *T. thermosaccharolyticum* previously isolated from dung as an anaerobic and thermophilic strain. Strains of *B. amyloliquefaciens* were investigated because of deviation as *B. subtilis*, previously isolated from ropy bread (Tab. 2.1).

### 3.1.2 Effect of pressure and temperature on the inactivation of spores from *B. licheniformis* and *B. subtilis*

To determine the inactivation of endospores in food, spores of the strains *B. subtilis* TMW 2.485 and *B. licheniformis* TMW 2.492 isolated from pasteurized carrots were subjected to treatments in mashed carrots with pressures ranging from 200 to 800 MPa and temperatures ranging from 60 to 80 °C. For each parameter combination the inactivation kinetics with 8 different pressure holding times was determined. Figure 3.2A and 3.2B shows the spore counts after 16 min pressure holding time. Spores of *B. licheniformis* TMW 2.492 showed a higher resistance to the pressure compared to *B. subtilis* TMW 2.485. An inactivation by less than 2 log cycles was observed for both strains at 200 or 400 MPa and 70°C and an further increase in pressure or temperature resulted in an enhanced inactivation of the spores. Spores of both strains were completely inactivated at 80°C and 600 or 800 MPa after 16 min pressure holding time.

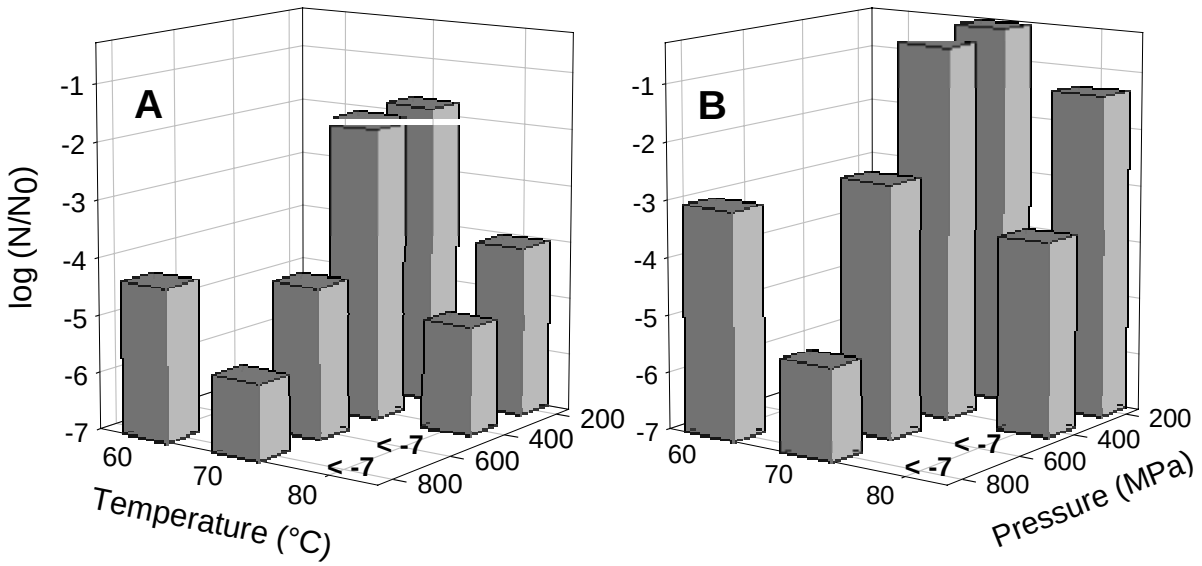


**Figure 3.2.** Log spore counts (N) of *Bacillus subtilis* TMW 2.485 (A) and *B. licheniformis* TMW 2.492 (B) after pressure / temperature treatment in mashed carrots. Spore counts are depicted relative to the spore counts of untreated samples ($N_0$). Data shown are means of at least two independent experiments and the standard deviation was generally less than 0.66 log units. Spore counts below detection limit ($\log(N/N_0) = -7$.) are indicated.

### 3.1.3 Variation of pressure resistance among strains of *B. subtilis* and *B. amyloliquefaciens*

To determine the variability of pressure resistance within strains of one species, 13 isolates from food and 5 strains from strain collections of the species *Bacilus subtilis* and *B. amyloliquefaciens* were subjected to pressure treatment in mashed carrots. The inactivation kinetics of 10 strains of *B. subtilis* at 800 MPa and 70°C in carrots are displayed in Fig. 3.3A. High variations of pressure resistance were observed. Three laboratory strains formed spores that are highly pressure sensitive and were reduced by more than 6 orders of magnitude within one minute. Four food isolates and one laboratory strain formed more pressure resistant spores that were reduced by more than 4 orders of magnitude after 16 min. Two strains isolated from ropy bread formed spores highly resistant to pressure that were inactivated by less than 2 orders of magnitude after 16 min pressure holding time. These strains were more pressure resistant than strain *B. licheniformis* TMW 2.492 (compare Fig. 3.2B and 3.3A).

The inactivation of spores from 7 strains of *B. amyloliquefaciens* is shown in Figure 3.3B. All strains were previously isolated from the same source, ropy bread, and these strains essentially exhibited the same resistance to pressure. After 16 min of pressure holding time, 1

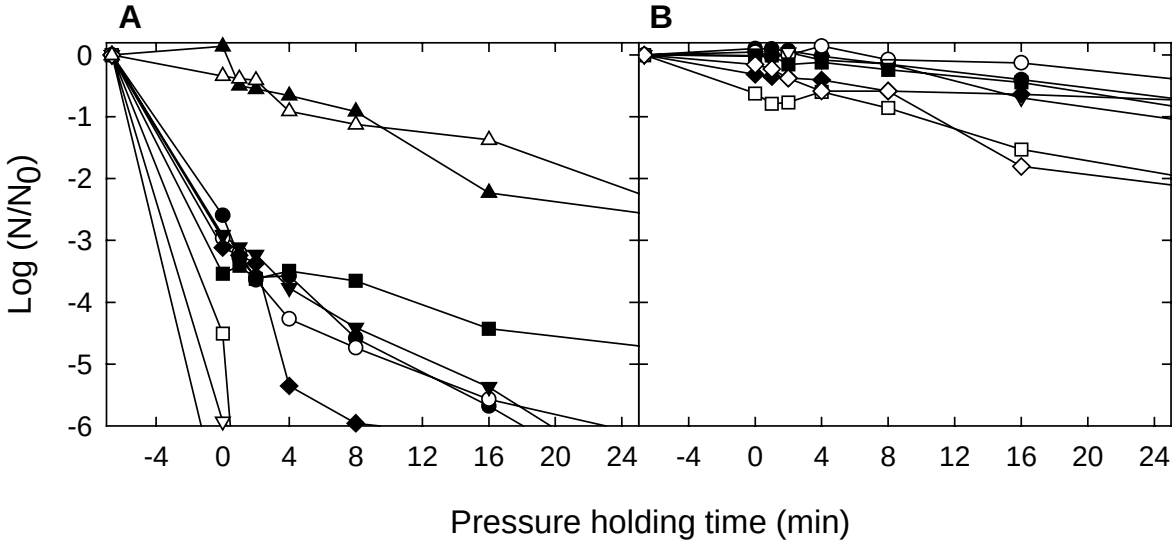


**Figure 3.3.** Log spore counts of *B. subtilis* and *B. amyloliquefaciens* spores after treatment with 800 MPa and 70°C in mashed carrots. Panel A: *B. subtilis* TMW 2.485 ( ●), TMW 2.484 ( ○), DSM 347 ( ▼), DSM 6405 ( ▽), TMW 2.480 ( ■), DSM 618 (□), TMW 2.469 (◆), DSM $10^T$ (◇), TMW 2.476 (▲), TMW 2.483 (△). Panel B: *B. amyloliquefaciens* TMW 2.479 ( ●), TMW 2.482 ( ○), TMW 2.474 ( ▽), TMW 2.478 ( ■), TMW 2.481 (□). TMW 2.477 (◆), TMW 2.475 (◇). Data shown are means of at least two independent experiments and the standard deviation was generally less than 0.66 log units. Lines dropping below the x-axis indicate spore counts below the detection limit, $\log(N/N_0) = -6$.

to 90% of the spores remained active. *B. amyloliquefaciens* formed the most pressure resistant spores among the strains in this study and tolerated 16 min at 800MPa and 70°C (Fig. 3.3B) in mashed carrots without reduction of spore counts. After 64 min at 800MPa and 70°C, the log cycle reduction of TMW 2.479 and TMW 2.482 was 2.07 and 2.93, respectively.

### 3.1.4 Effect of sporulation conditions on pressure resistance of endospores

The sporulation temperature and the mineral content of spores influence the resistance of spores of *B. subtilis* ATCC 19659 to heat and pressure (Igura et al., 2003). It was the aim to evaluate whether comparable effects govern the pressure resistance of spores of a food isolate of *B. subtilis*. In Figure 3.4 the effect of the sporulation temperature, and of the composition of the sporulation medium on the resistance of *B. subtilis* TMW 2.485 spores to treatment with 800 MPa and 70°C is shown. The sporulation conditions strongly affected pressure resistance. For instance, after one second pressure holding time, spores from broth cultures at 30°C were inactivated by less than 0.5 orders of magnitude, whereas spores of the same strain from agar cultures at 48°C were inactivated by 6 orders of magnitude. Cultures of *B. subtilis* grown in broth yielded more pressure resistant spores compared to spores grown on agar plates. An increase of the sporulation temperature resulted in a decreased resistance to pressure. Spores from cultures at 30°C had the highest pressure resistance and spores obtained from cultures at 48°C had the lowest pressure resistance. Addition of 5 mM $CaSO_4$ to the sporulation medium also resulted in a decreased pressure resistance of the spores (Fig. 3.4), likewise, addition of 5 mM $ZnSO_4$ or $MnSO_4$ decreased the spores pressure resistance (data not shown).

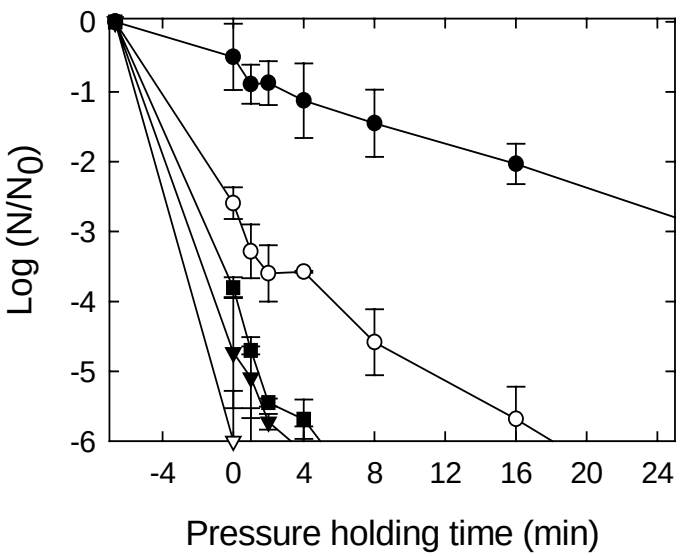


**Figure 3.4.** Inactivation in mashed carrots at 800 MPa and 70°C of *B. subtilis* TMW 2.485 spores after sporulation at various temperatures and mineral contents. Sporulated at 30°C in broth (●), 30°C on agar plates (○),44°C on agar plates (▼), 48°C on agar plates (▽), 30 °C on agar plates supplemented with 5 mM $CaSO_4$ (■). Data shown are means of at least two independent experiments and error bars indicate the standard deviation. Lines dropping below the x-axis indicate spore counts below the detection limit, $\log(N/N_0) = -6$.

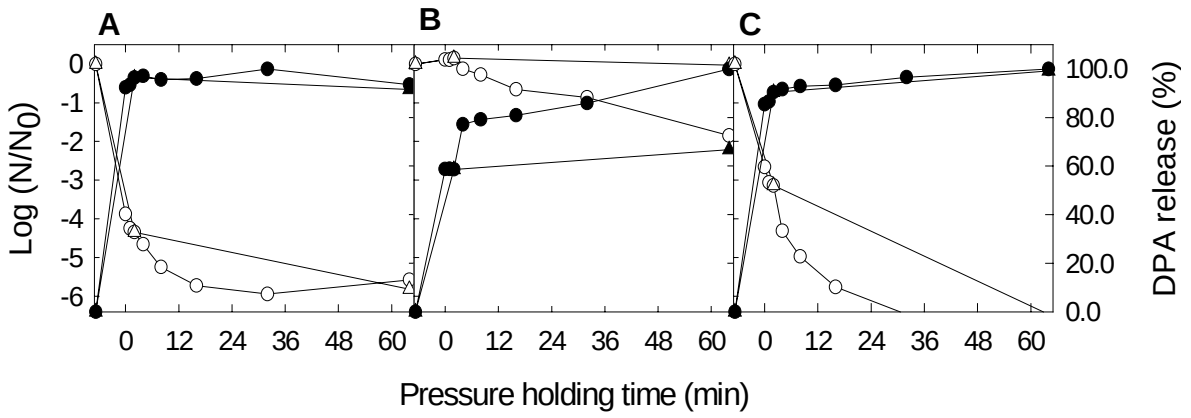


**Figure 3.5.** Effect of continuous pressurization and pressure pulse treatment at 70°C in THB on spore counts of *Bacillus* spores, and the release of DPA from the spores. Panel (A), *B. subtilis* TMW 2.485, Panel (B), *B. amyloliquefaciens* TMW 2.479, Panel (C) *B. licheniformis* TMW 2.492. (○, △), spore counts, (●, ▲) DPA release relative to the initial DPA content of the spores. (●, ○), continuous pressurization at 800 MPa; (▲, △), pressure pulse treatment, 800 MPa for 2 min followed by 0.1 MPa. The DPA content of *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis* spores was 1.45±0.15, 0.96±0.13 and 0.39±0.05 mM/$10^9$ spores, respectively. Data shown are means of at least two independent experiments. The standard deviation for the determination of spore counts and the DPA-release was generally less than 0.66 log units and 5%, respectively. Lines dropping below the x-axis indicate spore counts below the detection limit, $\log(N/N_0) = -6.4$.

### 3.1.5 Pressure induced release of DPA from bacterial endospores

The high levels of DPA in bacterial endospores are an important factor in their resistance towards chemical and physical stressors, and the pressure-induced release of DPA is considered a trigger for nutrient-receptor independent spore germination. To determine whether the variation of pressure resistance of bacterial endospores corresponds to the pressure-induced release of DPA from the respective spores, the release of DPA was determined from spores with low, intermediate, and high pressure resistance, i.e. *B. subtilis* TMW 2.485, *B. licheniformis* TMW 2.492 and *B. amyloliquefaciens* TMW 2.479, after pressure treatment at 800 MPa and 70°C. Experiments were performed in THB, because compounds from the carrots interfered with the quantification of DPA. The release of DPA from the spores is compared to the decrease of spore counts in Figure 3.5. No correlation of the total DPA content and pressure resistance was found (Fig. 3.5). No significant differences were observed when the inactivation of spores in THB was compared to the inactivation in mashed carrots with the same pH. The DPA release of the spores took place at the same time or prior to inactivation. For example, after 0 min pressure holding time, spore counts of *B. licheniformis* TMW 2.494 were reduced by 2.6 log cycles and 85% of total DPA was released from the spores. Spores of *B. subtilis* and *B. licheniformis* with low and intermediate pressure

resistance released 96 and 90% DPA, respectively, after 2 min pressure holding time. Spores that were pressure-treated for 2 min and that lost essentially all of their DPA could be inactivated at 70°C and 0.1 MPa. Following this short pressure pulse for the release of DPA, the kinetics of inactivation at 70°C and 0.1 MPa was not different from the inactivation at 70°C and 800 MPa. Thus, the generation of pressure-induced DPA free spores was accompanied with the loss of their heat resistance, and pressure did not further influence spore inactivation once the spores had lost more than 90% of their DPA. In contrast, spores of the highly pressure resistant *B. amyloliquefaciens* TMW 2.479 released only 58% of their DPA after 2 min at 800 MPa, and spores having lost 58% of their DPA were not heat sensitive. Pressure germination at moderate pressures results also in a release of DPA from the spores and phase-dark spores are obtained which exhibit sensitivity to heat and pressure comparable to vegetative cells (Wuytack et al., 1998). Therefore, phase-contrast microscopy of spores of *B. subtilis* TMW 2.485 before and after lethal pressure applications was performed. Figure 3.6 shows, that spores remained phase-bright.

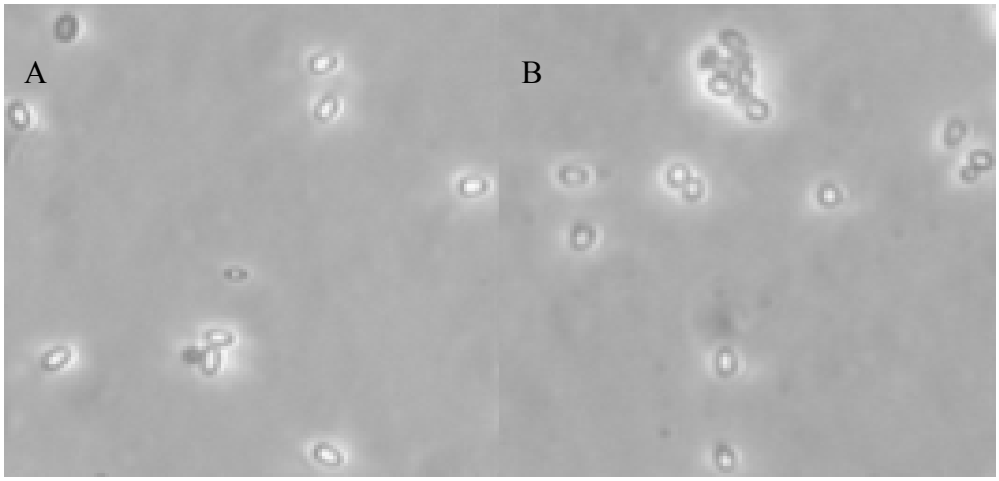


**Figure 3.6.** Phase-contrast microscopy of *B. subtilis* TMW 2.485 spores. Panel A: Untreated spores. Panel B: Spores after 5 min at 800 MPa and 70°C.

### 3.1.6 Role of DPA in heat- and pressure resistance of the DPA-deficient mutant *B. subtilis* CIP 76.26

To elucidate the role of DPA in pressure resistance of bacterial endospores in more detail, the inactivation of the DPA-deficient mutant strain *B. subtilis* CIP 76.26 by heat and/or pressure was determined. The level of DPA in spores from cultures of this strain grown in the absence of DPA is only 16% of the DPA levels in spores obtained from media with DPA (Balassa et al., 1979). Results of Balassa et al. (1979) were verified in this study (data not shown). DPA-

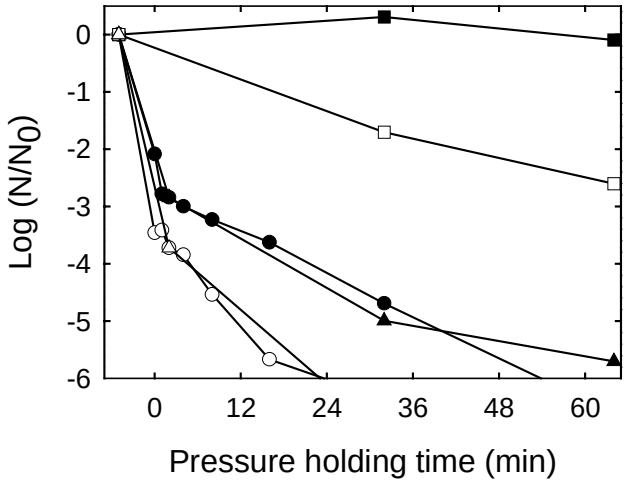


**Figure 3.7.** Effect of heat (65°C / 0.1 MPa), combined heat / pressure treatment (65°C / 600 MPa), or pressure-pulse treatment (65°C / 600 and 0.1 MPa) on the inactivation of spores of the DPA deficient *B. subtilis* mutant CIP 76.26. Spores were obtained using medium with external DPA (●, ■, ▲), or medium without external DPA (○, □, △). (●, ○), continuous pressurization at 600 MPa and 65°C; (▲, △), pressure pulse, 600 MPa and 65°C for 2 min, followed by 0.1 MPa and 65°C; ( ■, □), 0.1 MPa and 65°C. Data shown are means of at least two independent experiments and the standard deviation was generally less than 0.66 log units. Lines dropping below the x-axis indicate spore counts below the detection limit, $\log(N/N_0) = -6$.

free spores of CIP 76.26 were inactivated at 65°C and 0.1 MPa (Fig. 3.7). Leaving aside the spore inactivation in the fist 3 minutes of the pressure treatments, during which the temperature exceeds 65°C due to adiabatic heating, inactivation with 65°C and 600 MPa was not substantially accelerated compared to the inactivation at ambient pressure. Spores with DPA were heat stable; however, the inactivation with 65°C and 600 MPa was comparable to the inactivation of DPA free spores when first 3 minutes of the inactivation kinetics, during which accelerated inactivation under pressure is attributable to the temperature rose due to adiabatic heating, were neglected. Comparable to the wild type strains of *B. subtilis*, and *B. licheniformis*, DPA-containing spores of CIP 76.26 were inactivated at 65 °C and 0.1 MPa after a treatment at 65°C and 600 MPa for two min. In short, DPA containing spores were heat resistant and DPA free spores were heat sensitive, independent on whether the DPA free spores were obtained by cultivation on a DPA free medium, or after a pressure pulse to release DPA from the spores.

