Effect of High-Pressure Carbon Dioxide on Desiccated *Escherichia coli* and *Salmonella*

by

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Abstract

The safety of low water activity (a_W) foods is a concern due to the survival of low-infectious dose pathogens such as *Escherichia coli* and *Salmonella*. Desiccation of non-heat resistant *E. coli* and *Salmonella* increases their heat resistance; therefore, a non-thermal alternative is necessary to ensure the safety of low a_W foods. High-pressure CO_2 (HPCD) is a well-established food processing method for high a_W foods, but low a_W foods present a challenge due to the reliance of the inactivation mechanism on the presence of water. This study aimed to identify conditions which could achieve a 5-log reduction of pathogenic *E. coli* and *Salmonella* on dry food. Four strains of Shiga toxin-producing *E. coli* (STEC) and 1 strain of enteropathogenic *E. coli* (EPEC) were treated together as a cocktail, and five strains of *Salmonella* were treated individually. Non-pathogenic surrogate organisms are necessary for validation of treatment processes in the food industry without the risk of introducing pathogens. The suitability of *E. coli* AW1.7, *Pediococcus acidilactici* FUA 3072, *Enterococcus faecium* NRRL B-2354 and *Staphylococcus carnosus* R6 FUA 2133 as surrogate organisms was evaluated. Treatments were also validated in two low a_W foods, beef jerky and almonds. Samples were equilibrated to various a_W and treated with heat, HPCD or pressurized N_2. Selective agars and selective enrichments were used after HPCD and N_2 treatments to differentiate healthy and injured cells of *E. coli*, *Salmonella* and *E. faecium*.

Comparisons between isogenic strains of *E. coli* and *Salmonella* demonstrated that at a a_W of more than 0.80, the locus of heat resistance (LHR), a 14 kbp genomic island conferring resistance to wet heat, becomes detrimental to the survival of *S.* Typhimurium ATCC 13311 pLHR, but not *E. coli* AW1.7 pLHR, whereas strains without the LHR remain heat resistant at all a_W. Treatment of desiccated *E. coli* AW1.7 and the STEC cocktail with dry gaseous CO_2 (5.7

MPa and 65 °C) did not reduce cell counts; however, treatment with gaseous CO_2 saturated with water (5.7 MPa and 65 °C) reduced cell counts of all *E. coli*. Treatment of beef jerky inoculated with *E. coli* and *Salmonella* with saturated gaseous CO_2 resulted in greater than 5-log reductions for all *E. coli* and *Salmonella*. *E. coli* AW1.7 is an acceptable surrogate for dry STEC cells and STEC on beef jerky treated with HCPD. *P. acidilactici* FUA 3072 was more sensitive than some strains of *Salmonella* but *E. faecium* NRRL B-2354 and *S. carnosus* R6 are suitable surrogates for dry *Salmonella* cells and *Salmonella* on beef jerky or almonds treated with HPCD. Treatment time did not affect treatment efficacy. Increasing the a_W of beef jerky samples from 0.75 to 0.9 did not affect treatment efficacy. Treatment of beef jerky with water-saturated gaseous CO_2 was more effective than treatment with supercritical CO_2 or treatments with N_2 at the same temperature and pressure. Overall, the treatment of low a_W foods with saturated gaseous HPCD can meet industry standards by achieving a greater than 5-log reduction of *E. coli* and *Salmonella*. Additionally, surrogate organisms to represent pathogenic *E. coli* and *Salmonella* have been validated in beef jerky and almond food matrices.

Preface

This thesis is an original work by Danielle Schultze. The construction of the Mobile Pasteurization Apparatus was done by Dr. Ricardo Couto. Some of the research conducted in this thesis is part of a collaboration with the Fraunhofer UMSICHT institute in Oberhausen, Germany. Results in Figures 7, 9, 10 and 11a were presented on a poster at the 10^{th} International High Pressure Bioscience and Biotechnology Conference as "Influence of high pressure CO_2 on the survival of desiccated *Escherichia coli* and *Salmonella* spp. cells and on beef jerky" by Schultze D., Temelli F., Couto R., McMullen L.M. and Gänzle M.

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List of Abbreviations

ANOVA	Analysis of variance
aw	Water activity
CFA	Cyclopropane fatty acid
CFU	Colony forming unit(s)
DNA	Deoxyribonucleic acid
EHEC	Enterohaemorrhagic *E. coli*
FDA	Food and Drug Administration
FSIS	Food Safety Inspection Services
HC	Haemorrhagic colitis
HPCD	High pressure carbon dioxide
HUS	Hemolytic uremic syndrome
kbp	Kilobase pairs
LB	Luria-Bertani
LEE	Locus of enterocyte effacement
LHR	Locus of heat resistance
MRS	De Man, Rogosa and Sharpe
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
STEC	Shiga toxin-producing *E. coli*
SPI	*Salmonella* pathogenicity island
TS	Tryptic soy
T3SS	Type III secretion system
USDA	United States Department of Agriculture
VRBGA	Violet red bile glucose agar

1. Introduction

1.1 Safety of low water activity foods

Low moisture foods have a water activity (a_W) of 0.85 or less [1]. These products are generally shelf-stable at ambient temperature [2]. The shelf life of low-moisture foods like nuts may be a year or more [3], therefore tracing outbreak sources can be difficult and outbreaks can span several months. The a_W is defined as the ratio between the partial pressure of water in a product and the saturation vapour pressure of pure water when the temperature is constant [4]. Decreasing the a_W places osmotic stress on bacteria due to the change in turgor pressure [5]. It also results in less available water for bacteria to use for growth [5]. Therefore, low a_W foods ($a_W < 0.85$) inhibit the growth of contaminants. However, although bacteria are unable to grow in dry conditions, low-infectious dose pathogens may survive at low a_W and do not require growth in the food to cause severe illness. Numerous studies have determined that *Escherichia coli* and *Salmonella* can survive in low a_W foods for extended periods of time [5–11]. This presents a serious problem because the organisms can grow and cause infections upon reaching the gastrointestinal tract after ingestion [5]. Additionally, it is hypothesized that *Salmonella* in low a_W foods has a lower infectious dose due to factors such as low moisture and high fat content that protect cells from harsh conditions during digestion [5,12]. Both *E. coli* and *Salmonella* are difficult to eliminate from low a_W foods due to the increased heat resistance of dry cells [5]. Seeras [5] determined that both heat-resistant and non-heat resistant desiccated *E. coli* survive thermal treatments that are lethal to hydrated cells. D-values for the reduction of desiccated *Salmonella* with heat are by orders of magnitude higher than those for wet cells. For example, the $D_{60\text{-}62}$ for *S.* Weltevreden in wheat flour was 875 min at a_W of 0.4 and the $D_{63\text{-}65}$ was 29 min at a_W 0.5 [13]. This presents a challenge to the food industry because pathogens are frequently

controlled by thermal interventions [14], thus their presence in dry foods places consumers at risk. Therefore, it is imperative to develop an intervention technique, which can effectively reduce highly resistant *E. coli* and *Salmonella* in low a_W food products.

1.2 *E. coli* and *Salmonella* pathogenesis and relevance

E. coli and *Salmonella* are closely related *Enterobacteriaceae*. They are both Gram-negative bacilli, and facultative anaerobes with many properties in common.

1.2.1 *E. coli*

Most *E. coli* are non-pathogenic; however, there are 6 main virotypes characterized by virulence genes, cellular adhesion mechanisms and the site of colonization: enteropathogenic *E. coli* (EPEC), diffusely adherent *E. coli* (DAEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), *Shigella*/enteroinvasive *E. coli* (EIEC), and Shiga toxin-producing *E. coli* (STEC) [15]. The STEC group also contains enterohaemorrhagic *E. coli* (EHEC), such as *E. coli* O157:H7. Although *E. coli* O157:H7 is the most frequently implicated STEC serotype in North America [15], data from the EU determined that most infections between 2007 and 2011 were linked to serotypes O157, O26, O103, O91, O145, O111 and O128 [16]. The USDA (United States Department of Agriculture) identified the most common non-O157:H7 STEC that are frequently associated with severe illness in humans, known as the big six: O26, O45, O103, O111, O121, and O145 [17].

STEC are characterized by the presence of genes encoding for the Shiga toxin that are located on lambdoid prophages [15]. STEC may carry variants of the *stx2* gene (*stx2a*, *stx2b*, *stx2c*, *stx2d*, *stx2e*, *stx2f*, and *stx2g*) and/or variants of the *stx1* gene (*stx1a*, *stx1c*,and *stx1d*), although presence of *stx2* genes are typically associated with more severe illness [15]. EHEC were

originally classified based on the development of haemorrhagic colitis (HC) [15]. Genomic analysis later determined that EHEC usually possess the locus of enterocyte effacement (LEE), which encodes adhesins and plays a role in the attachment to the intestinal epithelium and food surfaces using fimbriae [15]. The LEE encodes for a type III secretion system (T3SS), which injects proteins into host cells and causes attachment and effacement lesions [15,18]. The gene *eae* is also involved in attachment to intestinal cells with effacement of the underlying microvilli [18]. Overall the presence of the LEE is associated with severe illness and higher risk of complications associated with EHEC infections [15]. However, not all HC-inducing STEC possess the LEE, which resulted in the formation of 4 categories of STEC: EHEC 1, EHEC 2, STEC 1 and STEC 2 [15]. EHEC 1 contains *E. coli* O157:H7 and EHEC 2 contains non-O157 serotypes such as O111:H8 and O26:H11 [15]. EHEC 1 and 2 are usually LEE-positive and are frequently associated with HC [15]. STEC 1 contains LEE-negative serotypes such as O113:H21 and O91:H21, and STEC 2 contains serotypes such as O45:H2 and O103:H2/H6 [15]. The Shiga-toxin is an AB_5 type toxin composed of one A subunit and five B subunits. The B subunits facilitate binding of the toxin to the globotriacylceramide (Gb_3) receptor on the vascular endothelium and renal cells of the host [15]. The A subunit exerts the toxic effect by internalizing within the cell and causing the ribosomal subunit to inhibit protein synthesis and induce cell apoptosis [19,20]. Endothelial damage causes cells to swell, which narrows capillaries thus reducing blood supply to kidneys impairing renal function, which can lead to hemolytic uremic syndrome (HUS) and acute kidney failure [15,21]. The gene for the enterohemolysin toxin (*Ehx*) is encoded on the plasmid pO157 and causes lysis of red blood cells by forming a pore in the cytoplasm of the cell [15,22,23]. Plasmids in non-O157 STEC strains contain similar genes [15,24], which may also contribute to the development of HUS.

STEC are a concern to public health due to their ability to cause severe illness with a remarkably low infectious dose of as low as 10 to 100 cells [15]. For example, Paton et al. [25] determined that as few as 1 CFU of *E. coli* O111:NM per 10 g of dry sausage may have caused illness. Symptoms of STEC infections include bloody or non-bloody diarrhea and abdominal pain, which typically develop 3-4 days after the consumption of a contaminated food product [15,26]. *E. coli* O157:H7 strains are more frequently associated with severe complications such as HUS, which typically begins after the resolution of gastrointestinal symptoms [15]. HUS occurs in approximately 5-10% of STEC infections and can result in long term sequelae including permanent kidney damage, hemolytic anemia, cardiac and gastrointestinal complications, neurological disorders, hypertension, diabetes mellitus and cognitive and behavioural changes [15,26]. An estimated 265,000 STEC infections occur in the US every year with 36% of them linked to *E. coli* O157:H7 and the remaining 64% linked to non-O157 STEC [26]. In 2011, FoodNet reported 463 *E. coli* O157:H7 cases and 521 non-O157 STEC cases, but the hospitalization and mortality rates were over twice as high for the *E. coli* O157:H7 [27]. The use of antibiotics to treat STEC infections increases Stx production, and is therefore not recommended [15,26]. The illness is self-limiting or fatal; there are no known therapies to prevent or treat HUS [15,26]. The cultivation of STEC from contaminated foods that have been implicated in illness can be challenging due to the low number of cells necessary for illness. Additionally, agar-based detection methods cannot differentiate non-O157 STEC from generic *E. coli* because they can both ferment sorbitol, whereas *E. coli* O157:H7 cannot [15]. However, classic serotyping provides useful data and is based on the identification of the O-polysaccharide and H surface antigens [15,26]. In the US, there were over 175,000 STEC illnesses in 2013, with a financial burden of an estimated $2.9 million [28]. Six percent of all hospitalizations and 8%

of deaths due to foodborne illness in Canada were linked to *E. coli* O157:H7 [29]. STEC transmission can be fecal-oral and STEC can contaminate food products, which makes them a relevant food safety concern. It is estimated that 68% of *E. coli* O157:H7 and 82% of non-O157 STEC illnesses in the US are food-related [30].

1.2.2 *Salmonella*

The genus *Salmonella* includes two species: *Salmonella enterica* and *Salmonella bongori*, and over 2,500 serotypes [31]. *S. enterica* is separated into six subspecies based on genomic similarity, the most relevant of which in food safety is *S. enterica* subsp. *enterica*, which is responsible for approximately 99% of *Salmonella* infections [31]. Within the subspecies *enterica*, there are over 1,300 serotypes [31]. *Salmonella* pathogenicity islands (SPIs), located on the chromosome or plasmids, contain numerous virulence factors such as adhesion, invasion and toxin genes [31,32]. Host invasion and intracellular proliferation are both necessary for *Salmonella* infection and are facilitated by genes in the SPI. SPI-1 contains invasion genes and SPI-2 is necessary for intracellular pathogenesis and is involved in the development of systemic infections [32]. T3SS are encoded on SPI and can inject over 30 different proteins inside the cytoplasm of intestinal epithelial cells [32]. *S. enterica* have two different T3SS, one that is linked to cellular invasion (SPI-1), the other that is related to intracellular pathogenicity (SPI-2) [32]. The SPI-1 T3SS induces cytoskeletal rearrangement along the epithelium, triggering membrane ruffling in which the bacteria is engulfed and internalized within a vacuole [31,32]. Eventually, the *Salmonella*-containing vacuole is internalized within macrophages associated with Peyer's patches [32]. One effector protein is SopB, which prevents the fusion of lysosomes to the vacuole, preventing the degradation of the *Salmonella* [31,32]. This ultimately permits the intracellular survival and replication of *Salmonella* within macrophages, thus allowing infection

to proceed [31,33]. The SPI-2 T3SS is responsible for invasive *Salmonella* infections [32]. Environmental conditions such as low osmolarity, lack of certain nutrients and decreased pH in the *Salmonella*-containing vacuole have been associated with the induction of the expression of SPI-2 T3SS genes [34,35]. The SPI-2 T3SS is responsible for the transfer of effector proteins from the *Salmonella*-containing vacuole to targets in the host cells [32]. Effector proteins include SpiC, which disrupts vesicular transport and SrfT, which causes cell apoptosis [32]. Systemic infections occur when *Salmonella* in immune cells are transported to other parts of the body [32]. Dendritic cells facilitate the spread of *Salmonella* throughout the body, and genes on the SPI-2 T3SS inhibit their normal immune function [32,36]. Some strains possess virulence plasmids containing the *Salmonella* plasmid virulence genes [32], which are involved in bacterial multiplication in extra-intestinal infections [37]. Virulence plasmids may also contain genes encoding for fimbriae [32].

Symptoms such as diarrhea, nausea, vomiting, headache, fever and abdominal cramps typically develop 12-72 h after consuming a food contaminated with *Salmonella* [31,38]. Symptoms are typically self-limiting, lasting approximately 7 days [39], but severity depends on the serotype of *Salmonella* and immunity of the individual [31]. Complications include cholecystitis, pancreatitis, appendicitis, cellulitis, urinary tract infections, pneumonia, endocarditis, meningitis, and Reiter's syndrome [31,39,40]. Individuals with more severe illness have an increased risk of developing reactive arthritis [41]. However, immune-compromised individuals may develop severe illness such as sepsis, which can cause death if not treated [41]. Approximately 5% of patients develop sepsis [31]. The treatment of *Salmonella* infections with antibiotics was associated with a small risk of developing reactive arthritis [41]. Antibiotic resistance is an increasing concern for *Salmonella*, many of which contain genes encoding for resistance toward

chloramphenicol, tetracycline, ampicillin and streptomycin [42]. Additionally, the infectious dose is lower in immune-compromised individuals, children, and the elderly where symptoms can develop with as few as 10-1,000 CFU/g [43,44]. A small fraction of individuals may become asymptomatic carriers, shedding *Salmonella* in their stool for up to 1 year after symptoms resolve [31]. Reservoirs for *Salmonella* include poultry, cattle, reptiles, and insects [31,39]. Common sources of *Salmonella* on farms include feed, soil, litter, and fecal matter [39]. *Salmonella* is the foodborne pathogen that causes the most deaths and has the highest cost burden in the US [45], resulting in an estimated $3.6 billion for over one million cases in 2013 [28]. In the US, 23% of all food outbreaks are linked to *Salmonella*. In Canada non-typhoidal *Salmonella* infections are implicated in about 5% of all foodborne illnesses, 24% of hospitalizations and 16% of deaths [29]. *Salmonella* was responsible for approximately 6,500 cases of salmonellosis each year between 2009 and 2013 [29].

Together, *Salmonella* and O157 STEC are thought to cause 30% of hospitalizations and 24% of deaths from food-borne illness each year [29]. Therefore, it is crucial to destroy STEC and *Salmonella* prior to ingestion in order to reduce the risk of serious illness.

1.3 Stress resistance of *E. coli* and *Salmonella*

E. coli and *Salmonella* possess resistance mechanisms to protect them against environmental stressors, but overall resistance is highly strain-specific [46]. The majority of *E. coli* have D_{60} values less than 1 min and highly heat resistant strains have D_{60} values greater than 10 min [47–50]. *E. coli* AW1.7 has a D_{60} value greater than 60 min [49], making it extremely heat resistant, which has been attributed to the presence of the locus of heat resistance (LHR) and the accumulation of compatible solutes [46,47]. Heat resistance in *S.* Senftenberg was also linked to the LHR [5]. The LHR is a 14 kbp genomic island that contains genes encoding for heat shock

proteins and proteases, which prevent aggregation [46]. The extreme heat stress sigma factor, *rpoE*, facilitated the survival of *E. coli* and *Salmonella* exposed to heat stress [51], but was downregulated during desiccation stress [52]. Expression of *rpoS* triggers a stress response to multiple types of stress such as heat, pressure, starvation, desiccation, oxidative and osmotic stress [5,53]. Gene expression changes significantly when cells are stressed [8]. Mild heat treatments resulted in the upregulation of multiple virulence genes in *Salmonella* [54]. Only about 50% of the *S.* Enteritidis genome was expressed under stress-inducing conditions, and less than 5% was expressed under conditions of dry stress [8]. Therefore, *Salmonella* may become metabolically dormant under low a_W conditions [8].

E. coli and *Salmonella* can accumulate or synthesize compatible solutes to survive in low a_W environments [5,55]. A decrease in a_W results in higher levels of solutes outside of the cell, which would cause a loss in turgor pressure within cells [5,55]. However, bacteria can increase their internal levels of solutes with compatible solutes, which maintain turgor pressure and prevent cell dehydration during desiccation stress [5,55,56]. Compatible solutes function to protect proteins through preferential hydration around the surface of molecules, which decreases the overall free energy, thus increasing the stability [56–58]. For example, the disaccharide trehalose replaces water molecules to form a layer around cellular components, preventing damage to proteins and the cell membrane during desiccation [5,55]. Trehalose has been associated with increased resistance of *E. coli* to osmotic stress and increased growth and resistance of *S.* Typhimurium to heat stress [55,56,59]. *E. coli* can also accumulate sucrose, glycerol, glycine betaine and proline to accommodate osmotic stress [56]. In the presence of NaCl, *E. coli* AW1.7 accumulates higher levels of trehalose than heat sensitive *E. coli* strains [50,56].

