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Culture

Using nano-LC-MS/MS to investigate the toxicity of outbreak *E. coli* O104:H4 strain

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Introduction

The deadliest *E. coli* outbreak recorded worldwide began in Germany on May 1, 2011 with a trickle of patients presenting bloody diarrhea[1]. By May's end, the number of reported cases surged to 1240, including cases reported in eight other European countries[2]. The source for the epidemic was first associated with cucumbers from Spain[3], but then subsequent investigation revised that conclusion in June to beansprout seeds from a farm in Egypt[4]. The whole outbreak resulted in more than 4000 reported cases, 50 deaths[5], and strained hospital dialysis and plasmapheresis resources[6].

An unusually high number of patients suffered from haemolytic-uraemic syndrome (HUS), a devastating and rare disease characterized by disintegration of red blood cells, acute kidney failure, and impaired ability to clot blood[7]. The majority of HUS outbreaks are caused by enterohemorrhagic *E. coli* (EHEC), which can contain a prophage that produces Shiga toxin. The toxin enters cells in the gastrointestinal tract, inhibits mRNA translation and causes cell death. This damage manifests for the patient as cramping and diarrhea, first watery and then bloody. Some patients experience acute renal damage that leads to potentially fatal HUS[8].

Shiga toxin is released only at one point in the phage's life cycle, the lytic cycle, which is induced by stress from a human host's neutrophils or prescribed antibiotics[8]. If laboratory tests identify Shiga toxin-producing *E. coli*, physicians will not prescribe antibiotics because rapidly eliminating bacteria can trigger the release of a potentially lethal dose of Shiga toxin.

While the outbreak's high rate of HUS in patients implicated a Shiga toxin-producing *E. coli*, confirmation and identification of the etiological agent was not straightforward. Clinical laboratory tests for patients with HUS detect the most common HUS-associated strains, primarily those of the O157:H7 serotype, through a bacteriological assay for sorbitol fermentation. Unlike more than 90% of *E. coli*, O157:H7 cannot ferment sorbitol for survival. The outbreak strain, however, could ferment sorbitol, and the laboratory tests reported an unexpected absence of Shiga toxin-producing EHEC.

The lack of identification of the pathogen hampered accurate clinical diagnoses and potential efforts to trace the source of the outbreak. The primary contamination source for *E. coli* of the O157:H7 serotype is cattle fecal matter, suggesting a close farm–produce association for the outbreak's origin. The identified serotype of the outbreak strain, however, does not necessarily originate from cattle.

The outbreak strain was not positively identified until May 25. Helge Karch and colleagues at the University of Münster and the Robert Koch Institute identified the culprit strain through clever serotyping and PCR assays. Multilocus sequence typing was used to confirm that the outbreak was caused by a single clone, HUSEC041, and that it had the rare serotype of O104:H4. This serotype is normally associated with enteroaggregative *E. coli* (EAEC) that are known to cause persistent diarrhea, but not hemorrhaging or HUS[3]. Only one instance of an outbreak involving *E. coli* O104 had been reported previously[9].

Karch and his colleagues' analysis suggested that the outbreak strain contained an unusual mosaic of features from EAEC and EHEC that made outright identification difficult. The strain lacked features typical of O157:H7, such as an enterocyte effacement pathogenicity island and an intimin positive gene, but produced aggregative factors typical of EAEC. However, the strain did exhibit high resistance to third-generation cephalosporins, trimethoprim/sulfamethoxazole, and tetracycline that is typical of O157:H7. The strain also possessed the rarer and more potent Shiga toxin 2 gene[3]. A polymerase chain reaction

assay was soon disseminated online to enable screening for the outbreak clone by the O104:H4 gene[10].

DNA sequencing would provide the initial blueprint for understanding the pathogen's novel set of characteristics. In June, two independent groups completed DNA sequencing of the outbreak isolate's 5.2 million base pair genome and two large plasmids using short-read DNA sequencers[11,12]. Both groups released the sequencing data to the scientific community, which rapidly performed bioinformatics to explain the strain's pathogenicity and evolutionary origin[13]. It was also suggested that the strain may harbor genes unique from those in other strains.

After patients displaying symptoms of the outbreak strain arrived in the United Kingdom, the Health Protection Agency (HPA) obtained samples and sequenced the strain's genome using a long-read DNA sequencing platform. This analysis confirmed the mosaic nature indicated in previous analyses and identified virulence factors that may account for higher incidence of HUS.