### 3.1.7 Distribution of detection times of pressure-treated spores: germination or sublethal injury?

The release of DPA from spores following pressurization at 550 MPa or greater was interpreted as a consequence of spore germination by some authors (Wuytack et al., 1998), whereas other authors suggested that pressure induced, unphysiological release of DPA acts as a trigger for germination after pressure release (Paidhungat et al., 2002). To discriminate between these two hypotheses, detection times of individual spores before and after pressure treatment were determined. In all samples a pronounced distribution of spore germination and outgrowth was detected, indicating large differences in the physiological states of individual spores within a sample. A small fraction of spores germinated faster than the average, whereas another small fraction exhibited prolonged detection times (Fig 3.8). Fig. 3.8A shows the detection times of single, untreated spores of *B. subtilis* TMW 2.485, and of single spores treated at 200 MPa and 70°C for 16 min. Pressure-treated spores exhibited a distribution of detection times shifted to longer detection times compared to untreated spores, indicating pressure induced sublethal injury rather than pressure induced germination. A comparable result was obtained with spores of *B. licheniformis* TMW 2.492 (Fig. 3.8B), untreated and treated at 800 MPa and 70°C for 4 min. The shortest detection time observed was 12h and the detection times of 90% of untreated spores were less than 31h. When spores of strain TMW 2.492 were treated with 70°C, 0.1 MPa for 10 min 90% of the spores had a detection time of 23h or less (Fig. 3.8B), indicating activation of spore germination by heat treatment. In

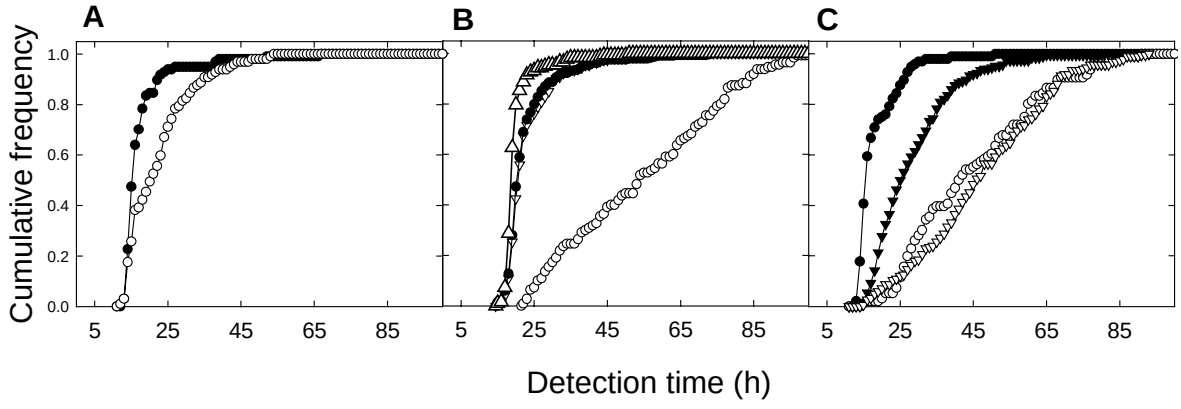


**Figure 3.8.** Detection times of spores of *B. subtilis* TMW 2.485 (A), *B licheniformis* TMW 2.492 (B) and the DPA deficient *B. subtilis* mutant CIP 76.26 (C). A: Untreated spores (●, n=97) and spores treated with 200 MPa / 70°C for 16 min (○, n=97). B: Untreated spores (●, n=282), and spores treated with 800 MPa / 70°C for 4 min (○, n=110), 100 MPa / 20°C for 30 min (▽, n=183), and 0.1 MPa / 70°C for 10 min (△, n=177). C: DPA-containing spores (●, ○, n=96), DPA-free spores (▼,▽, n=184), untreated (●, ▼) and 600 MPa / 65°C for 8 min (○, ▽).

comparison, germination and outgrowth of 90% of the spores was observed only after 81h in the pressure-treated (800MPa, 70°C) sample. None of the spores exhibited detection times of less than 20h, indicating that all spores of the population were injured by the treatment. Treatment of strain TMW 2.492 with 100 MPa and 20°C for 30 min, followed by storage at –20°C did not shift the distribution of detection times.

To investigate the influence of DPA on spore germination, the detection times of untreated spores of the DPA deficient *B. subtilis* CIP 76.26 with and without DPA were determined, and compared to the distribution of detection times after pressure treatment (Fig. 3.8C). Spores containing DPA exhibited much shorter detection times compared to DPA-free spores, indicating that the lack of DPA in the absence of any other physical stressors delays spore germination. After pressure treatment at 600 MPa and 65°C for 8 min, no difference was observed between the distribution of detection times of DPA-free and DPA-containing spores.

## 3.2 Behavior of endospores concerning food poisoning in low acid pressurized food

Strains of *C. botulinum* and *B. cereus* TMW 2.383 (Tab. 2.1) were used to determine the effect of pressure and temperature on the inactivation of bacterial endospores concerning food poisoning. Furthermore, strains were subjected to combined pressure / temperature treatments in order to compare their inactivation with that of food spoilers.

### 3.2.1 Development of a sporulation medium for *C. botulinum* strains

To obtain spores from each of the seven strains used in this work, three sporulation media were evaluated. Strains TMW 2.356, 2.358 and 2.359 did not form spores on ST1 or RCM agar. To obtain a medium supporting spore formation of all strains, the WSH medium was formulated. Using this medium, spores could be obtained from all 7 strains of *C. botulinum*.

Because the sporulation medium can strongly influence the heat resistance of *Bacillus* spores (Cazemier et al., 2001), the resistance of spores of *C. botulinum* TMW 2.357 obtained from these three different media was determined (Table 3.1). Spores obtained from cultures on WSH medium were more resistant to treatments with wet heat or pressure compared to spores obtained from STI and RCM media. Likewise, spores of *C. botulinum* TMW 2.359 obtained from WSH medium were more resistant to treatments with wet heat or pressure compared to spores obtained from egg-meat medium. The difference in spore counts after pressurization was approximately one order of magnitude (Fig. 3.9), and after wet heat treatment for 5 min in THB 0.9 log cycles. In this work, all further studies were performed with spores obtained from WSH-grown cultures, i. e. with the most resistant spores obtainable.

**Table 3.1.** Effect of the sporulation medium on heat (5 min at 100°C in THB) and pressure (16 min at 600 MPa and 80°C in mashed carrots) resistance of *C. botulinum* TMW 2.357.

| | **Spore counts log ($N/N_0$)[1)]** | |
|---|---|---|
| **Medium** | **Pressure treatment** | **Temperature treatment** |
| WSH | -1.2 ± 0.0 | -2.9 ± 0.4 |
| ST1 | -3.1 ± 0.2 | -6.0 ± 0.5 |
| RCM | -3.5 ± 0.2 | -6.3 ± 0.4 |

[1)] Log Spore counts (N) are depicted relative to the spore counts of untreated samples ($N_0$).

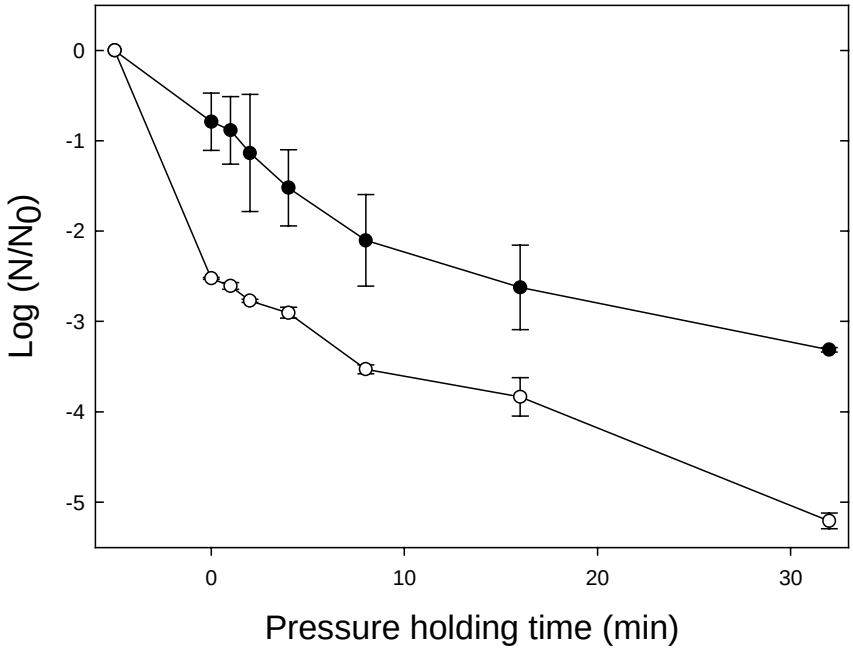


**Figure 3.9.** Log spore counts (N) of *C. botulinum* TMW 2.359 spores after treatment with 600 MPa and 80°C in mashed carrots. Spore counts are depicted relative to the spore counts of untreated samples ($N_0$). Spores were obtained from WSH medium (●), or egg-meat medium (❍). Data shown are means of two independent experiments and error bars indicate standard deviation.

### 3.2.2 Variation in resistance of *C. botulinum* strains to heat and pressure

All seven strains of *C. botulinum* were used to determine the resistance of their spores to combined pressure / temperature treatments. Spores obtained on WSH-medium were subjected to treatments in mashed carrots at 600 MPa and 80°C and spore inactivation was monitored over a period of 64 min (Fig. 3.10). Great differences in the pressure resistance of these seven strains were observed. The two non-proteolytic strains formed pressure sensitive spores and spore counts were reduced by more than 5.5 orders of magnitude within 1 s pressure holding time. In comparison, the spore counts of the proteolytic strain ATCC 19397 were reduced by more than 5 orders of magnitude after 12 minutes, and spores of the strain TMW 2.357 were inactivated by less than 3 orders of magnitude after 60 min pressure holding time.

The pressure resistance of the five proteolytic strains of *C. botulinum* was compared to their resistance to wet heat (100°C, Tab. 3.2). Strains of *C. botulinum* differed greatly in their resistance to heat, however, the heat resistance did not correlate with the pressure resistance.

*C. botulinum* TMW 2.359 was the most heat resistant strain and strain TMW 2.357 was the most pressure resistant strain.

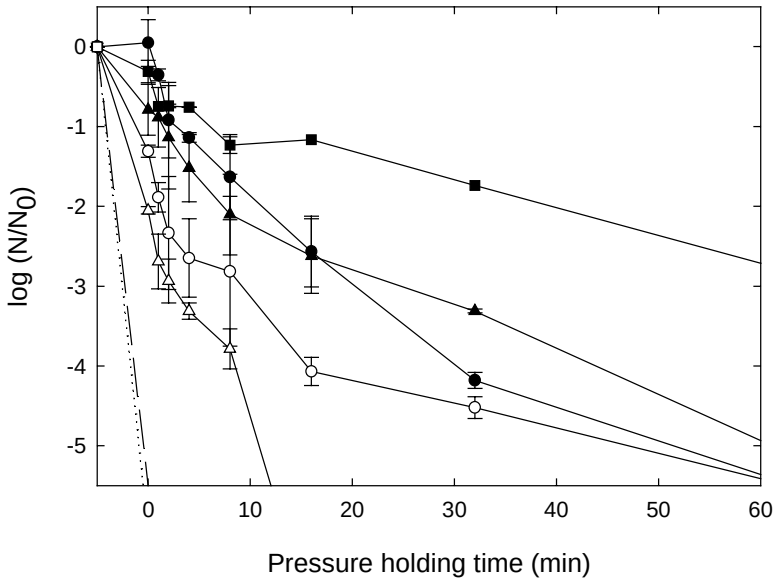


**Figure 3.10.** Log spore counts (N) of *C. botulinum* spores after treatment with 600 MPa and 80°C in mashed carrots. Spore counts are depicted relative to the spore counts of untreated samples ($N_0$). TMW 2.357 (■), TMW 2.356 (●), TMW 2.359 (▲), TMW 2.358 (❍), ATCC 19397 (Δ), ATCC 25765 (dotted line), TMW 2.518 (dashed line). Data shown are means of two independent experiments and error bars indicate standard deviation. Lines dropping below the x-axis indicate spore counts below the detection limit, $\log(N/N_0) = -5.5$.

### 3.2.3 Effect of the acidity on pressure inactivation and DPA release of *C. botulinum* spores

Low pH values were reported to accelerate the pressure-induced inactivation of bacterial endospores (Roberts et al., 1996). Because the resistance of spores to combined pressure / temperature treatments correlates to their ability to retain DPA (see chapter 3.1), the effect of pH on the pressure-induced release of DPA from *C. botulinum* endospores was further determined. The inactivation and the release of DPA was monitored following pressure treatments at 800 MPa, 80°C and at pH-values of 4.0, 5.15, or 6.0. Experiments were performed in THB to obtain a pressure-independent buffer system, and because compounds from the carrots interfered with the quantification of DPA. The release of DPA from the spores is compared to the decrease of spore counts in Fig. 3.11. A decrease in pH from 6.0 to 5.15 did not affect inactivation of *C. botulinum* TMW 2.357. When the pH was further decreased to 4.0, an accelerated inactivation of the spores was observed.

**Table 3.2.** Comparison of heat (10 min at 0.1 MPa and 100°C) and pressure (16 min at 600 MPa and 80°C) resistance of WSH spores of proteolytic strains of *C. botulinum* in mashed carrots.

| | Spore counts log $(N/N_0)^{1)}$ | |
|---|---|---|
| **Strain** | **Pressure treatment** | Temperature treatment |
| TMW 2.356 | -2.6 ± 0.4 | -2.3 ± 0.0 |
| TMW 2.357 | -1.2 ± 0.0 | -3.6 ± 0.0 |
| TMW 2.358 | -4.1 ± 0.2 | -5.6 ± 0.3 |
| TMW 2.359 | -2.6 ± 0.5 | -0.5 ± 0.1 |
| ATCC 19397 | -7.2 ± 0.2 | -4.4 ± 0.1 |

[1)] Log Spore counts (N) are depicted relative to the spore counts of untreated samples ($N_0$).

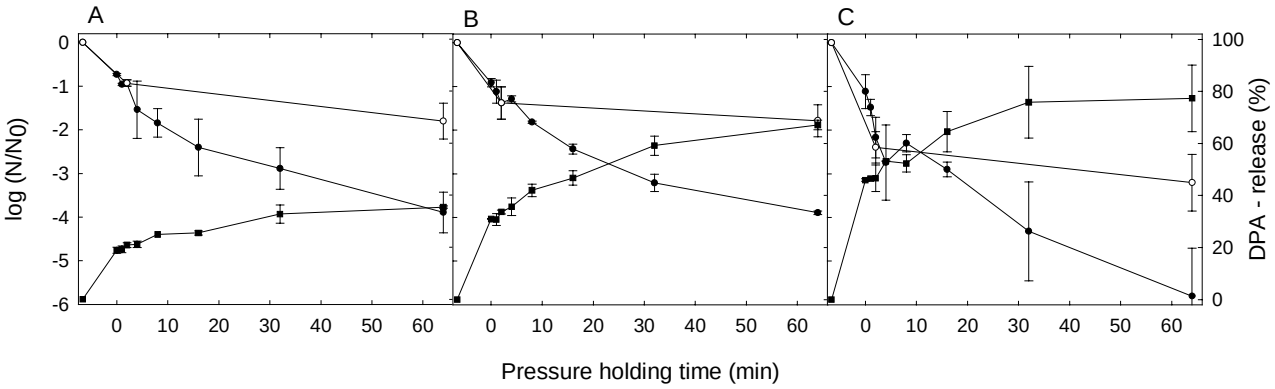


**Figure 3.11.** Effect of continuous pressurization or pressure pulse treatment at 80°C in THB on spore counts of *C. botulinum* TMW 2.357 as well as the release of DPA from spores. Experiments were performed at pH 6.0 (A), pH 5.15 (B) and pH 4.0 (C). Spore counts are indicated by circles (●, ❍) and the DPA release relative to the initial DPA content of the spores is indicated by squares (■). Closed symbols indicate the respective results of continuous pressurization at 800 MPa and open symbols indicate pressure pulse treatment, i.e. 800 MPa for 2 min at 80°C, followed by incubation at 0.1 MPa and 80°C. Data shown are means of duplicate or triplicate independent experiments and error bars indicate standard deviation. Lines dropping below the x-axis indicate spore counts below the detection limit, $\log(N/N_0) = -6.0$.

A release of DPA from the spores was observed after pressure treatment. Treatments at low pH values resulted in an increased release of DPA. After one hour pressure holding time at pH 4.0, 5.15 and 6.0, the release of DPA was 77%, 67% and 35%, respectively. It was evaluated whether a short pressure pulse can generate DPA free, heat sensitive spores of *C. botulinum*. After treatments for 2 min at 800 MPa, at pH 4.0, 5.15 and 6.0, spores of *C. botulinum* TMW

2.357 released only 47, 33, and 21% of their DPA corresponding to log 2.4 ± 0.4, 1.4 ± 0.4, and 0.9 ± 0.1 reduction of spore counts, respectively, and these spores remained heat resistant (Fig. 3.11).

### 3.2.4 Effect of the temperature on pressure inactivation and DPA release of *C. botulinum* spores

The temperature is an important factor to control the pressure-induced inactivation of bacterial endospores (Reddy et al., 2003). To affect the temperature during pressure inactivation at 800 MPa, WSH spores of *C. botulinum* TMW 2.357 were subjected to pressure treatment in mashed carrots at compression rates of 2 and 6 MPa $s^{-1}$. Starting at 80°C, the temperature in the mashed carrots rose to 100°C and 116 °C following compression with 2

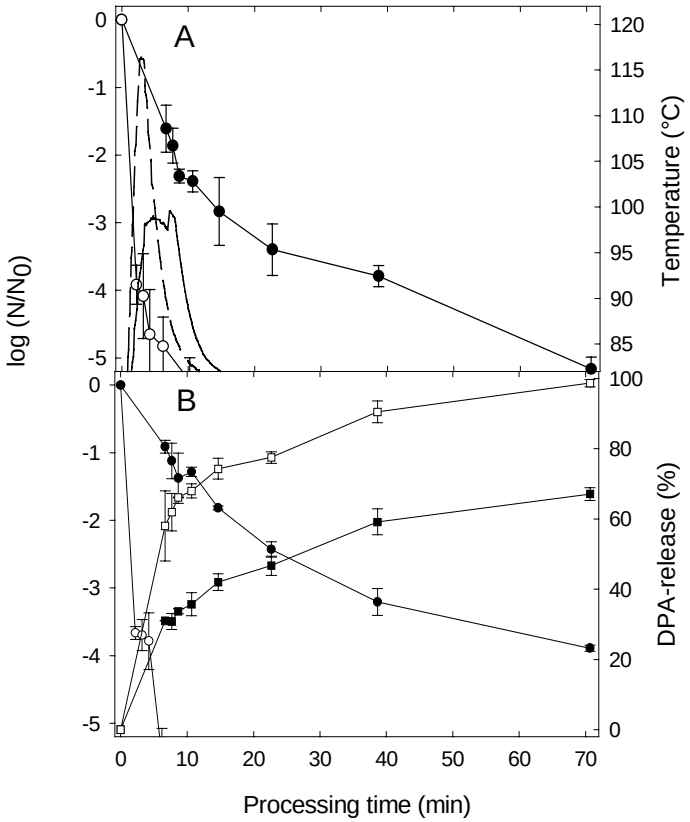


**Figure 3.12.** Effect of the compression and decompression rates of 2 or 6 MPa $s^{-1}$ on inactivation of spores of *C. botulinum* TMW 2.357 in mashed carrots (A) and THB (B). Furthermore shown is the temperature during pressure treatments in either mashed carrots or THB (panel A), and the DPA release after treatments in THB (Panel B). Spore counts are indicated by circles (●, 2 MPa $s^{-1}$; ❍, 6 MPa $s^{-1}$) and the DPA release relative to the initial DPA content of the spores is indicated by squares (■, 2 MPa $s^{-1}$; ❑, 6 MPa $s^{-1}$). The solid and dashed lines indicate the sample temperature during treatments with 2 and 6 MPa $s^{-1}$ compression and decompression rates, respectively. Data shown are means of duplicate or triplicate independent experiments and error bars indicate standard deviation. Lines dropping below the x-axis indicate spore counts below the detection limit, $\log(N/N_0) = -5.1$.

MPa $s^{-1}$ and 6 MPa $s^{-1}$, respectively (Fig. 3.12A). After reaching the maximum pressure level, temperature decreased rapidly by conduction to the pressure vessel. This transient difference in temperature strongly accelerated spore inactivation. The processing time for a spore count reduction of 5.5 log units shortened from over 70 min (compression with 2 MPa $s^{-1}$) to 10 min (compression are 6 MPa $s^{-1}$). To compare the inactivation kinetics with the DPA release, the experiment was repeated with THB as pressurization medium (Fig. 3.12B). The temperature profile during treatments in THB did not differ from those treatment in mashed carrots (data not shown). As observed during treatments in mashed carrots, the transient increase in temperature resulted in a strongly accelerated inactivation. This accelerated inactivation was reflected by an accelerated release of DPA in treatments with a compression rate of 6 MPa $s^{-1}$. Virtually quantitative release of DPA was observed after 64 min pressure holding time whereas spores counts were reduced below the detection limit already after 4 min.