The accumulation of compatible solutes also contributes to cross-resistance, in which resistance to one type of stress imparts resistance to other types of stress. For example, heat, pressure and oxidative stress resistance are related in *E. coli* [56]. Heat and pressure resistance were also improved by the osmotic stress-induced accumulation of solutes [60,61]. Additionally, the *rpoH* mediated heat shock response and the *rpoS*-mediated general stress response contribute to heat and pressure resistance in *E. coli* [62]. Some STEC survived after pressure treatment up to 600 MPa [50], but pressure-resistant *E. coli* became pressure-sensitive when *cfa*, which is responsible for the synthesis of cyclopropane fatty acids (CFA) for modification of the membrane composition [63], or *rpoS* were knocked out [64]. Therefore, CFA synthesis contributes to the cross-resistance to heat and pressure in *E. coli* [63,65]. Bacteria may also alter their membrane fluidity in response to decreased temperature, pH or a_W [66]. However, heat and pressure resistance are not always related [48,50], therefore cross-resistance to stress may be strain-specific. Cross-resistance may occur because different stressors activate similar underlying mechanisms [64]. Heat, pressure and drying can cause osmotic stress, protein denaturation, oxidative stress and membrane damage, and although the source of the stress varies, bacteria may respond through the same stress response system for different stressors [64]. For example, trehalose synthesis is regulated by *rpoS*, so it is only synthesized when *rpoS* is expressed during stress [66]. Therefore, exposure to an initial stress activates *rpoS* and results in the synthesis of trehalose, which could provide resistance to a second different stress through the accumulation of compatible solutes that were already being synthesized, thus resulting in cross-resistance [66]. Mutations to *rpoS* resulted in the loss of osmotic-stress-dependent heat resistance [55]. Cells can also resist stress by utilizing nutrients from surrounding dead cells; therefore, the initial number of cells can affect stress resistance. Desiccation killed 50% of *Salmonella*, but extracellular

polysaccharides from dead cells provided nutrients and solutes, which helped the remaining *Salmonella* to survive [67]. Degraded rRNA may also be a nutrient source for bacteria during conditions of stress or starvation [8]. Both *E. coli* and *Salmonella* are also frequently resistant to low pH, which facilitates survival in the stomach and colonization in the gastrointestinal tract [15,66]. Acid-adapted *Salmonella* were highly resistant to heat and salt [66], and desiccated *Salmonella* were more resistant to ethanol, hydrogen peroxide, sodium hypochlorite, and quaternary ammonium chloride than non-desiccated *Salmonella* [67]. However, acid-adapted *Salmonella* became more sensitive to desiccation stress, indicating that not all stress-responses are related, and cross-resistance does not always occur, likely due to difference response pathways [67]. Ultimately, there are fundamental differences in the heat resistance of wet or dry cells, and resistance mechanisms permit pathogenic *E. coli* and *Salmonella* to survive treatments expected to kill them. Cross-resistance plays a role in bacterial resistance, but the complexity of interactions requires further study. Cross-resistance in *E. coli* and *Salmonella* are relevant concerns in the food industry because food products frequently undergo more than one treatment to reduce bacterial cell counts; therefore, highly cross-resistant strains may be more likely to cause foodborne illness.

1.4 HPCD as a food processing technique

High-pressure CO_2 (HPCD) was first reported to disrupt the growth of bacteria in 1927 [68]. HPCD is classified as a cold pasteurization method that inhibits microorganisms through molecular effects of CO_2 [69]. Supercritical CO_2 exists when the temperature and pressure are above its critical points (T_C = 31 °C, P_C = 7.4 MPa) [70]. Supercritical CO_2 has properties in between those of gases and liquids with a gas-like diffusivity and a liquid-like density and ability to dissolve other compounds [70]. Subcritical CO_2 exists when the temperature and/or pressure

are below their critical values. CO_2 possesses antimicrobial characteristics and non-pressurized CO_2 is frequently used to prevent microbial growth in modified atmosphere packaging [71]. HPCD technology is used as an extraction technique [72,73], but it possesses several advantages for the food industry as a method to inactivate pathogens. Compared to heat treatments, HPCD results in fewer changes to taste, colour, texture and nutritional quality in liquid foods [69]. Additionally, CO_2 is non-toxic, non-flammable, has Generally Recognized as Safe status, and leaves no residue in foods [69,70]. Therefore, it can be applied to foods as a processing aid. According to Health Canada, a processing aid is "a substance that is used for a technical effect during food processing or manufacture but, unlike food additives, its use does not affect the intrinsic characteristics of the food and it results in no or negligible residues of the substance or its by-products in or on the finished food" [74]. Processing aids do not need to be disclosed on labels, making HPCD an attractive clean-label technology [70,73,74].

The antimicrobial effects of HPCD have been well documented in various liquids and food systems using supercritical [72,73,75–80] and subcritical conditions [76,77,80–83]. For example, Wei et al. [82] effectively reduced *Listeria* in shrimp, orange juice and egg yolk and *Salmonella* in chicken and egg yolk by treating with HPCD at 6.18 MPa and 35 °C for 2 h. However, only few studies have investigated the effects of HPCD on solid foods and very little on low a_W foods. Since pathogens such as *E. coli* and *Salmonella* are more resistant in low a_W environments, some studies indicate that HPCD treatments are not effective on low a_W foods [70]. Haas et al. [75] achieved reductions of bacteria, molds and yeasts on fresh herbs and spices treated with HPCD, which increased with increasing temperature but decreased with decreasing a_W. However, Jung et al. [78] achieved a 7-log reduction of STEC and *S.* Typhimurium on dry alfalfa seeds. Therefore, in the small amount of existing literature, HPCD does not consistently achieve

adequate reductions of bacteria in low a_W foods. The absence of research on dry foods may be due to the perceived ineffectiveness of treatments at low a_W due to the current understanding of the inactivation mechanisms of HPCD.

1.5 Mechanism of HPCD-induced bacterial inactivation

1.5.1 Decreased external pH

Hydrated CO_2 is polyprotic and can undergo more than one dissociation reaction to produce carbonic acid and bicarbonate, carbonate and hydrogen ions in aqueous media. This decreases the extracellular pH through the following dissociation reactions [69,70].

$$\mathrm{CO_2(g)} \leftrightarrow \mathrm{CO_2(aq)}[\mathrm{CO_2}]_{\mathrm{aq}} = \mathrm{H} \times p_{\mathrm{CO_2}}$$
$$\text{with } \mathrm{H} = 3.3 \times 10^{-2}\mathrm{mol/(L\ atm)(25°C)} \quad (1)$$

$$\mathrm{CO_2(aq)} + \mathrm{H_2O} \leftrightarrow \mathrm{H_2CO_3}$$
$$[\mathrm{H_2CO_3}]/[\mathrm{CO_2}]_{\mathrm{aq}} = 1.7 \times 10^{-3}\ \mathrm{mol/L(25°C)} \quad (2)$$

$$\mathrm{H_2CO_3} \leftrightarrow \mathrm{H^+} + \mathrm{HCO_3^-}$$
$$[\mathrm{HCO_3^-}][\mathrm{H^+}]/[\mathrm{H_2CO_3}] = 2.5 \times 10^{-4}\ \mathrm{mol/L(25°C)} \quad (3)$$

$$\mathrm{HCO_3^-} \leftrightarrow \mathrm{H^+} + \mathrm{CO_3^{2-}}$$
$$[\mathrm{CO_3^{2-}}][\mathrm{H^+}]/[\mathrm{HCO_3^-}] = 5.61 \times 10^{-11}\ \mathrm{mol/L(25°C)} \quad (4)$$

The pH of phosphate buffer solution consistently decreased by 3 units regardless of the pressure [84]. Deletions of genes involved in acid resistance, specifically *rpoS*, resulted in higher sensitivity to HPCD treatments [85]. The decrease in external pH necessitates increased energy consumption by bacteria to maintain homeostasis [76]. However, the reduction of external pH

cannot fully explain the effect of HPCD on the reduction of cells [75] due to larger reductions observed with HPCD than with acid alone [83]. Additionally, stronger acids like hydrochloric acid or phosphoric acid cannot exert a comparable reduction [82,83], likely due to the decreased solubility through the cell wall and accumulation within the cytoplasm compared to CO_2 [69,75,83]. Although some studies have found that desiccated *Salmonella* are more acid-resistant [66], Gruzdev et al. [67] found that desiccated *Salmonella* were more sensitive to acetic or citric acid at pH 3.0.

1.5.2 Decreased internal pH

Being non-polar, unhydrated CO_2 can diffuse through the phospholipid layers of the cell membrane and accumulate within the cytoplasm [69,70]. Inside the cell, its dissociation is governed by equilibrium laws where the accumulation of CO_2, a reactant, shifts the equilibrium to the products, resulting in the formation of carbonic acid and carbonate, bicarbonate and hydrogen ions (Equations 1-4) [70]. Initially, the cell attempts to maintain homeostasis using pH buffering systems such as cytoplasmic buffering, proton pumps, membrane-bound H^+-ATPases, and the production of acids or bases [70,79,86]. However, the buffering ability of the cell is eventually exceeded, resulting in a decrease of the intracellular pH [69,70]. The decreased internal pH is hypothesized to play the largest role in the inactivation of bacteria [69,85]. However, the acidification of the extra- and intra-cellular environment through dissociation reactions is dependent on the presence of water; therefore, the effect may be limited in low a_W environments.

1.5.3 Effects on enzymes

Enzyme activity is significantly impacted by fluctuations in pH. Therefore, a decrease of the internal pH may inactivate or inhibit enzymes necessary for metabolic reactions and regulatory

processes [69,86]. HPCD treatments on *E. coli* resulted in minor changes in some enzymes and complete inactivation of others, suggesting that enzyme inactivation by HPCD is selective and enzymes with acidic isoelectric points will precipitate first [81]. Proteins can also be inactivated by low pH due to interactions of protein-bound arginine with CO_2 [69]. Decarboxylases enzymes contribute to the acid resistance of *E. coli*, but can be inactivated themselves by low pH, further disrupting the acid resistance of cells [69,85].

1.5.4 Effects on reaction kinetics and metabolism

Reaction rates are dependent on the concentration of substrates, products and cofactors, which allow cells to regulate enzymatic activity [70]. The concentration of HCO_3^- increases due to CO_2 dissociation and interferes with enzymatic activity by either promoting or inhibiting reactions [87]. Additionally, CO_2 is a substrate in carboxylation reactions and a product in decarboxylation reactions; therefore, the equilibrium balance of these metabolic reactions is disrupted by increasing concentrations of CO_2 [70]. The disruption of chemical reactions within the cell can significantly impact cell metabolism.

1.5.5 Effects on electrolyte balance

Increasing concentrations of CO_3^{2-} can cause the precipitation of electrolytes including Ca^{2+} and Mg^{2+} [70]. These electrolytes are involved in osmotic regulation; therefore, this can greatly disrupt cell activity [70]. In the absence of Ca^{2+}, cell division ceased, indicating the requirement of Ca^{2+} for growth [88]. The concentration of Ca^{2+} in the cytoplasm was exponentially less than the extracellular concentration, therefore precipitation of Ca^{2+} by CO_3^{2-} could disrupt cellular functions [89]. Additionally, in *E. coli* and *Salmonella*, Ca^{2+} is removed from the cell via a Ca^{2+}/H antiporter driven by the proton motive force, which may also be disrupted by the acidification of the extra- and intra-cellular environment [89]. Starved cells could not remove

Ca^{2+}, but regained the ability to remove it with the addition of glucose [89]. Magnesium is also essential for cell division and the growth of bacteria [90].

1.5.6 Physical disruption of cells

The physical disruption of cells was the first explanation for the inactivation of bacteria with HPCD and it was attributed to rapid depressurization, resulting in the expansion of CO_2 within cells [69]. The concentration of protein in solution after HPCD treatments was comparable to the concentration when cells were broken using other methods [91]. Additionally, although some cells had holes or were completely ruptured, others were inactivated yet remained intact [14]. *E. coli* and yeast exposed to HPCD resulted in 25% intact cells yet only 1% or 2.2% viable cells, respectively [81,92]. Therefore, physical damage to cells is not solely responsible for the loss of viability.

1.5.7 Modification of cell membrane and extraction of cellular components

CO_2 exhibits hydrophobic properties, therefore unhydrated CO_2 can accumulate within the cell membrane and interact with phospholipids [87,93]. Known as the anesthesia effect, these interactions cause an increase in membrane fluidity and therefore membrane permeability [87]. HCO_3^- ions may also alter the surface charge of cells by interacting with phospholipid heads and proteins on the surface, effectively decreasing the permeability of the cell to water [87]. Supercritical CO_2 has a high solvent power; therefore, extraction of non-polar compounds may occur during treatments using HPCD in the supercritical region. This contributes to microbial inactivation through the disruption of the membrane which can lead to the leaking of cytoplasmic contents [70,72,80].

1.5.8 Depressurization rate

Depressurization at high rates may result in CO_2 expansion within the cell, thus resulting in the rupture of cell walls [80]. Fraser et al. [94] found that slow depressurization resulted in better survival compared to fast depressurization. However, Debs-Louka et al. [83] found that depressurization from 4 MPa over 0.4 s, 15 min and 50 min did not differentially affect the reduction of cell counts and suggested that it depends on the state of CO_2: subcritical or supercritical. Gradual depressurization did not consistently result in less reduction in yeast cells, possibly because slower depressurization results in longer contact time between cells and CO_2 [95]. Due to the rupture of bacterial cells during depressurization, it was thought that multiple pressurization cycles during treatments could increase cell death [80]. However, treatments with fewer pressurization cycles resulted in higher reductions of yeast cells, and most bacterial inactivation occurred during the initial pressurization, rather than the depressurization [95].

1.6 Different types of HPCD systems

HPCD systems can be batch, semi-continuous or continuous. Batch systems treat products without the flow of CO_2 or the food product. Semi-continuous systems treat products with a flow of CO_2 through the chamber, but the food product does not move. In continuous systems, both the CO_2 and the food product move through the system [69]. Typically, batch systems are the least efficient at reducing bacterial cell counts due to the decreased contact between CO_2 and cells, therefore requiring longer treatment times [69]. Treatments with a semi-continuous system were more efficient and needed only 10-min treatments, compared to a batch system where 40-60 min treatments were needed [96]. Use of a semi-continuous system achieved triple the amount of enzyme inactivation and higher levels of CO_2 dissolved in the sample [69,97]. The pasteurization of liquid foods with HPCD is close to being applied at the commercial scale [70].

However, new and effective strategies must be identified for solid and low a_W food products to achieve a greater than 5-log reduction of *E. coli* and *Salmonella*.

1.7 HPCD-assisted drying

Although sun-drying is the cheapest available drying method, it can result in microbial contamination and is time-consuming [98]. Vacuum-drying techniques are superior at preserving flavour and textural properties, but are expensive [99,100]. HPCD could overcome these problems by being implemented as a combined drying and microbial decontamination technique. Benali & Boumghar [101] suggested that HPCD-assisted drying could prevent thermal drawbacks because the water is not removed by vaporization or sublimation as in heating processes. Several studies have proposed using HPCD to dry foods [101–103]. Bourdoux et al. [98] used HPCD-assisted drying to treat fresh coriander, and achieved a 4.61 log reduction of *Enterobacteriaceae*, a greater than 5-log reduction of *Salmonella*, and a greater than 5-log reduction of *E. coli* O157:H7 with counts below the detection limit. HPCD-assisted drying more effectively reduced bacterial cells compared to freeze-drying [98]. Overall, HPCD-assisted drying shows potential for use as a processing aid in the production of low a_W food products.

1.8 Resistance to HPCD

The development of resistant sub-populations of pathogenic organisms to HPCD is a possible concern which must be acknowledged. Considering the development of resistance toward other types of stress, it is possible for *E. coli* and *Salmonella* to develop stress resistance mechanisms to HPCD. Garcia-Gonzalez et al. [104] identified the resistance of *E. coli* and *L. monocytogenes* after treatment with fifteen HPCD cycles. However, the increasing resistance of *E. coli* and *L. monocytogenes* occurred gradually between cycles [104], therefore consistent testing in an industrial environment could provide suitable information of treatment efficacy. Tailing was

observed in graphs from a study by Hong et al., [76] which may indicate the presence of a resistant sub-population of *L. plantarum* treated with HPCD. However, treatment of surviving isolates resulted in similar sensitivity as previously determined [76]. Overall, the adaptation of bacteria to HPCD is a relevant concern. Therefore, these factors must be considered during process design. Additionally, the incorporation of the hurdle technology may produce synergistic effects which increase the efficiency of HPCD treatments.

1.9 Synergistic effects of HPCD

In this context, synergistic effects occur when the reduction due to several factors together is greater than the reduction due to each factor individually. Therefore, it is possible that the combination of heat, pressure and acidification during HPCD treatments results in synergy. Bacteria exposed to low pH are more stressed, which may increase their sensitivity to other processing factors through the denaturation of enzymes and increased proton permeability [66,105]. Additionally, increased cell permeability from the effects of pressurized CO_2 on the cell membrane may increase the concentration of CO_2, carbonic acid and hydrogen ions entering the cell [79]. However, at low pH conditions the equilibrium of the dissociation reaction will shift toward CO_2, therefore decreasing its solubility [71]. Haas et al. [75] observed synergistic effects when treating *E. coli* in broth with gaseous CO_2.

Additionally, hurdle technology involves the combination of HPCD with other inactivation techniques, such as essential oils or bacteriocins, which may also improve the reduction of bacteria with HPCD. This strategy could be applied to limit the development of resistant subpopulations, which may develop over time [104]. The hurdle effect may also inactivate pathogens synergistically [106]. For example, Bi et al. [107] found that HPCD treatments resulted in improved reduction of *E. coli* with nisin, which is typically ineffective against Gram-

negative organisms due to the outer membrane. *E. coli* also became sensitive to nisin after treatment with high hydrostatic pressure [108]. Additionally, *Salmonella* and *S. aureus* became sensitive to nisin and pediocin after treatment with high hydrostatic pressure [109]. Overall, proof of synergistic effects and better understanding of the interactions requires further research due to the complexity of the mechanisms.