As the outbreak continued, significant questions remained unanswered: What functional proteins is the clone capable of producing? Is this a novel genetic structure, or is it an EAEC that acquired Shiga toxin or an EHEC that acquired aggretins? To answer those questions, the HPA's Department for Bioanalysis and Horizon Technologies investigated how a novel proteomic, mass spectrometry-based approach could be applied to detect this mosaic outbreak strain, identify the composition of its expressed protein complement, and elucidate the strain's origins.

The proteomic approach

Mass spectrometry-based proteomic approaches have been used over the past decade to reliably identify bacteria based upon detected amino acid sequences. Unlike PCR, ELISA, Western blots, or bacteriological assays, identification of an etiological agent by mass spectrometry does not rely upon predictions about the culprit pathogen's identity nor require pathogen-specific reagents.

Multiple mass spectrometry techniques exist. In brief, protein extracts may be first digested by proteases before delivery to the mass spectrometer by an inlet or vaporizer, which is either a chromatography device or a solid vehicle for matrix assisted laser desorption/ ionization (MALDI). The mass spectrometer then ionizes and further fragments the sample, separates ions by unique mass-to-charge ratios, and detects the resultant ions, often by a quantitative method. Each compound produces a unique fragmentation pattern. Bioinformatic algorithms compare these patterns, called spectra, to spectra from reference compounds in a database and produce a list of matched peptides and proteins that are used for identification and characterization of the sample.

A widespread mass spectrometry technique in microbiology labs is MALDI time-of-flight mass spectrometry (MALDI-TOF-MS). Due to its ease of use and a database of mass spectral profiles of more than 500 pathogenic strains of bacteria developed over the past decade[14], MALDI-TOF-MS is excellent for rapid identification of routine bacteria. However, MALDI-TOF-MS cannot identify the individual ions that characterize the mass spectrum of a species. If a mass spectral profile for a strain is not yet present in a database, the strain cannot be identified. The HPA would require another mass spectrometry technique to detect and characterize the mosaic outbreak strain.

The HPA collaborated with Thermo Fisher Scientific to explore a novel mass spectrometry approach for simultaneous microbial identification and characterization of the proteome based on nano-liquid chromatography tandem mass spectrometry (nano-LC-MS/ MS). Nano-LC-MS/MS provides ultra-high resolution and accurate mass for differentiation of similar peptide sequences, such as unique features of closely related strains of Enterobacteriaceae. Nano-LC-MS/MS also concentrates peptides for greater detection sensitivity than MALDI-TOF-MS. This approach would reveal not only a subspecies level identification but also a comprehensive profile of the culprit agent's individual mosaic of pathogenic markers and virulence characteristics, whether novel or existing.

A bottom-up proteomics approach was used (shown in Figure 1), beginning with lysing and extracting the proteins of the unknown microorganism(s) of interest. After solubilization (of the proteins), a proteolytic enzyme is added that digests the proteins into peptide fragments which are optimal for chromatographic separation and analysis via tandem mass spectrometry (nano-LC/MS/MS). The most successful instrument utilized to date for this procedure is the hybrid Orbitrap mass spectrometer, developed by Makarov[15]. The Orbitrap works by detecting the oscillation/motion of the peptides in the Z-direction. The resulting peptide tandem mass spectra produced are high resolution/mass accuracy data of currently unsurpassed quality. After chromatographic separation and tandem mass spectrometry, the resulting peptide MS/MS data are searched against known microbial protein databases in order to identify the proteins from which they were derived. The identified proteins can then be used to identify the microorganism as well as the individual proteins of the cell.

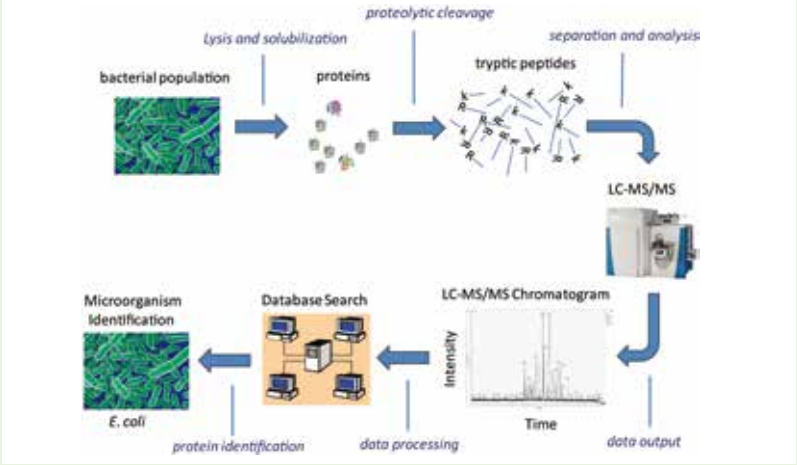

Figure 1

Illustration of the typical bottom-up proteomics experiment beginning with an unknown microorganism. Here the cell population is lysed, solubilized and extracted to optimize the protein content. Next, the proteins are digested into peptides using a proteolytic enzyme like trypsin which cleaves on the C-terminal side of arginine (R) and lysine (K) residues. The resulting peptide is analysed via chromatography and tandem mass spectrometry, and the resulting peptides are sequenced and identified using database search algorithms. The peptides identified are mapped back to the individual proteins and the microorganism can be identified.