The inactivation of *C. botulinum* spores in THB was slightly retarded compared to treatments in carrots. The difference was one log unit or less. The pH value of the THB buffer was set to match the pH of mashed carrots at ambient pressure. Because the $pK_A$ values of Tris and Histidine are much less dependent on pressure than the main buffering components in carrots, carboxylic acids and phosphates, this discrepancy may be attributable differences in pH during pressure treatment.

### 3.2.5 Comparison of the pressure resistance of spores from *C. botulinum* with other bacterial endospores

To compare the pressure resistance of spores of *C. botulinum* with that one of other bacterial endospores, spores of strain TMW 2.357 obtained from cultures on WSH medium and spores of *B. cereus* TMW 2.383, *B. subtilis* TMW 2.485, *B. licheniformis* TMW 492, *B. smithii* TMW 2.487, *B. amyloliquefaciens* TMW 2.479, and *T. thermosaccharolyticum* TMW 2.299 were subjected to pressure treatment at 800 MPa and 80°C in mashed carrots. Spores from *B. amyloliquefaciens* TMW 2.479 were more resistant to pressure / temperature treatments than spores from 20 other bacilli (see also chapter 3.1). After treatment for 64 min at 800 MPa and 70°C, spore counts of this strain were only reduced by 2.1 ± 0.2 log. Spores from *C. botulinum* TMW 2.357 were more resistant to pressure than spores of 6 other strains of *C. botulinum* (Fig. 3.13). Moreover, spores from *C. botulinum* TMW 2.357 obtained from WSH medium were more resistant to heat or pressure than spores obtained from other culture media. *T. thermosaccharolyticum* TMW 2.299 was selected because its heat resistance generally exceeds that of *C. botulinum* more than 10-fold (Jay, 1992) and the spores of strain

TMW 2.299 used in this work withstood treatments with 0.1 MPa, 100°C for 10 min without reduction of viable cell counts. The study in chapter 3.1 on the inactivation of strain *B. licheniformis* TMW 2.492 by pressure and temperature in the range of 200 – 800 MPa and 60 – 80 °C has shown that it exhibited a higher resistance compared to other strains of *B. subtilis* for which data on their pressure resistance is available (Furukawa et al., 2003; Furukawa et al., 2001; Hayakawa et al., 1994b; Heinz and Knorr, 1996; Igura et al., 2003, Moerman et al., 2001, Stewart et al., 2000) with the exception of two strains of *B. subtilis* isolated from ropy bread. The inactivation kinetics of these strains are displayed in Fig. 3.13. Spores from *B. amyloliquefaciens* TMW 2.479 were the only spores exhibiting a higher resistance to pressure / temperature treatment than *C. botulinum* TMW 2.357. Other strains, including *T. thermosaccharolyticum* TMW 2.299, were less resistant to pressure. Spore counts of *B. subtilis*, *B. licheniformis*, and *B. cereus* were reduced below the detection limit following one compression / decompression cycle without pressure holding time (Fig. 3.13 and data not shown).

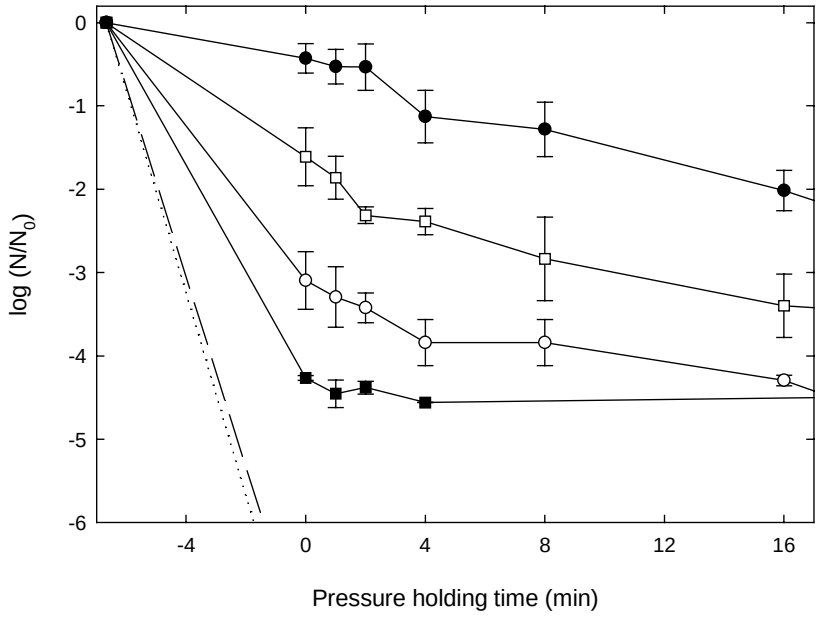


**Figure 3.13.** Log spore counts of *Bacillus subtilis* TMW 2.485 (dotted line), *B. licheniformis* TMW 2.492 (dashed line), *B. amyloliquefaciens* TMW 2.479 (●), *B. smithii* TMW 2.487 (❍), *T. thermosaccharolyticum* TMW 2.299 (■) and *C. botulinum* Typ B TMW 2.357 (❑) after treatment with 800 MPa and 80°C in mashed carrots. Data shown are means of two, three, four or five independent experiments and error bars indicate standard deviation. Lines dropping below the x-axis indicate spore counts below the detection limit, $\log(N/N_0) = -6.0$.

### 3.3 Effect of pressurization with isothermal holding times on bacterial endospores

Spores of *C. botulinum* TMW 2.357 (WSH) and *B. amyloliquefaciens* TMW 2.479 were used to investigate the effect of combined pressure (0.1 to 1400 MPa) / temperature (70 to 120°C) treatment with isothermal holding times on bacterial endospores. These strains were selected, because spores of *B. amyloliquefaciens* TMW 2.479 and WSH spores of *C. botulinum* TMW 2.357 were the most pressure resistant spores concerning spoilage and food safety, respectively. For these experiments, performed with the micro-system, spore suspensions in THB (pH 5.15) were more concentrated, in order to be able to observe at least 6.5 log cycles of inactivation. The inactivation kinetics of WSH spores of *C. botulinum* TMW 2.357 at temperatures ranging from 70 to 120°C and pressures ranging from 600 to 1400 MPa are displayed in Fig. 3.14. At 70°C, pressurization with 1000, 1100, and 1200 MPa resulted in a respective reduction of 0.5, 1.1, and 1.7 log cycles after 8 min pressure holding time. Enhancing the temperature by 10°C, inactivation after 8 min at 900, 1000, 1100, and 1200 MPa was between 0.5 and 1.5 log cycles. Likewise, inactivation at 90°C and 900, 1000, 1100, and 1200 MPa was low, with a reduction of 1 to 2.5 orders of magnitude. In contrast, pressurization at 100°C and 1400 MPa accelerated spore inactivation leading to a 4.2 log cycles reduction even after 1 min pressure holding time. Remarkably, further pressure treatment had no additional effect. Although, the rise of the pressure level from 600 to 1400 MPa at 100°C accelerated inactivation, incubation at ambient pressure resulted in a faster spore reduction than treating with 600 or 800 MPa. This pressure mediated protection was also observed at 110 and 120°C. Likewise, the tailing already noticed at 100°C is even more pronounced at 110°C. At 120°C, it is also indicated. The relation of pressure resistance spores in the samples was reduced to 1 in $10^5$ and $10^6$, respectively. In contrast, treatment with 110 or 120°C at 0.1 MPa did not show any tailing and led to a spore inactivation below the detection limit. The inactivation kinetics of the *B. amyloliquefaciens* TMW 2.479 spores with isothermal holding times at temperatures ranging from 100 to 120°C and pressures ranging from 800 to 1200 MPa exhibited similar results (Fig. 3.15). Although log cycles reduction at 100 or 110°C could be accelerated trough pressure application, at 120°C a pressure mediated protection was also observed. Likewise, the already described pressure-tailing was observed at 100, 110 and 120°C. In contrast to the results of *C. botulinum* TMW 2.357, pressure level between 800 and 1200 MPa, showed almost no varying effect of inactivation. Likewise, comparison of combined pressure temperature treatment of *T. thermosaccharolyticum* TMW 2.299 spores at 200 and 800 MPa with non-isothermal holding times showed almost no difference (Fig. 3.16). The results indicated above, leads to a different behavior of the spores

of strain TMW 2.357 and strain TMW 2.479 in respect of their resistance to a combined pressure / temperature treatment (Fig. 3.17). Whereas *B. amyloliquefaciens* TMW 2.479 spores were more pressure resistant at 1200 MPa and 100°C or 800 MPa and 120°C than WSH-spores of *C. botulinum* TMW 2.357, they were simultaneously less resistant at 800 MPa and 100°C, and similar resistant at 1200 MPa and 120°C.

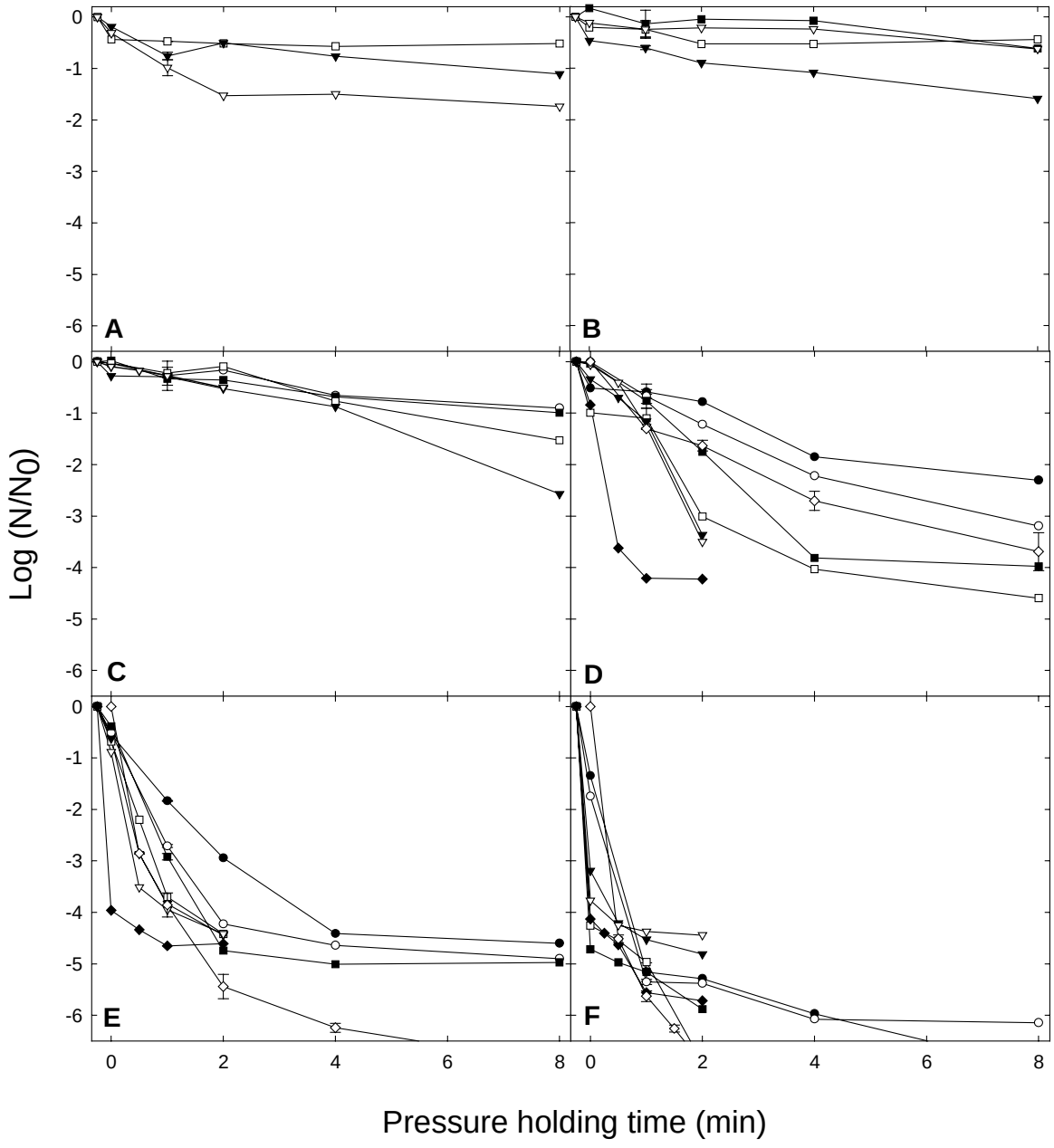


**Figure 3.14.** Log spore counts (N) of *C. botulinum* TMW 2.357 spores after combined pressure / temperature treatment with isothermal holding times in THB (pH 5.15). Spore counts are depicted relative to the spore counts of untreated samples ($N_0$). Panel A: 70°C, panel B: 80°C, panel C: 90°C, panel D: 100°C, panel E: 110°C, and panel F: 120°C. Pressure level was: 600 MPa (●), 800 (❍), 900 MPa (■), 1000 MPa (❑), 1100 MPa (▼), 1200 MPa (▽), 1400 MPa (◆), and 0.1 MPa (◇).Lines dropping below the x-axis indicate spore counts below the detection limit, $\log(N/N_0) = -6.5$.

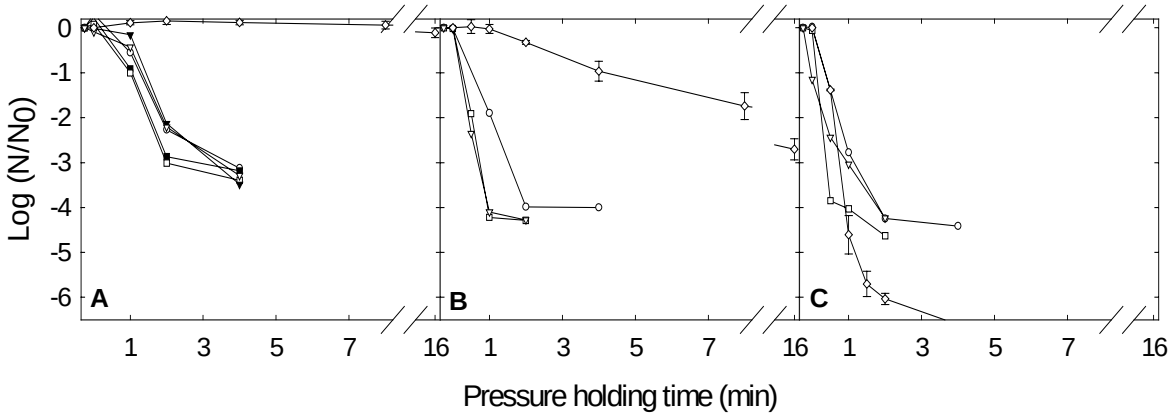


**Figure 3.15.** Log spore counts (N) of *B. amyloliquefaciens* TMW 2.479 spores after combined pressure / temperature treatment with isothermal holding times in THB (pH 5.15). Spore counts are depicted relative to the spore counts of untreated samples ($N_0$). Panel A: 100°C, panel B: 110°C, and panel C: 120°C. Pressure level was: 800 (❍), 900 MPa (■), 1000 MPa (❑), 1100 MPa (▼), 1200 MPa (▽), and 0.1 MPa (◇).Lines dropping below the x-axis indicate spore counts below the detection limit, $\log(N/N_0) = -6.5$.

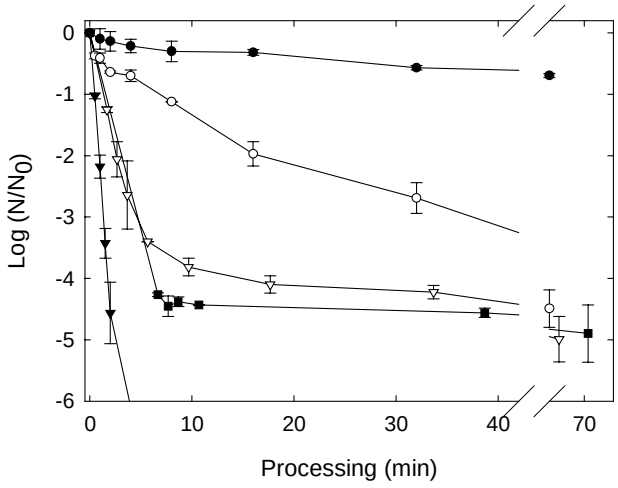


**Figure 3.16.** Log spore counts (N) of *T. thermosaccharolyticum* TMW 2.299 spores after combined pressure / temperature treatment with non-isothermal holding times in mashed carrots. Spore counts are depicted relative to the spore counts of untreated samples ($N_0$). 0.1 MPa / 100°C (●), 0.1 MPa / 110°C (❍), 0.1 MPa / 120°C (▼), 200 MPa / 80°C (▽), and 800 MPa / 80°C (■). Data shown are means of two independent experiments and error bars indicate standard deviation. Lines dropping below the x-axis indicate spore counts below the detection limit, $\log(N/N_0) = -6.0$.

To investigate, if the pressure-tailing is caused by the heterogeneity of the spore population, the log cycle reduction at 600 and 800 MPa after 2 min pressure holding time was compared with the reduction after twofold 2 min and 4 min, respectively. Regarding to the twofold 2 min treatment, temperature equilibration was awaited between first and second pressure cycle.

As these experiments were performed with the equipment, which results in non-isothermal holding times, the compression rate was used to adjust the maximum temperature to 108°C. The effect of these pressure / temperature combinations on WSH spores of *C. botulinum* TMW 2.357 were determined in duplicate and standard deviation was less than 1 log cycle. After 2 min, pressurization at 800 MPa and 108°C resulted in a reduction of 2.1 orders of magnitude, whereas of twofold 2 min and 4 min reduced the log spore count of 2.7 and 2.2, respectively. At 600 MPa and 108°C the corresponding values were 1.3, 1.7, and 1.4. Values from experiments at 600 MPa / 800 MPa and 118°C were also as closely (data not shown).

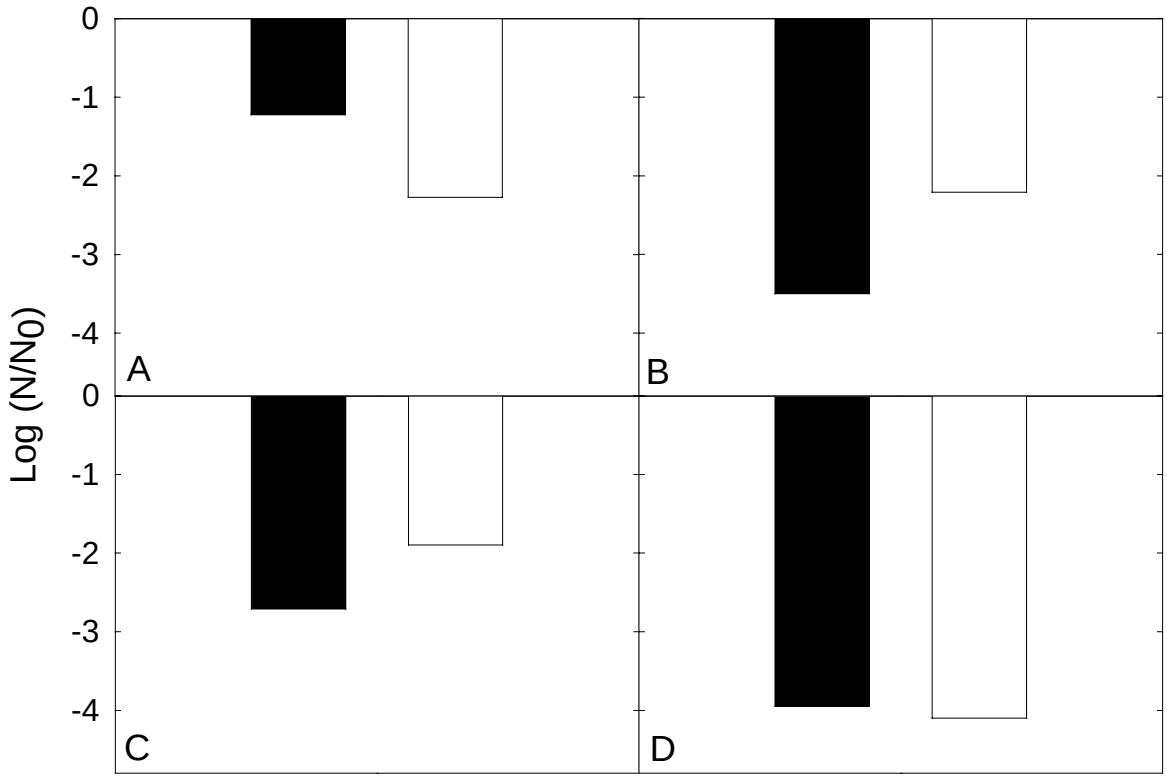


Figure 3.17. Log spore counts (N) of *C. botulinum* TMW 2.357 (■) and *B. amyloliquefaciens* TMW 2.479 (❑) spores after combined pressure / temperature treatment with isothermal holding times in THB (pH 5.15). Panel A: 800 MPa / 100°C for 2 min. Panel B: 1200 MPa / 100°C for 2 min. Panel C: 800 MPa / 120°C for 1 min. Panel D: 1200 MPa / 120°C for 1 min. Spore counts are depicted relative to the spore counts of untreated samples ($N_0$).