1.10 Decontamination of beef jerky and almonds

Beef jerky is a low a_W food product, the popularity of which is increasing. Consumption of beef jerky in the US increased by 20% between 2011 and 2018 [110]. As a ready-to-eat product, the safety of beef jerky depends on the quality of beef used in its production. The primary reservoir for STEC is ruminant animals, such as cattle [15,111]. While 10-80% of cattle are suspected to carry STEC, cattle are asymptomatic to pathogenic effects because they lack the Gb_3 receptor [112,113]. Therefore, it is impossible to identify carriers of STEC. Additionally, some cattle are super-shedders and shed as many as 10^4 CFU/g pathogens in their feces, which increases rates of contamination [111]. The most common source of carcass contamination is the hide, which can cross-contaminate other areas during slaughter and processing [18,111]. For these reasons beef products are frequently implicated in STEC outbreaks and recalls [114]. Additionally, beef products are one of the top five groups of products related to *Salmonella* foodborne outbreaks [39] and *Salmonella* was isolated from approximately 2% of beef carcasses [115]. Therefore, as a result the USDA Food safety and Inspection Services (FSIS) started testing for *Salmonella* when STEC sampling is conducted [111]. It is important to note that the FSIS only began testing for non-O157 STEC in meat in 2012 [17]. Hide decontamination, knife trimming, chemical washes and sprays and fast chilling are often implemented to reduce bacterial contamination on beef carcasses [111]. No combination of trimming and steam vacuuming was able to remove all

E. coli O157:H7 or *Salmonella* [111]. Additionally, bacteria may survive acid treatments due to protection from fat, uneven meat surfaces and small cuts [111]. Highly resistant beef isolate *E. coli* AW1.7 was isolated from carcasses after decontamination [49]. Typically, more effective treatments are also more detrimental to the quality of the meat [111]. Several outbreaks of *E. coli* and *Salmonella* linked to beef jerky [116–118] prompted the FSIS to intervene [17,119]. More than 250 clinically determined cases of foodborne illness were linked to beef jerky consumption between 1966 and 2003, with the actual number estimated to be significantly higher due to under-reporting of gastroenteric illnesses [118,119]. The FSIS recommended that manufacturers ensure pathogen reduction through testing and use a_W as the indicator for shelf-stability rather than the moisture:protein ratio [119]. Due to the established heat resistance of *Salmonella* in low a_W conditions, the USDA FSIS determined that *Salmonella* was more heat-resistant than other pathogens of concern and therefore processes must achieve a 5-log reduction of *Salmonella* [2,117,120]. *E. coli* also exhibit remarkable heat and desiccation resistance, and although there is no USDA standard for the required reduction of *E. coli* O157:H7, the industry standard is also a 5-log reduction [120].

Almonds are a nutritious snack high in vitamin E [121]. The popularity of almonds is increasing likely due to the health benefits and applications in dairy-free and gluten-free products [121]. The consumption of almonds in Canada increased by almost 30% between 2011 and 2018 [122] and in 2017, the US produced 80% of the world's almond harvest [121]. Outbreaks of *Salmonella* were linked to almonds in the US, Canada, Australia, and Sweden within the past 20 years [123]. Prior to an outbreak of *Salmonella* on almonds in Canada and the US between 2000 and 2001, almonds had never been associated with a foodborne illness outbreak and dried products such as nuts were considered low-risk foods due to an a_W below 0.7 [124]. The strain

responsible for the outbreak, PT30, was isolated from almonds 8 months after harvest and from orchard surfaces 1 year after harvest [125]. Although treatments achieving 4-log reduction of bacteria or greater became mandatory for almonds in California in 2007 [121], there have still been outbreaks of *Salmonella* in almond-products such as almond butter [123]. Other nut and seed products such as pistachios, cashews and cashew products, pine nuts, peanut butter, and coconut are frequently implicated in *Salmonella* outbreaks [123,126]; however, almonds are the only nut or seed product with a required *Salmonella* reduction [127]. The most common *Salmonella* serovars isolated from almonds are Montevideo, Thompson, Enteritidis and Typhimurium [128]. Coliforms and *E. coli* have been isolated from unprocessed almonds [129], and *E. coli* O157:H7 outbreaks have been linked to walnuts and hazelnuts [123]. Cattle are the primary reservoir for STEC, but contamination of other foods occurs through water, air, dust, soil or manure [15]. *Salmonella* contamination can occur on the tree from birds, insects or dust [12]. Additionally, during harvest, almonds are shaken off the trees and may sit on the ground for up to 2 weeks to reach the target kernel and hull moisture content [12] because the a_W of nuts is typically less than 0.7 [130]. However, this provides an opportunity for contamination from soil, rodents or birds [12]. Any rain or irrigation during this time provides a moist environment which facilitates rapid growth of bacteria to high numbers on the shell, and permits transfer of bacteria from the shell to the inside [12]. Almonds may also be held for up to several weeks at the de-shelling facility [125]. Cross-contamination may occur during the de-shelling process where high amounts of dust can transport *Salmonella* from shells to the surface of the kernel, or due to direct contact [12]. It has become clear that bacteria can survive for extended periods of time on almonds and other nuts. No reductions of *Salmonella* PT 30 were observed after storage at 4 °C for up to 18 months [130]. Kimber et al. [3] isolated multiple strains of *Salmonella* on almonds

and pistachios after storage at room temperature for 1 year even though greater than 2-log reductions were observed. *Salmonella* cell counts were reduced slower compared to *E. coli* O157:H7 and *L. monocytogenes* after storage at room temperature [3]. Tailing was observed for *E. coli* O157:H7 and *L. monocytogenes*, indicating a possible resistant sub-population of cells [3]. This information is relevant because untreated almonds and pistachios may be stored for a year or more by handlers during processing [3].

Previously, a 5-log reduction of *Salmonella* was required by the FDA to label almonds as pasteurized, and all almonds must be treated to achieve a 4-log reduction [12]. The FDA approved four almond decontamination processes; however, each has drawbacks. Propylene oxide is effective, but the process is too time-consuming for industrial scale procedures and consumers are concerned about chemical residues on almonds [12]. Hot oil can be used, but leaves residual oil on almonds, affecting the sensory characteristics [12]. Hot water treatments result in the loss of almond skin, affecting product quality [12]. Finally, steam treatments result in the loosening of skin and the formation of wrinkles on the surface of the almonds, affecting their quality [12]. A risk assessment by Lambertini et al. [127] determined that treatment method, followed by storage time had the largest impact on the risk of illness from almond consumption. Interestingly, in 2017, the FDA determined that a 4-log reduction of *Salmonella* is sufficient for companies to label almonds as pasteurized [121], potentially lowering the required efficacy of almond decontamination processes. It is important to note that roasting alone cannot achieve a 4-log reduction of *Salmonella* [12], likely due to its high heat resistance in low a_W environments.

1.11 Hypothesis and objectives

It has been clearly established that pathogenic *E. coli* and *Salmonella* are significant health concerns and may possess remarkable resistance to a variety of stressors, including heat, which is most frequently utilized in the food processing industry [14]. They are able to contaminate low a_W foods and have been implicated in outbreaks. Therefore, current methods to inactivate these pathogens in low a_W food products are not sufficient, and alternative strategies must be developed. HPCD is a well-characterized technique which shows potential to reduce *E. coli* and *Salmonella* in low a_W foods and offers attractive advantages to food processors in terms of product quality and clean labelling. However, the USDA FSIS compliance guidelines for beef jerky production are specific for only a few processes, which result in decreased product quality [2,119]. While the guidelines indicate that producers may validate their individual process to ensure a 5-log reduction of *Salmonella* and *E. coli*, it is not feasible for processors to perform scientific challenge studies in external facilities, nor is it acceptable to introduce pathogens into their processing facilities. Therefore, a non-pathogenic surrogate organism is necessary for effective in-plant validation of process efficacy. Due to high variation of stress resistance between strains of *E. coli* and *Salmonella*, surrogate organisms must be validated with relevant pathogens in the food matrix in which it will be used. Although *E. coli* O157:H7 is highly studied as a prototype for EHEC, it does not represent all STEC; therefore, a cocktail of STEC will be designed. A surrogate will be deemed acceptable if it displays resistance to treatments that is equal to or greater than that of the target organism(s).

It was hypothesized that HPCD reduced pathogens on beef jerky and almonds by 5 log and that *E. coli* AW1.7, due to its remarkable resistance toward desiccation and heat, is an acceptable surrogate organism to represent STEC and *Salmonella*. Therefore, the objectives of this research

were to: (1) Identify conditions that can achieve a 5-log reduction of desiccated *E. coli* and *Salmonella* cells with HPCD, (2) Identify a non-pathogenic surrogate organism that displays equal or higher resistance than pathogens and (3) Validate the HPCD technology and non-pathogenic surrogate organism in low a_W food matrices (beef jerky and almonds). Ultimately, the long-term goals of this research are to increase the industrialization potential of HPCD such that it can be implemented to inactivate pathogenic *E. coli* and *Salmonella* in solid and low a_W food products in a larger scale.

2. Materials and Methods

2.1 Bacterial strains and culture conditions

All strains used in this study are listed in Table 1. Stock solutions were prepared by mixing strains with 50% glycerol in media and stocks were stored at -80 °C. All stocks of *E. coli* and *Salmonella* were prepared for use in experiments, unless otherwise specified, by streaking onto Luria-Bertani (LB) (Fisher Scientific, Ottawa, ON) agar and incubated aerobically at 37 °C for 16-18 h. A single colony forming unit (CFU) was inoculated into 5 mL LB broth and incubated aerobically with agitation (200 rpm) at 37 °C for 16-18 h. Growth media for *S.* Typhimurium ATCC 13311 pLHR and *S.* Typhimurium ATCC 13311 pRK767 were supplemented with 15 mg/L tetracycline-HCl to select for the plasmid, which contained tetracycline resistance genes [5]. Differentiation between healthy and sublethally injured cells and selective enrichments of *Salmonella* and *E. coli* were done by plating onto Violet Red Bile Glucose Agar (VRBGA; Oxoid, Nepean, ON) and incubation at 37 °C ovenight. *S. carnosus* R6 FUA 2133 and *E. faecium* NRRL B-2354 were prepared similarly, but with Tryptic Soy (TS) agar or broth (Difco, Becton Dickinson, Mississauga, ON). Differentiation between healthy and sublethally

injured cells and selective enrichments of *E. faecium* were done using mEnterococcus Agar (Difco). *P. acidilactici* FUA 3072 was prepared similarly, but with De Man, Rogosa and Sharpe (MRS) agar or broth (Fisher Scientific) and incubated anaerobically. All overnight subcultures were plated onto agar and incubated at 37 °C for 16-18 h. The resulting bacterial lawn was harvested using 1 mL of 0.1% peptone water (Difco). Cells were centrifuged at 9,000 x *g* for 2 min and resuspended in 1 mL of 0.1% peptone water. The cell-suspension obtained here was used as the inoculum. The inoculum of 4 STEC, 03-2832 O121:H19, 05-6544 O26:H11, C0283 O157:H7, and 03-6430 O145:NM and 1 EPEC, PARC 449 O145:NM, were combined in equal volumes for a final volume of 3.5 mL and was hereafter referred to as the STEC cocktail. All other strains were treated individually. All subsequent resuspension of samples and dilutions were done in 0.1% peptone water and all subsequent incubations were done at the temperature and time conditions described above.

2.2 Confirmation of *Salmonella* isolates

Salmonella FUA 1917, FUA 1934, FUA 1946, FUA 1955, and FUA 1984 (Table 1) were isolated from a water treatment facility. To confirm the identity of these strains, DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI), according to the manufacturers protocol for Gram-negative bacteria. Concentration and quality of extracted DNA was measured using a NanoDrop (Thermo Scientific, Waltham, MA). Polymerase chain reaction (PCR) was done using an Eppendorf Mastercycler™ thermocycler (Eppendorf, Hamburg, Germany) with the following protocol: 94 °C for 3 min, 94 °C for 60 s, annealing temperature of 58 °C for 60 s, and extension at 72 °C for 90 s all for 35 cycles, followed by a final extension at 72 °C for 3 min, and cooling to 4 °C. *Salmonella*-specific primers [131] were used, with the forward primer sequence being 5'-TGCGGCTGGATCACCTCCTT-3' and the

reverse primer sequence being 5'-TATAGCCCCATCGTGTAGTCAGA-3'. PCR products were visualized with gel electrophoresis, using a 1% agarose gel at 115 V with SYBR safe 10,000X (Thermo Fisher, Ottawa, ON). The ladder used was 5 μL of the GeneRuler 1 Kb Plus (Thermo Fisher).

Table 1: Bacterial strains used in this study, source, and reference.

Strain	Source	Reference
E. coli AW1.7	Slaughter facility	[112]
E. coli AW1.7 ΔpHR1	Laboratory strain	[132]
E. coli AW1.7 Δ*cfa*	Laboratory strain	[133]
E. coli K-12 MG1655 ATCC 700926	Sensitive laboratory strain	ATCC
E. coli 03-2832 O121:H19	Human	[50]
E. coli 05-6544 O26:H11	Human	[50]
E. coli C0283 O157:H7	Cattle	[50]
E. coli PARC 449 O145:NM	Unknown	[50]
E. coli 03-6430 O145:NM	Human	[50]
Enterococcus faecium NRRL B-2354	Dairy	[134]
Pediococcus acidilactici FUA 3072	Sausage	[135]
S. enterica Typhimurium ATCC 13311	Human	ATCC
S. enterica Typhimurium ATCC 13311 pLHR	Laboratory strain	[5]
S. enterica Typhimurium ATCC 13311 pRK767	Laboratory strain	[5]
S. enterica Senftenberg ATCC 43845	Eggs	[136]
Salmonella FUA 1917	Water treatment facility	This study
Salmonella FUA 1934	Water treatment facility	This study
Salmonella FUA 1946	Water treatment facility	This study
Salmonella FUA 1955	Water treatment facility	This study
Salmonella FUA 1984	Water treatment facility	This study
Staphylococcus carnosus R6 FUA 2133	Meat starter culture	[137]

2.3 Preparation of beef jerky

Beef jerky was prepared using 2 beef inside rounds obtained from a federally inspected facility and were stored at -20 °C until use. Inside rounds (8.30 kg) were thawed at -1 °C until slightly frozen, sliced into 6 mm thick slices using a Berkel Model X13 meat slicer (Berkel, Chicago IL). Beef jerky seasoning (without MSG) (Unipac, Edmonton, AB) and cure COOAE1 (Newly Weds Foods, Yorkville, IL) were added according to the manufacturer's recommendations for the

preparation of a marinated product, and sliced beef was marinated in the brine for 16-18 h at 4 °C. Slices were placed on racks, transferred into an ALKAR processing oven and smokehouse (ALKAR-RapidPak Inc., Lodi, WI) and treated using the conditions described in Table 2. Temperature probes monitored 4 areas in the smokehouse, and the a_W of beef jerky was measured with a water activity meter (CX-2, Aqualab by Meter, Pullman, WA) every 0.5 h after the first hour until the product reached a final a_W around 0.75. The beef jerky remained in the smokehouse for a total of 3.75 h with a final a_W of 0.74-0.75. After processing, beef jerky was double bagged in 3 mm and 4 mm vacuum bags (Unipac, Edmonton, AB) with 5 slices per bag and sealed using a vacuum packager (Multivac Inc. Model C200, Kansas City, MO). Samples were stored at 0 °C until further use.

Table 2: Conditions used during beef jerky processing. DB, dry bulb; WB, wet bulb; RH, relative humidity.

Step	Step Time (h)	DB Temp. (°C)	WB Temp. (°C)	%RH	Exhaust Fan	Exhaust Damper	Smoke Preheat
Cook	00:30	35	25	45	On	Auto	10
Smoke cook	01:00	35	24	40	Off	Closed	---
Smoke cook	01:00	45	29	30	Off	Closed	---
Cook	01:00	55	34	25	On	Auto	---
Cook	02:00	80	48	20	On	Auto	---

2.4 Sample preparation

2.4.1 Preparation of desiccated bacteria for heat treatments

E. coli AW1.7 & AW1.7 ΔpHR1, and *S.* Typhimurium ATCC 13311 pLHR & pRK767 were grown in TS agar or broth. Samples (20 µL) of inoculum were dried in glass vials (12x30 mm; Supelco, Sigma Aldrich, Oakville, ON) for 6-8 h inside a biosafety cabinet. A sample of the liquid inoculum was diluted and plated to determine initial cell counts. After drying, samples were stored for 16-18 h 37 °C in air-tight containers with different desiccants to equilibrate

samples to a specific a_W (Table 3) [138]. One set of samples was resuspended, diluted and plated after drying to determine cell counts after the drying process. Samples were exposed to 60 °C for 5 or 15 min using a water bath (Thermo Scientific NESLAB EX 7, Waltham, MA). After treatments, samples were resuspended, diluted and plated to determine the cell counts.

Table 3: Saturated salt solutions used as desiccants, and their respective water activities.

Desiccants	Corresponding water activity
Silica gel beads	0.1
Sodium chloride	0.75
Ammonium sulfate	0.8
Potassium nitrate	0.9-0.95
Potassium sulfate	0.96-0.98
Water	0.99-1.0

2.4.2 Preparation of desiccated bacteria for HPCD treatments

Samples (20 µL) of inoculum of all strains were dried in individual lids which had been removed from 1.5 mL eppe tubes (Fisher Scientific) and transferred to a biosafety cabinet for 6-8 h. A sample of the liquid inoculum was diluted and plated to determine initial cell counts, which ranged from 10-12 log CFU/mL. After drying, samples were stored for 16-18 h at 37 °C in an air-tight container with silica gel beads or a saturated solution of sodium chloride to equilibrate cells to a_W 0.1 or 0.75, respectively. One set of samples was resuspended, diluted and plated after drying and equilibration to determine the cell counts after the drying process. Remaining samples were treated with HPCD using the SITEC Phase Equilibria Apparatus or the Mobile Pasteurization Apparatus as described below. After treatments, samples were resuspended, diluted and plated to determine the cell counts.

2.4.3 Preparation of beef jerky and almond samples for HPCD treatments

Whole beef jerky slices were removed from vacuum packages and cut into rectangular samples with an average surface area of 2 cm^2. Whole almonds with the skin attached were purchased

from a local supermarket (The Real Canadian Superstore, Edmonton, AB) for use in this experiment. Individual beef jerky samples were vortexed in the inoculum for 30 s to inoculate samples with different strains. Almonds were inoculated the same way, but only with strains of *Salmonella* and *S. carnosus*. After inoculation, samples were air-dried for 10 min in a biosafety cabinet to remove surface moisture. Initial cell counts from beef jerky samples and almonds after inoculation were determined by resuspending each sample in 1 mL of 0.1% peptone water, vortexed twice for 20 s with a 1-min rest period between each vortex, and diluted and plated. Initial cell counts on beef jerky ranged between 9-10 log CFU/2cm^2. Initial cell counts on almonds ranged between 8-10 CFU/almond. Remaining beef jerky samples were transferred to an air-tight container containing either a saturated solution of sodium chloride or water and stored at room temperature (22 °C) for 2 weeks or stored for 1 week to equilibrate samples to a_W 0.75 or 0.9, respectively. Almonds were transferred to air-tight containers containing water and stored at room temperature (22 °C) for 1 week to equilibrate samples to a_W 0.9. Cell counts from one set of samples was determined after equilibration to identify the reduction of cell counts from the equilibration/drying process. Remaining samples were treated with HPCD or pressurized N_2 in the Mobile Pasteurization Apparatus. Half of almond samples were dipped in sterile water immediately prior to treatment with HPCD. After treatments, samples were resuspended in 0.1% peptone water, which was diluted and plated to determine the reduction of cell counts. For samples inoculated with *E. coli* and *Salmonella* the number of sub-lethally injured cells was determined by comparing cell counts obtained from TS agar and VRBGA. The same comparison was done for samples inoculated with *E. faecium* but with TS agar and mEnterococcus Agar. Beef jerky samples and almonds inoculated with *E. coli*, *Salmonella*, and *E. faecium* also underwent a selective enrichment after HPCD treatment in their respective

selective media to detect growth when cell counts were below the detection limit (1 log CFU/2cm^2). Samples were incubated in TS broth at 37 °C for 16-18 h. The overnight culture was streaked onto TS agar and VRBGA or mEnterococcus agar for samples inoculated with *E. coli* and *Salmonella* or *E. faecium*, respectively.