Methods

Proteomic analysis was performed on five *E. coli* strains of serotype O104: three clinical isolates from patients affected by the German outbreak and two other isolates that were previously characterized as serotype O104, but have EAEC and EHEC genetic composition respectively. The genomes of the three German outbreak isolates were sequenced to confirm they were from the same strain.

All strains were cultured using LB broth and agar and then harvested prior to employing two parallel approaches for reducing complexity of the mixture and mass spectrometry analysis. In the first approach, lysates were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and gel slices were digested with trypsin. Peptides were analysed using nano-LC-MS/MS. In the second approach, the entire cell lysate was digested directly with trypsin in solution and injected on to two LC-MS/MS systems (Thermo Scientific LTQ Orbitrap and Thermo Scientific LTQ Orbitrap Velos, each with a front-end Ultimate 3000 Dionex nano/capillary liquid chromatography system, Thermo Fisher Scientific) that provided ultra-high resolution and accurate mass for differentiating closely related peptides.

The recorded peptide MS/MS spectra were matched to both protein and *in silico* genome-translated databases to identify expressed proteins. The peptides were then fed into a bioinformatics pipeline[15] to acquire unique signatures at the genus and species level.
An extensive list of identified peptides was then searched, using Blast and Scaffold, for virulence determinants, *E. coli* virulence factors, and putative EHEC and EAEC-specific virulence markers.

Results

An extensive list of protein signatures was produced from cells grown using plates or broth. These peptide lists identified proteins that covered a significant percentage of the predicted open reading frames of the sequenced outbreak strain genome, indicating the sensitivity and reliability of the nano-LC-MS/MS method to yield protein profiles using selective or enriched culture preparation.

Peptides resulting from high-abundance proteins were then analysed for markers and signatures that uniquely identified genus, species or virulence characteristics. The mosaic outbreak strain's virulence signatures were compared to both EAEC and EHEC protein signatures, all obtained using the same proteome approach.

Similar profiles were detected. Approximately 2500 proteins from the outbreak isolates were identified. A collection of 68 peptide signatures were unique to the outbreak *E. coli* isolates and not shared by EAEC or EHEC, separating the outbreak strain from other closely related Enterobacteriaceae. Species-level peptide signatures were also detected, including those for the AggR transcription factor, haemolysin protein, Aaf fimbriae protein, and Iha adhesion protein. In total, 3031 peptides were identified as unique to the outbreak strains when compared against control isolates.

In addition, the technique detected features that were expected based upon prior laboratory tests and genomic data, including the production of Shiga toxin, Pic serine protease (autotransporter toxin), and tellurium resistance.

The list of peptides was then filtered to exclude physiological and regulatory proteins. Search of the simplified list for *E. coli* pathotype virulence determinants and virulence factors resulted in a definitive list of expressed virulence determinants of the outbreak strain.

The results also support the view that the background genome (biome) came from an EAEC progenitor that acquired plasmids and prophages, and exchanged chromosomal loci leading to the emergence of an aggressive strain with a distinctive profile.
All strains shared 89% of the expressed proteins. The two large plasmids encoded 31 proteins. Peptide signatures for adhesion and multidrug resistance (including ß-lactamase, CTX-M extended spectrum ß-lactamase and Metallo-ß-lactamase enzymes) were observed.

Conclusions

Detecting and accurately identifying pathogens in an efficient manner minimizes the human and economic impact of outbreak strains. Experimental results demonstrate that a proteomic approach, based on nano-LC-MS/MS and comparison against a database of known pathogenic markers, accelerates the identification and characterization of the sources of *E. coli*-related illnesses and diseases.

In the HPA's study of the O104:H4 outbreak strain, the nano-LC-MS/MS technique was able to identify a significant number of pathogenic markers with no requirement for enrichment, selective media or antibiotic incorporation that can otherwise delay analysis. The protein signatures detected provide definitive characterization at the genus, species, and often strain level, as well as detection of expressed pathogenic determinants and antibiotic resistance mechanisms.

This mass spectrometry-based approach enables clinical laboratories investigating outbreak strains to rapidly design screening and verification tests directly and in an unbiased manner, rather than performing multiple, potentially futile detection approaches while the outbreak is underway.