## 3.4 Effect of high pressure and heat on bacterial toxins

### 3.4.1 Effect of pressurization on the reactivity of heat-stable enterotoxin STa of *E. coli* in the EIA

Data were obtained of the smallest of the tested toxins after combined pressure / temperature treatment for 30 min in the range of 0.1 to 800 MPa at 5 and 80°C. Pressurization from 200 to 800 MPa at 5°C leads to a slightly increase of the reactivity. However, reactivity decreased to 66 ± 21% at 800 MPa and 80°C (Fig. 3.18). Furthermore, the reactivity of STa in the EIA was monitored at 80°C at 0.1 MPa and 800 MPa over a period of 128 min (Fig. 3.19). At ambient pressure no decrease in EIA reactivity could be observed even after 128 min. Likewise, treatment at 121°C for 30 min showed no effect (data not shown). In contrast, reactivity decreased at 800 MPa and 80°C to 44.0 ± 0.3 % after 128 min pressure holding time.

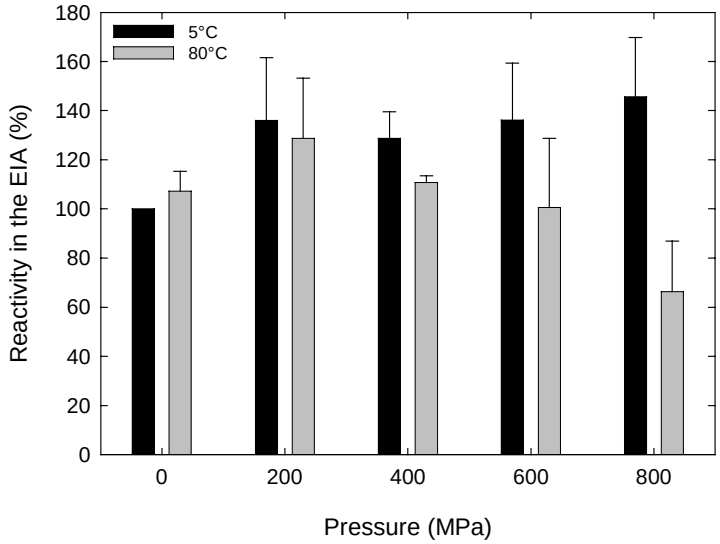


**Figure 3.18.** Effect of pressurization on the reactivity of heat-stable enterotoxin STa of *E. coli* in the EIA after 30 min at 5 and 80°C. Data shown are means of two independent experiments and error bars indicate standard deviation. An untreated sample was used as a control, representing 100% reactivity, and gave absorbance values at 485 nm of 0.56 ± 0.1. TE buffer as negative control showed values of 1.38 ± 0.23.

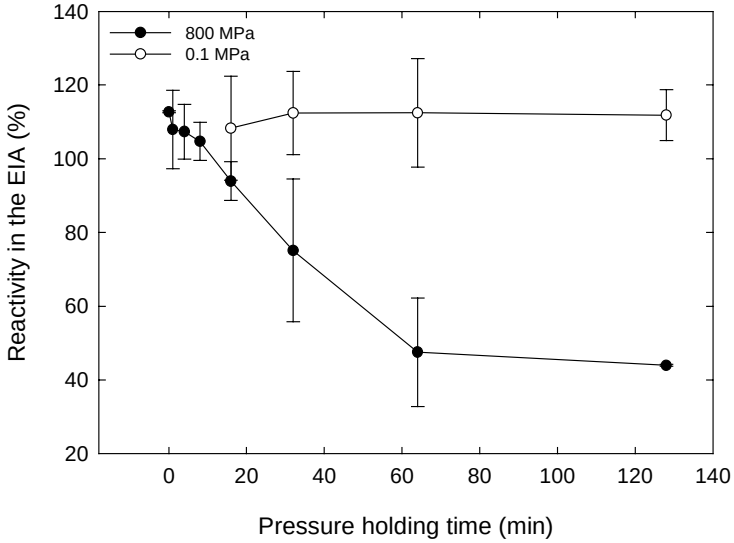


**Figure 3.19.** Effect of pressurization on the reactivity of heat-stable enterotoxin STa of *E. coli* in the EIA at 80°C. Data shown are means of two independent experiments and error bars indicate standard deviation. An untreated sample was used as a control, representing 100% reactivity, and gave absorbance values at 485 nm of 0.56 ± 0.1. TE buffer as negative control showed values of 1.38 ± 0.23.

### 3.4.2 Reactivity of pressurized staphylococcal enterotoxins in EIA's

One of the most important properties of SEs in terms of food safety are their heat stability. To examine whether these relatively small toxins exhibit also a high pressure stability, the effect of combined pressure / temperature treatment on the reactivity of SEA to SEE in the EIA after 30 min at 5°C and 20°C, and after 30 min and 120 min at 80°C was determined. The results for SEC are shown in Fig. 3.20. Pressurization at 5°C and 20°C in the range of 0.1 MPa to 800 MPa showed no effect. At ambient pressure EIA reactivity of SEC decreased by 35% after 30 min and by 63% after 120 min at 80°C. Pressure treatment at 80°C for 30 min in the range of 0.1 MPa to 400 MPa showed a slightly increase and from 400 MPa to 800 MPa again a slightly decrease in the immuno-reactivity. Pressurization for 120 min at 80°C had almost no additional effect. Only after 120 min at 800 MPa and 80°C pressurization leads to a further decrease in reactivity (Fig. 3.20C). The effect of pressure on SEA to SEE did not differ (Fig. 3.20A, B, D, E). However, thermal stability varied strongly. The order of heat resistance at 80°C was SEA = SEC = SEE > SED > SEB.

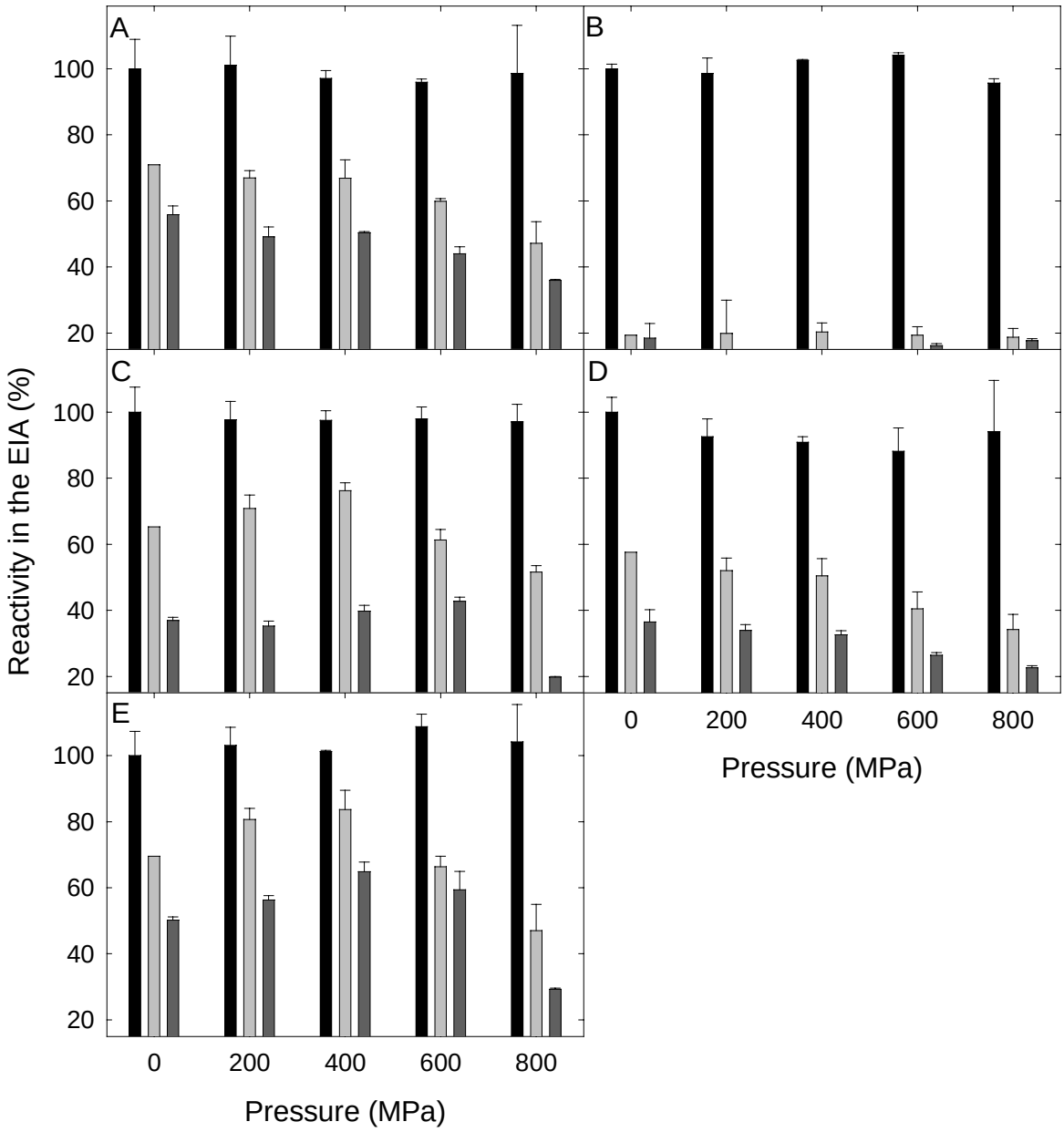


**Figure 3.20.** Effect of pressurization on the reactivity of staphylococcal enterotoxin in the EIA after 30 min at 20°C, and after 30 and 120 min at 80°C. Panel A: SEA, panel B: SEB, panel C: SEC, panel D: SED, and panel E: SEE. Data shown are means of two independent experiments and error bars indicate standard deviation. An untreated sample was used as a control, representing 100% reactivity, and gave absorbance values at 450 nm of 1.036 and 1.061, respectively.

### 3.4.3 Effect of pressure on the detection of cholera toxin by reversed passive latex agglutination

The multimeric CT was subjected to combined pressure / temperature treatment for 30 min in the range of 5 to 121°C and 0.1 to 800 MPa. At 5, 40, and 60°C and a pressure level from 0.1 to 800 MPa all samples could be classified at a titer of 1:128 as positive (+++) so that no difference of the agglutination pattern to the untreated sample could be observed (data not shown). At 80°C the detectable toxin concentration did slightly decrease, leading at 800 MPa to a negative reaction (±) at a titer of 1:128 (Table 3.3). Therefore, kinetics were determined. Although CT was still detectable after a period of 90 min at 800 MPa and 80°C, toxin concentration decreased close to the detection limit (Table 3.4). Incubation at 80°C and 0.1 MPa for the same period caused no reduction. However, after 30 min at 121 °C the concentration of the detectable cholera toxin decreased under the detection limit.

**Table 3.3.** Detection of *Vibrio cholerae* enterotoxin after combined pressure / temperature treatment for 30 min[1)].

| | **Pressure (MPa)** | | | | | |
|---|---|---|---|---|---|---|
| | **80°C** | | | | | **121°C** |
| LT (titer) | 0.1 | 200 | 400 | 600 | 800 | 0.1 |
| 1:2 | +++ | +++ | +++ | +++ | +++ | ± |
| 1:4 | +++ | +++ | +++ | +++ | +++ | - |
| 1:8 | +++ | +++ | +++ | +++ | +++ | - |
| 1:16 | +++ | +++ | +++ | +++ | +++ | - |
| 1:32 | +++ | +++ | +++ | +++ | ++ | - |
| 1:64 | +++ | +++ | +++ | ++ | + | - |
| 1:128 | +++ | ++ | ++ | ++ | ± | - |

[1)] Interpretation of the test results was performed by the methods recommended by the manufacturers of the kit. Results classified as (+), (++) and (+++) are considered to be positive.

**Table 3.4.** Detection of *Vibrio cholerae* enterotoxin after combined pressure / temperature treatment at 80°C[1)].

| | **Pressure holding time (min)** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **800 MPa** | | | | | | | | | **0.1 MPa** | | |
| LT (titer) | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 30 | 60 | 90 |
| 1:2 | +++ | +++ | +++ | +++ | +++ | ++ | ++ | ++ | + | +++ | +++ | +++ |
| 1:4 | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | ± | +++ | +++ | +++ |
| 1:8 | +++ | +++ | +++ | +++ | ++ | + | + | ± | - | +++ | +++ | +++ |
| 1:16 | +++ | +++ | +++ | +++ | ++ | + | + | ± | - | +++ | +++ | +++ |
| 1:32 | +++ | +++ | ++ | ++ | + | ± | ± | - | - | +++ | +++ | +++ |
| 1:64 | +++ | ++ | + | + | ± | - | - | - | - | +++ | +++ | +++ |
| 1:128 | ++ | + | ± | ± | - | - | - | - | - | +++ | ++ | ++ |

[1)] Interpretation of the test results was performed by the methods recommended by the manufacturers of the kit. Results classified as (+), (++) and (+++) are considered to be positive.

### 3.4.4 Reactivity in EIA and cytotoxicity of the supernatant of toxigenic *B. cereus* after pressure treatment

The diarrheal type of intoxication of *B. cereus* is caused by multimeric enterotoxins that are characterized as heat labile. In order to determine the pressure resistance of the largest toxin of this study, the effect of pressure treatment on the reactivity in the EIA of the supernatant of *B. cereus* DSM 4384 was investigated (Fig. 3.21). EIA reactivity was slightly enhanced as pressure increased. Thereby, the pressure induced increase was more pronounced at higher temperatures, leading to a maximum reactivity of 182 ± 63%. Additionally, the cytotoxicity of the samples as a measure of biological activity was determined (Fig. 3.22). Likewise, pressure treatment had almost no effect on *B. cereus* enterotoxins, but in contrast to the results obtained by the EIA, an increase of the pressure level in the range of 0.1 MPa to 800 MPa at 5°C resulted in a slightly decrease of the toxicity to 81% at maximum pressure. Even if the temperature was increased up to 30°C at the same pressure level, no further significant decrease of the toxicity could be observed.

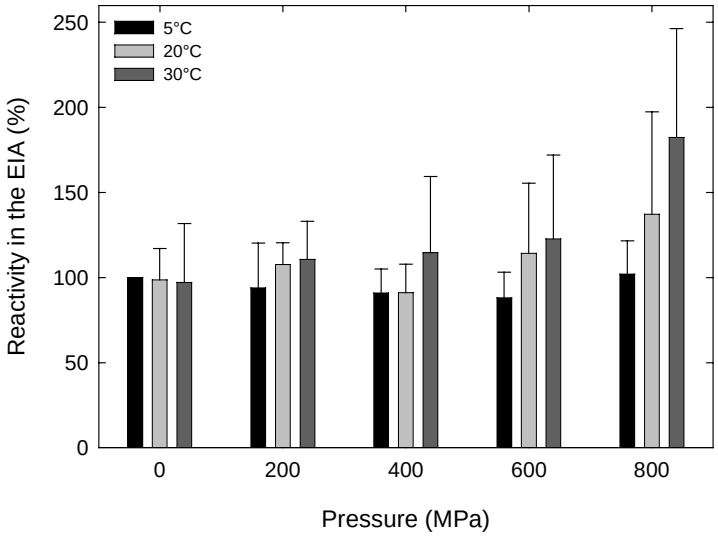


**Figure 3.21.** Effect of pressurization at 5, 20 and 30°C on the reactivity of the supernatant of *B. cereus* DSM 4384 in the EIA after 30 min pressure holding time. Data shown are means of three independent experiments and error bars indicate standard deviation. An untreated sample was used as a control, representing 100% reactivity, and a dilution of 1:320 gave absorbance values at 450 nm of 1.12, 0.96, and 0.94, respectively.

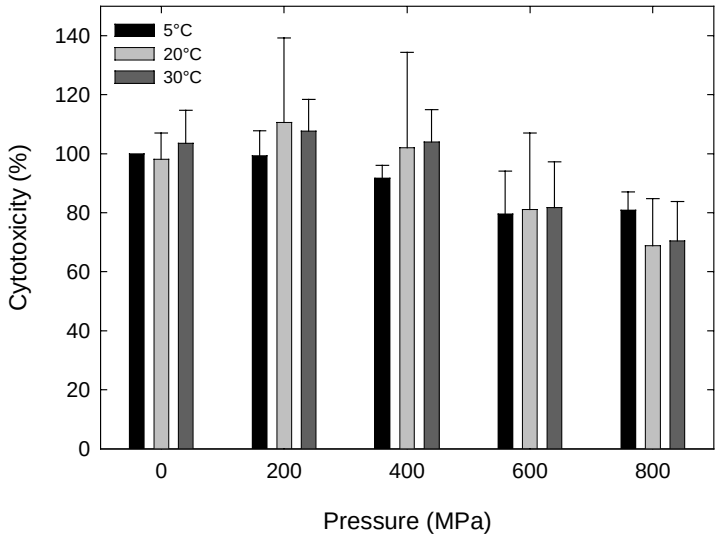


**Figure 3.22.** Effect of pressurization at 5, 20 and 30°C on the cytotoxicity of the supernatant of *B. cereus* DSM 4384 after 30 min pressure holding time. Data shown are means of three independent experiments and error bars indicate standard deviation. An untreated sample was used as a control, representing 100% cytotoxicity, and the dilution that gave a 50% reduction in the survival rate of the Vero cells was 1:348, 1:575, and 1:758, respectively.

# 4 Discussion

The expected heterogeneity of the determined flora of carrots corresponds to the one of a soil sample. The impossibility to isolate any germs from the mashed carrots, obtained out of a commercial process just before the usual heating step at 121°C, shows, that the required treatment to ensure food safety and to prevent spoilage depends also highly on the pre-treatment of the respective food. The need of an adequate inactivation of bacterial endospores was emphasized by the fact, that all of the samples showed microbial growth after incubation at 30°C or 60°C for several weeks. On the basis of the flora-analysis, the following species concerning food spoilage were used to determine the effect of pressure and temperature on the inactivation of bacterial endospores: Mesophilic and aerobic strains of *B. subtilis* and *B. licheniformis* as multible isolated, *B. smithii*, which caused industrial problems (carrot juice), as representative of thermophilic and aerobic strains, and *T. thermosaccharolyticum* previously isolated from dung as an anaerobic and thermophilic strain. Strains of *B. amyloliquefaciens* were investigated because of deviation as *B. subtilis*, previously isolated from ropy bread.

## 4.1 Variability of pressure resistance in spores of *Bacillus* species

The resistance of 14 food isolates and 5 laboratory strains of *Bacillus* species to combined pressure / temperature treatments was compared. In agreement with literature data, appreciable inactivation of spores of *B. subtilis* TMW 2.485 and *B. licheniformis* TMW 2.492 was observed when the pressure exceeded 400 MPa and the temperature exceeded 60°C and both, an increase of pressure and an increase in temperature, enhanced spore inactivation (Hayakawa et al., 1994 Lee et al., 2002; Raso et al., 1998; Reddy et al., 2003; Rovere et al., 1998). To date, kinetic data for the inactivation of spores of *Bacillus* species are available for a few laboratory strains only. A large variability of pressure resistance in food isolates of the closely related species *B. subtilis, B. licheniformis,* and *B. amyloliquefaciens* was observed. Using two strains of *Clostridium botulinum* Type E, Reddy et al. (1999) also observed differences in pressure resistance within one species. Remarkably, the strain *B. licheniformis* TMW 2.492 used in this study exhibited a intermediate pressure resistance compared to other food isolates of *B. subtilis* and *B. amyloliquefaciens* but a higher resistance compared to other strains of *B. subtilis* for which literature data is available (Furukawa et al., 2003; Furukawa et al., 2001; Heinz and Knorr, 1996; Igura et al., 2003; Moerman et al., 2001; Stewart et al., 2000). This finding highlights the need for studies with food isolates to establish pressure processes in food preservation.

The highest resistance to pressure was observed in strains of *B. subtilis* and *B. amyloliquefaciens* previously isolated from ropy bread. The spores of rope forming bacilli are more heat resistant compared to other strains of *B. subtilis* and *B. amyloliquefaciens* because these spores survive the baking process, i.e. heat-treatment at 100°C for 45 to 60 min (Röcken and Spicher, 1993). This finding may implicit a correlation between heat resistance and pressure resistance. However, the heat resistance of different strains of rope forming bacilli (Röcken and Spicher, 1993) does not correlate to their pressure resistance. Furthermore, spores of *B. amyloliquefaciens* are considerably more pressure resistant when compared to spores of *Geobacillus stearothermophilus*, which exhibits a higher resistance to wet heat (Ananta et al., 2001). Likewise, the pressure resistance of spores of six *Bacillus* strains did not correlate to their heat resistance (Nakayama et al., 1996). Therefore, those target organisms used to determine suitable process conditions for the thermal treatments of foods are not suitable target organisms for pressure processes. Spores of *B. amyloliquefaciens* TMW 2.482, TMW 2.478, TMW 2.479, TMW 2.474 and TMW 2.477 are the most pressure resistant spores compared to other published data for spores of *Bacillus*, *Geobacillus, Alicyclobacillus* or *Clostridium* species, including strains of *C. botulinum* Type A and Type E (Ananta et al., 2001; Cléry-Barraud et al., 2004; Fujii et al., 2002; Furukawa et al., 2003; Furukawa et al., 2001; Hayakawa et al., 1994; Igura et al., 2003; Lee et al., 2002; Moerman et al., 2001; Raso et al., 1998a; Raso et al., 1998b; Reddy et al., 1999; Reddy et al., 2003; Stewart et al., 2000; Wuytack et al., 1998). Therefore, they must currently be considered as relevant target organisms for the pressure sterilization of foods.