2.4.4 Preparation of beef jerky and almond samples for HPCD treatments at Fraunhofer UMSICHT

Whole beef jerky slices (The Meat Makers, Beef Steak: Original, Siauliai, Lithuania) were cut into rectangular samples with an average surface area of 2 cm^2 and vortexed with inoculum for 30 s to inoculate the surface with *S. carnosus*. Whole almonds with the skin attached were purchased from a local supermarket (Alesto kalifornische Mandeln naturbelassen, Lidl, Oberhausen, Germany) and were inoculated according to the same procedure as described in Section 2.4.3. After inoculation, samples were dried in a biosafety cabinet for about 1 h to remove surface moisture. For beef jerky, 5 samples were resuspended in 5 mL of 0.1% peptone water, and for almonds 10 whole almonds were resuspended in 10 mL of 0.1% peptone water. Samples were vortexed twice for 20 s with a 1-min rest period between each vortex. Samples were resuspended in 0.1% peptone water, which was diluted and plated onto TS agar to determine the initial counts of bacteria on samples after inoculation. Remaining samples were loosely covered with sterile aluminium foil and transferred to a humidity chamber (Weiss Technik Typ 100/+10, Oberhausen, Germany) set to a temperature of 20 °C and a relative humidity of 75% or 90% to equilibrate samples to a_W 0.75 or 0.90, respectively. Equilibration took approximately 3 d, and samples were stored at these conditions in the relative humidity chamber until used for HPCD treatments. Cell counts from one set of samples was determined

after equilibration to identify the cell reduction due to the equilibration/storage process. After treatments, samples were resuspended, diluted and plated to determine cell counts.

2.5 HPCD units and treatments

2.5.1 SITEC Phase Equilibria Apparatus

Figure 1 depicts the schematic diagram of the SITEC unit (SITEC-Sieber Engineering AG, Ebmatingen, Switzerland). Briefly, a CO_2 cylinder with syphon for liquid withdrawal was connected to a CO_2 pump (Model 260D, Teledyne ISCO Inc., Lincoln, NE) and was cooled with a refrigeration bath (VWR Model 1162A, Radnor, PA) to ensure the CO_2 remained in the liquid phase during compression. A heating bath (Lauda RMT RM6, Delran, NJ) was used to circulate a 1:1 water antifreeze mixture to heat the vessel to the desired temperature. The internal volume of the vessel was 10 mL. The vessel was equipped with a temperature probe (Thermolyne Digital Pyrometer, Sigma Aldrich, Oakville, ON). A micrometering valve was used at the outlet to allow for a controlled depressurization rate. Vials were placed directly inside the vessel, which was preheated to 65 °C, with the lid slightly loosened to allow for maximum CO_2 exposure. The vessel was hand-tightened, and all exit valves were closed until hand-tight. The vessel was first flushed with CO_2 at pressures not exceeding 1 MPa to remove oxygen from the system prior to fully pressurizing to the desired pressure. Preliminary experiments were conducted at 5.7 MPa and 65 °C for 15 min based on previous research [139]. The treatment time was considered to start after pressurization was completed and all valves were closed. After the treatment, the system was depressurized at a rate of 6 MPa/min. The samples were removed from the vessel for microbiological analysis. Only preliminary experiments were conducted in the SITEC Phase Equilibria Apparatus, therefore no data analysis was done.

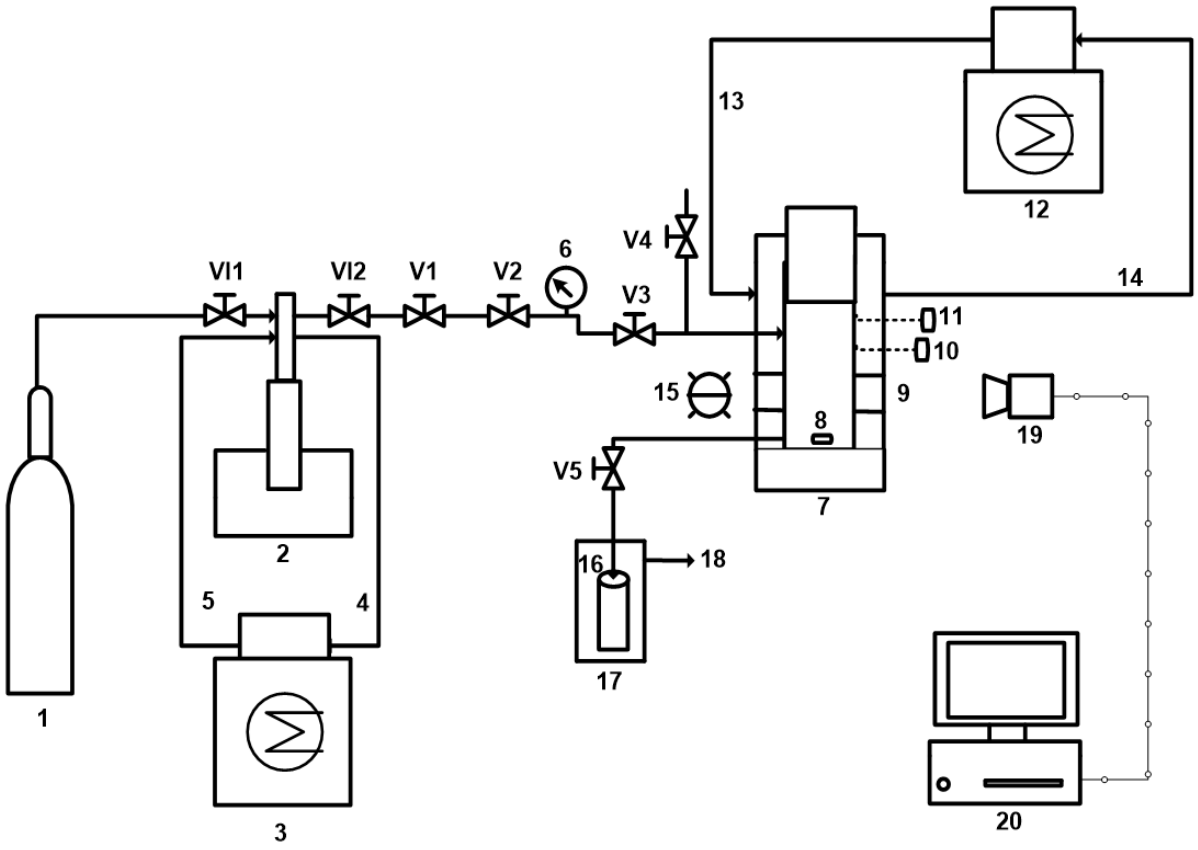


Figure 1: Schematic diagram of the SITEC Phase Equilibria Apparatus. 1, CO_2 cylinder; 2, ISCO syringe pump; 3 and 12, cooling and heating water bath, respectively; 4, cooling water outlet line; 5, cooling water inlet line; 6, pressure gauge (gauge 2); 7, magnetic stirrer; 8, stir bar; 9, sapphire window; 10, temperature sensor; 11, pressure sensor; 13, heating water inlet line; 14, heating water outlet line; 15, cold light source; 16, sampling vial; 17, cold trap; 18, CO_2 outlet ; 19, camera; 20, computer. VI1, VI2, inlet and outlet needle valves for the ISCO pump, respectively; V1 – V5, needle valves.

2.5.2 Mobile Pasteurization Apparatus

Figure 2 shows the schematic diagram of the Mobile Pasteurization Apparatus which was designed and constructed at the University of Alberta using high-pressure parts. Briefly, a CO_2 or N_2 cylinder with syphon for liquid withdrawal was connected to a pump (Model 260D, Teledyne ISCO Inc., Lincoln, NE). Another high-pressure liquid pump (Model Reaxus 6010R, Teledyne ISCO Inc., Lincoln, NE) was used to pump water into the CO_2 line through a T-connection. CO_2 and water flowed into the vessel, which had an internal volume of 42.53 mL. The CO_2 and N_2

pump was cooled with a refrigeration bath (Lauda Alpha R8, Delran, NJ) to ensure the CO_2 remained in the liquid phase for compression. Electrical heating bands (Brisk Heat, Columbus, OH) surrounded the vessel, as well as the lines before and after the vessel to heat the system to the desired temperature. The vessel contained temperature probes from Maxthermo (Taipei, Taiwan) for the line before and after the vessel, and one from Omega (Spectris Canada, St-Eustache, QC) which measured the temperature in the vessel. The system also contained several pressure gauges (Swagelok, Cleveland, OH). A relative humidity probe (JUMO Process Control Inc., Syracuse, NY) was added to the system and located at the bottom of the vessel, extending to the inside of the vessel to take measurements, however it was later removed since it was unable to give accurate measurements under high pressure CO_2 conditions. A micrometering valve was installed after the outlet of the vessel to control the depressurization rate. An in-line 0.2 μm pore filter was installed after the micrometering valve to prevent the exhaust of bacteria from the unit. A metal wire basket with 3 shelves was constructed to allow for simultaneous treatment of multiple samples during one cycle. The unit was designed with 2 pumps for simultaneous pumping of CO_2 and water followed by a static mixer to be able to maintain a constant relative humidity in the system during potential continuous treatments; however, the water pump was not used for batch treatments performed in this study due to the difficulty in the uniform delivery of the small amounts of water that were needed. Instead, an alternative method for moisture addition was developed in which sterile water was pipetted onto filter paper (Whatman, Pore Size: 8 μm, 110 mm diameter, Sigma Aldrich, Oakville, ON), which was placed on the top shelf of the sample basket, directly below the inlet. Enough water was added such that the gaseous environment in the vessel was saturated with water based on the solubility of water in the gas at 65 °C and 5.7 MPa. Therefore, 30 μL was added to treatments using CO_2, and since the

solubility of water in N_2 was calculated to be 3.5 times that in CO_2 [140,141], 105 µL was added to treatments using N_2. Bacterial samples in lids were placed in the basket with one sample per shelf. Beef jerky and almond samples were placed directly on the basket shelves with one sample per shelf, and the basket was dipped in 100% ethanol and flamed between treatments. The basket was placed into the vessel, which was preheated to 65 °C. The vessel was sealed using wrenches, and all exit valves were closed until hand-tight. In experiments using the water pump for the addition of moisture, the vessel was first flushed with CO_2 at pressures not exceeding 1 MPa to remove oxygen from the system. In experiments where the addition of water was done using filter paper it was not possible to flush the system first with CO_2 or N_2. The system was pressurized to 5.7 MPa or 12.0 MPa, where treatments were conducted at 65 °C for 1, 4, 8, or 15 min. The treatment time was considered to start after pressurization was completed and all valves were closed. After the treatment was completed, the system was depressurized at a rate of 6 MPa/min. The samples were then removed from the vessel for microbiological analysis.

2.5.3 HPCD unit at Fraunhofer UMSICHT

Figure 3 depicts the schematic diagram of the HPCD unit used at the Fraunhofer UMSICHT institute. The unit was installed by Premex (Premex Solutions GmbH, Lyss, Germany) and slightly modified as follows. Briefly, a CO_2 pump (LEWA GmbH, Leonberg, Germany) compressed the CO_2, which then passed through a heat exchanger (Natex Prozesstechnologie, Innoweld Metallverarbeitung GmbH, Ternitz, Austria), to increase the temperature prior to reaching the vessel, which had an internal volume of 500 mL. The vessel was heated from the bottom, and contained a stirrer (Twister HPM, Premex Solutions GmbH, Lyss, Germany) for agitation of samples during treatment, which was connected to the top of the vessel. The system contained pressure gauges from WIKA (WIKA Alexander Wiegand SE & Co. KG, Klingenberg,

Germany). A metal basket was constructed for the treatment of multiple samples during one cycle. Beef jerky or almond samples were placed directly in the basket, and the basket was decontaminated with 70% ethanol between treatments. The basket was attached to the vessel, which was preheated to 40 °C or 60 °C. The vessel was closed, and all exit valves were closed until hand-tight. To maintain the moisture level inside the system during treatments, 1.5 mL of water was added directly into the bottom of the vessel. The system was pressurized to 6, 10 or 12 MPa, where treatments were conducted for 4 or 15 min. The treatment time was considered to start after pressurization was completed and all valves were closed. After the treatment was completed, the system was depressurized. Beef jerky samples were depressurized at a rate of 6 MPa/min. However, almonds had to be depressurized slower to prevent cracking due to CO_2 expansion. They were depressurized at 6 MPa/min until the pressure reached 4 MPa, 0.5 MPa/min until the pressure reached 2 MPa, 0.25 MPa/min until the pressure reached 1 MPa, and 0.1 MPa/min until the pressure reached ambient pressure. The samples were then removed from the vessel for microbiological analysis.

2.6 Statistical analysis

All experiments were conducted in triplicate independent experiments. Raw data underwent a log transformation and the log cell reduction was calculated by subtracting log cell counts after treatments from initial log cell counts. Data were subjected to Analysis of Variance using the PROC GLM procedure of the University Edition of SAS (SAS Institute Inc., Cary, NC). Tukey's posthoc test was used to determine differences among means. The significance value was $P<0.05$.

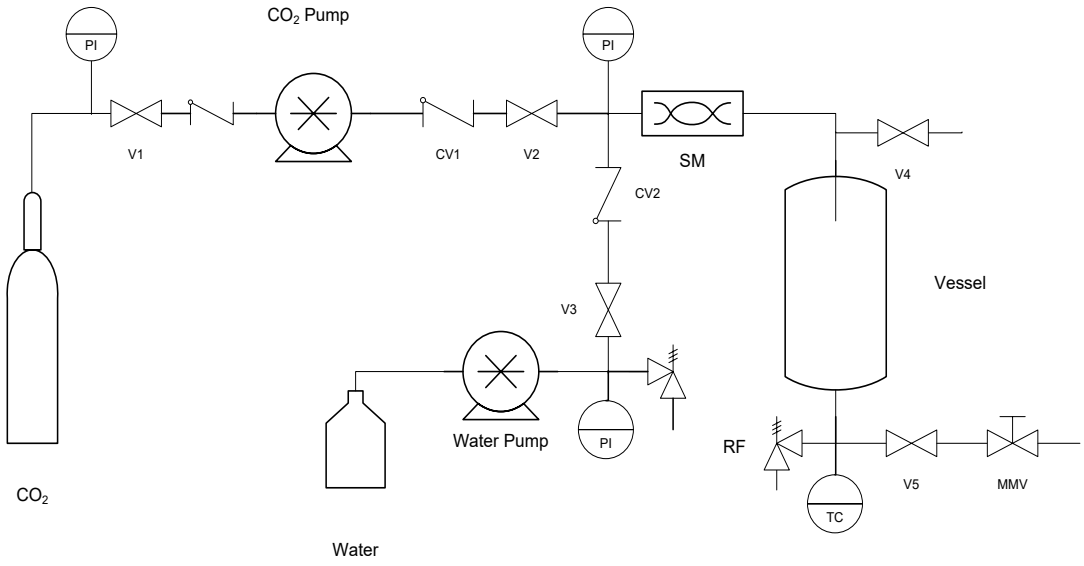


Figure 2: Schematic diagram of the Mobile Pasteurization Apparatus (MPA). V1 – V5, needle valves; CV1 – CV2, check valves; SM, static mixer; PI, pressure gauges; RF, safety release valve; MMV, micrometering valve; TC, thermocouple.

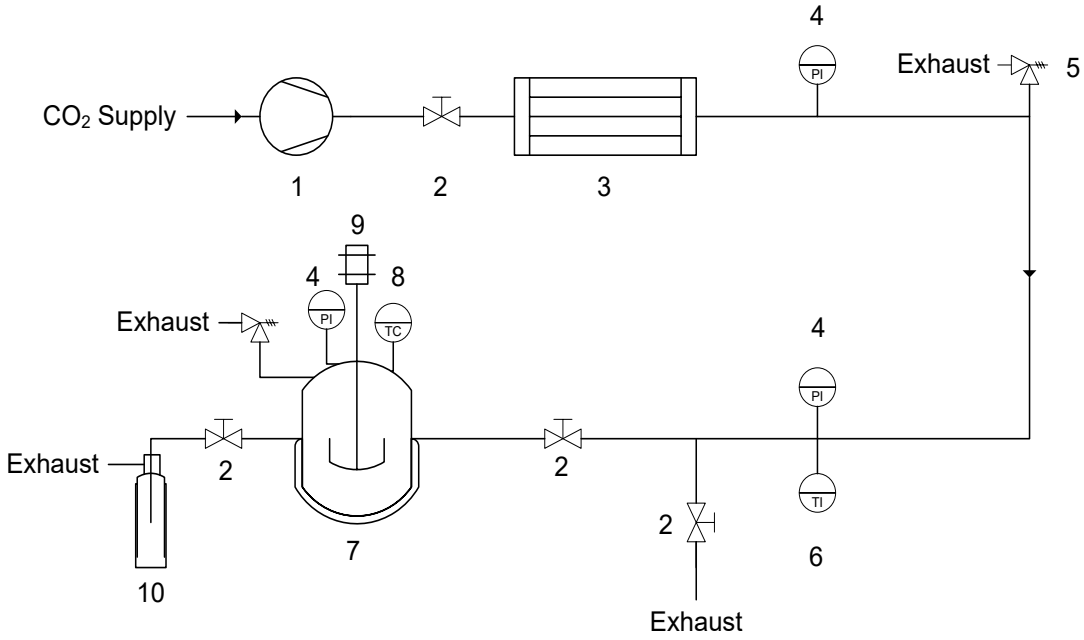


Figure 3: Schematic diagram of the Microbial Inactivation Unit used at Fraunhofer UMSICHT. 1, gas compressor; 2, needle valve; 3, heat exchanger; 4, pressure indicator; 5, safety relief valve; 6, temperature indicator; 7, vessel; 8, temperature controller; 9, magnetic mixer; 10, ethanol bath to disinfect used CO_2.

3. Results

3.1 Confirmation of novel *Salmonella* isolates

Isolates were obtained from a local waste water treatment facility, an environment containing a diverse range of bacteria, therefore it was necessary to confirm their identity as *Salmonella.* Results from PCR and gel electrophoresis (Appendix, Figure S1) successfully confirmed the 5 unknown strains as *Salmonella.* The presence of a strong band with a size of approximately 312 bp is consistent with the size of the primers [131]. The results are strengthened by the lack of amplification of the two negative controls, one of which contained no template, and the other contained *E. coli* DNA. Additionally, the positive control containing DNA from a known *Salmonella* strain had the same size band. Therefore, the unknown strains were identified as *Salmonella*.

3.2 Effect of heat treatments on desiccated bacteria

Initial experiments were necessary to quantify the heat tolerance of desiccated *E. coli* and *Salmonella* at different a_W before comparisons with those treated with HPCD. This was done using isogenic strains of *E. coli* and *Salmonella* with and without the LHR. *E. coli* AW1.7, *E. coli* AW1.7 ΔpHR1, *S.* Typhimurium ATCC 13311 pLHR and *S.* Typhimurium ATCC 13311 pRK767 were equilibrated to various water activities and heated at 60 °C for 5 or 15 min. After treatment for 5 min, the reduction of cell counts of *S.* Typhimurium ATCC 13311 pRK767 and *E. coli* AW1.7 at any a_W were not different compared to their respective desiccated controls. *E. coli* AW1.7 ΔpHR1 re-equilibrated to a_W 0.99-1.0 was reduced by heat treatment for 5 min compared to its desiccated control. *S.* Typhimurium ATCC 13311 pLHR was reduced when re-equilibrated to a_W of 0.9 or higher. The maximum reduction was observed at a_W 0.96-0.98 with a

greater than 7-log CFU/mL reduction (Figure 4). At a_W 0.96-0.98 and at a_W 0.99-1.0 *S.* Typhimurium ATCC 13311 pLHR was more sensitive to heat treatment for 5 min compared to the other 3 strains (Figure 4).