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Progress and potential for high-pressure thermal processing of foods

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Introduction

High pressure processed (HPP) products meet the increasing consumer desire for convenience foods that are fresh tasting, reduced in chemical additives, microbiologically safe and have an extended shelf life. In Asia, Europe, U.S.A. and Australia, the number of commercial HPP products, including fruit jams, fruit juices, fruit preparations, yogurt smoothies, guacamole, salsa, oysters, ready-to-eat rice and sliced ready-to-eat poultry and meats, on the market is steadily growing[1,2]. In commercial food industry practice, high pressure processing uses pressures of approximately 300 to 600 MPa for periods from about thirty seconds to a few minutes to destroy pathogenic bacteria, such as *Listeria monocytogenes, Salmonella, Escherichia coli* and *Vibrio* spp., as well as bacteria, yeasts and moulds that cause food spoilage.

The required pressure treatment for microbiologically safe and stable products is dependent on the target microorganism(s) to be inactivated and the desired conditions for storage (see Figure 1). One MegaPascal (MPa) is equivalent to approximately 9.87 atmospheres, 10 Bar, 0.1kgcm^2 or 145 lb.in.^{2}. Pressures at the bottom of the Mariana sea trench are about 8 ton cm^2 or 110 MPa, and considerably higher pressures are required for inactivation of microorganisms. At low or near ambient temperatures, vegetative bacterial cells, yeasts and moulds are sensitive to pressures between 200–700 MPa, however, bacterial spores may survive pressurization above 1500 MPa [3,4].

Spoilage of HPP pasteurized food due to the outgrowth of bacterial spores can be controlled via complementary means, such as refrigeration and/or acidification, however, low-acid foods (LAF) that are microbiologically safe and stable are not obtainable by HPP at low or ambient temperature. Preservation of LAF is traditionally achieved by thermal processing, which eliminates (directly) or prevents (in combination with other treatments) the growth of spores or vegetative cells in the final product during normal conditions of distribution and storage. High-pressure thermal (HPT) processing can inactivate bacterial spores through a combination of high-pressure treatment with elevated temperatures (see Table 1).

The advantage of HPT processing lies in the reduced thermal load applied to products due to (1) reduced heating and cooling times obtained by the rapid heating/cooling developed in the product during (de)pressurization (see Figure 2), and potentially (2) reduced processing temperatures and/or times through synergistic effect of pressure and heat on spore inactivation. Compared with conventional thermal processing, the accelerated and homogeneous heating and cooling of foods during HPT processing enables more precise delivery of heat to all food packs and reduces the need for excessively long heating of conduction to ensure the minimum thermal process is delivered. This is expected to result in improved food quality attributes, such as flavor, texture, nutrient content and color, as HPT products receive less heat damage. HPT processing is, therefore, a potential alternative to conventional thermal processing, to deliver quality benefits to a range of processed foods, such as soups, sauces, and chilled or shelf-stable multicomponent meals with extended shelf life.

CSIRO Food & Nutritional Science's 35L high pressure processing plant. Image: CSIRO

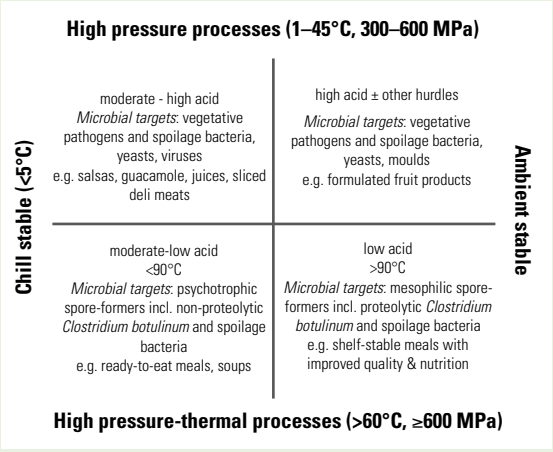

Figure 1
High pressure processes, target microorganisms for inactivation and example food categories.

Basic HPT principles

As a food processing parameter, pressure behaves very differently to temperature. Pressure is instantaneously transmitted to all points within a pressure vessel due to the isostatic principle, unlike in thermal processing where heat transfer relies on conduction and/or convection. Therefore, assuming uniform thermal distribution within a sample, process time is independent of sample size, shape or packaging material. Additionally, physical compression decreases the volume of a product which is accompanied by an increase in temperature[5]. The magnitude of temperature change within a product depends mainly on its compressibility and its specific heat. Compression heating of water has been reported to range from 2.5, 3.0 and 5.3°C/100 MPa at initial temperatures of 15, 25 and 90°C[6]. Fats and oils are more compressible than water and increase 3.2 to 8.7°C/100 MPa at initial temperatures of 25°C[5]. Product composition (especially heterogeneous or multicomponent foods), initial product temperature and the applied pressure will all affect the increase in temperature developed in a pressurized product.