### 4.2 Effect of sporulation conditions on pressure resistance

A large variation of pressure resistance of spores of *B. subtilis* TMW 2.485 depending on the sporulation conditions was observed. The observed decrease in pressure resistance with increasing sporulation temperature is consistent with results from Igura et al. (2003). It could further be shown that addition of minerals to the sporulation medium reduced the pressure resistance of spores. The effect of sporulation temperature and spore mineralization on pressure resistance was opposite to the effect on heat resistance (Igura et al., 2003). However, spores obtained from broth cultures were more resistant to pressure and more resistant to heat compared to spores obtained from agar cultures (Figure 3.4).

### 4.3 Detection times as a measure of physiological heterogeneity and sublethal injury

The determination of detection times of individual vegetative bacterial cells was proposed as a suitable measure for the physiological heterogeneity of a population (Baranyi and Pin, 1999)

and has been used to determine sublethal injury in heat stressed cells of *Lactobacillus plantarum* (Smelt et al., 2002b). In this study, the method was applied to determine population heterogeneity in untreated and pressure-treated spores of *Bacillus* species. Physiological heterogeneity within an isogenic bacterial culture occurs because of chemical and physical gradients in the culture vessel, and because of statistic events in gene expression (Elowitz et al., 2002). The knowledge of the physiological heterogeneity of bacterial cultures is a prerequisite for the mathematical modeling of bacterial growth and inactivation (Heinz and Knorr, 1996; McKellar et al., 2002). As reported for vegetative cells of *L. plantarum*, a strong increase of the detection times after application of sublethal stress was observed. Moreover, upon pressure treatment, a broad distribution of detection times was noted and spores from a given sample required 24 to 96 h for germination and growth. This results in a systematic error in the determination of spore counts by surface plating as shown here and in most other studies dealing with inactivation of bacterial endospores by pressure. Incubation of the agar plates for more than 96 h is required to achieve outgrowth of more than 99% of the surviving spores and shorter incubation times underestimate the spore counts.

However, with the rope-forming *Bacillus* isolates used in this work, longer incubation times also result in systematic errors. Those spores that germinate in less than 24h rapidly cover the entire agar plate and thus make enumeration of those spores that germinate later impossible.

### 4.4 Pressure induced loss of DPA and heat resistance: germination or sublethal injury?

Moderate pressures up to 250 MPa at ambient temperature initiate spore germination in a similar way as during nutrient-induced germination (Clouston and Wills, 1969; Gould and Sale, 1970; Heinz and Knorr, 1998; Wuytack et al., 1998). Pressure germination at moderate pressures results in a release of DPA from the spores and phase-dark spores are obtained which exhibit sensitivity to heat and pressure comparable to vegetative cells. Germination at pressure exceeding 500 MPa and ambient temperature (25 – 40°C) is explained by a different mechanism compared to low pressures (Wuytack et al., 2000, Wuytack et al., 1998). Pressure application causes the unphysiological loss of DPA from the spore and allows spore germination independent of the presence of nutrient receptors after decompression (Paidhungat et al., 2002; Paidhungat et al., 2001; Wuytack et al., 2000).

In this work, the loss of DPA and an enhanced heat sensitivity of spores of *Bacillus* species was observed after treatments with 800 MPa and 70°C, which may be interpreted either as a

consequence of a physiological process, germination, or as a result of the physico-chemical loss of DPA from the spores. Spores of *B. subtilis* TMW 2.485 remained phase-bright after lethal pressure applications, arguing against pressure induced germination. To further differentiate between pressure induced germination and pressure induced sublethal injury, the distribution of detection times of single spores was determined. Induction of spore germination with heat reduced detection times, indicating that the experimental setup is suitable to detect spore activation. Treatment of spores with moderate pressure (100 MPa and 20°C) did not affect the detection times, however, an activation of spore germination by pressure (Wuytack et al., 2000) may have been reversed by frozen storage following pressure treatment (Collado et al., 2003). Treatment of spores with 200 MPa / 70°C, or 800 MPa / 70°C increased the detection times by a factor of two to four, indicating that combined application of heat and pressure did not induce germination, but inflicted sublethal injury. Experiments with the DPA-deficient mutant *B. subtilis* CIP 76.26 demonstrated that the enhanced detection times could be partially explained by the lack of DPA in pressure-treated spores. Other injuries inflicted by pressure may include the inactivation of cortex lytic enzymes. Therefore, the loss of DPA during combined application of heat and pressure must be considered a result of a physico-chemical process. In contrast to treatments at high pressure and low temperature, the loss of DPA after high pressure / high temperature treatment does not lead to initiation of spore germination after decompression. The release of DPA may be caused by an increased permeability of the plasma membrane, the cortex, or the outer membrane of the spores. It is well established that pressure application increases the permeability of bacterial membranes and compromises the function of integral membrane proteins (Pagán and Mackey, 2000; Ulmer et al., 2000).

The release of DPA from the spores was accompanied with an increased heat sensitivity of the spores. The comparison of the DPA release and the heat sensitivity of pressure-treated spores of *B. subtilis*, *B. licheniformis* and *B. amyloliquefaciens* indicates that a complete loss of DPA (> 90% of untreated spores) is required to obtain heat-sensitive spores. These DPA free, phase bright spores are less heat resistant than dormant spores, but are much more heat- and pressure resistant compared to vegetative cells of bacilli (Heinz and Knorr, 1996). The combined application of pressure and heat was required to result in DPA-free, heat-sensitive spores. However, once more than 90% of the DPA were released from the cells, the inactivation of spores was not further influenced by pressure. Following a pressure-pulse with 800 MPa and 70°C for 2 min to fully release DPA from the spores, further treatments with 800 MPa and 70°C, or 0.1 MPa, 70°C had an equivalent effect on the spores of *B. subtilis* and

*B. licheniformis*. Comparable effects were obtained with the DPA-deficient mutant *B. subtilis* CIP 76.26. In *B. amyloliquefaciens*, treatment with 2 min at 800 MPa / 70°C released only 58% of the DPA from the spores and these remained heat resistant. Therefore, the inactivation of spores of bacilli may be considered a two stage process. First, as a result of combined application of pressure and heat, sublethally injured, DPA-free spores are generated that are heat sensitive. Second, these spores are heat-inactivated independent on the pressure level. This proposed mechanisms of inactivation of spores by heat and pressure may provide an explanation why in some cases, a correlation between the heat- and pressure resistance of spores is found, whereas such a correlation is absent in other cases.

In conclusion of chapter 3.1, a strong variability of the resistance to pressure / temperature treatments within bacilli was observed. Relevant target organisms for pressure / temperature treatment of foods are proposed, i.e. the five strains of *B. amyloliquefaciens* (TMW 2.482, TMW 2.479, TMW 2.478, TMW 2.474 and TMW 2.477) which form highly pressure resistant spores. These data indicate a two stage mechanisms of spore inactivation in the pressure / temperature range used in this study, i.e. T> 60°C and p>600 MPa. First, pressure and temperature act to generate sublethally injured DPA-free and phase bright spores. Second, these spores are inactivated by moderate heat independent of the pressure. Therefore, the resistance of spores to combined pressure / temperature treatments depends on their ability to retain DPA, and on the heat resistance of DPA free spores. This mechanism may explain why some of the spore properties with importance for wet heat resistance of spores are also relevant for pressure resistance, whereas others are not. Furthermore, it may enable pressure-pulse treatments of foods to safely inactivate bacterial endospores with a minimal treatment intensity.

### 4.5 Comparison of pressure and heat resistance of *Clostridium botulinum* and other endospores in mashed carrots

The resistance of spores of *C. botulinum*, as target strain concerning food safety, to combined pressure / temperature treatments at various pH values was determined. The strains were selected to obtain a “worst case scenario” by choosing the most resistant types of spores by appropriate choice of sporulation conditions from the most pressure resistant strain. The pressure resistance was compared to heat resistance. To provide a rationale for the resistance of *C. botulinum* spores to pressure, the release of DPA from the spores after pressure treatment was determined. The comparison of the resistance of *C. botulinum* spores to that one of spores from other bacteria relevant in preservation of low-acid foods provides a first

step towards the identification of a suitable target organism for the development and evaluation of industrial high pressure processes.

The comparison of the resistance of spores from seven strains of *C. botulinum* to heat and to combined pressure / temperature treatments has shown a strong effect of sporulation conditions on the heat or pressure resistance of *C. botulinum* endospores. This result corroborates previous observations obtained with spores of *B. subtilis* (Cazemier et al., 2001; Igura et al., 2003). The resistance of spores to physical treatments was increased particularly by the use of soil extract. Moreover, medium containing soil extract was the only medium that supported sporulation by all strains employed in this study. The effect of soil extract on spore pressure resistance is possibly mediated by the content of metal ions in soil as divalent cations are known to affect heat and pressure resistance of spores (Cazemier et al., 2001; Igura et al., 2003). Because endospores present in food are likely to originate from soil, these types of spores are relevant in food processing.

A high variation of pressure and heat resistance within various spores of strains of *C. botulinum* was observed. Remarkably, the $D_{120°C}$ value for thermal inactivation of *C. botulinum* TMW 2.359 was determined as 1.2 min (Wittmann and Hennlich, 2003), which exceeds D-values for other *C. botulinum* strains by a factor of 6. Data were verified in this study (data not shown). In chapter 3.1, spores from 18 strains of *B. subtilis* and *B. amyloliquefaciens* also exhibited a high variation in pressure resistance. Taken together, these results highlight the need to study a large number of strains to provide reliable data on the inactivation of spores in pressure / temperature processes for food preservation.

In accordance with studies of Reddy et al. (1999; 2003) it was observed that proteolytic strains were substantially more pressure resistant than non-proteolytic strains. It is difficult to compare the resistance of spores of proteolytic *C. botulinum* strains used in this work with that one of *C. botulinum* strains BS-A and 62-A (Reddy et al., 2003) because the temperature profiles during processing differ strongly, and different suspension media were used. However, the resistance of WSH-derived spores of *C. botulinum* strain TMW 2.357 (this work) can be considered to be higher as compared to strain BS-A and 62-A (Reddy et al., 2003). Spore counts of strain *C. botulinum* BS-A and 62-A were reduced in phosphate buffer by 2 ± 0.6 and 3± 0.6 log after treatments for 20 min at a pressure of 827 MPa, an average temperature of about 75°C and a maximum temperature of 92°C. Strain TMW 2.357 was reduced in THB by 2.4 ± 0.1 log after treatments for 23 min at a pressure of 800 MPa, an average temperature of 87.0°C and a maximum temperature of 100°C. It must be taken into

account that spore counts of pressure treated samples obtained by plating techniques are underestimated when compared to spore counts obtained by MPN techniques and long incubation times (see chapter 3.1).

In agreement with literature data for spores of *C. botulinum* and other bacteria, the inactivation of spores observed here was strongly enhanced upon an increase of temperature or pressure (this study, Reddy et al., 2003, Rovere et al., 1998). In contrast to most other spores, a reduction of spore counts of *C. botulinum* by more than 5 log is attained only at pressure and temperature levels exceeding 600 MPa and 100 °C. Furthermore, a decrease of spore pressure resistance when the pH was decreased from pH 5.15 to pH 4.0 in a pressure-independent buffer system was observed. Likewise, the pressure-induced inactivation of spores of *Bacillus coagulans* was independent of the pH in the range of 5.0 to 7.0, whereas a further reduction of the pH to 4.0 accelerated the spore-inactivating effect of the pressure treatment (Roberts et al., 1998). The pH value of food is a function of pressure, and in aqueous systems buffered with phosphates or carboxylic acids, the pH is depressed by 1.0 pH unit upon compression from 0.1 to 300 MPa (Molina-Gutierrez et al., 2002). Therefore, pH values of 4.5 or below occur during pressure treatments even in foods with a pH above 4.5 at ambient pressure.

Pressure treatment opens channels of spores of *B. subtilis* that permit the release of DPA from the spores (Paidhungat et al., 2002). Following pressure treatment at ambient temperature, this release of DPA results in an activation of the germination pathway (Paidhungat et al., 2002; Wuytack et al., 1998). However, the inactivation of spores from *B. subtilis* and *B. licheniformis* by pressure processing at temperatures > 70°C is achieved by a two-stage mechanism that does not involve spore germination (see chapter 3.1). First, pressure causes DPA release and concomitant loss of heat resistance. Second, the DPA-free spores are inactivated by wet heat independent of the pressure level. In accordance with this model, a short pressure pulse generated DPA free, viable spores of *B. subtilis* and *B. licheniformis*, which lost their heat resistance. In this chapter, the pressure-induced inactivation of *C. botulinum* spores was determined at various levels of pH and temperature and compared to the release of DPA from the spores. Generally, pressure / temperature treatments resulted in a partial release of DPA from the spores and a quantitative release of DPA from spores was observed only after treatments resulting in a reduction of spore counts by more than 5 log. Compared to treatments at 800 MPa, 80°C and pH 5.15, the release of DPA from spores was enhanced when the pH was reduced to 4.0, or when the temperature during treatment was

increased. Likewise, the inactivation of spores by low pH is caused by a drastic change in the spore permeability barrier which leads to the loss of DPA and a concomitant hydration of the core (Setlow et al., 2002). These findings support the results of chapter 3.1 that pressure inactivation of bacterial endospores by combined pressure / temperature treatments does not involve spore germination and that the release of DPA during p/T treatments is a physico-chemical process. Spores of *C. botulinum* TMW 2.357 released their DPA much more slowly than spores of *B. subtilis* and *B. licheniformis*. Remarkably, spores of the highly pressure resistant strain *B. amyloliquefaciens* TMW 2.479 also retained DPA during pressure treatment, and remained heat resistant following a short pressure pulse. Therefore, a possible explanation for the high resistance of *B. amyloliquefaciens* and *C. botulinum* to pressure is the property of their spores to retain the DPA during pressure treatments.

### 4.6 Proposal for a suitable target or surrogate strain for pressure / temperature processing of foods

The development and assessment of high pressure food processes requires a target or surrogate strain which should have a higher resistance to pressure as compared to other food spoilage organisms (target strain for spoilage) and organisms relevant for food safety, especially *C. botulinum* (surrogate strain) (Sizer et al., 2002). Moreover, the organism should be non-toxinogenic and non-pathogenic and should not require specific equipment or growth media for cultivation and handling to be suitable for use with pilot plant and industrial scale equipment.

This chapter has clearly shown that heat resistance of various species does not relate to their high pressure resistance. As expected from literature data, spores of *T. thermo-saccharolyticum* were more resistant to wet heat than spores of other strains, including *C. botulinum* and *B. amyloliquefaciens* (this work, Jay, 1992; Röcken and Spicher, 1993). In contrast, *B. amyloliquefaciens* and *C. botulinum* exhibited a much higher resistance to pressure compared to *T. thermosaccharolyticum*. Likewise, the most pressure resistant strain of *C. botulinum*, strain TMW 2.357, exhibited only a intermediate heat resistance compared to other *C. botulinum* strains (this work). Thus, heat and pressure resistance of spores neither correlated within strains of *C. botulinum* nor in comparison with spores of *T. thermosaccharolyticum* TMW 2.299. This observation is in general agreement with previous studies performed with other bacteria (Nakayama et al., 1996). It is most noteworthy that spores of proteolytic strains of *C. botulinum* are among the most pressure resistant bacterial endospores identified so far.

Therefore, the target organisms used to design and control thermal processing in food production are unsuitable as target or surrogate organisms in high pressure processes. Based on the data from the non-isothermal experiments, *B. amyloliquefaciens* TMW 2.479 could be suggested as such a target organism for high pressure / high temperature processing of low acid foods. This strain is a mesophilic, aerobic, non-pathogenic and non-toxinogenic microorganism growing on standard laboratory media. It was previously isolated from spoiled food (Röcken and Spicher, 1993), and exhibits a higher resistance to combined heat and pressure treatments than spores from *C. botulinum* and spores from other organisms for which literature data is available (*Alicyclobacillus acidoterrestris*, *B. anthracis*, *B. cereus*, *B. coagulans*, *B. licheniformis*, *B. smithii*, *B. subtilis*, *C. botulinum, C. sporogenes*, *Geobacillus stearothermophilus*, and *T. thermosaccharolyticum*; this study, Ananta et al., 2001; Cléry-Barraud et al., 2004; Crawford et al., 1996; Fujii et al., 2002; Furukawa et al., 2003; Furukawa et al., 2001 Gould and Sale, 1972; Hayakawa et al., 1998; Hayakawa et al., 1993; Hayakawa et al., 1994a; Hayakawa et al., 1994b; Heinz and Knorr, 1996; Igura et al., 2003; Lee et al., 2002, Moerman et al., 2001, Oh and Moon, 2003, Raso et al., 1998a; Raso et al., 1998b; Reddy et al., 2003; Reddy et al., 1999; Roberts and Hoover, 1996; Rovere et al., 1998; Shearer et al., 2000; Stewart et al., 2000; Watanabe et al., 2003; Wuytack et al., 1998). However, the 12D-concept was established based on heat resistance data from 109 strains of *C. botulinum* (Esty and Meyer, 1922). Because only 9 strains of *C. botulinum* and only a limited number of strains (< 50) of other sporeformers have been evaluated with respect to their pressure resistance, additional strains need evaluation as well to allow the establishment of criteria for high pressure processes in food production. Furthermore, strains from *Clostridium baratii* and *Clostridium butyricum*, also being dedicated as potential botulinum toxin producers (Simpson, 2004), may also have to be considered.

In conclusion of chapter 3.2, these results support the hypothesis of chapter 3.1 that the resistance of spores to combined pressure / temperature treatments depends on their ability to retain DPA. Combined pressure / temperature treatments effectively reduced spore counts of *C. botulinum* by more than 5.5 log within 2 min pressure holding time (approx. 5 min processing time) at pressure and temperature levels above 600 MPa and 100°C, respectively. Therefore, pressure processing seems to be a suitable process to destroy *C. botulinum* spores in food at reduced temperatures helping to retain aroma compounds and functional ingredients of foods even in sterilized foods. As the experiments of chapter 3.1, the results of chapter 3.2 also showed that *B. amyloliquefaciens* TMW 2.479 may be suggested as a target organism for the pressure processing of low acid, although additional pressure-death time data for a larger

number of strains and a larger number of pressure / temperature combinations is required to establish target or surrogate organisms. Also further experiments are required to determine the influence of the pressure / temperature regime and the respective construction of the high pressure plant. As a first step in this direction, isothermal pressurization experiments were performed.

### 4.7 Effect of pressurization with isothermal holding times on bacterial endospores

The effect of combined pressure (0.1 to 1400 MPa) / temperature (70 to 120°C) treatment with isothermal holding times on most resistant spores concerning spoilage (spores of *B. amyloliquefaciens* TMW 2.479) and food safety (WSH spores of *C. botulinum* TMW 2.357) was determined. That way, it was possible to investigate a temperature independent pressure inactivation. Furthermore, it was possible to extend the investigated spectrum of pressure / temperature combinations of these two most important strains of this study. It has to be highlighted that spores of *C. botulinum* never were investigated under such pressures up to now. An increase of pressure (600 to 1400 MPa) and an increase in temperature (90 to 110°C) accelerated inactivation of TMW 2.357-spores, which is in accordance with the results from the non-isothermal treatments. But, incubation at 100°C and ambient pressure resulted in a faster spore reduction than treating with 600 or 800 MPa at the same temperature. This pressure mediated spore protection was also observed at 110 and 120°C and also at 120°C for *B. amyloliquefaciens* TMW 2.479 spores. These results are in contrast to the current opinion (Ananta et al., 2001, Hayakawa et al., 1994 Lee et al., 2002; Raso et al., 1998; Reddy et al., 2003; Rovere et al., 1998), that spore inactivation is generally accelerated by pressurization compared to atmospheric conditions. This apparent contradiction may be easily explained by the use of different pressure / temperature regimes and equipment with various temperature transfers between vessel and content. Furthermore, both strains showed a pronounced pressure dependent tailing, which means that a small fraction of the spore population may have remained highly resistant. In accordance to the results from the non-isothermal treatments (600 or 800 MPa at 115°C), pressurization of spores of *C. botulinum* TMW 2.357 at 600 or 800 MPa at 120°C reduced spore counts by more than 5.5 log within 4 min processing time. But in the case of the non-isothermal treatments, observation of the tailing would have not been possible as the reduction was below the detection limit. This spore behavior to pressure inactivation was also noticed in other studies (Cléry-Barraud et al., 2004; Lee et al., 2002; Reddy et al., 2003; Crawford et al., 1996). Another investigation (Mallidis and Drizou, 1991) also found, that the spore population was heterogeneous with regard to its sensitivity to heat

and pressure. Although the tailing and the upward concavity of survival curves of bacterial spores after heat treatment at moderate pressure is discussed in an number of publications (Cerf, 1977), in this study, this tailing was absent at ambient pressure. Thus, it is possible that pressure treatment, even at high doses, results in a small percentage of survivors. Cléry-Barraud et al. (2004) hypothesized that these highly resistant spores were spontaneous mutants induced by pressure as Ludwig et al. (2002) obtained a spontaneous mutant of a *Bacillus thuringiensis* strain by a single pressure treatment. But the formation of spontaneous 'mutants' is unlikely during a 20 sec ramp and the following inactivation. The higher resistance of the remaining spores is obviously displayed by the comparison of the log cycle reduction after 2 min pressure holding time with that one of twofold 2 min and 4 min, as the twofold 2 min and the 4 min treatment, respectively, by far not resulted in a twofold reduction of spores. Furthermore, the assumption of other authors (Hayakawa et al., 1994a, b; Furukawa et al., 2003), that repetitive pressure treatment is a more effective method of spore inactivation than continuous pressurization, could not be confirmed.