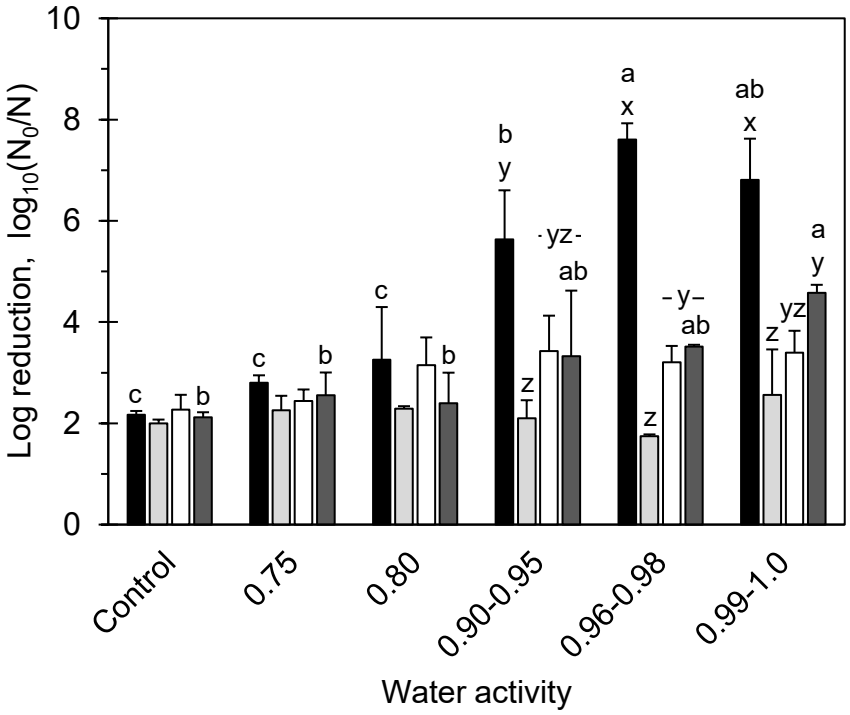


Figure 4: Influence of a_W on the reduction of *S.* Typhimurium ATCC 13311 pLHR (black bars), *S.* Typhimurium ATCC 13311 pRK767 (light grey bars), *E. coli* AW1.7 (white bars), and *E. coli* AW1.7 ΔpHR1 (dark grey bars) cells after drying, equilibration, and heat treatment at 60 °C for 5 min. Data are means ± standard deviation (n=3). Letters a, b and c denote significant differences among treatments for the same strain ($P<0.05$). Letters x, y and z denote significant differences among strains in the same treatment group ($P<0.05$).

After treatment for 15 min the reduction of cell counts of *S.* Typhimurium ATCC 13311 pRK767 were not different from its desiccated control at any a_W (Figure 5). *E. coli* AW1.7 re-equilibrated to a_W of 0.9-0.95, 0.96-0.98 and 0.99-1.0 was reduced by heat treatment for 15 min compared to its desiccated control, but *E. coli* AW1.7 ΔpHR1 was only reduced when samples were re-equilibrated to a_W 0.99-1.0. *S.* Typhimurium ATCC 13311 pLHR samples re-equilibrated to a_W 0.9-0.95, 0.96-0.98 and 0.99-1.0 were reduced by heat treatment for 15 min (Figure 5).

S. Typhimurium ATCC 13311 pLHR was more sensitive compared to the other 3 strains at a_W at or above 0.9. Additionally, at a_W 0.96-0.98, *S.* Typhimurium ATCC 13311 pRK767 was more resistant to heating than the other 3 strains. Overall, re-equilibration to a_W at or above 0.90 was detrimental for LHR-containing *Salmonella*, while *Salmonella* without the LHR continue to survive. However, the presence or absence of the LHR had less effect on the survival of *E. coli* AW1.7 at various a_W.

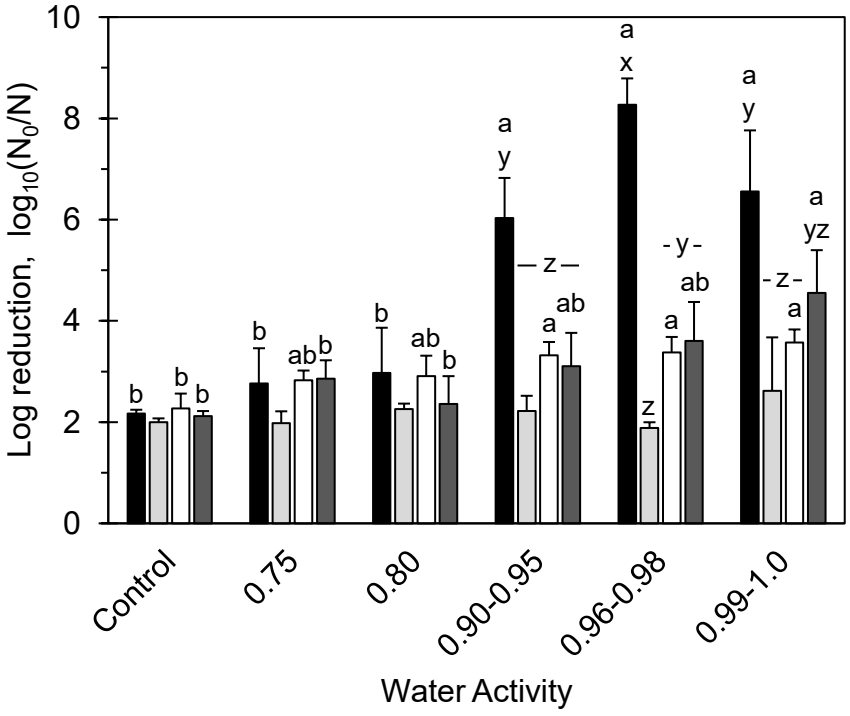


Figure 5: Influence of a_W on the reduction of *S.* Typhimurium ATCC 13311 pLHR (black bars), *S.* Typhimurium ATCC 13311 pRK767 (light grey bars), *E. coli* AW1.7 (white bars), and *E. coli* AW1.7 ΔpHR1 (dark grey bars) cells after drying, equilibration, and heat treatment at 60 °C for 15 min. Data are means ± standard deviation (n=3). Letters a and b denote significant differences among treatments for the same strain ($P<0.05$). Letters x, y and z denote significant differences among strains in the same treatment group ($P<0.05$).

3.3 Response of desiccated novel *Salmonella* isolates to heat

Experiments were conducted with the novel *Salmonella* isolates to determine their desiccation and heat resistance before treatment with HPCD. Heating desiccated cells of *Salmonella* FUA

1934, FUA 1946 and FUA 1955 for 1, 4 or 15 min at 60 °C consistently resulted in about 2 log-reduction (Figure 6). There were no differences among strains treated for the same amount of time. Changing the treatment time did not affect cell reductions for any strain. Overall, the three *Salmonella* isolates behaved similarly to each other, and all three strains demonstrated heat resistance after desiccation.

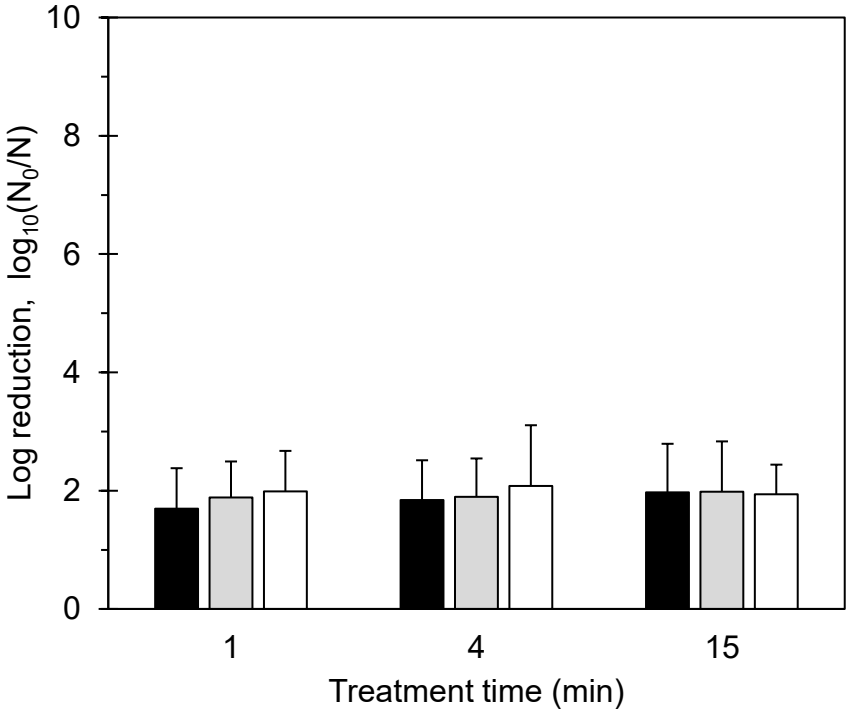


Figure 6: Effect of heating at 60 °C for 1, 4, or 15 min on *Salmonella* FUA 1934 (black bars), *Salmonella* FUA 1946 (grey bars), and *Salmonella* FUA 1955 (white bars) after desiccation and equilibration to a_W 0.75. Data are means ± standard deviation (n=3).

3.4 Impact of HPCD on reduction of desiccated cells

3.4.1 Influence of sample container on the reduction of *E. coli*

It was necessary to compare different sample containers because samples used in preliminary experiments conducted in the SITEC unit were prepared in glass vials, whereas the construction of the Mobile Pasteurization Apparatus allowed for the treatment of uncontained samples.

Additionally, beef jerky and almond samples could not be treated in vials due to the sample size. Figures S2 and S3 in the Appendix compare the effect of drying *E. coli* AW1.5, MG1655, and STEC cocktail samples in glass vials and plastic lids, followed by equilibration to a_W 0.1 or 0.75, respectively and treatment with dry CO_2 at 5.7 MPa and 65 °C for 15 min. The drying container had no effect on the reduction of *E. coli* AW1.7 or the STEC cocktail at a_W 0.1 or 0.75, therefore the results obtained using the SITEC Phase Equilibria Apparatus and the Mobile Pasteurization Unit can be directly compared, and differences observed among results are not due to the container.

3.4.2 Influence of water addition on the reduction of *E. coli*

The addition of water increased the reduction of *E. coli* AW1.7, MG1655 and the STEC cocktail when cells were equilibrated to a_W 0.75 and treated at 5.7 MPa and 65 °C for 15 min (Figure 7). Cell counts of *E. coli* MG1655 were reduced by treatment with dry and saturated CO_2. However, treatment with dry CO_2 did not reduce cell counts of *E. coli* AW1.7 and the STEC cocktail compared to their respective desiccated controls. In contrast, treatment with gaseous CO_2 saturated with water decreased cell counts for all strains compared to the treatment without water and the desiccated untreated control. These results highlight the crucial role that water plays in the reduction of cell counts with pressurized CO_2.

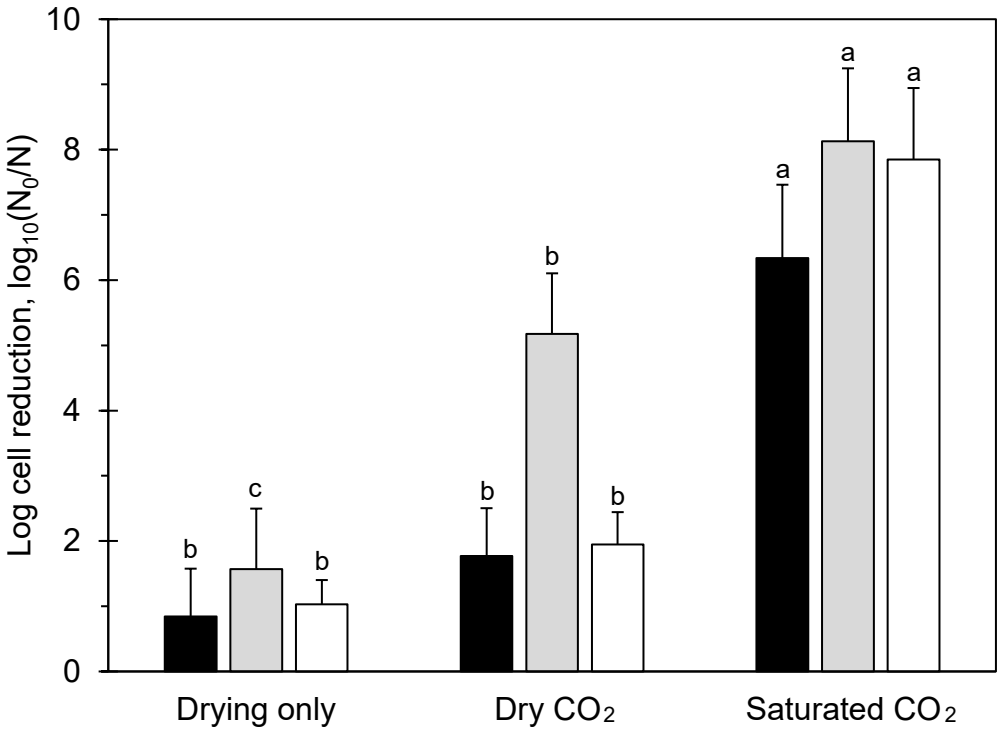


Figure 7: Effect of water on the reduction of *E. coli* AW1.7 (black bars), *E. coli* MG1655 ATCC 700926 (grey bars), and the STEC cocktail (white bars) desiccated to a_W 0.75, followed by CO_2 treatment at 5.7 MPa and 65 °C for 15 min. In the "Dry CO_2" treatment no water was added to the vessel. In the "Saturated CO_2", water was added to saturate gaseous CO_2. Data are means ± standard deviation (n=3-6). Letters a, b and c denote significant differences among treatments for the same strain ($P<0.05$).

3.4.3 Influence of treatment time on the reduction of *E. coli*, *Salmonella*, *P. acidilactici*, *E. faecium* and *S. carnosus* subjected to HPCD treatment

Experiments were done to determine if the length of the HPCD treatment affected the reduction of cell counts, and if so to identify the treatment time that provided the greatest reduction in cell counts. Reductions in numbers of *E. coli* AW1.7, AW1.7 Δ*cfa*, MG1655 and the STEC cocktail were determined after treatment with saturated gaseous CO_2 at 5.7 MPa and 65 °C for 4, 8 or 15 min (Figure 8). There were no differences between reductions of *E. coli* AW1.7 and the STEC cocktail at any time, therefore *E. coli* AW1.7 can be a surrogate for STEC under these

conditions. All treatments reduced cell counts compared to the control group (dried cells); however, changing the treatment time did not affect the reduction of cell counts.

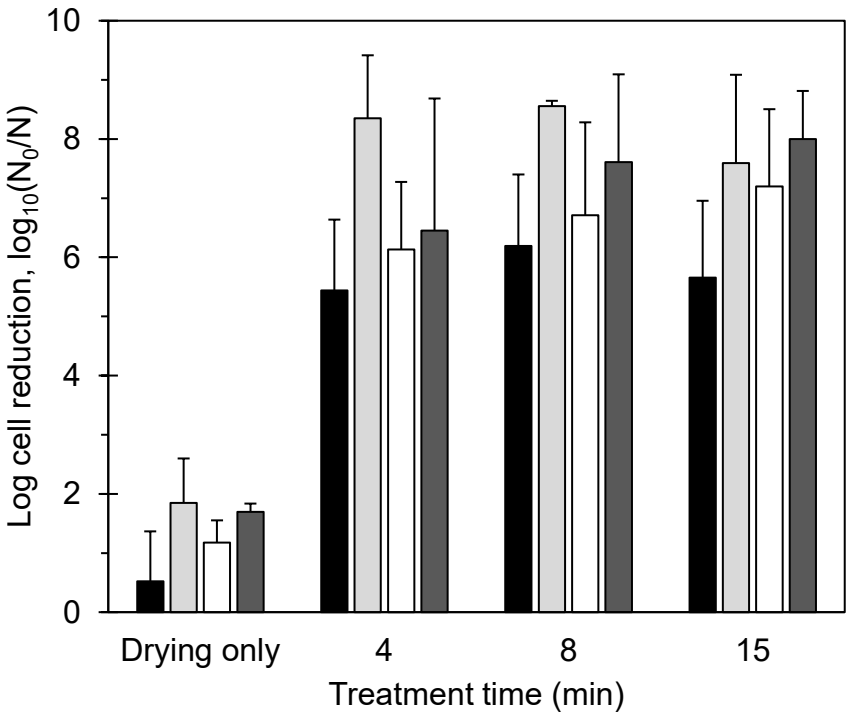


Figure 8: Effect of treatment time on the reduction of *E. coli* AW1.7 (black bars), *E. coli* MG1655 ATCC 700926 (light grey bars), the STEC cocktail (white bars), and *E. coli* AW1.7 Δ*cfa* (dark grey bars) desiccated to a_W 0.75, followed by treatments with saturated gaseous CO_2 at 5.7 MPa and 65 °C. Data are means ± standard deviation (n=3).

Reductions in cell numbers of *S.* Typhimurium ATCC 13311, *S.* Senftenberg ATCC 43845, *Salmonella* FUA 1934, *Salmonella* FUA 1946, *Salmonella* FUA 1955, *P. acidilactici*, *E. faecium* and *S. carnosus* were determined after treatment with saturated gaseous CO_2 at 5.7 MPa and 65 °C for 1, 4, 8 or 15 min (Figure 9). It was hypothesized that the behaviour of all 5 *Salmonella* strains subjected to HPCD with saturated gaseous CO_2 mimics the behaviour of *E. coli* AW1.7. No significant differences were identified between cell reductions of *E. coli* AW1.7 and reductions of strains of *Salmonella* when they were treated as desiccated cells. Other bacteria were introduced as potential surrogate organisms. *P. acidilactici* FUA 3072 was tested because

Pediococcus was previously used as a surrogate organism in beef jerky production [2]; however, when desiccated, it was more sensitive to HPCD compared to *Salmonella* FUA 1934 at all treatment times, *Salmonella* FUA 1946 after 1, 4 and 8 min of treatment, and *Salmonella* FUA 1955 after 8 min of treatment (Figure 9); thus, it was not a suitable surrogate for *Salmonella*. *E. faecium* NRRL B-2354 was evaluated as it has been used as a surrogate for *Salmonella* by other researchers [134]. *E. faecium* was more resistant than *S.* Typhimurium ATCC 13311 and *S.* Senftenberg ATCC 43845 after 1 and 15 min of treatment and *Salmonella* FUA 1955 after 15 min of treatment (Figure 9). Therefore, *E. faecium* NRRL B-2354 is a suitable surrogate in terms of its behaviour compared to the target organisms when treated with HPCD at these conditions. However, due to the controversial pathogenicity of enterococci and national importation legislation, this strain was not permitted for use in experiments conducted at the Fraunhofer UMSICHT institute in Germany. Therefore, *S. carnosus* R6 FUA 2133 was introduced because it is a non-pathogenic starter culture. *S. carnosus* was more resistant to treatment with HPCD than *S.* Typhimurium ATCC 13311 after all treatments, *S.* Senftenberg ATCC 43845 after 1 and 15 min of treatment, and *Salmonella* FUA 1946 and *Salmonella* FUA 1955 after 15 min of HPCD treatment (Figure 9). Thus, *S. carnosus* R6 FUA 2133 is an acceptable surrogate for the five *Salmonella* strains used in this study when treated with HPCD at these conditions because it is equally or more resistant than the target organisms. Ultimately, *S. carnosus* was selected as a surrogate for *Salmonella* strains in experiments conducted at the Fraunhofer UMSICHT institute because of its comparable behaviour, and confirmed non-pathogenicity.