The typical pressure and temperature profiles of an HPT process shown in Figure 2 illustrate the preheating of a product to a target temperature, product temperature increase due to compression heating (T_s–T_{p1}), slight cooling of the product during pressure hold time (T_{p1}– T_{p2}) and product cooling due to decompression (T_{p2}–T_f). Upon decompression, and under adiabatic (perfectly insulated conditions, the product will return to its prepressurized temperature. However, the steel walls of pressure vessels do not undergo compression heating, and maintain their core temperature near the initial temperature resulting in heat transfer from the contents of the vessel (pressure transmitting fluid and packaged products) to the vessel wall and a subsequent cooling of the product during processing[5]. Spore inactivation by HPT processing is reliant on higher temperatures. It is therefore critical that temperature gradients throughout the vessel and the product are accounted for to achieve the minimum process performance in every product unit in the process[7,8,9].

Equipment for HPT processing must be able to heat and maintain vessel temperatures of up to 90°C at pressures of 600–800 MPa, or higher. Vessels of varying scale are in use including laboratory, 0.02–1.5L, pilot plant, 2–50L, and industrial (although not yet in commercial use), up to 150L[10]. Equipment design considerations reflect the desire for uniform temperature distributions throughout the vessel during processing and process efficiency. Features such as vessel wall insulation, internal vessel wall heating or polymeric product carriers will reduce heat transfer from product to the vessel wall; faster pumping systems will reduce pressure come-up time, enabling maximum compression heating; pressurizing fluid inlet temperature and geometry will also influence temperature conditions within a pressure process[11].

Spore inactivation by pressure

Low acid food products that are microbiologically safe and stable are not obtainable by high-pressure processing at near-ambient temperatures, as bacterial spores can survive pressures above 1,500 MPa[3,4], which exceeds the pressure capabilities of current commercial-scale HPP equipment. Sufficient high pressure inactivation of bacterial spores is generally achieved in combination with initial process temperatures that exceed 60°C (reviewed extensively for *Bacillus* and *Clostridium* spp. in Wilson *et al.* 2008). Of particular interest for LAF is the ability of a combined HPT process to synergistically inactivate spores of the major bacterial spore-forming pathogens of concern, which are proteolytic strains of the neurotoxigenic species *Clostridium botulinum*.

Differences in response to HPT processing between strains of the same species have been regularly reported[12,13,14]. Figure 3 illustrates the range of spore inactivation our lab observed following a HPT process with a selection of 29 *C. botulinum* strains and one *Clostridium sporogenes* PA3679 strain. As others have found, nonproteolytic *C. botulinum* were less HPT-resistant than proteolytic *C. botulinum* strains[13, 15, 16].

Several studies have established that the pressure and heat resistance of spores does not correlate with their heat-only resistance[12,13,17]. In their study of spore-forming, spoilage-associated

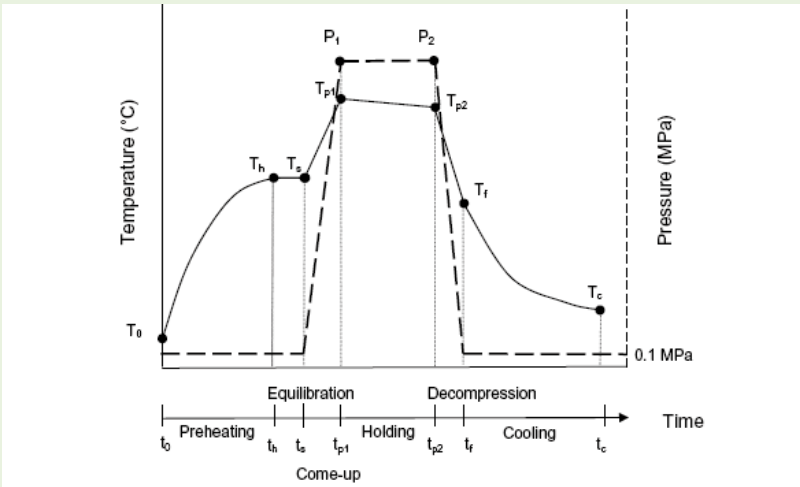

Figure 2
Typical temperature and pressure profiles of a high pressure thermal process (Juliano, Knoerzer, and Versteeg 2011).