Inactivation of *B. amyloliquefaciens* TMW 2.479 spores with isothermal holding times differed strongly to that of spores of *C. botulinum* TMW 2.357, as pressure level between 800 and 1200 MPa showed almost no varying effect in respect of a faster spore reduction. This was also found for spores of *T. thermosaccharolyticum* TMW 2.299 and other authors described a similar behavior (Crawford et al., 1996; Lee et al., 2002). As consequence, the order of the resistance of spores to combined pressure / temperature treatment is not fixed. This different behavior makes it difficult if not impossible to suggest a general target organism for the pressure processing of low acid foods. Thus, for each combination of a pressure with a temperature level, a target organism must be defined, as the rearrangement of one parameter can lead to another most resistant target strain.

In conclusion of chapter 3.3, the approaches proposed by Sizer et al. (2002), which should be used for the validation of low-acid pressure processes in terms of food safety can reconsidered with respect to their suitability to address the microbial safety achieved in pressurization: (I) "Consider pressure processes as conventional thermal process; the enhanced lethality due to the contribution of pressure is not taken into account." The observed pressure mediated spore protection shows, that spore inactivation is not generally accelerated by pressurization compared to atmospheric conditions. (II) "Demonstration of a 12-D-process with biological validation using *C. botulinum* spores; the process should be demonstrated with the most resistant strain of *C. botulinum*." As the 12D-concept was established based on the heat

resistance of 109 strains, and as pressure resistance varies with pressure and temperature level, for each process design, a "most resistant strain of *C. botulinum*" must defined. (III) "Demonstration of a 12-D process using inactivation kinetics for *C. botulinum* after development of a suitable kinetic model which demonstrates that inactivation of *C. botulinum* is linear over a range of values." The effect of combined pressure (600 to 1400 MPa) / temperature (70 to 120°C) on spores of TMW 2.357 and TMW 2.479 indicates, that over a broad range of values, inactivation is not linear. (IV) "Demonstration of a 12D process using a surrogate organism; identification of a surrogate organism with pressure resistance greater than that of *C. botulinum*." In chapter 3.2, such a target organism, *B. amyloliquefaciens* TMW 2.479, appeared to be suitable for non-isothermal treatment. However, in chapter 3.3 it was shown, that this bacilli are not generally more resistant to pressure over the whole range of values. Whenever this approach seems theoretically useful, it is not suitable for evaluation of high pressure processes and the postulates have also not been proven for thermal treatments.

### 4.8 Evaluation of established processes in respect of safety concepts

As already noted above, data of Esty and Meyer (1922) were used to calculate that a thermal process at 121°C for 2.45 min would eliminate a population of 1 x $10^{12}$ spores of *C. botulinum*, which was the origin of the 12-D concept. The inactivation of this amount of spores have never been demonstrated. Likewise, strains from *Clostridium baratii* and *Clostridium butyricum*, also being described as potential *botulinum*-toxin producers (Simpson, 2004), have never been considered. The heat resistance of the *C. botulinum* TMW 2.359 spores exceed by far the heat resistance of *C. botulinum* strains on which the 12-D concept was originally based. As consequence, the common 12-D concept of 2.45 min (12 x 0.204) would reduce TMW 2.359 spores by 2.0 log cycles. Even if doubling the required treatment time to 5 min, which is the commonly industrial used 5-D concept in respect of *C. sporogenes*, inactivation would be less than log cycle reduction of WSH spores of TMW 2.357 caused through pressure treatment at 110°C / 600 MPa after 4 min or at 110°C / 1400 MPa after 1 min. The use of the 12-D thermal process has a long history of safe use, as botulism from commercially canned foods has been virtually eliminated since the implementation of these regulations (Anonymous, 2003). Thus, the reduction of 5 log cycles of most resistant spores seems to be sufficient to consider canned foods as safe. Likewise, a process capable of achieving a minimum 5-D reduction of *E. coli* O157:H7 should eliminate the risk of disease from consumption of fruit juices (Anonymous, 2003). A reduction of six orders of magnitude in respect of pasteurized seafood is also believed to be sufficient (FDA,

2001). Both cases were calculated on the basis of a suitable contamination, regarding to juice with a concentration of *E. coli* < 10 cfu/mL. The concentration of spores in mashed carrots just before heat treatment was even determined with < 1 cfu/mL. Thus, pressure processing appears to be a suitable process to reduce contamination in the same dimension as conventional heat treatment. Still, the behaviour of the relevant organisms must be proven for any single product and process until enough data are available to possibly come to more general conclusions.

The pressure mediated tailing, which was not observed at moderate pressure in this study, is also numerously reported for heat treatments (Cerf, 1977). Hence, the basic assumption that thermal inactivation of microbial spores follows first-order kinetics has been challenged (Anonymous, 2003). If there are actually differences in respect of such a tailing of survival curves of bacterial spores after heat or pressure treatment is to be further studied. Thus, a closer look at the safety of novel food processing techniques may enable a fruitful revision of safety concepts for established (thermal) processes.

### 4.9 Effect of high pressure and heat on bacterial toxins

As a thermodynamic parameter, pressure is known for many years to act on biological materials in a differently way as temperature (Lullien-Pellerin and Balny, 2002). Many bacterial pathogens produce toxic exoproteins which serve as primary virulence factors. If these proteins act on intestinal cells they are usually named enterotoxins. These enterotoxins display a broad variety of structures, which influence their properties, e.g. low or high stability against heating steps, and differ also in their mode of action.

Generally the selection of enterotoxins for such studies is limited by the availability of toxins and suitable detection methods. For the purpose of this study it would have been preferable to test the biological activity for the other toxins, too, which was, however, not possible for experimental or ethical reasons, e.g. testing the activity of SEs´ would require monkey feeding studies.

The four bacterial enterotoxins chosen show different structures and also resemble different biological activities. SEs are single polypeptides of approximately 25 to 28 kDa (Dinges et al., 2000). Inactivation of this type of enterotoxins by high pressure would be of utmost interest because SEs are classical “preformed” toxins, which means that they are produced by bacteria in food and act in the intestine after a relatively short incubation time. The enterotoxins of *B. cereus*, *V. cholerae* and *E. coli* are mainly produced in the intestine after ingestion of food

contaminated with the respective bacteria, but if the bacteria have grown to high numbers in the food production of toxins can not be excluded. The main reason, however, for the choice of these toxins was that they resemble different secondary structures (see introduction), so one could expect differing effects of high pressure with or without heat treatment.

Summarizing the effects observed on immuno-reactivity in the respective assays, there was no effect of pressurization of up to 800 MPa at ambient (20°C) or lower (5°C) temperatures. Some reduction of immuno-reactivity could be demonstrated, when high pressure treatment was combined with a heating step. Particularly for STa (1.2 kDa), a significant reduced EIA result was obtained using 80 °C and a pressure of 800 MPa, while incubation at 121°C for 30 min or at 80°C for 124 min at ambient pressure showed no effect. In contrast, comparatively small effects of pressure and strong effects of heat treatment on SEC (25-28 kDa) could be observed. The different behavior of the two monomeric proteins indicate that heat and pressure resistance of bacterial toxins does not correlate. Likewise, no correlation was found for the resistance of vegetative cells (Benito et al, 1999; Garcia-Graells et al., 2002) and bacterial endospores to pressure and heat, respectively (Nakayama et al., 1996). Furthermore, SEC was stabilized at 80°C in the middle pressure range. Pressure / temperature diagrams of other proteins also show that there is an optimum pressure at which proteins are most resistant to heat treatment (Smeller, 2002).

In a similar way as the monomeric STa, the multimeric cholera toxin (86 kDa) became negative in the RPLA after a combined pressure (800MPa) and heat (80 °C) treatment for 90 min, whereas this was not observed without pressurization. The additive effect of the temperature rise due to adiabatic heating can be disregarded, as the starting temperature is reached again after a pressure holding time of 11 min (Table 1). As expected (see introduction), heat resistance at 121 °C of STa and CT differed strongly. The concentration of the detectable CT decreased under the detection limit after 30 min. The observed relative high resistance of CT in the RPLA to both, heat and / or pressure treatment, could be explained by the use of polyclonal antibodies.

It can be generally assumed that the loss of immuno-reactivity is mainly due to changes in tertiary structure und that the biological activity should also be decreased. Particularly, studies on heat inactivation of SEs widely used the assumption that loss of reaction with the specific antibodies indicates inactivation (Bergdoll, 1983). To verify this thesis, the results of an immuno assay of the pressure treated supernatant of toxigenic *B. cereus* was compared to its cytotoxicity. The results of both methods showed that pressurization in the range of 0.1 to 800

MPa at 5, 20 and 30°C have almost no effect even on the largest toxin of this study (119 kDa). There was, however, a slight increase of the immunoassay result for the L2 component of the HBL enterotoxin complex at 20 and 30 °C and a pressure of 800 MPa, whereas the respective cytotoxicity decreased. This demonstrates that the immuno-reactivity does not necessarily correlate to the biological activity of the protein. One explanation for this observation could be that increasing pressure leads to a dissociation of the HBL complex and therefore to a better accessibility of the L2 molecules for the antibodies, which might partly be hidden in the test mixture used for this study under normal conditions. Oligomeric proteins such as glyceraldehydes-3-phosphate dehydrogenase (GAPDH) from yeast, malate dehydrogenase, and lactate dehydrogenase (LDH) were also found to dissociate through pressure treatment (Groß and Jaenicke, 1994).

Overall these results indicate that pressure application may increase inactivation by heat treatment and combined treatments may be effective at lower temperatures and/or shorter incubation time. However, pressurization may not eliminate the toxins from food to the same extent as temperature treatment. Still, it must be emphasized that pressurization does not mask bacterial toxins, which admits their detection after pressure based food processing.

## 5 Summary

The inactivation of bacterial endospores by hydrostatic pressure requires the combined application of heat and pressure. After analyzing the flora of carrots and mashed carrots, strains concerning food spoilage were isolated from the carrot habitat to obtain practice relevant "wild strains". The resistance of spores of 14 food isolates and 5 laboratory strains of *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis* to treatments with pressure and temperature (200 to 800 MPa, and 60 to 80°C) in mashed carrots was determined. A large variation was observed in the pressure resistance of spores and their reduction by treatments with 800 MPa / 70°C for 4 min ranged from more than 6 log to no reduction. The sporulation conditions further influenced their pressure resistance. The loss of DPA from spores varying in their pressure resistance was determined and spore sublethal injury was assessed by determination of the detection times of individual spores. Treatment of spores with pressure and temperature resulted in DPA-free, phase bright spores. These spores were sensitive to moderate heat and exhibited strongly increased detection times as judged by the time required for single spores to grow to visible turbidity of the growth medium. The role of DPA in heat and pressure resistance was further substantiated by the use of the DPA-deficient mutant strain *B. subtilis* CIP 76.26. Taken together, these results indicate that inactivation of spores by combined pressure / temperature processing is achieved by a two stage mechanism that does not involve germination. At a pressure between 600 – 800 MPa, and a temperature greater 60 °C, DPA is released predominantly by a physico-chemical rather than a physiological process, and the DPA-free spores are inactivated by moderate heat independent of the pressure level.

Furthermore, the effect of pressurization on endospores of *Clostridium botulinum*, as the target organism concerning the safety of low acid, canned food, was investigated. The resistance of seven strains was compared to that of *Bacillus cereus*, *B. subtilis*, *B. licheniformis*, *B. smithii*, *B. amyloliquefaciens*, and *Thermoanaerobacterium thermosaccharolyticum* with respect to treatments with pressure and temperature in the range of 600 to 800 MPa and 80 to 116°C in mashed carrots. A large variation was observed in the pressure resistance of *C. botulinum* spores. Using treatments with 600 MPa, 80°C for 1 s their reduction ranged from more than 5.5 log cycles to no reduction. Spores of the proteolytic *C. botulinum* TMW 2.357 exhibited a greater resistance to pressure than spores from all other bacteria examined with the exception of *B. amyloliquefaciens*. The heat resistance of spores did not correlate with the pressure resistance, neither within strains of *C. botulinum* nor when

*C. botulinum* spores were compared to spores of *T. thermosaccharolyticum*. A quantitative release of DPA was observed from *C. botulinum* spores upon combined pressure / temperature treatments only after inactivation of > 99.999% of the spores. Thus, it was confirmed, that DPA is released by a physico-chemical rather than a physiological process. The resistance of spores to combined pressure / temperature treatments correlated with their ability to retain DPA

The behavior of spores of *B. amyloliquefaciens* TMW 2.479 and WSH-spores of *C. botulinum* TMW 2.357, as most resistant spores concerning spoilage and food safety, to combined pressure (600 to 1400 MPa) / temperature (70 to 120°C) treatment with isothermal holding times was further investigated. At 100, 110 and 120 °C incubation at moderate pressure could result in a faster spore reduction than if simultaneous pressurizing. Both strains showed a pronounced pressure dependent tailing, which was absent at moderate pressure. Repetitive pressure treatments confirmed, that the spore population was heterogeneous with regard to its sensitivity to pressure, as treatment of twofold 2 min by far not resulted in a twofold reduction of spores. Inactivation of the clostridia differed strongly to that of the bacilli, as latter showed between 800 and 1200 MPa almost no varying effect in respect of a faster spore reduction. As consequence, the order of the resistance of spores to combined pressure / temperature treatment is not fixed. This different behavior makes it impossible to suggest a generally valid target organism for the pressure processing of low acid foods. A closer look at the safety of novel food processing techniques enabled the evaluation of safety concepts, also for established (thermal) processes.

Even though the inactivation of microorganisms by high pressure treatment is the subject of intense investigations, the effect of high pressure on bacterial toxins has not been studied so far. In this study, the influence of combined pressure / temperature treatment (0.1 to 800 MPa and 5 to 121°C) on bacterial enterotoxins from *Staphylococcus aureus*, *Bacillus cereus*, *Vibrio cholerae* and *Escherichia coli* (STa) was determined.

Structural alterations were monitored in enzyme immunoassays (EIA's). Cytotoxicity of the pressure treated supernatant of toxigenic *B. cereus* DSM 4384 was investigated in order to compare its toxicity with the results obtained in the immunoassay. Reduction of the immunochemical reactivity could be demonstrated, when high pressure was combined with heat. At lower temperatures, there was almost no effect of pressurization of up to 800 MPa in the respective assays. The biological activity of proteins does not necessarily correlate with their immuno reactivity. Likewise, heat and pressure resistance of bacterial toxins did not

correlate. The results indicate that pressurization can increase inactivation observed by heat treatment, and combined treatments may be effective at lower temperatures and/or shorter incubation time. However, pressurization may not eliminate the toxins from food to the same extent as temperature treatment. Still, it must be emphasized that pressurization does not mask bacterial toxins, which admits their detection after pressure based food processing.

# 6 Zusammenfassung

In dieser Arbeit wurde die mikrobielle Sicherheit schwach saurer, hochdruckbehandelter Lebensmittel bewertet. Relevante Leitorganismen für Verderb in Karottenbrei konnten anhand einer Florenanalyse des Habitates “Karotte” bestimmt werden. Nach der Isolierung natürlich auftretender Endosporenbildner wurden 14 Lebensmittelisolate und 5 Laborstämme von *Bacillus subtilis*, *Bacillus amyloliquefaciens* und *Bacillus licheniformis* ausgewählt, um die Reduktion von keimfähigen Endosporen in Karottenbrei in Abhängigkeit der Zeit unter verschiedenen Druck- (200 bis 800 MPa) und Temperaturbedingungen (60 bis 80°C) zu bestimmen. Die Abtötung bakterieller Endosporen durch hydrostatischen Druck ist nur in Kombination mit Hitze möglich. Dabei wurde eine große Variabilität der Resistenz gegenüber Hochdruck festgestellt. So reichte ihre Abtötung bei 800 MPa / 70°C nach vier Minuten von über 6 logarithmischen Einheiten bis zu keiner Reduktion. Zusätzlich wurde die Hochdruckresistenz durch die Sporulationsbedingungen stark beeinflußt. Die Bestimmung der Lag-Phasen einzelner Sporen zur Ermittlung der Populationsheterogenität und der subletalen Schädigungen zeigte eine Verschiebung zu erheblich längeren Detektionszeiten. Daneben wurde auch die Freisetzung von Dipicolinsäure (DPA) unterschiedlich druckresistenter Stämme bestimmt. Eine kombinierte Druck-/ Temperaturbehandlung führte zu DPA freien, aber immer noch lichtbrechenden Sporen, was jedoch mit dem Verlust ihrer Hitzeresistenz einher geht. Die Rolle der DPA wurde darüber hinaus durch Druck- und Temperaturinaktivierungskinetiken einer *B. subtilis* Mutante bestätigt, deren DPA Gehalt der Sporen durch Zusatz zum Sporulationsmedium gesteuert werden kann. Zusammenfassend konnte gezeigt werden, dass die Inaktivierung bakterieller Endosporen mit einer kombinierten Druck-/ Temperaturanwendung durch einen zweistufigen Prozess gekennzeichnet ist, welcher unabhängig von einer Keimung ist. Bei einem Druck zwischen 600 und 800 MPa und einer Temperatur von über 60 °C wird die DPA vor allem durch einen physikalisch-chemischen und nicht durch einen physiologischen Prozeß freigesetzt. Eine weitere Druckanwendung zeigt bei solchen Sporen keinen zusätzlichen Abtötungseffekt.

Darüber hinaus wurden Inaktivierungskinetiken der Sporen von *Clostridium botulinum* bestimmt, der als Leitkeim zum Schutz vor Lebensmittelvergiftungen bei schwach sauren Konserven gilt. Auf diese Weise konnte die Resistenz der Sporen von 7 *C. botulinum* Stämmen mit derer von *Bacillus cereus*, *B. subtilis*, *B. licheniformis*, *Bacillus smithii*, *B. amyloliquefaciens* und *Thermoanaerobacterium thermosaccharolyticum* in Karottenbrei im Druck- und Temperaturbereich zwischen 600 bis 800 MPa und 80 bis 116°C verglichen

werden. Auch hier zeigten sich große Schwankung innerhalb der Spezies *C. botulinum* bezüglich der Druckresistenz. Nach einer Sekunde bei 600 MPa und 80°C wurden Unterschiede von keiner Abtötung bis zu einer Inaktivierung von über 5.5 logarithmischen Einheiten festgestellt. Dabei wiesen die Sporen des proteolytischen Stammes *C. botulinum* TMW 2.357 eine größere Hochdruckresistenz auf, als die aller anderen in dieser Arbeit untersuchten Stämme. Einzigste Ausnahme waren Sporen von *B. amyloliquefaciens*. Es konnte keine Korrelation zwischen Hitze- und Druckresistenz festgestellt werden, weder innerhalb der *C. botulinum* Stämme, noch wenn diese Sporen mit Sporen von *T. thermosaccharolyticum* verglichen wurden. Bei *C. botulinum* ließ sich der vollständige Verlust von DPA nach kombinierter Druck-/ Temperaturanwendung erst feststellen, nachdem über 99.999% der Sporen inaktiviert waren. Dies bestätigte die Annahme, dass die DPA eher durch einen physikalisch-chemischen und weniger durch einen physiologischen Prozeß freigesetzt wird. Dabei korrelierte die Resistenz der Sporen gegenüber einer kombinierten Druck-/ Temperaturbehandlung mit ihrer Fähigkeit DPA zurückzuhalten.

Zusätzlich wurden mit den druckresistentesten Endosporen im Hinblick auf Verderb (*B. amyloliquefaciens* TMW 2.479) und Vergiftung (*C. botulinum* TMW 2.357) Experimente im Druck- und Temperaturbereich 600 bis 1400 MPa und 70 bis 120°C bei isothermen Druckhaltezeiten durchgeführt. Bei beiden Stämmen gab es bestimmte Druck- und Temperaturkombinationen bei denen bei Normaldruck eine schnellere Inaktivierung zu beobachten war als bei gleichzeitiger Druckanwendung. Zusätzlich wurde ein ausgeprägter, druckabhängiger Sockel nachgewiesen. Im Unterschied dazu war ein solcher Sockel bei Normaldruck nicht vorhanden. Im Verhältnis zu einem zweiminütigen Druckprozeß ergab eine Behandlung von 2 mal 2 Minuten keine zweifache Inaktivierung. Bezüglich des Sockels spricht dies für eine starke Populationsheterogenität. Im Bereich 800 bis 1200 MPa und 100 bis 120°C wurde die Abtötung der *B. amyloliquefaciens* Sporen im Unterschied zu *C. botulinum* Sporen durch Druck kaum beeinflußt. Folglich ist die Reihenfolge beider Stämme bezüglich ihrer Hochdruckresistenz nicht immer gleich, weshalb es nicht möglich ist, einen generell druckresistentesten Stamm zu definieren. Eine genauere Sicherheitsbetrachtung neuer Lebensmittelverfahrenstechniken ermöglichte auch die Beurteilung bestehender Sicherheitskonzepte für gängige (thermische) Prozesse.