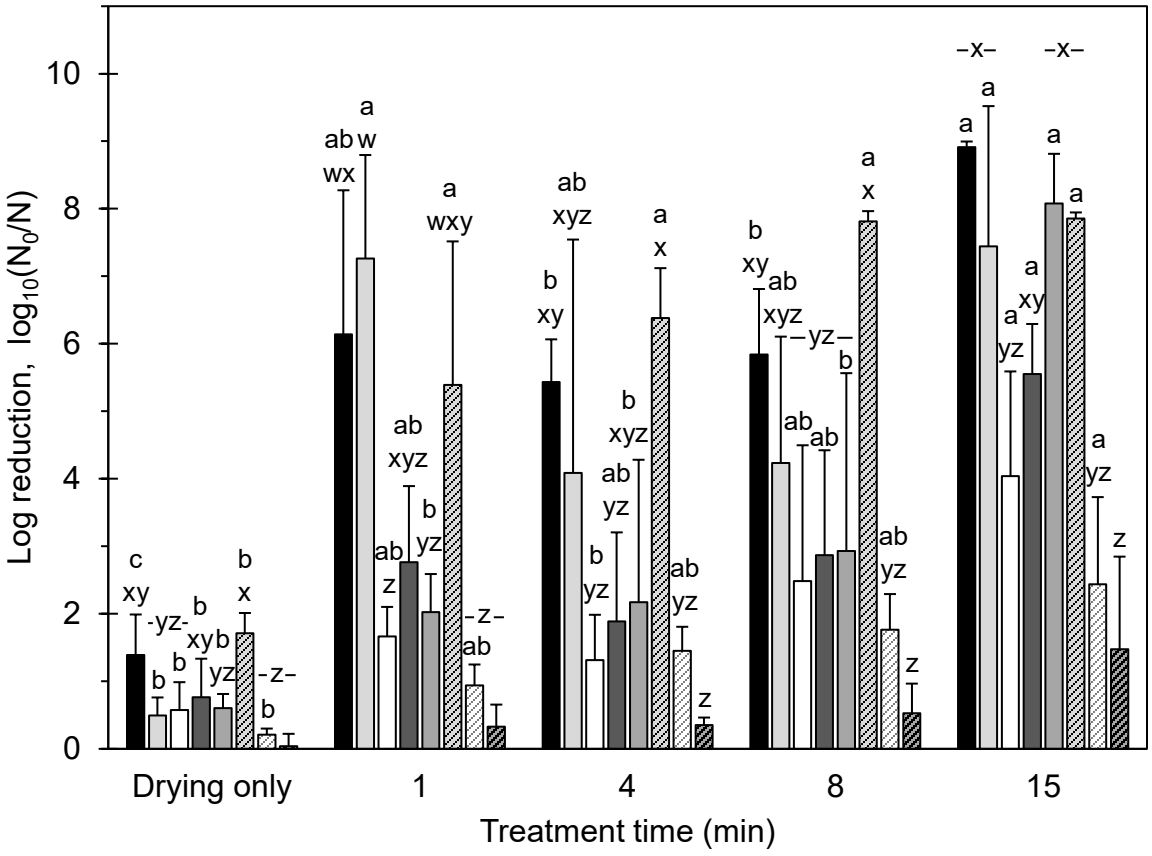


Figure 9: Effect of treatment time on the reduction of *S.* Typhimurium ATCC 13311 (black bars), *S.* Senftenberg ATCC 43845 (light grey bars), *Salmonella* FUA 1934 (white bars), *Salmonella* FUA 1946 (dark grey bars), *Salmonella* FUA 1955 (medium grey bars), *P. acidilactici* FUA 3072 (hatched medium grey bars), *E. faecium* NRRL B-2354 (hatched light grey bars), and *S. carnosus* R6 FUA 2133 (hatched dark grey bars) after desiccation to a_W 0.75, followed by treatment with saturated gaseous CO_2 at 5.7 MPa and 65 °C. Data are means ± standard deviation (n=3). Letters a and b denote significant differences among treatments for the same strain ($P<0.05$). Letters w, x, y and z denote significant differences among strains in the same treatment group ($P<0.05$).

3.5 Reduction of desiccated bacteria inoculated onto beef jerky and treated with HPCD

To validate the antimicrobial efficacy of HPCD in a food system, *E. coli* AW1.7, MG1655 and the STEC cocktail were inoculated onto beef jerky and equilibrated to a_W 0.75 followed by treatment with saturated gaseous CO_2 at 5.7 MPa and 65 °C for 15 min (Figure 10). The counts

for all strains of *E. coli* were reduced below the detection limit. There were no differences among the cell reductions of strains after treatment with CO_2. Since *E. coli* AW1.7 was consistently equally or more resistant than the STEC cocktail, it was concluded that *E. coli* AW1.7 is an acceptable surrogate for dry STEC cells or on beef jerky treated with HPCD under these conditions.

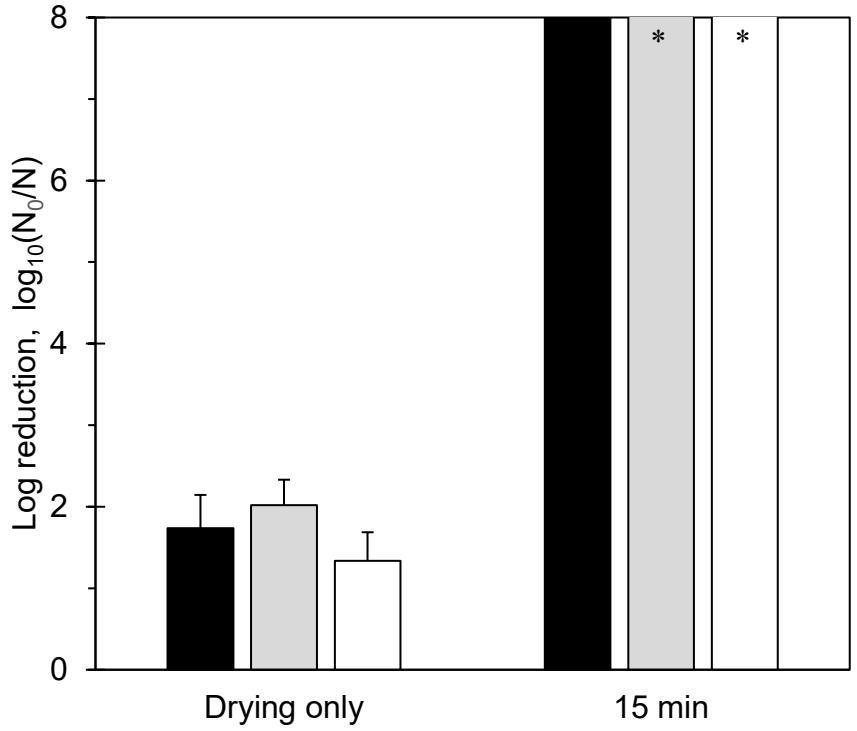


Figure 10: Effect of saturated gaseous CO_2 at 5.7 MPa and 65 °C for 15 min on the reduction of *E. coli* AW1.7 (black bars), *E. coli* MG1655 ATCC 700926 (grey bars), and the STEC cocktail (white bars) inoculated onto beef jerky and equilibrated to a_W 0.75. Y-axis was limited to detection limit. Data are means ± standard deviation (n=3). * indicates no growth after enrichment.

However, this does not apply to *Salmonella* due to the large variability in the cell reductions. *S.* Typhimurium ATCC 13311, *S.* Senftenberg ATCC 43845, *Salmonella* FUA 1934, *Salmonella* FUA 1946, *Salmonella* FUA 1955, *E. faecium* and *S. carnosus* were inoculated onto beef jerky and treated at 5.7 MPa (Figures 11a and 11b) or 12 MPa (Figure 11c) and 65 °C for 4, 8 or 15

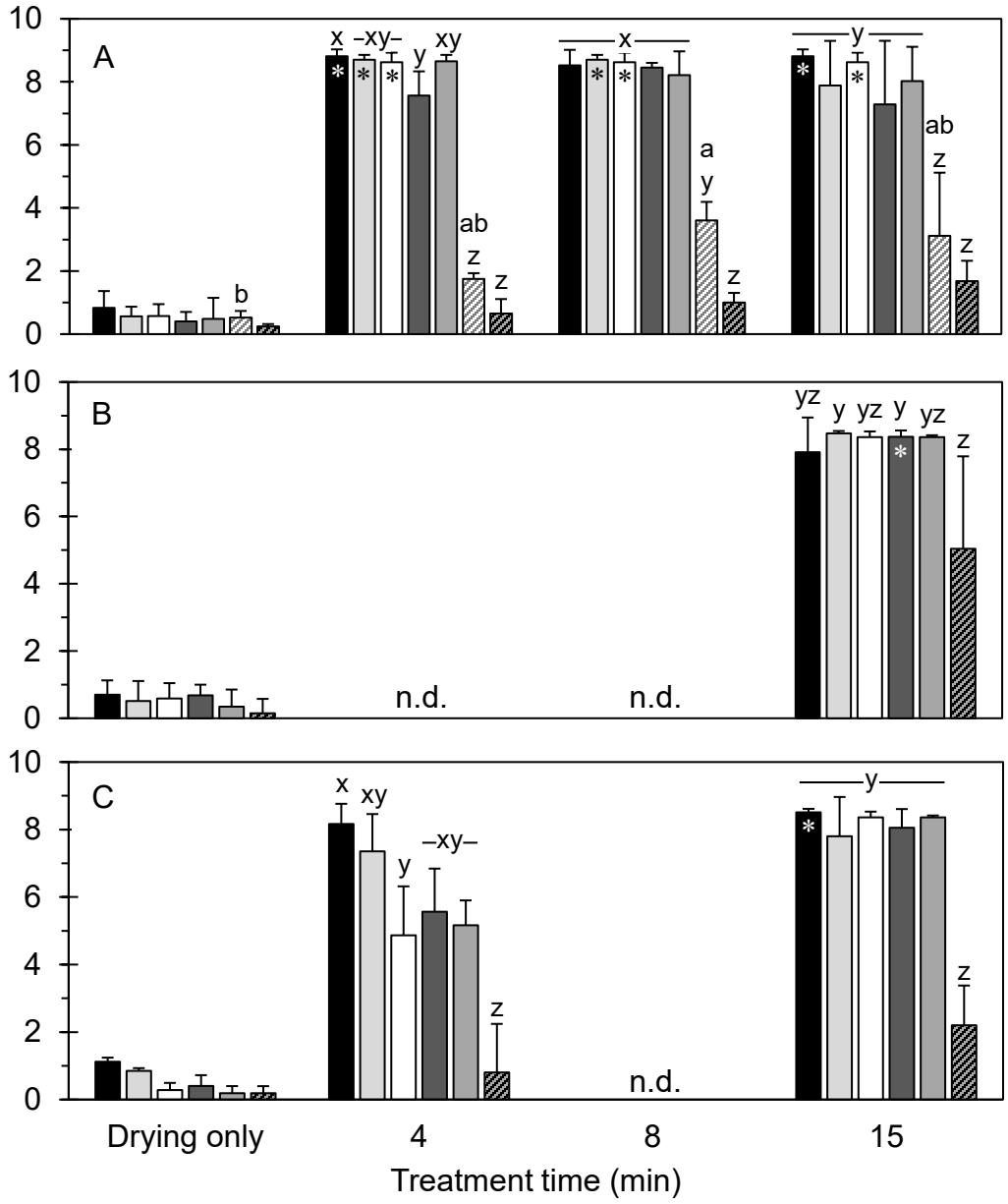
min. Beef jerky samples were equilibrated to a_W 0.75 (Figures 11a and 11c) or 0.9 (Figure 11b). Results in Figure 11a show that changing the treatment time did not affect the cell reductions of any strains of *Salmonella*. *E. faecium* and *S. carnosus* were more resistant than all 5 strains of *Salmonella* after 4 min of treatment. After 8 of 15 min of HPCD treatment, cell reductions of *E. faecium* and *S. carnosus* were lower than that for all strains of *Salmonella*. These results exclude *E. coli* AW1.7 as a surrogate organism for *Salmonella* because cell counts of *E. coli* AW1.7 on beef jerky were consistently below the detection limit, but those for *Salmonella* were not. Therefore, a complete reduction of *E. coli* AW1.7 could not indicate a complete reduction of *Salmonella*, making it unsuitable as a surrogate.

Experiments conducted at Fraunhofer UMSICHT compared gaseous CO_2 (5.7 MPa) and supercritical CO_2 (10.0 and 12.0 MPa), at a_W 0.75 and a_W 0.9 (data not shown). However, due to time limitations, the experiments were not completed. Therefore, treatments where samples were equilibrated to a higher a_W or exposed to CO_2 at a higher pressure were repeated using the Mobile Pasteurization Apparatus. Comparison between Figure 11a and 11b revealed that increasing the a_W beef jerky samples from 0.75 to a_W 0.9 did not result in an increased reduction of *Salmonella* or *S. carnosus*. However, when the a_W of samples was increased to 0.9, *S. carnosus* was only more resistant than *S.* Senftenberg ATCC 43845 and *Salmonella* FUA 1946, whereas at a_W 0.75, *S. carnosus* was more resistant than all strains of *Salmonella* after 15 min of treatment. Therefore, *S. carnosus* is a more suitable surrogate organism when the a_W is increased. To determine if an increase in pressure would increase lethality, the pressure was increased from 5.7 MPa to 12 MPa, changing conditions from gaseous to supercritical CO_2 (Figure 11c). Comparison between data in Figure 11a and 11c revealed that 15 min of treatment with supercritical CO_2 resulted in similar lethality as gaseous CO_2. Gaseous CO_2 was more

effective at reducing cell counts after 4 min of treatment for *Salmonella* FUA 1934 and *Salmonella* FUA 1955. Differences among strains treated at 12 MPa were comparable to treatments at 5.7 MPa. Overall, the a_W of samples did not affect the reduction of cell counts and gaseous CO_2 was more effective than supercritical CO_2 after 4 min of treatment. Additionally, the effect of CO_2 treatment on the lethality of cells on beef jerky appears to be more effective than that observed when dry cells were treated with HPCD.

3.6 Reduction of desiccated bacteria inoculated onto beef jerky with pressurized N_2

Experiments were conducted using pressurized N_2 to determine if the reductions of cell counts are caused by heat, pressure, or pressurized CO_2. *S.* Typhimurium ATCC 13311, *Salmonella* FUA 1934 and *S. carnosus* were inoculated onto beef jerky and equilibrated to a_W 0.75 (Figure 12a) or 0.9 (Figure 12b) followed by treatment with saturated supercritical N_2 at 5.7 MPa and 65 °C for 15 min. *S.* Typhimurium ATCC 13311 was almost completely reduced after treatment with N_2 at a_W 0.75 and 0.9. Treatment with CO_2 resulted in cell counts of *S.* Typhimurium ATCC 13311 below the detection limit, but treatments with N_2 did not. Greater reductions were achieved with CO_2 than with N_2 for *Salmonella* FUA 1934 and *S. carnosus* at both a_W. The a_W did not affect the reduction of cells with CO_2 or N_2.



* indicates no growth after enrichment

Figure 11: Reduction of *S.* Typhimurium ATCC 13311 (black bars), *S.* Senftenberg ATCC 43845 (light grey bars), *Salmonella* FUA 1934 (white bars), *Salmonella* FUA 1946 (dark grey bars), *Salmonella* FUA 1955 (medium grey bars), *E. faecium* NRRL B-2354 (hatched light grey bars), and *S. carnosus* R6 FUA 2133 (hatched dark grey bars) inoculated onto beef jerky. Panel A; equilibrated to a_W 0.75 and treated at 5.7 MPa and 65 °C for 4, 8, and 15 min. Panel B; equilibrated to a_W 0.9 and treated at 5.7 MPa and 65 °C for 15 min. Panel C; equilibrated to a_W 0.75 and treated at 12.0 MPa and 65 °C for 4 and 15 min. Y-axis for all panels represents the log cell reduction (N_0/N). n.d. = not determined. Data are means ± standard deviation (n=3). Letters a and b denote significant differences among treatments for the same strain ($P<0.05$). Letters x, y and z denote significant differences among strains in the same treatment group ($P<0.05$).

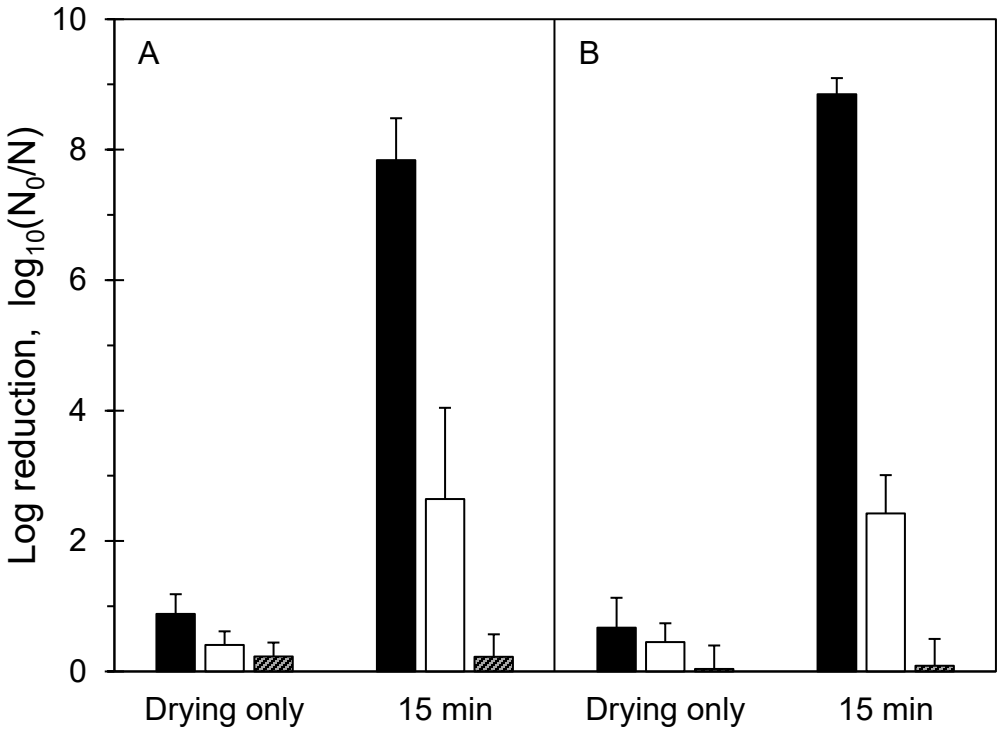


Figure 12: Effect of saturated supercritical N_2 on the reduction of *S.* Typhimurium ATCC 13311 (black bars), *Salmonella* FUA 1934 (white bars), and *S. carnosus* R6 FUA 2133 (hatched dark grey bars) inoculated onto beef jerky and equilibration to a_W 0.75 (A) or 0.9 (B), followed by treatment at 5.7 MPa and 65 °C for 15 min. Data are means ± standard deviation (n=3).

3.7 Reduction of desiccated bacteria inoculated onto almonds with HPCD

Validation of *S. carnosus* R6 FUA 2133 as a surrogate for *Salmonella* was also completed on whole almonds. *S.* Typhimurium ATCC 13311, *S.* Senftenberg ATCC 43845, *Salmonella* FUA 1934, *Salmonella* FUA 1946, *Salmonella* FUA 1955, and *S. carnosus* were inoculated onto whole almonds and equilibrated to a_W 0.9 followed by treatment with saturated gaseous CO_2 at 5.7 MPa and 65 °C for 15 min (Figure 13). Dipping the almonds in water immediately prior to treatment resulted in increased reductions compared to when almonds were not dipped in water for *S.* Senftenberg ATCC 43845 and *Salmonella* FUA 1946. When almonds were not dipped in water, only cell reductions for *S.* Typhimurium ATCC 13311 and *S. carnosus* were different. However, when almonds were dipped in water, there were no differences among the cell

reductions for any of the strains. Therefore, *S. carnosus* is a suitable surrogate organism for *Salmonella* on almonds when samples are dipped in water prior to treatment.