Table 1
Factors influencing spore inactivation by high pressure-thermal processing

Direct effects	Indirect effects (via thermal component)
Pressure magnitude and hold time	Design of HP unit • temperature uniformity (insulation, active heating of vessel wall, product carrier material) • compression/decompression rates
Process temperatures • temperature at pressure on (T_0) • maximum temp. (T_{max}), • temperature under pressure (T_{hold})	Pressure liquid composition • influence on compression heating
Product composition • intrinsic hurdles (pH, a_w) • protective factors	Product composition • temperature uniformity due to differences in compression heating
Number of pressure cycles	Material properties of product packaging

bacteria, Olivier *et al.*[18] found the very heat-resistant strains *Bacillus amyloliquifaciens* FRR B2782 and *Geobacillus stearothermophilus* FRR B2792, were highly pressure HPT-sensitive; conversely, the most heat-sensitive strain, *Bacillus coagulans* FRR B2723, proved to be the most HPT-resistant strain under most HPT conditions studied (Table 2). It was also shown that, despite the high heat resistance of *Clostridium thermosaccharolyticum* TMW 2.299, (selected because strains of *C. thermosaccharolyticum* are generally more than 10-fold more heat resistant than *C. botulinum*), it was less pressure and heat resistant than the most resistant *C. botulinum* strains. Koutchma *et al.*[17] found that while the heat only resistance of *G. stearothermophilus* ATCC 7953 was much greater than that of *C. sporogenes* PA3679, its pressure and heat resistance was significantly lower[18].

Many studies of HPT spore inactivation have focused on the use of *C. sporogenes* PA3679 and *G. stearothermophilus* due to their traditional use in thermal inactivation studies as an appropriate surrogate for *C. botulinum*, in the case of the former, or as the most heat-resistant, thermophilic spoilage microorganism, in the case of the latter. However, with the lack of correlation between heat-only and HPT-resistance, it is appropriate to focus research on other *Bacillus* spp. that exhibit particularly high-pressure and heat resistance, e.g. *B. coagulans* or *B. amyloliquefaciens* TMW 2.479 has been shown to be a highly pressure- and heat-resistant, mesophilic spoilage microorganism[13,19]. This strain is apparently more pressure and heat resistant than strains of *C. botulinum*, *C. sporogenes* PA3679 or *G. stearothermophilus* strains in the literature and has been suggested as a potential HPT non-pathogenic surrogate for proteolytic *C. botulinum*. Olivier *et al.*[18] showed that *B. coagulans* FRR B2723, processed in either Bolognese or cream sauce in a pilot-scale unit, produced the most highly HPT-resistant spores of the species they examined (see Table 2).

Most studies comparing the heat-only and HPT resistance of bacterial spores have concluded that, in most cases, pressure and heat do act synergistically to deliver lethality[16,17,18,19,20,21]. Margosch *et al.*[20] observed a protective effect of pressure against thermal inactivation for some HPT treatments, in particular under close to isothermal and isobaric conditions. Pressure protective effects for *C. botulinum* TMW 2.357 were observed at temperatures of 100, 110 or 120°C, at pressures of 600–800 MPa, and for *B. amyloliquefaciens* TMW 2.479 at 120°C at pressures 800–1000 MPa. Spore inactivation in this study was conducted in Tris-His buffer, where pH is essentially pressure independent, while other published studies of spore inactivation have been conducted in phosphate buffer, the pH of which is affected by pressure[22,23], or in water, the ionic dissociation of which is enhanced under pressure resulting in a decrease in pH[3], or in model food products, where pH is also likely to be reduced under pressure[13]. The isothermal, pH-independent nature of these conditions means that the protective effects observed may not be reproducible in commercial HPT equipment or in actual food products. Bull *et al.* found that for five proteolytic *C. botulinum* strains in three model food products, strain to strain variation in synergy between high pressure and heat was observed[12]. Recently, the heat resistance of *C. sporogenes* ATCC 7955 spores was shown to increase under pressure, although under the conditions employed, HPT processes always resulted in smaller D-values than conventional heat-only D-values[24].

The effect of the intrinsic properties of food on spore inactivation by HPT processing has been explored[13,21,23,25]. Ananta *et al.* found that *B. stearothermophilus* ATCC 7953 was protected from inactivation when inoculated in cocoa mass with 10% water and subjected to HPT treatments of 600 MPa/90°C/60 min[25]. The same protective effect was not observed when *B. stearothermophilus* ATCC 7953 was inoculated into cocoa mass with 20 or 30% water, where inactivation levels of 2 to 6-$\log_{10}$ were obtained following HPT at 600 MPa for 60 min as process temperatures increased from 70 to 90°C. Ananta *et al.* suggested that the protective effect was due to the low water activity of the fat-rich product, rather than the fat itself[25]. No significant difference in the level of inactivation of *B. stearothermophilus* ATCC 7953 was shown in egg patties or deionized water[21]. Although the egg patties had a fat content of ca. 11% w/w, their water activity was high (ca. 0.99), which supports the proposal of Ananta *et al.*[25].