Obwohl die Abtötung von Mikroorganismen durch Druck bisher intensiv erforscht wurde, gibt es keine Daten hinsichtlich des Verhaltens bakterieller Toxine unter Hochdruck. Aus diesem Grund wurden Enterotoxine von *Staphylococcus aureus*, *Bacillus cereus*, *Vibrio*

*cholerae* und *Escherichia coli* im Druck- und Temperaturbereich von 0.1 bis 800 MPa und 5 bis 121°C untersucht.

Während Enzymimmunoassays zur Untersuchung struktureller Veränderungen dienten, wurde die Bestimmung der Zytotoxizität des druckbehandelten Kulturüberstandes von *B. cereus* DSM 4384 dazu verwendet, um dessen Toxizität mit den Ergebnissen aus dem Immunoassay zu vergleichen. Durch die Kombination von Hochdruck und Hitze wurde die immunochemische Reaktivität reduziert. Bei niedrigeren Temperaturen hatten Drücke bis 800 MPa dagegen keine Einfluß auf die jeweiligen Assays. Die biologische Aktivität von Proteinen korreliert nicht notwendigerweise mit ihrer immunologischen Reaktivität. Die Hitze- und Druckresistenz bakterieller Toxine korrelierte ebenfalls nicht. Diese Untersuchungen zeigten, dass die thermische Inaktivierung dieser Proteine durch Hochdruck verstärkt werden kann und eine kombinierte Druck-/ Hitzebehandlung bei niedrigeren Temperaturen und/oder kürzeren Behandlungszeiten wirkungsvoll ist. Es muss hervorgehoben werde, dass keine druckinduzierte Maskierung bakterieller Enterotoxine beobachtet wurde. Dadurch ist ihr Nachweis auch nach einer auf Hochdruck basierenden Lebensmittelverarbeitung weiterhin möglich.

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# 8 Appendix

## Re-classification of strain TMW 2.480

Strain TMW 2.480 was re-classified as *Bacillus sp.* based on the sequence (1506 bp) of the 16S rRNA. The alignment shows an accordance to *Bacillus vallismortis* DSM 11031$^{T}$, AB021198 of 99.73% (4 mismatches) and to *Bacillus subtilis* DSM 10$^{T}$, AJ276351 of 99.67% (5 mismatches). The sequence was submitted to the EMBL Nucleotide Sequence Database. Data have been assigned the accession number AJ809499.

```
DSM11031T GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTC 60
DSM10T    GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTC 60
TMW2.480  GACGAACGCTGGCGGSGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTC 60
          ********** ********************************************

DSM11031T CCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGAT 120
DSM10T    CCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGAT 120
TMW2.480  CCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGAT 120
          ************************************************************

DSM11031T AACTCCGGGAAACCGGGGCTAATACCGGATG**C**TTGTTTGAACCGCATGGTTCA**A**ACATAA 180
DSM10T    AACTCCGGGAAACCGGGGCTAATACCGGATG**G**TTGTTTGAACCGCATGGTTCA**A**ACATAA 180
TMW2.480  AACTCCGGGAAACCGGGGCTAATACCGGATG**G**TTGTTTGAACCGCATGGTTCA**G**ACATAA 180
          ****************************** ******************** ******

DSM11031T AAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGT 240
DSM10T    AAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGT 240
TMW2.480  AAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGT 240
          ************************************************************

DSM11031T AA**T**GGCTCACCAAGGC**A**ACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGA 300
DSM10T    AA**C**GGCTCACCAAGGC**A**ACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGA 300
TMW2.480  AA**C**GGCTCACCAAGGC**G**ACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGA 300
          ** ************* ****************************************

DSM11031T CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGA 360
DSM10T    CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGA 360
TMW2.480  CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGA 360
          ************************************************************

DSM11031T AAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTG 420
DSM10T    AAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTG 420
TMW2.480  AAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTG 420
          ************************************************************

DSM11031T TTAGGGAAGAACAAGT**G**CCGTTC**A**AATAGGGCGG**C**ACCTTGACGGTACCTAACCAGAAAG 480
DSM10T    TTAGGGAAGAACAAGT**A**CCGTTC**G**AATAGGGCGG**T**ACCTTGACGGTACCTAACCAGAAAG 480
TMW2.480  TTAGGGAAGAACAAGT**G**CCGTTC**A**AATAGGGCGG**C**ACCTTGACGGTACCTAACCAGAAAG 480
          ********** ****** ********** ************************

DSM11031T CCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAA 540
DSM10T    CCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAA 540
TMW2.480  CCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAA 540
          ************************************************************

DSM11031T TTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTC 600
DSM10T    TTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTC 600
TMW2.480  TTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTC 600
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DSM11031T AACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCC 660
DSM10T    AACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCC 660
TMW2.480  AACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCC 660
          ************************************************************

DSM11031T ACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCT 720
DSM10T    ACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCT 720
TMW2.480  ACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCT 720
          ************************************************************


DSM11031T GGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGG 780
DSM10T    GGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGG 780
TMW2.480  GGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGG 780
          ************************************************************

DSM11031T TAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGC 840
DSM10T    TAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGC 840
TMW2.480  TAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGC 840
          ************************************************************

DSM11031T AGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAA 900
DSM10T    AGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAA 900
TMW2.480  AGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAA 900
          ************************************************************

DSM11031T TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAAC 960
DSM10T    TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAAC 960
TMW2.480  TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAAC 960
          ************************************************************

DSM11031T CTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAG 1020
DSM10T    CTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAG 1020
TMW2.480  CTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAG 1020
          ************************************************************

DSM11031T AGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG 1080
DSM10T    AGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG 1080
TMW2.480  AGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG 1080
          ************************************************************

DSM11031T CAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTG 1140
DSM10T    CAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTG 1140
TMW2.480  CAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTG 1140
          ************************************************************

DSM11031T CCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTG 1200
DSM10T    CCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTG 1200
TMW2.480  CCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTG 1200
          ************************************************************

DSM11031T GGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAA 1260
DSM10T    GGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAA 1260
TMW2.480  GGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAA 1260
          ************************************************************

DSM11031T TCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAA 1320
DSM10T    TCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAA 1320
TMW2.480  TCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAA 1320
          ************************************************************

DSM11031T TCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC 1380
DSM10T    TCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC 1380
TMW2.480  TCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC 1380
          ************************************************************

DSM11031T GCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCC 1440
DSM10T    GCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCC 1440
TMW2.480  GCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTWWGGAGCC 1440
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DSM11031T AGCCGCCGAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAA 1500
DSM10T    AGCCGCCGAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAA 1500
TMW2.480  AGCCGCCGAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAA 1500
          ************************************************************

DSM11031T GGTGCG 1506
DSM10T    GGTGCG 1506
TMW2.480  GGTGCG 1506
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**Classification of strain TMW 2.383**

Strain TMW 2.383 was classified as *Bacillus cereus* based on the sequence (1508 bp) of the 16S rRNA and according to Sacchi et al. (2002). The alignment shows one mismatch to *Bacillus thuringiensis* (AY138289), no mismatch to *Bacillus cereus* (AY138277) and also no mismatch to *Bacillus anthracis* (AY138383). As strain TMW 2.383 is haemolytic (data not shown), classification as *B. anthracis* could be excluded, because latter is a non haemolytic bacilli (Reber, 2001). The sequence was submitted to the EMBL Nucleotide Sequence Database. Data have been assigned the accession number AJ809498.

```
TMW2.383  GATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCT 60
AY138289  GATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCT 60
AY138277  GATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCT 60
AY138383  GATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCT 60
          ************************************************************

TMW2.383  CTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGG 120
AY138289  CTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGG 120
AY138277  CTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGG 120
AY138383  CTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGG 120
          ************************************************************

TMW2.383  ATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACYGCATGGTTCGAAATT 180
AY138289  ATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACYGCATGGTTCGAAATT 180
AY138277  ATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATT 180
AY138383  ATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATT 180
          ****************************************** ***************

TMW2.383  GAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAG 240
AY138289  GAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAG 240
AY138277  GAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAG 240
AY138383  GAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAG 240
          ************************************************************

TMW2.383  GTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGG 300
AY138289  GTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGG 300
AY138277  GTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGG 300
AY138383  GTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGG 300
          ************************************************************

TMW2.383  GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGAC 360
AY138289  GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGAC 360
AY138277  GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGAC 360
AY138383  GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGAC 360
          ************************************************************
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TMW2.383  GAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGT 420
AY138289  GAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGT 420
AY138277  GAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGT 420
AY138383  GAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGT 420
          ************************************************************

TMW2.383  TGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAA 480
AY138289  TGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAA 480
AY138277  TGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAA 480
AY138383  TGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAA 480
          ************************************************************

TMW2.383  AGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGG 540
AY138289  AGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGG 540
AY138277  AGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGG 540
AY138383  AGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGG 540
          ************************************************************

TMW2.383  AATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGC 600
AY138289  AATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGC 600
AY138277  AATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGC 600
AY138383  AATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGC 600
          ************************************************************

TMW2.383  TCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATT 660
AY138289  TCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATT 660
AY138277  TCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATT 660
AY138383  TCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATT 660
          ************************************************************

TMW2.383  CCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTT 720
AY138289  CCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTT 720
AY138277  CCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTT 720
AY138383  CCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTT 720
          ************************************************************

TMW2.383  CTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT 780
AY138289  CTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT 780
AY138277  CTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT 780
AY138383  CTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT 780
          ************************************************************

TMW2.383  GGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCT 840
AY138289  GGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCT 840
AY138277  GGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCT 840
AY138383  GGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCT 840
          ************************************************************

TMW2.383  GAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGG 900
AY138289  GAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGG 900
AY138277  GAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGG 900
AY138383  GAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGG 900
          ************************************************************

TMW2.383  AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGA 960
AY138289  AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGA 960
AY138277  AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGA 960
AY138383  AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGA 960
          ************************************************************
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TMW2.383  ACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCTCCTTCGGGAGC 1020
AY138289  ACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGC 1020
AY138277  ACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCTCCTTCGGGAGC 1020
AY138383  ACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCTCCTTCGGGAGC 1020
          ************************** ******************************

TMW2.383  AGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC 1080
AY138289  AGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC 1080
AY138277  AGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC 1080
AY138383  AGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC 1080
          ************************************************************

TMW2.383  CGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGAC 1140
AY138289  CGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGAC 1140
AY138277  CGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGAC 1140
AY138383  CGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTWAGTTGGGCACTCTAAGGTGAC 1140
          ************************************ ********************

TMW2.383  TGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACC 1200
AY138289  TGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACC 1200
AY138277  TGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACC 1200
AY138383  TGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACC 1200
          ************************************************************

TMW2.383  TGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCT 1260
AY138289  TGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCT 1260
AY138277  TGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCT 1260
AY138383  TGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCT 1260
          ************************************************************

TMW2.383  AATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGG 1320
AY138289  AATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGG 1320
AY138277  AATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGG 1320
AY138383  AATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGG 1320
          ************************************************************

TMW2.383  AATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACA 1380
AY138289  AATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACA 1380
AY138277  AATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACA 1380
AY138383  AATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACA 1380
          ************************************************************

TMW2.383  CCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGGGGTAACCTTTTTGGAG 1440
AY138289  CCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGGGGTAACCTTTTTGGAG 1440
AY138277  CCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGGGGTAACCTTTTTGGAG 1440
AY138383  CCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGGGGTAACCTTTTTGGAG 1440
          ************************************************************

TMW2.383  CCAGCCGCCTAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGG 1500
AY138289  CCAGCCGCCTAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGG 1500
AY138277  CCAGCCGCCTAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGG 1500
AY138383  CCAGCCGCCTAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGG 1500
          ************************************************************

TMW2.383  AAGGTGCG 1508
AY138289  AAGGTGCG 1508
AY138277  AAGGTGCG 1508
AY138383  AAGGTGCG 1508
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**Classification of strain TMW 2.552**

Strain TMW 2.552 was classified as *Bacillus gelatini* based on the sequence (1528 bp) of the 16S rRNA. The alignment shows an accordance to *B. gelatini* DSM 15865$^{T}$, AJ551329 of 100%, and an accordance of 95% to the second relative *Bacillus flexus* IFO 15715, AB021185. The sequence was submitted to the EMBL Nucleotide Sequence Database. Data have been assigned the accession number AJ809500.

```
TMW2.552  GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACGGAAGGAGAGCTTGCT 60
DSM15865T GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACGGAAGGAGAGCTTGCT 60
IFO15715  GATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACTGATTAGAAGCTTGCT 60
          ** ************************************ ** **     ******** 

TMW2.552  CTCTGGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTCATAGATGGGG 120
DSM15865T CTCTGGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTCATAGATGGGG 120
IFO15715  TCTATGACGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGG 120
                ** ****************************************   ***  *** 

TMW2.552  ATAACACCGAGAAATCGGTGCTAATACCGAATAATAGAGCGGAGCGCATGCTCCGCGCTT 180
DSM15865T ATAACACCGAGAAATCGGTGCTAATACCGAATAATAGAGCGGAGCGCATGCTCCGCGCTT 180
IFO15715  ATAACTCCGGGAAACCGGAGCTAATACCGGATAACATTTTCTCTTGCATAAGAGAAAATT 180
          ***** *** **** *** ********** **** *         ****         ** 

TMW2.552  GAAAGTCGGCTTTSAGCTGACACTATGAGATGGGCCCGCGGCGCATTAGCTAGTAGGTGA 240
DSM15865T GAAAGTCGGCTTTGAGCTGACACTATGAGATGGGCCCGCGGCGCATTAGCTAGTAGGTGA 240
IFO15715  GAAAGATGG-TTTCGGCTATCACTTACAGATGGGCCCGCGGTGCATTAGCTAGTTGGTGA 239
          *****  ** ***  ***  ****   ************** ************ ***** 

TMW2.552  GGTAACGGCTCACCTAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTG 300
DSM15865T GGTAACGGCTCACCTAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTG 300
IFO15715  GGTAACGGCTCACCAAGGCAACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTG 299
          ************** **** ******* ********************************** 

TMW2.552  GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGA 360
DSM15865T GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGA 360
IFO15715  GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGA 359
          ************************************************** ******** 

TMW2.552  CGAAAGTCTGACCGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTG 420
DSM15865T CGAAAGTCTGACCGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTG 420
IFO15715  CGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTG 419
          ************ ************************** ****** ************ 

TMW2.552  TTGTCAGGGAAGAACAAGTACGAGAGTAACTGCTCGTACCTTGACGGTACCTGACCAGAA 480
DSM15865T TTGTCAGGGAAGAACAAGTACGAGAGTAACTGCTCGTACCTTGACGGTACCTGACCAGAA 480
IFO15715  TTGTTAGGGAAGAACAAGTACAAGAGTAACTGCTTGTACCTTGACGGTACCTAACCAGAA 479
          **** ***************** ************ ****************** ******* 

TMW2.552  AGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGG 540
DSM15865T AGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGG 540
IFO15715  AGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGG 539
          ********************************************************* ***** 

TMW2.552  AATTATTGGGCGTAAAGCGCGCGCAGGCGGTTCTTTAAGTCTGATGTGAAAGCCCACGGC 600
DSM15865T AATTATTGGGCGTAAAGCGCGCGCAGGCGGTTCTTTAAGTCTGATGTGAAAGCCCACGGC 600
IFO15715  AATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGC 599
          ******************************  ************************** 

TMW2.552  TCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGGAGAGAAAAGTGGAATT 660
DSM15865T TCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGGAGAGAAAAGTGGAATT 660
IFO15715  TCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGAAAAGCGGAATT 659
          ****************************************** ********* ****** 
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TMW2.552  CCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTT 720
DSM15865T CCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTT 720
IFO15715  CCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTT 719
          ************************************************************


TMW2.552  TTGGCCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT 780
DSM15865T TTGGCCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT 780
IFO15715  TTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT 779
          **** ********************************************************


TMW2.552  GGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGGGGGTTCCA--CCCTCAGTGCT 838
DSM15865T GGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGGGGGTTCCA--CCCTCAGTGCT 838
IFO15715  GGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCT 839
          ****************************** ***** * ***** *   ** * ******


TMW2.552  GACGTTAACACATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGG 898
DSM15865T GACGTTAACACATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGG 898
IFO15715  GCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGG 899
          *  * **** ***************************  ******  **************


TMW2.552  AATTGACGGGGGCCCGCACAAGCAGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGA 958
DSM15865T AATTGACGGGGGCCCGCACAAGCAGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGA 958
IFO15715  AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGA 959
          ********************** ************************************


TMW2.552  ACCTTACCAGGTCTTGACATCCTCTGACCACTTGAGAGATCAAGCTTTCCCCTTCGGGGG 1018
DSM15865T ACCTTACCAGGTCTTGACATCCTCTGACCACTTGAGAGATCAAGCTTTCCCCTTCGGGGG 1018
IFO15715  ACCTTACCAGGTCTTGACATCCTCTGACAACTCTAGAGATAGAGCGTTCCCCTTCGGGGG 1019
          **************************** ***  ******  *** **************


TMW2.552  ACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT 1078
DSM15865T ACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT 1078
IFO15715  ACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT 1079
          ************************************************************


TMW2.552  CCCGCAACGAGCGCAACCCTTGACCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTG 1138
DSM15865T CCCGCAACGAGCGCAACCCTTGACCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTG 1138
IFO15715  CCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGTG 1139
          ********************** ********** ******************


TMW2.552  ACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGA 1198
DSM15865T ACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGA 1198
IFO15715  ACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGA 1199
          ************************************************************


TMW2.552  CCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCTGCGAAACCGCAAGGTGGAG 1258
DSM15865T CCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCTGCGAAACCGCAAGGTGGAG 1258
IFO15715  CCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCTGCAAGACCGCGAGGTCAAG 1259
          ****************************************** * ***** ****  **


TMW2.552  CCAATCCCAAAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCC 1318
DSM15865T CCAATCCCAAAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCC 1318
IFO15715  CCAATCCCATAAAACCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCT 1319
          ********* *** ******************************************


TMW2.552  GGAATTGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACA 1378
DSM15865T GGAATTGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACA 1378
IFO15715  GGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACA 1379
          ***** ************************************************************


TMW2.552  CACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCKYW-WGG 1437
DSM15865T CACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTT-TGG 1437
IFO15715  CACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGGGGTAACCTTTATGG 1439
          ******************************************* *******     **


TMW2.552  AGCCAGCCGCCGAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATC 1497
DSM15865T AGCCAGCCGCCGAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAA------------ 1485
IFO15715  AGCCAGCCGCCTAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATC 1499
          *********** ************************************
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TMW2.552  GGAAGGTGCGGCTGGATCACCTCCTTTCTGG 1528
DSM15865T ------------------------------
IFO15715  GGAAGGTGCGGCTGGAT-------------- 1516
```

**Classification of strains**

Strains were classified based on a partial sequence of the 16S rRNA. Inside the parenthesis the nearest relative strain, its accession number and its proportional probability is indicated. Furthermore, the number of mismatches (x) and sequenced bases (y) are displayed as (x/y).

**TMW 2.531 *Geobacillus sp.*** (96% *G. caldoxylosilyticus* DSM 12041, AY608951.1 (29/757)).

```
GACGAACGCTGGSGGCRTGCCTAATACATGCAAGTCGAGCGGACCGAATAGAAGCTTGCTTCTGTTTGGTTAGCG
GCGGACGGGTGAGTAACACGTGGGCAACCTGCCCGTAAGACGGGGATAACTWCGGGAAACCGGAGCTAATACCCG
ATAACCCTSAAGACCGCATGGTCTTTAGTTGAAAGGCGGCTTCGGCTGTCACTTACGGATGGGCCCGCGGCGCAT
TAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTRGCCGACCTGAGAGGGTGATYGGCCACACTGG
GACTGASACWCGGMCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAG
CAACGCCGCGTGAGCGAAGAAGGTCTTCGGATTGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGYGGTTCGAATA
GGGCCGTACCTTGACGGTACCTAACGAGAAAGCCACGGATAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTG
GCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTCCTTAAGTCTGATGTGAAAGCCCACGGC
TCAACCGTGGAGGGTCATTGGAAACTGGGGGACTTGAGTGCAGAAGAGGAGAGCGGAATTSCACGTGTAGCGGTG
AAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGGCTGTAACTGACGCTGAGGCGCGAAA
GCGTGGG (H18)
```

**TMW 2.532 *Bacillus sp.*** (98% *B. licheniformis* DSM 13, X68416.1 (12/766)).

```
GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGC
GGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGAT
GCTTGATTGAACCGCATGGTTCAATTATTAAAGGTGGCTTTCAGCTACCACTTACAGATGGACCCGCGGCGCATT
AGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACCATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGG
ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTTCGCAATGGACGAAAGTCTGACGGAGC
AACGCCGCGTGAGTGATGAAGGTTTTCGGATCGGAAAACTCTGTTGTTAGGGAAGAACAAGTGCCGGTCCAATAG
GGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGG
CAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCT
CAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGA
AATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGGCTGTAACTGACGCTGAGGCGCGAAAG
CGTGGGGAGCGAACAG (H29)
```

**TMW 2.533 *Bacillus subtilis*** (100% *B. subtilis* DSM $10^T$, AB042061.1 (0/756)).

```
GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGC
GGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGAT
GGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTA
GCTAGTTGGTGAGGTAAYGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGA
CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCA
ACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGG
GCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGC
AAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTC
AACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAA
ATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGC
GTGGGG (H41)
```

**TMW 2.534 *Bacillus licheniformis*** (99% *B. licheniformis* DSM 13, X68416.1 (3/766)).

GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGC
GGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGAT
GCTTGATTGAACCGCATGGTTCAATCATAAAAGGTGGCTTTTAGCTACCACTTACAGATGGACCCGCGGCGCATT
AGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGG
ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGARC
AACGCCGCGTGAGTGATGAAGGTTTTCGGATCGKAAAACTCTGTTGTTAGGGAAGAACAAGTGCCGTTCGAATAG
GGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG
CAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCT
CAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGA
AATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGGCTGTAACTGACGCTGAGGCGCGAAAG
CGTGGGGAGCRAACAG (A12)

**TMW 2.535 *Bacillus simplex*** (99% *B. simplex* LMG 11160$^{T}$, AJ628743.1 (8/756)).

GACGAACGCTGGSGGCGTGCCTAATACATGCAAGTCGAGCGAATCGAYGGGAGCTTGCTCCCTGAGATTAGCGGC
GGACGGGTGAGTAACACGTGGGCAACCTGCCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGAT
ACGTTCTTTTCTCSCATGAGAGAAGATGGAAAGACGGATTACGCTGTCACTTATAGATGGGCCCGTGGCGCATTA
GCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATGCGTRGCCGACCTGAGAGGGTGATCGGCCACACTGGGA
CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCA
ACGCCGCGTGAACGAAGAAGGCCTTCGGGTCGGAAAGTTCTGTTGTTAGGGAAGAACAAGTGCCAGAGYAACTGC
TGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCA
AGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTCCCTTAAGTCTGATGTGAAAGCCCACGGCTCA
ACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGACGAGGAAAGTGGAATTSCAAGTGTAGCGGTGAAA
TGCGTAGAGATTTGGAGGAACACCAGTGGCGACGGCGACTTTCTGGGCTGTAACTGACACTGAGGCGCGAAAGCG
TGGGGAG (A20)

**TMW 2.536 *Geobacillus thermodenitrificans*** (100% *G. thermodenitrificans* DSM 465$^{T}$, AY608960.1

(0/767)).

GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACCGAACGAGAGCTTGCTCTTGTTTGGTCAGCG
GCGGACGGGTGAGTAACACGTGGGCAACCTGCCCGCAAGACCGGGATAACTCCGGGAAACCGGAGCTAATACCGG
ATAACACCAAAGACCGCATGGTCTTTGGTTGAAAGGCGGCTTCGGCTGCCACTTGCGGATGGGCCCGCGGCGCAT
TAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAG
CGACGCCGCGTGAGCGAAGAAGGCCTTCGGGTCGTAAAGCTCTGTTGTGAGGGACGAAGGAGCGCCGTTTGAATA
AGGCGGCGCGGTGACGGTACCTCACGAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGG
GCGAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGTGAAAGCCCACGGC
TCAACCGTGGAGGGTCATTGGAAACTGGGGGACTTGAGTGCAGGAGAGGAGAGCGGAATTCCACGTGTAGCGGTG
AAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGCCTGTAACTGACGCTGAGGCGCGAAA
GCGTGGGGAGCAAACAG (B5)

**TMW 2.487 *Bacillus smithii*** (100% *B. smithii* DSM 4216, Z26935.1 (2/761)).

GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACTTTCAAGAAGCTTGCTTTTGAAAGTTAGCGG
CGGACGGGTGAGTAACACGTGGGCAACCTGCCTGCAAGACGGGGATAACTCCGGGAAACCGGGGCTAATACCGGA
TAAYATCTTTCTTCGCATGAAGGAAGGTTGAAAGGCGGCGCAAGCTGCCGCTTGCAGATGGGCCCGCGGCGCATT
AGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGG
ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGC
AACGCCGCGTGAGCGAAGAAGGTCTTCGGATCGTAAAGCTCTGTTGTCAGGGAAGAACAAGTACCGTTCGAACAG
GGCGGTACCTTGACGGTACCTGACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG
CAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCTCTTAAGTCTGATGTGAAAGCCCACGGCT
CAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGCGGAATTCCACGTGTAGCGGTGA
AAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAA
GCGTGGGGAG (N1)

**TMW 2.537 *Geobacillus stearothermophilus*** (100% *G. stearothermophilus* DSM 2027, AY608933.1

(0/757)).

CACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACCGRAYKRGGGCTTGCYYTKRTTYGGTCAGCG
GCGGACGGGTGAGTAACACGTGGGCAACCTGCCCGCAAGACCGGGATAACTCCGGGAAACCGGAGCTAATACCGG
ATAACACCGAAGACCGCATGGTCTTCGGTTGAAAGGCGGCCTTTGGGCTGTCACTTGCGGATGGGCCCGCGGCGC
ATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGGCCTGAGAGGGTGACCGGCCACACT

GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGG
AGCGACGCCGCGTGAGCGAAGAAGGCCTTCGGGTCGTAAAGCTCTGTTGTGAGGGACGAAGGAGCGCCGTTCGAA
GAGGGCGGCGCGGTGACGGTACCTCACGAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
GGGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCTCTTAAGTCTGATGTGAAAGCCCACG
GCTCAACCGTGGAGGGTCATTGGAAACTGGGGGACTTGAGGGCAGGAGAGGAGAGCGGAATTCCACGTGTAGCGG
TGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGCCTGCACCTGACGCTGAGGCGCGA
AAGCGTGG (H11)

**TMW 2.538 *Geobacillus stearothermophilus*** (100% *G. stearothermophilus* DSM 2027, AY608933.1 (0/758)).

GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACCGGATTGGGGCTTGCCTTGATTCGGTCAGCG
GCGGACGGGTGAGTAACACGTGGGCAACCTGCCCGCAAGACCGGGATAACTCCGGGAAACCGGAGCTAATACCGG
ATAACACCGAAGACCGCATGGTCTTCGGTTGAAAGGCGGCCTTTGGGCTGTCACTTGCGGATGGGCCCGCGGCGC
ATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGGCCTGAGAGGGTGACCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGG
AGCGACGCCGCGTGAGCGAAGAAGGCCTTCGGGTCGTAAAGCTCTGTTGTGAGGGACGAAGGAGCGCCGTTCGAA
GAGGGCGGCGCGGTGACGGTACCTCACGAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
GGGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCTCTTAAGTCTGATGTGAAAGCCCACG
GCTCAACCGTGGAGGGTCATTGGAAACTGGGGGACTTGAGGGCAGGAGAGGAGAGCGGAATTCCACGTGTAGCGG
TGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGCCTGCACCTGACGCTGAGGCGCGA
AAGCGTGG (H5)

**TMW 2.484 *Bacillus subtilis*** (100% *B. subtilis* DSM 10$^{T}$, AB042061.1 (0/756)).

GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGC
GGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGAT
GGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTA
GCTAGTTGGTGAGGTAAYGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGA
CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCA
ACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGG
GCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGC
AAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTC
AACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAA
ATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGC
GTGGGG (H2)

**TMW 2.485 *Bacillus subtilis*** (100% *B. subtilis* DSM 10$^{T}$, AB042061.1 (0/756)).

GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGC
GGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGAT
GGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTA
GCTAGTTGGTGAGGTAAYGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGA
CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCA
ACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGG
GCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGC
AAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTC
AACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAA
ATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGC
GTGGGG (H3)

**TMW 2.539 *Paenibacillus lautus*** (100% *P. lautus* DSM 3035, X60621.1 (0/730)).

GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTYAGCGGCGGA
CGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCT
TGATTGAACCGCATGGTTCAATTATAAAAGGTGGCTTTTAGCTACCACTTACAGATGGACCCGCGGCGCATTAGC
TAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACT
GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC
GCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGC
GGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAA
GCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAA
CCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAAT
GCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTG (D3)

**TMW 2.540 *Bacillus sp*** (100% *B. sp.* LMG 20240, AJ316310.1 (0/726)).

GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACTGATTAGAAGCTTGCTTCTATGACGTTAGCGGCG
GACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGGGAAACCGAAGCTAATACCGGATA
GGATCTTCTCCTTCATGGGAGATGATTGAAAGATGGTTTCGGCTATCACTTACAGATGGGCCCGCGGTGCATTAG
CTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGAC
TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAA
CGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACGAGAGTAACTGCT
CGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAA
GCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAA
CCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGAAAAGCGGAATTCCACGTGTAGCGGTGAAAT
GCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTTTGGTCTGTA (D5)

**TMW 2.541 *Staphylococcus warneri*** (100% *S. warneri* ATCC 27836, Z26903.1 (0/733)).

GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACAGATAAGGAGCTTGCTCCTTTGACGTTAGCGGCG
GACGGGTGAGTAACACGTGGATAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATA
ACATATTGAACCGCATGGTTCAATAGTGAAAGGCGGCTTTGCTGTCACTTATAGATGGATCCGCGCCGTATTAGC
TAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACT
GAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAAC
GCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATCAGGGAAGAACAAATGTGTAAGTAACTGTGC
ACATCTTGACGGTACCTGATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAG
CGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTCAAC
CGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATG
CGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGC (D17)

**TMW 2.542 *Staphylococcus epidermidis*** (100% *S. epidermidis* ATCC 12228, AE016751.1 (0/727)).

GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACAGACGAGGAGCTTGCTCCTCTGACGTTAGCGGCG
GACGGGTGAGTAACACGTGGATAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATA
ATATATTGAACCGCATGGTTCAATAGTGAAAGACGGTTTTGCTGTCACTTATAGATGGATCCGCGCCGCATTAGC
TAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACT
GAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAAC
GCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGGAAGAACAAATGTGTAAGTAACTATGC
ACRTCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAG
CGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTCAAC
CGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATG
CGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAAC (D20)

**TMW 2.543 *Bacillus sp.*** (95% *B. pseudofirmus* DSM 8715, X76439.1 (36/732)).

GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGATCKATGGGAGCTTGCTCCCTGAGATCAGCGGCGGA
CGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACYGGATAAC
ACCTACCCCCKCATGGGGGAAGGTTGAAAGGTGGCTTCGGCTATCACTTACAGATGGACCCGCGGCGCATTAGCT
AGTTGGTGAGGTAAYGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTG
AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACG
CCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCCGTTCGAATAGGGCG
GCGCCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAG
CGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAAC
CGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCAAGTGTAGCGGTGAAATG
CGTAGATATTTGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACT (D72)

**TMW 2.544 *Bacillus macroides*** (99% *B. macroides* LMG 18474, AJ628749.1 (4/727)).

GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACAGAAAAGGAGCTTGCTCCTTTGACGTTAGCGGCG
GACGGGTGAGTAACACGTGGGCAACCTACCCTATAGTTTGGGATAACTCCGGGAAACCGGGGCTAATACCGAATA
ATCTCTTTTGCTTCATGGYRARAGACTGAAAGACGGYWTCKSCTGTCGCTATAGGATGGGCCCGCGGCGCATTAG
CTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGAC
TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAA
CGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTAAGGGAAGAACAAGTWCAGTAGTAACTGGC
TGTACCTTGACGGTACCTTATTAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAA
GCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGTGAAAGCCCACGGCTCAA
CCGTGGAGGGTCATTGGAAACTGGGGGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCAAGTGTAGCGGTGAAAT
GCGTAGAGATTTGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAA (D73)

**TMW 2.545 *Bacillus licheniformis*** (100% *B. licheniformis* DSM 13, D31739.1 (2/733)).

GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGA
CGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCT
TGATTGAACCGCATGGTTCAATCATAAAAGGTGGCTTTTAGCTACCACTTRCAGATGGACCCGCGGCGCATTAGC
TAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACT
GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC
GCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGC
GGYACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAA
GCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAA
CCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAAT
GCGTAGAGATGTGGAGGAACACCGATGGCGAAGGCGACTCTCTGGTCTGTAACTGACG (D75)

**TMW 2.546 *Bacillus sp.*** (95% *B. cohnii* DSM 6307$^{T}$, X76437.1 (34/648)).

GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGCAGGAAACCAGATGACCCCTTCGGGGTGATTCTGGYG
GAATGAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGGGAAACCGGAGC
TAATACCGGATAGTATTTCCTTTCTCCTGATTGGAAATGGAAAGACGGTTTCGGCTGTCACTTACAGATGGGCCC
GCGGTGCATTAGCTAGTTGGTGGGGTAATGGCCCACCAAGGCGACGATGCATAGCCGACCTGAGAGGGTGATCGG
CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGT
CTGACGGAGCAACGCCGCGTGAGCGATGAAGGCCTTCGGGTCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACG
AGAGTAACTGCTCGTACCTTGACGGTACCTAACCAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATA
CGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGTAGGCGGTTTCTTAAGTCTGATGTGAAAG
CCCACAGCTCAACTGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTG
TAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCT (D77)

**TMW 2.547 *Bacillus sp***. (95% *B. pseudofirmus* DSM 8715, X76439.1 (35/732)).

GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGATCGATGGGAGCTTGCTCCCTGAGATCAGCGGCGGA
CGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAAC
ACCTACCCCCKCATGGGGGAAGGTTGAAAGGTGGCTTCGGCTATCACTTACAGATGGACCCGCGGCGCATTAGCT
AGTTGGTGAGGTAAYGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTG
AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACG
CCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCCGTTCGAATAGGGCG
GCGCCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAG
CGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAAC
CGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCAAGTGTAGCGGTGAAATG
CGTAGATATTTGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTG (D98)

**TMW 2.548 *Staphylococcus haemolyticus*** (100% *S. haemolyticus* ATCC 29970$^{T}$, D83367.1 (1/729)).

GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACAGACAAGGAGCTTGCTCCTTTGACGTTAGCGGCG
GACGGGTGAGTAACACGTGGGTAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATA
ATATTTCGAACCGCATGGTTCGATAGTGAAAGATGGTTTTGCTATCACTTATAGATGGACCCGCGCCGTATTAGC
TAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACT
GAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAAC
GCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGGAAGAACATACGTGTAAGTAACTATGC
ACGTCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAG
CGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTCAAC
CGTGGAGGGTCATTGGAAACTGTAAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATG
CGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTG (D99)

**TMW 2.549 *Bacillus psychrodurans*** (100% *B. psychrodurans* DSM 11713, AJ277984.1 (0/734)).

GAACGCTGGCGGCATGCCTAATACATGCAAGTCGAGCGAATGACGAAGAAGCTTGCTTCTTCTGATTTAGCGGCG
GACGGGTGAGTAACACGTGGGCAACCTGCCCTGTAGATTGGGATAACTCCGGGAAACCGGGGCTAATACCGAATA
ATCCATTTCCTCWCATGGGGAGATGTTAAAAGACGGCATCTCGCTGTCACTACAGGATGGGCCCGCGGCGCATTA
GCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGA
CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCA
ATGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTGAGGGAAGAACAAGTAYGAGAGTAACTGC
TCGTACCTTGACGGTACCTCATTAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCA
AGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGTGAAATCCCACGGCTCA
ACCGTGGAAGGTCATTGGAAACTGGGGGACTTGAGTACAGAAGAGGAAAGCGGAATTCCAAGTGTAGCGGTGAAA
TGCGTAGAGATTTGGAGGAACACCAGTGGCGAAGGCGGCTTTCTGGTCTGTAACTGACG (D101)

**TMW 2.492 *Bacillus licheniformis*** (100% *B. licheniformis* DSM 13, X68416.1 (0/730)).

GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGA
CGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCT
TGATTGAACCGCATGGTTCAATYATAAAAGGTGGCTTTYAGCTACCACTTRCAGATGGACCCGCGGCGCATTAGC
TAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACT
GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC
GCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGC
GGYACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAA
GCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAA
CCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAAT
GCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTG (D102)

**TMW 2.550 *Bacillus mycoides*** (100 % *B. mycoides* according to Sacchi et al., 2002).

GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAARTTAGCGGCG
GACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATA
AYATTTTGCACCGCATGGTGCGAAATTCAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAG
CTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGAC
TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAA
CGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGC
TGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCA
AGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCA
ACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAA
TGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACT (D112)

**TMW 2.551 *Bacillus licheniformis*** (100% *B. licheniformis* DSM 13, X68416.1 (0/730)).

GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGA
CGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCT
TGATTGAACCGCATGGTTCAATCATAAAAGGTGGCTTTTAGCTACCACTTRCAGATGGACCCGCGGCGCATTAGC
TAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACT
GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC
GCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGC
GGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAA
GCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAA
CCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAAT
GCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTG (D115)

**TMW 2.482 *Bacillus amyloliquefaciens*** (100% *B. amyloliquefaciens* CMB01, AF489591.1[*)] (0/758)).

GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGRACAGATGGGAGCTTGCTCYCTGATGTTAGCGGCGGA
CGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGRCTGGGRTAACTCCGGGAAACCGGGGCTAATACCGGATGCT
TGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCT
AGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTG
AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACG
CCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGCG
GCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAG
CGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAAC
CGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATG
CGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTG
GGGAGCGA (Fad 11/2)

[*)] Ban et al., 2003

**List of publications that resulted from this dissertation:**

**Original papers**

**Margosch, D.**, M. G. Gänzle, M. A. Ehrmann, and R. F. Vogel. 2004. Pressure inactivation of *Bacillus* endospores. Appl. Environ. Microbiol. 70:7231-8.

**Margosch, D.**, M A. Ehrmann, M. G. Gänzle, and R. F. Vogel. 2004.Comparison of pressure and heat resistance of *Clostridium botulinum* and other endospores in mashed carrots. J. Food Protect. 67:2530-7.

**Margosch, D.**, M. Moravek, M. A. Ehrmann, M. G. Gänzle, E. Märtlbauer, and R. F. Vogel. Effect of high pressure and heat on bacterial toxins. Submitted for publication.

(Sample preparation of toxigenic *B. cereus* and determination of its cytotoxicity and reactivity in the EIA of was performed by M. Moravek, Lehrstuhl für Hygiene und Technologie der Milch, Ludwig-Maximilians Universität München.)

**Margosch, D.**, M. A. Ehrmann, M. G. Gänzle, V. Heinz, and R. F. Vogel. Inactivation of *Clostridium botulinum* and other endospores by combined pressure / temperature treatment at isothermal holding times. In preparation.

(Pressure treatment was performed together with A. Ardia, Fachgebiet für Lebensmittel-biotechnologie und –prozesstechnik, TU Berlin.

**Oral presentations**

Vogel, R. F., **D. Margosch**, M. G. Gänzle, V. Heinz, and M. A. Ehrmann. Behaviour of *Clostridium botulinum* as safety determinant in low acid pressurized food. EHPRG meeting on Advances in High Pressure Research, Lausanne, September 2004.

**Margosch, D.**, und R. F. Vogel. Inaktivierung von Endosporen von *Clostridium botulinum* und anderen Sporenbildnern. Vorstellung der Hochdruckforschungsbereiche in Weihenstephan gegenüber dem Bundesamt für Wehrtechnik und Beschaffung. März 2004.

Gänzle, M. G., **D. Margosch**, M. A. Ehrmann, and R. F. Vogel. Recent development in high pressure relating to food safety. Vortrag bei dem Seminar über Novel (mild) preservation technologies in relation to food safety, Brüssel, Januar, 2004.

**Margosch, D**., M. G. Gänzle, M. A. Ehrmann, und R. F. Vogel. Inaktivierung sporenbildender Lebensmittelverderber und –vergifter in Hochdruckprozessen. Gemeinsames Statusseminar von BMBF-, DBU- und DFG-geförderten Forschungsprojekten „Hochdruckbehandlung von Lebensmitteln“, Berlin, Oktober, 2003.

Gänzle, M. G., **D. Margosch**, M. A. Ehrmann, und R. F. Vogel. Frisch gepresst – Untersuchungen und Überlegungen zur mikrobiologischen Sicherheit hochdruckbehandelter Lebensmittel. GDL-Kongress Lebensmitteltechnologie in Stuttgart-Hohenheim, September, 2003.

**Poster Presentations**

Baars, A., N. Pereyra, A. Delgado, **D. Margosch**, R. F. Vogel, M. Cerny, P. Schieberle, and F. Meußdörffer. High hydrostatic pressure – energy saving technology for preservation of food. Presentation at the conference „ICEF9“, Montpellier, March, 2004.

**Margosch, D**., M. A. Ehrmann, M. G. Gänzle, and R. F. Vogel. Rolle der Dipicolinsäure bei der druckinduzierten Inaktivierung bakterieller Endosporen. 5. Fachsymposium Lebensmittel-mikrobiologie der VAAM und DGHM, Seeon, Mai, 2003.

**Margosch, D**., C. Beimfohr, F. Meußdörffer, M. G. Gänzle, M. A. Ehrmann, and R. F. Vogel. Identifizierung von Leitkeimen und Verhalten unter Hochdruck. 36. Technisches Seminar, Weihenstephan, Januar, 2003.

Baars, A., N. Pereyra, A. Delgado, **D. Margosch**, R. F. Vogel, M. Cerny, P. Schieberle, F. Meußdörffer, C. Beimfohr. Entwicklung eines innovativen Hochdruckverfahrens zur umweltfreundlichen Sterilisation von Lebensmitteln. 36. Technisches Seminar, Weihenstephan, Januar, 2003.