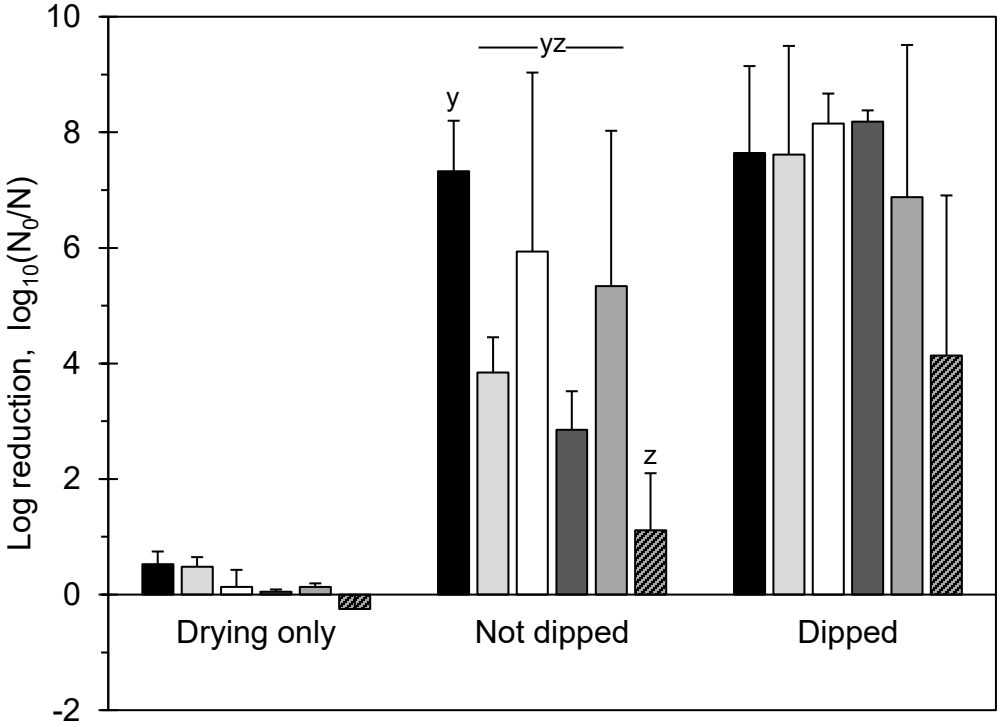


Figure 13: Effect of saturated gaseous CO_2 at 5.7 MPa and 65 °C for 15 min on the reduction of *S.* Typhimurium ATCC 13311 (black bars), *S.* Senftenberg ATCC 43845 (light grey bars), *Salmonella* FUA 1934 (white bars), *Salmonella* FUA 1946 (dark grey bars), *Salmonella* FUA 1955 (medium grey bars), and *S. carnosus* R6 FUA 2133 (hatched dark grey bars) inoculated onto whole almonds and equilibrated to a_W 0.9. In the "Not dipped" group the treatment was conducted following the same procedure as beef jerky treatments. In the "Dipped" group, each almond was dipped in sterile water prior to the CO_2 treatment. Data are means ± standard deviation (n=3). Letters y and z denote significant differences among strains in the same treatment group ($P<0.05$).

4. Discussion

4.1 Comparison between CO_2 and N_2 treatments on the reduction of *Salmonella* and *S. carnosus*

Several studies have compared the efficacy of HPCD with other gases including N_2 [14,75,77,80,82,91,95]. Figures 11a and Figure 12 compared the effect of treating beef jerky

inoculated with *Salmonella* or *S. carnosus* with pressurized CO_2 or N_2 at the same temperature and pressure. Considering to the critical temperature and pressure of N_2 (T_C = −147 °C, P_C = 3.39 MPa), the treatment conditions were in the supercritical region. There was no difference in the reduction of cell counts achieved from samples equilibrated to a_W 0.75 or 0.9 after treatment of samples with N_2. Treatment of samples at a_W 0.75 with CO_2 resulted in the reduction of cell counts of *S.* Typhimurium ATCC 13311 below the detection limit, but treatment with N_2 did not. Treatments with CO_2 were more effective at reducing *Salmonella* FUA 1934 and *S. carnosus* R6 compared to N_2. Strain-specific variation was observed in the resistance of different *Salmonella* strains, which has also been observed toward other types of stress such as desiccation [67]. Previous studies have also found supercritical N_2 to be less effective at reducing cell counts compared to CO_2. For example, treatments with N_2 did not reduce cell counts of yeast, *E. coli* or *Salmonella* at all [14,77], which was attributed to the low solubility of N_2 in water [142]. Dillow et al. [77] hypothesized that because the conditions were so far from the critical point, N_2 did not maintain the gas-like mass transport properties and liquid-like density associated with supercritical fluids. Additionally, unlike CO_2, N_2 lacks the ability to acidify the environment through dissociation reactions. Comparison between CO_2 and N_2O, which both have high solubility in water and similar physical characteristics with the exception that CO_2 acidifies in solution while N_2O neutralizes, resulted in increased reduction of cell counts when treated CO_2 than with N_2O [142]. Treatments with N_2O resulted in higher reductions compared to those with N_2 [142]. Therefore, reductions from N_2 may be strictly due to physical factors, such as pressure-induced damage to the cell membrane, whereas reductions from N_2O are due to both physical factors and its ability to penetrate the cell wall because it can still induce anesthesia effects on the membrane [142]. However, CO_2 was the most effective due to the combined effects of

pressure, solubilization and acidification. The results in Figures 6, 12 and 11a provide insight toward the effects of heat only, heat with pressure, and heat, pressure and CO_2, respectively. Although CO_2 is not responsible for the entire effect, it is still more effective than using N_2.

4.2 Effect of treatment time on the reduction of *E. coli*, *Salmonella*, *E. faecium* and *S. carnosus* with HPCD

Treatment time did not affect the reduction of *E. coli* cells (Figure 8). The literature is inconsistent because some studies found that increasing the treatment time increased the reduction [83], but others found that longer treatments did not consistently result in higher reductions [73]. However, it is possible that differences could not be observed between *E. coli* strains in this study because they were all close to the detection limit, and therefore showed relatively similar reductions because the treatments were highly effective. Results for *Salmonella* were inconsistent among strains (Figure 9). Treatment time did not affect the reduction of cell counts for *S.* Senftenberg 43845, *Salmonella* FUA 1934, *E. faecium* and *S. carnosus*. This was not surprising for *E. faecium* and *S. carnosus* because they were highly resistant to all treatments. Additionally, the large variation in the data may prevent significant differences from being observed among other strains. Cell reductions after HPCD treatments of beef jerky samples inoculated with *Salmonella* were not affected by time (Figure 11a), but since all strains were either completely reduced or close to the detection limit it was not possible to determine differences in their resistance. The effect of treatment time may be related to the solubilization of CO_2 in water; therefore, the effects of treatment time may still be dictated by the amount of water available in the environment. The effect of treatment time may also depend on the initial bacterial load. When the initial cell counts are higher, a longer treatment time was needed to achieve the same reduction as when starting with lower counts [143]. This is because dead cells

provide nutrients as well as a protective physical barrier to help remaining cells survive [144]. Since initial bacterial cell counts were high in this study, this may contribute toward the resistance of cells. However, Uesugi et al. [130] observed similar reductions of *Salmonella* on almonds regardless of the initial cell counts, which ranged from 8 log to 1 log CFU/almond. Additionally, the type of HPCD system affects treatment efficiency. Semi-continuous systems are more effective and allow for reduced treatment times. For example, treatment times of 40 min or more were required to completely inactivate *B. cereus*, *S. aureus*, *Listeria* and *Salmonella* using a batch HPCD system [77], but treatment times of only 10 min or less were needed to inactivate yeast and *B. subtilis* cells using a semi-continuous system [96]. Therefore, the development of a semi-continuous or continuous HPCD system could allow for decreased treatment times and/or a greater reduction of resistant organisms.

4.3 Recovery of injured cells after HPCD treatment

Numerous studies have acknowledged the possibility that HPCD treatments result in sublethally injured cells [70,84,145,146]. This is crucial because bacteria can survive in a dormant state for 1 year [9], 2 years [7] or even up to 10 years [11]. The presence of sublethally injured cells may result in the over-estimation of treatment efficacy because pathogens may be able to avoid detection [147]. Therefore, all cell counts were conducted on selective media, when possible, to differentiate between healthy and injured cells. Overall, counts on selective media were typically lower than counts on non-selective media by 1 log or less (data not shown). Therefore, a small fraction of cells was sublethally injured. Colony size was also variable after HPCD treatments (data not shown). Specifically, after incubation for the same length of time, agar plates frequently contained smaller colonies distributed within normal-sized colonies. This phenomenon was not observed for control treatments which were not exposed to HPCD. This

was observed in other studies where it was found that supercritical CO_2 affected the colony size distribution [93]. Selective enrichments were also conducted for beef jerky and almonds to differentiate between a complete or incomplete reduction when counts were below the detection limit (data not shown). In several cases, growth after the enrichment indicated surviving cells and therefore, an incomplete reduction. The occurrence of injured cells is critical for the application of this technology in the industry because an overestimation of treatment efficacy can result in foodborne illnesses due to the survival of low infectious dose pathogens. Although selective enrichments were conducted in this study, they only determined the presence or absence of growth, but not the number of recovered cells. However, other studies have quantified recovered cells. Injured *S. aureus* cells recovered to nearly the original number of 9 log CFU/mL after incubation for 18 h in recovery media [84]. Sublethally injured cells may be highly sensitive to oxidative stress, but survival is dependent on the state of the cells and their recovery conditions, which may account for the variability in survival between different studies [64]. Additionally, the storage of beef jerky resulted in decreased counts of *E. coli* O157:H7 and *Salmonella* after storage for 60 days, sometimes below detection limits [148,149]. However, for low-infectious dose pathogens such as *E. coli* and *Salmonella*, a small number of cells can illicit severe illness. Therefore, recommendations for producers could incorporate immediate storage after HPCD treatments for up to 60 days to increase the reduction of cell counts. This would not affect the quality of the product because food products with a a_W below 0.85 are considered to be shelf-stable [2].

RNA sequencing can identify changes in the expression of genes in *E. coli* O157:H7 after treatment with HPCD to better understand what happens in cells and how they may recover from treatments. Downregulation of a gene encoding for a transcriptional repressor of a gene that

inhibits cell division was detected in *E. coli* exposed to HPCD [146], which could result in delayed growth and smaller colonies. Additionally, central metabolic processes, gene replication, expression and protein synthesis are repressed [146]. Tricarboxylic acid cycle activity decreased, reducing ATP and CO_2 production by the cell [146]. The expression of enzymes involved in NADPH generation increased, which helps *E. coli* survive oxidative, acid and pressure stress [146]. The expression of genes involved in the maturation of cytochrome *c* increased, increasing the activity of the electron transport chain and genes involved in maintaining membrane integrity, helping the cell to maintain a barrier from exterior stressors [146]. Unsurprisingly, the expression of genes involved in general stress responses including heat shock proteins, protein folding chaperones and proteins preventing protein aggregation also increased [146]. Finally, expression of virulence genes was decreased, likely as a way to conserve energy for essential functions; however, pathogenicity can still occur once conditions become favourable [146]. Overall, the expression of cell differentiation and metabolic activity decreased in exchange for increased expression of stress response pathways, which may result in reduced cell counts due to the injury of cells and slow growth rate. However, bacteria can recover when conditions become favourable, which was confirmed in the current study by enriching samples after treatments. These results imply that treatments with HPCD may not guarantee the safety of food products even when cell counts are below the detection limit. Therefore, further studies focusing on the recovery of injured cells in specific food matrices are needed to confirm the effectiveness of HPCD treatments.

4.4 Effect of the LHR on the reduction of *E. coli* and *Salmonella* after dry heat treatments

The response mechanisms of *E. coli* and *Salmonella* toward dry stress are relatively similar [5,46,150]. However, this study determined that the presence of the LHR affected the survival of dry *Salmonella* above a certain a_W, but did not affect the survival of *E. coli* (Figures 4 and 5). At a_W of 0.80 and below, *E. coli* and *Salmonella* with or without the LHR are equally resistant to heat treatments, therefore the LHR does not play a role in the heat resistance of dry *E. coli* and *Salmonella*. This is in accordance with other literature where it has been identified that a lower a_W results in higher resistance towards heat, particularly in *E. coli* and *Salmonella* [5,55], and the LHR does not affect survival at low a_W [5]. Figure 6 confirmed that desiccation resulted in the heat resistance of LHR negative strains, *Salmonella* FUA 1934, FUA 1946 and FUA 1955. Additionally, other researchers have identified the increase in heat resistance of dry *E. coli* and *Salmonella* in the presence of solutes including glucose, sucrose, glycerol, fructose, sorbitol and NaCl [151,152], which is explained by the mechanisms of dry survival involving compatible solutes [56]. Hiramatsu et al. [6] hypothesized that heat resistance in desiccated bacteria was related to the inhibition of protein denaturation due to the absence of water. However, Gruzdev et al. [67] observed increased heat resistance of desiccated *S. enterica* cells that were rehydrated prior to heat treatments compared to non-desiccated cells.

However, in the current study, when the a_W was at or above 0.90, counts of *S.* Typhimurium ATCC 13311 pLHR, which contains the LHR on a plasmid, were reduced compared to *S.* Typhimurium ATCC 13311 pRK767, containing the empty plasmid, when treated at 60 °C for 5 or 15 min (Figures 4 and 5). Therefore, the LHR is somehow responsible for the decreased resistance when cells were dried and re-equilibrated to a_W of 0.9-0.95, 0.96-0.98 and 0.99-1.0.

Seeras [5] observed a similar effect starting at a_W 0.75; however, samples in those experiments were dried in TSB rather than 0.1% peptone and were treated at higher temperatures (110 °C). Although the exact mechanism remains unclear, it is possible that survival mechanisms for desiccation are prioritized over those for heat resistance during the drying process, thus resulting in sensitivity to heat once the desiccation stress is removed [5]. However, this does not fully explain why *Salmonella* without the LHR still exhibits high heat resistance at higher a_W. Additionally, *E. coli* with or without the LHR were equally resistant to heat treatments after drying and re-equilibration to all a_W. This is consistent with other research, where *E. coli* with and without the LHR were equally resistant to desiccation and heat treatment [5]. Therefore, the LHR functions differently in *E. coli* and *Salmonella* despite the organisms being so similar. Additionally, some genes within the LHR produce proteins with functions that are still unknown. These may somehow contribute to the sensitivity of LHR positive *Salmonella* at higher a_W that do not play the same role in *E. coli*. Another possibility is that the expression of the LHR when cells are re-hydrated takes energy away from other stress response mechanisms in *Salmonella*, which would help it to survive better, thus impairing cell survival. Overall, while it has been confirmed that the LHR confers heat resistance in *E. coli* and *Salmonella* at high a_W [46], the mechanism is more complex when bacteria are desiccated and re-equilibrated to high a_W.

4.5 Effect of the cell membrane composition and structure on the reduction of *E. coli* with HPCD

The cell membrane is a primary barrier of protection to the cell, and its composition is modified through the synthesis of CFAs, by the enzyme CFA synthase, encoded by the gene *cfa* [63]. CFAs have been proven to protect cells against osmotic stress [153,154], low pH [155,156] and pressure [64]. Therefore, a comparison was made between *E. coli* AW1.7 and a *cfa* knockout

mutant, *E. coli* AW1.7 Δ*cfa*. The Δ*cfa* mutant has an altered profile of fatty acids compared to the wild-type due to the absence of CFAs, which were replaced with their unsaturated fatty acid substrates [63]. Chen et al. [63] found that CFAs increased the resistance of *E. coli* AW1.7 in liquid broth to heat, pressure and acid. However, in this study no differences were identified between the cell counts of *E. coli* AW1.7 Δ*cfa* and the wild type after treatment with saturated gaseous CO_2 for up to 15 min (Figure 8). However, these results make sense because Chen et al. [157] determined that the wild type was more resistant at high a_W conditions and *E. coli* AW1.7 Δ*cfa* was more resistant at low a_W conditions. Therefore, dry cells treated with saturated CO_2 are in transition between wet and dry, therefore no differences were observed.

4.6 Validation of potential surrogate organisms to pathogens with HPCD

A surrogate organism should have resistance greater than or equal to that of the target organism, have similar characteristics, and be evaluated in the food system in which it will be used [2]. Additionally, it must be a non-pathogenic organism to avoid contaminating the food processing facility in which it will be applied. Since the resistance of *E. coli* AW1.7 was greater or equal to the resistance of the STEC cocktail at all tested conditions, *E. coli* AW1.7 is an acceptable surrogate organism for STEC on dry cells and beef jerky treated with HPCD, and therefore can be used in future challenge studies.

However, the search for a surrogate organism to represent *Salmonella* was more challenging. *E. coli* AW1.7 was rejected because although there were no significant differences between cell reductions for *E. coli* and the strains of *Salmonella*, when treated with HPCD on beef jerky for 15 min, numbers of *E. coli* AW1.7 were below the detection limit while some *Salmonella* strains survived. Therefore, the complete reduction of *E. coli* AW1.7 cannot guarantee the complete reduction of *Salmonella*. The high variability between cell reductions for strains of *Salmonella*

made it difficult to identify an organism to represent all 5 strains. Liu et al. [50] found that pressure resistance varies highly among strains of the same species; therefore, differences among strains in this study were not surprising. Differences among strains could be explained by differences in the membrane composition, thus influencing the membrane fluidity and ability of CO_2 to solubilize through the membrane [92]. However, these results reinforce the importance of validating a surrogate with more than one target organism due to variation among strains and the diversity of resistance to different types of stress.

Due to structural differences in cell membranes, a Gram-negative organism would have been preferred as a surrogate organism, but it was not possible to identify a non-pathogenic Gram-negative organism with similar or higher resistance to HPCD treatments than *Salmonella*. Therefore, Gram-positive organisms were considered. First, *P. acidilactici* FUA 3072 was tested. *Pediococcus* spp. was previously investigated by Borowski et al. [2] as a non-pathogenic starter culture organism to represent *E. coli* O157:H7 and *Salmonella* in ground-and-formed beef jerky. The reduction of *Pediococcus* spp. accurately predicted the reduction of *E. coli* O157:H7 and *Salmonella* in 100% of samples [2]; therefore, *P. acidilactici* showed promise as a surrogate organism in this study. However, *P. acidilactici* FUA 3072 proved to be more sensitive than several *Salmonella* strains, making it unsuitable as a surrogate organism in this study (Figure 9). A possible explanation for the differences is that Borowski et al. [2] used *Pediococcus* as a surrogate during the processing of beef jerky, starting with fresh ground meat, which has a high a_W. They determined that the largest reduction occurred during the initial processing before the a_W decreased. Therefore, it appears that while *Pediococcus* can represent *E. coli* and *Salmonella* at high a_W conditions, it was unsuitable for this study where bacteria were treated under dry conditions.