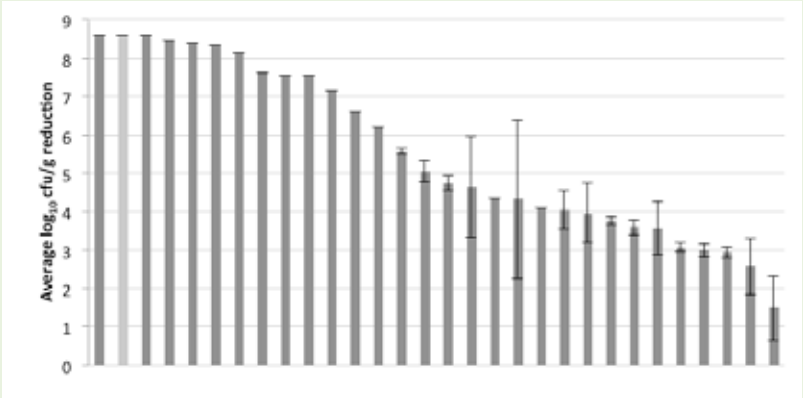

Figure 3
Inactivation of 29 *Clostridium botulinum* strains and *Clostridium sporogenes* PA3679 (light grey bar) by a HPT process of 600 MPa, 1 min with a pressure-on temperature of 95°C. Spores were pressure treated in MPA3679 broth, in a laboratory-scale, multi-vessel high-pressure unit (Model U111, Unipress Equipment, Poland).

Table 2
Relative heat vs HPT resistance of spoilage sporeforming bacteria[a]

Non-proteolytic strains		Proteolytic strains	
Heat resistance (most to least)	**Estimated average $\log_{10}$ reduction/min at $F_{121.1°C, 0.1\ MPa}$ (CFU/g)**	**HPT resistance (most to least)**	**Estimated average $\log_{10}$ reduction/min at $F_{121.1°C, 600\ MPa}$ (CFU/g)**
Bacillus amyloliquefaciens FRR B2782	No inactivation	*B. coagulans* FRR B2723	10 ± 4.1
Geobacillus stearothermophilus FRR B2792	0.51 ± 0.12	*B. amyloliquefaciens* FRR B2782	35 ± 2.9
B. sporothermodurans FRR B2706	17 ± 11	*G. stearothermophilus* FRR B2792	98 ± 30
B. coagulans FRR B2723	70 ± 29	*B. sporothermodurans* FRR B2706	110 ± 33

[a] in a cream sauce, in pilot-scale thermal-only and HPT systems (adapted from Olivier *et al.* 2011).

Margosch *et al.* found that inactivation of *C. botulinum* TMW 2.357 was not affected by decreasing the pH from 6.0 to 5.15[13], however, the inactivation rate was increased by a shift from pH 5.15 to 4.0. Under equivalent processing conditions, inactivation of *C. botulinum* TMW 2.357 in mashed carrots (pH 5.15) proceeded slightly more quickly than in Tris-His buffer set at the same pH, however the difference in inactivation levels was 1-$\log_{10}$ or less. Changes in pH under pressure may account for the discrepancy in inactivation levels, as Margosch *et al.*[13] describe the components of Tris-His buffer as being more pH stable under pressure than the main buffering components in carrots (carboxylic acids and phosphates[23]).
There is some difficulty in directly comparing the results of HPT inactivation studies conducted in different laboratories due to the varying thermal and pressure profiles of individual HP units. While HPT processes may be conducted at the same maximum pressure, using the same initial temperature or have the same apparent process temperature, the thermal history of a sample will be affected by variations in compression rates, pressure vessel sizes, compression fluids, vessel insulation capabilities, packaging materials and pack sizes. It has therefore been noted that details of the critical process parameters and product variables (pH, a_w, composition, packaging) should be reported for any HPT study to aid in establishing safe processing criteria for HPP/HPT foods[2].
Due to the above constraints, and combined with a lack of consistency of response to HPT conditions between strains of resistant microorganisms of public health significance, it is prudent to take a case-by-case approach to demonstrating the safety of HPT sterilized foods[9,12,19]. Further, as synergy has not been consistently observed among strains of *C. botulinum*, the prediction of inactivation of *C. botulinum* by HPT processing for the present must assume a lack of synergy. Therefore, any HPT process for LAF should be at least thermally equivalent to a F_0 process of 2.8 min in line with current good manufacturing practices.

