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Validation of High Pressure Processing (HPP) as a Suitable Technology for the Production of Safe Tender Coconut Water

Validación de las altas presiones hidrostáticas (HPP) como una tecnología apta para la producción segura de agua de coco verde

Mario González Angulo

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Validation of High Pressure Processing (HPP) as a Suitable Technology for the Production of Safe Tender Coconut Water

Validación de las altas presiones hidrostáticas (HPP) como una tecnología apta para la producción segura de agua de coco verde

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Mario González Angulo

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Dr. JORDI ROVIRA CARBALLIDO y Dra. CAROLE TONELLO SAMSON, Catedrático del área de Tecnología de los Alimentos del Departamento de Biotecnología y Ciencia de los Alimentos de la Universidad de Burgos y Directora del Departamento de Aplicaciones y Procesado de Alimentos de Hiperbaric.

Como Director y Codirectora de la Tesis Doctoral "Validation of high pressure processing (HPP) as a suitable technology for the production of safe tender coconut water/ Validación de las altas presiones hidrostáticas (HPP) como una tecnología apta para la producción segura de agua de coco verde" desarrollada por **D. Mario González Angulo**

INFORMAN

Favorablemente la presentación de dicha tesis, ya que reúne las condiciones necesarias para su defensa en cuanto a la realización de la fase experimental y la elaboración de la memoria.

Y para que así conste y a los efectos oportunos, se firma el presente informe,

En Burgos, a 20 de enero de 2021

Fdo.: Jordi Rovira Carballido

Fdo.: Carole Tonello Samson

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ABSTRACT

Tender coconut water is a traditional beverage of economic importance in tropical areas that is gaining popularity worldwide in recent years. It is defined as an isotonic juice, sterile while in the cavity of the coconut, and slightly acidic (pH 4.7 to 5.5). High pressure processing (HPP) is the most common nonthermal preservation method used to extend shelf-life and ensure food safety while maintaining organoleptic and nutritional qualities. This technology is widely used in Europe, Oceania and Asia to preserve tender coconut water. Nevertheless, bacterial spores survive HPP. For this reason, the risk of proteolytic *Clostridium botulinum* and nonproteolytic *Clostridium botulinum* growth should be assessed in tender coconut water due to its low acidity (pH >4.6). Additionally, other potential hazards that can be introduced by the manufacturing process of the beverage are vegetative pathogens such as *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella enterica*. Although HPP can effectively control these pathogens in juices and beverages, the three species exhibit great intraspecies variability in terms of their pressure resistance. Hence, the objective of this thesis was to evaluate safety of tender coconut water with respect to *C. botulinum* and to determine the factors that control growth and toxin formation. In addition, this work characterized the variable response to high pressure of multiple strains of *E. coli* O157:H7, *L. monocytogenes* and *S. enterica* in order to select representative strains for challenge tests based on their pressure resistance in acid and low-acid beverages. Multiple-strain cocktails were created and subsequently used in the validation of typical HPP parameters (600 MPa for 3 min) to produce safe tender coconut water during refrigerated storage.

To assess the risk associated with *C. botulinum*, tender coconut water (pH 5.2) was inoculated with nontoxigenic type E *C. botulinum* and *Clostridium* spp. spores and subjected to HPP (550 MPa for 3 min). A wide range of dissolved oxygen concentrations (<0.5 to 11 mg/L) and incubation temperatures following HPP (4 to 20 °C) were used to evaluate the effect on spore growth of these factors. Results showed that spores failed to grow after 61 days regardless of incubation temperature and dissolved oxygen concentration in coconut water. Additionally, pressure-resistant spoilage microorganisms grew and spoiled the beverage within a few days during storage at 10 and 20 °C, which is likely to hinder the growth of *C. botulinum* even if dissolved oxygen concentration depletes. To further investigate the parameters that control spore germination and growth, a “worst-case scenario” from a food safety perspective (but optimum for *C. botulinum* growth) was created. Total counts of *C. botulinum* and *Clostridium* spp. decreased in filter-sterilized coconut water adjusted to pH 7 for 61 days of anoxic incubation at 30 °C. This suggested that a fraction of spores germinated but failed to

resume vegetative growth. Similarly, addition of germinants and free amino acids only supported spore germination. Supplementation of coconut water with laboratory broth at low concentrations was sufficient to promote growth of *C. botulinum* and *Clostridium* spp., which presumptively evidenced that nutrient limitation in tender coconut water might prevent spore growth.

However, rather than plate counts, accurate risk assessment requires the evaluation of botulinum neurotoxin formation by proteolytic and nonproteolytic strains in multiple varieties of coconut water. Therefore, the safety of eight tender coconut waters from different geographical origins was evaluated on the basis of this criterion using multiple-strain cocktails of proteolytic *C. botulinum* and nonproteolytic *C. botulinum*. Under highly favorable conditions (anoxic sterile coconut water incubated for 50 days at 30 °C), slight toxin production by proteolytic *C. botulinum* was detected in only one type of coconut water, while none of the eight coconut waters studied supported growth by nonproteolytic *C. botulinum*. Further investigation demonstrated that rising the pH of coconut water and the addition of laboratory broth or mixtures of free amino acids, vitamins and minerals lead to proteolytic *C. botulinum* and nonproteolytic *C. botulinum* growth, but always under the same favorable incubation conditions. Future risk assessment of chilled and high pressure-processed tender coconut water (pH 4.7-5.3) should consider that the beverage is a poor substrate for growth and neurotoxin formation by *C. botulinum*.