As an alternative, *E. faecium* NRRL B-2354 was assessed as a surrogate. It was selected because it was validated by Kopit et al. [134] as a surrogate organism to represent *Salmonella* on almonds, and its heat resistance was comparable to *S.* Enteritidis [134]. *E. faecium* NRRL B-2354 was confirmed to be free of antibiotic resistance genes and virulence factors, and therefore was classified as a biosafety level 1 organism with the ATCC [134]. *E. faecium* was consistently more resistant than *Salmonella* both when dry cells were treated and on beef jerky (Figures 9 and 11a). This is consistent with literature where *E. faecium* NRRL B-2354 survived well with environmental stress [134] and it was more resistant to HPCD treatments than other bacteria [79], thus making it an attractive choice as a surrogate organism. Although this specific strain was determined to be free of virulence genes, *E. faecium* is associated with nosocomial infections, the frequency of which has increased in recent years [134]. For this reason, Germany does not consider *E. faecium* to be non-pathogenic, but rather classifies it as a biosafety level 2 organism. Therefore, it could not be imported into Germany within the time restraints, and could not be used at the Fraunhofer UMSICHT institute where only biosafety level 1 organisms are permitted. Overall, *E. faecium* NRRL B-2354 is a promising surrogate for *Salmonella* in North America.

As a result, *S. carnosus* R6 was investigated as an alternative surrogate. This strain was promising because it was isolated from a commercial meat starter culture [137]; therefore, it is non-pathogenic and can survive well in a meat matrix. Its resistance to HPCD treatments was comparable to *E. faecium* as it was more resistant than *Salmonella* both when dry cells were treated and on beef jerky (Figures 9 and 11a). Therefore, *S. carnosus* R6 was determined to be a suitable surrogate organism because it showed greater resistance to the treatments than the target organisms. Although there were concerns about differences in survival due to using a Gram-

positive organism to represent the behaviour of Gram-negative pathogens, Garcia-Gonzalez et al. [79] determined that there were no differences in survival between Gram-positive and Gram-negative organisms treated with HCPD, and that any differences were likely differences among species or strains. Additionally, *Listeria*, a Gram-positive organism, was reduced faster than *Salmonella*, a Gram-negative organism, with HPCD treatments on ground pork [73]. The D-value of Gram-positive *S. aureus* was significantly smaller than that of *E. coli* after treatment with supercritical CO_2 in ground beef [84]. Therefore overall, *S. carnosus* R6 can be implemented as a non-pathogenic surrogate organism to represent *Salmonella* in future studies applying HPCD to dry foods.

4.7 Effect of CO_2 phase on the reduction of *Salmonella* and *S. carnosus* on beef jerky with HPCD

Treatment of *Salmonella* and *S. carnosus* on beef jerky with supercritical CO_2 resulted in decreased reduction of cell counts after treatment for 4 min compared to gaseous, and there was no difference between cell reductions when samples were treated for 15 min (Figure 11a and 11c). Therefore, treatments for 4 min at a lower pressure were more effective than those at a higher pressure. While this is comparable to a study by Bae et al. [73] where increased pressure did not consistently lead to higher reductions of *Salmonella* and *Listeria*, these results contradict some literature which states that supercritical CO_2 is more effective than gaseous [76,93], and that increasing the pressure typically results in increased reductions of cell counts [69]. However, this was only observed when treatments were conducted with bacteria at high a_W. Chen et al. [157] confirmed that while supercritical CO_2 is more effective to inactivate wet cells, dry cells were more effectively reduced at sub-critical CO_2 conditions. While higher pressures increase the solubilization of CO_2 in water which increases penetration of CO_2 into the cell, gaseous CO_2

has increased mass transport properties and a lower density meaning that it diffuses into the cell faster [157]. Temperature also plays a role in treatment effectiveness because gaseous CO_2 was more effective at reducing cell counts of dry bacteria than liquid CO_2 [157]. Higher temperatures increased the diffusivity of CO_2 and the fluidity of the cell membrane, which improves CO_2 penetration into the cell [69]. However, Garcia-Gonzalez et al. [70] warn that treatment temperatures should not exceed the critical temperature because it will result in decreased density and therefore decreased solubilization. Overall, this study confirmed that gaseous CO_2 is more effective than supercritical to inactivate dry cells and bacteria inoculated onto low a_W foods.

4.8 Effect of dipping on the reduction of *Salmonella* and *S. carnosus* on almonds with HPCD

Experiments conducted on almonds at the Fraunhofer UMSICHT institute were not completed due to time constraints. Therefore, experiments that compared the effect of saturated gaseous CO_2 on the reduction of *Salmonella* and *S. carnosus* on almonds at a_W 0.9 were conducted using the Mobile Pasteurization Apparatus. Variability among strains was high, but the efficacy of the treatments was improved by dipping almonds in water immediately prior to HPCD treatment (Figure 13). Other researchers in the food microbiology lab at the University of Alberta have demonstrated that this method resulted in increased reduction of cell counts in dry nuts and seeds such as oats, barley, mung beans and soy beans (Fang, unpublished). Although the CO_2 is saturated in both treatments, dipping samples in water can increase the surface a_W prior to treatment. Therefore, the effectiveness of treatments with pressurized CO_2 is again related to the a_W of samples and the addition of water in treatments of dry foods.

4.9 Effect of water on the reduction of *E. coli* and *Salmonella* with HPCD

The literature has consistently claimed that HPCD is not an effective method to reduce the number of dry bacteria [70,72,75,157]. This is primarily justified by the inability of CO_2 to dissociate in the absence of water, thus preventing extra- and intracellular acidification. Additionally, in the presence of water, cell walls are more permeable to CO_2, thus resulting in increased absorption of CO_2 [158] and diffusion of CO_2 inside of the cell [77]. When the water content was less than 0.2 g/g dry matter, the sterilization rate constant was almost zero [158]. Specifically, with a low a_W, all water is inaccessibly bound to the cell, thus there is no free water in which the CO_2 is able to dissolve [158]. Additionally, a higher a_W increases the formation of HCO_3^- , which can influence membrane stability by interacting with phospholipids and proteins on the surface of the cell [157]. The limited reduction of cell counts of *E. coli* AW1.7 and the STEC cocktail in treatments with dry CO_2 can be attributed to the drying process rather than the CO_2 treatment itself because results were not different from control treatments (Figure 7). The significant reduction of *E. coli* MG1655 with dry CO_2 (Figure 7) was not surprising because as a K-12 laboratory strain, *E. coli* MG1655 is more sensitive to multiple types of stress including heat [159] and high hydrostatic pressure [160]. However, upon the addition of small quantities of water, which are capable of saturating the CO_2, a significant reduction of all *E. coli* was achieved (Figure 7). Although all samples were equilibrated to a_W 0.75 before treatments, it was hypothesized that the absence of water in the CO_2 source was actually causing a drying effect to occur during the treatment, which effectively lowered the a_W of the cells, and increased resistance of bacterial cells to CO_2 treatments. The problem was solved by saturating the CO_2 with a small quantity of water, which maintains the desired a_W of the samples during the treatment. As a result, treatments of dry cells at a_W 0.75 with gaseous CO_2 saturated with water

for 15 min resulted in a > 7 log reduction (Figure 7). This is important because previous studies were not able to effectively reduce dry bacteria using HPCD. This research not only confirmed the importance of water in the reduction mechanism of HPCD, but also established the amount of water that must be present to achieve significant reductions of cells, which goes beyond previous research.

4.10 Effect of the food matrix on the reduction of *E. coli* and *Salmonella* with HPCD

Differences were observed between reductions of cells from HPCD treatments on dry cells and treatments on beef jerky inoculated with bacteria. Bacteria were more sensitive to HPCD treatments when inoculated onto beef jerky (Figures 10 and 11) compared to when treated as dry cells (Figures 8 and 9). These results contradict the literature, where complex environments like food matrices exert protective effects on bacterial cells [70,84]. Protective effects may be due to the presence of fats [50,79,161] or proteins [83,161]. The addition of sunflower oil up to 30% increased the resistance of *P. fluorescens* to HPCD treatments [79]. It was hypothesized that fats may limit CO_2 penetration into the cell by altering the structure of the cell membrane [79]. Additionally, emulsifying agents, such as Tween 80 and sucrose stearate also increased the reduction of cells with HPCD [79]. However, the protective effects of the food matrix are based on comparisons between wet cells and cells in food matrices, with no comparisons using dry cells. Therefore, it is possible that dry cells are consistently more resistant than cells contained within a food matrix.

The increased presence of salt in beef jerky compared to peptone water may play a role in the survival of cells. Seeras [5] found that the addition of NaCl in the drying medium resulted in decreased survival compared to strains dried in 0.1% peptone water. A NaCl concentration of 2%

also resulted in increased sensitivity of *P. fluorescens* [79]. However, numerous studies claim that the presence of salt increases the resistance of cells [50,56,79]. *E. coli* was more resistant when cooked in ground beef containing 2% NaCl [50], and media supplemented with 2-6% NaCl resulted in increased heat resistance of non-pathogenic *E. coli* including *E. coli* AW1.7 [56]. Although the salt concentration of the beef jerky produced for this study is not known, the beef jerky used at the Fraunhofer UMSICHT institute contained approximately 2% NaCl. The relationship between solutes and a_W and their effects on resistance to HPCD treatments were also investigated. A study by Garcia-Gonzalez et al. [79] found that decreasing the a_W to 0.95 using sucrose protected *P. fluorescens*, but achieving the same a_W using NaCl or glycerol did not protect cells. However, decreasing the a_W to 0.924 using glycerol did protect the cells [79]. The protective effects of sucrose and glycerol were also observed with *S. aureus* [79]. These effects may be related to the accumulation of compatible solutes, which is known to improve the survival of dry *E. coli* and *Salmonella*. Glycerol and sucrose can cross the membrane at low a_W providing intracellular and extracellular protection [79]. Although the reason for decreased survival of bacteria on beef jerky in this study remains unknown, it may be related to the presence of salts and solutes within the beef jerky which are detrimental to cells at lower a_W. Alternatively, it is possible that the beef jerky is better able to hold on to the water added during the treatment, whereas the cells become drier during treatments, resulting in increased resistance of bacteria treated as dry cells but not on beef jerky. This is supported by previous studies using high a_W meat products which only achieve limited reduction with more extreme conditions. For example, Bae et al. [73] achieved just over 2 log reduction of *Salmonella* in ground pork after treatment at 14 MPa and 45 °C for 40 min. Another explanation could be the shift in the water sorption isotherms under the HPCD environment, which showed an increase in the a_W of samples

with an increase in temperature while water content remains constant (Ren, unpublished data). Therefore, beef jerky samples may reach a higher a_W during HPCD treatments compared to treatments on cells, which could improve the reduction due to the critical role of water in the bacterial inactivation mechanism of HPCD. The relationship between moisture sorption isotherms and samples during HPCD treatments could also be related to the apparent difference in the treatment efficacy between beef jerky and almonds. However, regardless of the cause, an increased reduction of bacteria in food products is beneficial.

4.11 Effect of increasing the a_W on the reduction of *Salmonella* and *S. carnosus* with HPCD

When the a_W of beef jerky samples was increased from 0.75 to 0.90, *S. carnosus* more closely represented *Salmonella* (Figure 11a and 11b), even though there were no significant differences between treatments at different a_W. These results contradict the literature because increasing the a_W increases the reduction of cell counts [69]. However, previous studies typically only differentiate between wet and dry cells or low and high a_W, whereas this study equilibrated samples to specific a_W and added known quantities of water. Additionally, effects due to the a_W of the samples may be overshadowed by the effect of adding water to the treatments to saturate CO_2, therefore resulting in no differences observed between samples at different a_W. If the a_W is increased it may be enough to reduce cell counts close to or below the detection limit for samples equilibrated to both a_W.

5. Conclusions

This research validated surrogate organisms to represent low-infectious dose pathogens in low a_W foods by treatment with HPCD. *E. coli* AW1.7 is a surrogate for the STEC strains used in this

study, which is important because most research focuses on *E. coli* O157:H7, but not other STEC strains. In addition, *E. faecium* NRRL B-2354 was validated to represent the *Salmonella* strains used in this study, and if the surrogate will be applied in a facility which treats *E. faecium* as biosafety level 2, then *S. carnosus* R6 may be used in its place. Overall, the effective treatment of low a_W foods can be accomplished using HPCD, but different considerations are required compared to the treatment of high a_W foods. High a_W foods are effectively treated with supercritical CO_2, whereas low a_W foods are more effectively treated with saturated gaseous CO_2 due to the differences in the inactivation mechanisms at different a_W and CO_2 state. Specific processes must be designed and validated for specific foods. Based on this study, the treatment of beef jerky could incorporate HPCD treatments into the drying process to allow for a high inactivation while the a_W remains high, followed by a drying step to achieve the desired a_W of the product. Almonds should be treated by dipping them in water immediately prior to treatment with HPCD. Additionally, the efficiency of both processes could be increased by developing a semi-continuous system in which CO_2 flows past the food product.

More research must be done to optimize treatments. The food matrix is not in equilibrium during HPCD treatments due to fluctuating temperature, a_W and pressure; therefore, better tools are needed to establish consistent conditions with respect to the moisture sorption isotherms and a_W during treatments to allow for a stronger comparison. Additionally, most studies focus on the microbial inactivation immediately after treatment, but very few determine the effects on microbial counts after storage, which may be relevant given the ability of injured cells to recover. Finally, there are limited studies investigating the effects of HPCD treatments on the physico-chemical properties, and flavour and nutritional quality of foods, specifically low a_W foods including beef jerky and almonds; therefore, additional research is necessary in this area.

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Appendix

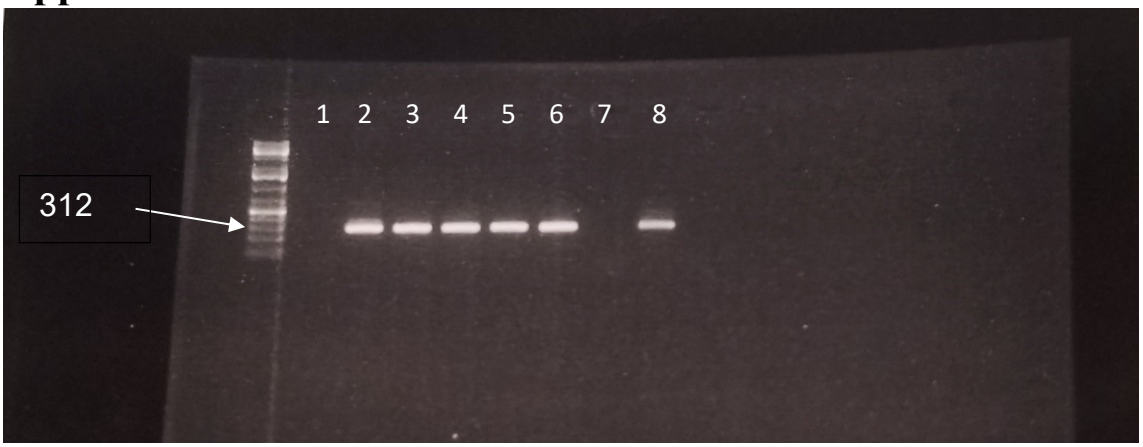

Figure S1: Image from gel electrophoresis and PCR from DNA obtained from the following *Salmonella* strains: FUA 1917, FUA 1934, FUA 1946, FUA 1955, and FUA 1984. Ladder used was GeneRuler 1Kb Plus. 1, negative control with no template DNA. 2, *Salmonella* FUA 1917. 3, *Salmonella* FUA 1934. 4, *Salmonella* FUA 1946. 5, *Salmonella* FUA 1955. 6, *Salmonella* FUA 1984. 7, negative control with *E. coli* DNA. 8, positive control with *Salmonella* DNA.

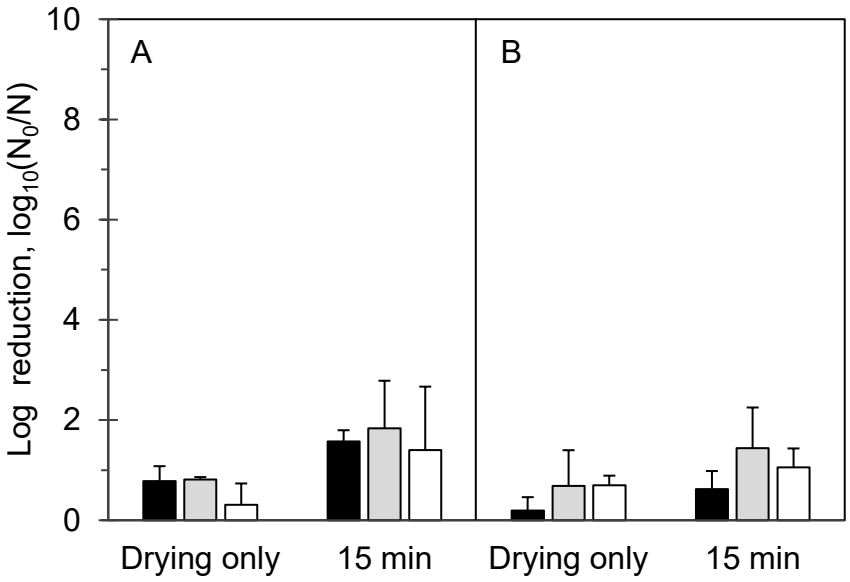

Figure S2: Effect of container on the reduction of *E. coli* AW1.7 (black bars), *E. coli* MG1655 ATCC 700926 (grey bars), and the STEC cocktail (white bars) desiccated to a_W 0.1, followed by CO_2 treatment at 5.7 MPa and 65 °C for 15 min. A, samples dried in vials; B, samples dried in lids. Data are means ± standard deviation (n=3).

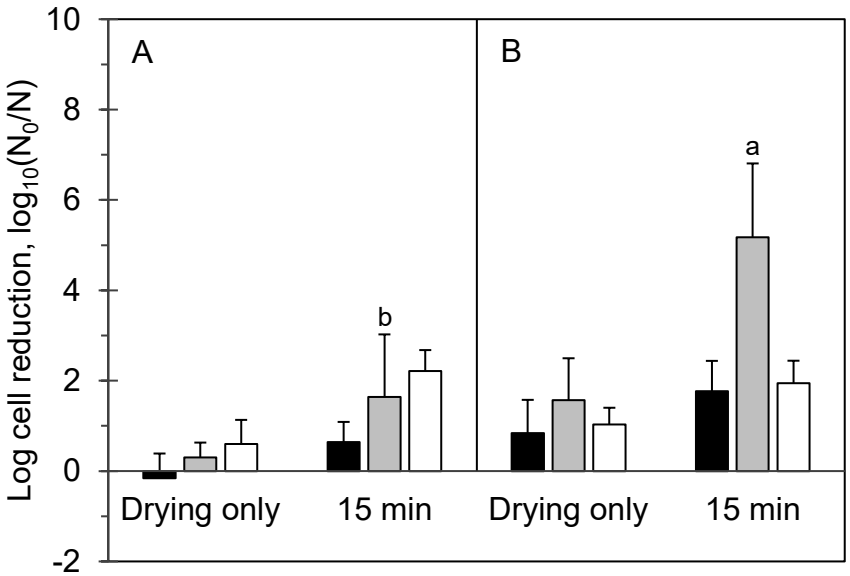

Figure S3: Effect of container on the reduction of *E. coli* AW1.7 (black bars), *E. coli* MG1655 ATCC 700926 (grey bars), and the STEC cocktail (white bars) desiccated to a_W 0.75, followed by CO_2 treatment at 5.7 MPa and 65 °C for 15 min. A, samples dried in vials; B, samples dried in lids. Data are means ± standard deviation (n=3). Letters a and b denote significant differences among treatments for the same strain ($P<0.05$).