An understanding of the physiological effects of HPT processing on spore inactivation would enable improvement of the efficacy of HPT treatments and process optimization. The mechanism of spore inactivation has been primarily studied in *Bacillus subtilis*; high pressure has been shown to initiate spore germination via at least two mechanisms: at moderately high pressures (50–300 MPa) and at very high pressures (400–800 MPa) (as reviewed by Black *et al.*[27]). At moderately high pressure, the spore nutrient receptors are activated and germination proceeds down the nutrient-triggered pathway[29 29,30]. Very high pressure directly causes the release of Ca-DPA+ (calcium dipicolinate, a key constituent of the spore core and proposed to be involved in their extreme dormancy and resistance mechanisms), possibly by opening the DPA channels in the inner membrane or via another action on the inner membrane, and subsequent germination[29,30,31]. Either mechanism of spore germination by pressure results in spores that may continue through to Stage II of germination; however, even those spores that only complete Stage I of germination are more heat-sensitive than dormant spores.

Toward commercial HPT Processing
In February 2009, the U.S. Food and Drug Administration accepted a petition filed by the National Center for Food Safety and Technology (NCFST) for the commercial production of a pressure assisted thermal sterilization (PATS)-processed mashed potato product in flexible pouches and processed in a 35L high-pressure sterilization vessel. This is the first HPT process to be accepted and is a demonstration of verifiable and reproducible inactivation of *C. botulinum* spores from ambient stable LAF. The approach taken by the research consortia was to consider the HPT process as primarily a thermal process with pressure utilized to rapidly heat and cool the product[31].

The potential food quality and nutritional benefits of foods processed under HPT conditions (>600 MPa, >90°C) has not been comprehensively determined. The main flavor components in fresh basil were profiled after various processes, and HPT processed basil retained up to 90% of flavor components compared to freezing, conventional heat sterilization and drying of fresh basil[32]. However, the texture and color of HPT processed basil were more similar to heat treated basil than to fresh basil. The retention of vitamin content after processing is also of interest, and Matser *et al.* determined that the effect of temperature and pressure on ascorbic acid is matrix dependent, but in general, the HPT processed samples had a significantly higher retention than the conventionally processed samples[33]. The effect of HPT treatment on color is also strongly product dependent with some HPT products (spinach and carrot) showing good color retention compared with thermal processing[33].
A recent review on the reaction kinetics of food quality chemicals found that while the pressure range mostly examined is sufficient, the temperature range is not[34].

The requirements for packaging of HPT processed foods are similar to those of thermally processed (e.g. retorted) products: seal integrity and barrier properties to oxygen, water vapor and carbon dioxide must be retained during and post processing[26]. Additionally, HPT processed packaging must withstand the stresses of rapid compression and decompression, and the associated volume and temperature changes without compromise to the aesthetic qualities of the packaging. Flexible, laminate films have been identified as potentially suitable packaging materials for HPT sterilized foods, however, the assessment of flexible packaging treated by HPT processes above 100°C has been scarcely reported. A PE/nylon/Al/PP delaminated under 690 MPa when temperatures approached 90°C, however, delamination was not observed when the temperature was reduced to 85°C[35]. The barrier (oxygen and water vapor) properties of vapor-deposited oxide- and nylon-containing films were compromised by a HPT treatment of 600 MPa and 110°ca. In contrast, the barrier properties of aluminium foil and PVDC–MA containing films were not significantly affected by HPT processing, although all materials suffered cosmetic deformation of the outer surface to some degree[36].

Conclusions
Commercial sterility of low-acid, shelf-stable foods (LASSF) by HPT processing remains one of the biggest challenges in high pressure applications. A more near-term goal is the application of HPT processing to extend the shelf life of low-acid chilled foods. Spores of non-proteolytic *C. botulinum* are the pathogen of most concern in extended shelf life, chilled foods because of their ability to germinate and produce toxin at refrigeration temperatures.
HPT processing conditions for the inactivation of non-proteolytic *C. botulinum* spores are more moderate than required for inactivation of proteolytic *C. botulinum*.

As the application of high pressure technology in the food industry progresses from pasteurization to the development of new LASSF products, considerable knowledge gaps remain to be filled before HPT processes can be designed with safety and process assurance. Advances in HPT equipment design and methodologies to accurately model temperature distributions will improve our ability to assess and control process performance; developments in packaging will ensure delivery of premium products; and continued research into inactivation kinetics and the mechanisms of spore inactivation by HPT will contribute to the validation of HPT processes.

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