From a prevalence perspective, the potential biological hazards that need to be controlled in tender HPP coconut water are vegetative pathogens such as enterohemorrhagic *E. coli*, *L. monocytogenes* and *Salmonella* spp. Process parameters optimization (pressure and holding time) and process validation (which is mandatory in some countries like the US) require the use of representative strains for challenge tests. However, there is a lack of consensus regarding the selection of representative strains to be used in challenge tests. To address this issue, the response to mild HPP conditions (500 MPa for 1 min at 10 °C) of multiple strains of *E. coli* O157:H7, *L. monocytogenes* and *S. enterica* was evaluated in juice model solutions consisting of tryptic soy broth + yeast extract adjusted to pH 4.5 and 6.0 with citric acid. Pressure resistance greatly varied within strains. The most resistant species were *L. monocytogenes* and *E. coli* O157:H7 as evidenced by minimum and maximum counts after HPP at pH 6.0. On the other hand, *S. enterica* was the least resistant pathogen with more than 82 % of the isolates tested showed non-detectable counts after HPP. Interestingly, more than 50 % of *E. coli* O157:H7 strains tested displayed survivors following HPP at pH 4.5, but did not adapt and registered non-detectable counts in the next sampling dates. None of the *L. monocytogenes* and *S. enterica* strains survived HPP at this pH. Recovery through storage at

12 °C was also variable for all pathogens at pH 6.0, but eventually most strains recovered and reached the stationary phase between days 7 and 14. Multivariate descriptive statistical analysis of the whole data set identified representative strains of each species with a pressure resistant phenotype able to adapt and recover after HPP in the model solutions. Each multiple-strain cocktails designed as a result of this characterization were used to validate the adequacy of standard industrial HPP parameters (600 MPa for 3 min at 10 °C) to ensure safety of four different varieties of tender coconut water. Reductions greater than 5 $\log_{10}$ CFU/mL were achieved for the three pathogens in all coconut waters immediately after HPP. The >5-$\log_{10}$ reduction was sustained for 60 days of refrigerated storage at 4 °C, except for *L. monocytogenes* in one of the coconut waters (pH 5.3), where survivors after HPP managed to adapt and grow during shelf-life. Evaluation of the growth of *L. monocytogenes* in inoculated but not HPP treated samples evidenced that the same coconut water variety was the only supporting growth of the pathogen, whereas it progressively died in the other varieties even though they were less acidic (pH 5.3-5.6). This suggests that some varieties of tender coconut water were a natural source of antimicrobial compounds against *L. monocytogenes*. The identification of such compounds would serve to define processing conditions more accurately.

In conclusion, growth of *C. botulinum* is highly unlikely in HPP tender coconut water under commercial conditions (presence of dissolved oxygen, occurrence of pressure-resistant competitive microbiota, pH 4.7-5.3) or even under temperature abuse. Hence, the pathogen should not be considered of concern in tender coconut water. Additionally, processing the beverage at 600 MPa for 3 min is effective in reducing vegetative pathogens (>5-$\log_{10}$ reductions), although further optimization is required to ensure that this inactivation is maintained throughout storage under refrigeration and temperature abuse conditions. Among vegetative pathogens, *S. enterica* showed the lowest pressure resistance and no ability to grow in the beverage, which suggests that it should not be considered of concern. The pertinent microorganisms are *E. coli* O157:H7 because of its pressure resistance and *L. monocytogenes* because of its pressure resistance and ability to grow at 4 °C in some coconut water varieties.

RESUMEN

El agua de coco verde es una bebida tradicional en zonas tropicales de creciente importancia económica que está ganando popularidad en todo el mundo en los últimos años. Es considerado un zumo isotónico, estéril en el interior de los cocos y ligeramente ácido (pH 4.7 a 5.5). El procesado por alta presión (HPP por sus siglas en inglés) es la tecnología de conservación no térmica más común que se emplea para prolongar la vida útil y garantizar la seguridad de los alimentos mientras se mantienen sus propiedades organolépticas y nutricionales. Este proceso es ampliamente utilizado en Europa, Oceanía y Asia para conservar el agua de coco. Sin embargo, las esporas bacterianas sobreviven al proceso HPP. Por esta razón, el riesgo de crecimiento de *Clostridium botulinum* proteolítico y *Clostridium botulinum* no proteolítico debe ser evaluado en el agua de coco verde debido a su baja acidez (pH >4,6). Además, otros peligros biológicos que pueden introducirse durante el proceso de fabricación de la bebida son patógenos vegetativos como *Escherichia coli* O157:H7, *Listeria monocytogenes* y *Salmonella enterica*. Aunque el proceso HPP ha demostrado ser eficaz en el control de estos patógenos en zumos y bebidas, las tres especies exhiben una gran variabilidad intra-especie en su resistencia frente la presión. Por lo tanto, el objetivo de esta tesis fue evaluar la seguridad del agua de coco verde con respecto a *C. botulinum* y establecer los factores que determinan su crecimiento y la formación de toxina botulínica. Además, este trabajo caracterizó la respuesta a la alta presión de múltiples cepas de *E. coli* O157:H7, *L. monocytogenes* y *S. enterica* para seleccionar cepas representativas que puedan emplearse en “estudios de desafío” en base a su resistencia a la alta presión sobre bebidas ácidas o ligeramente ácidas. Esto permitió diseñar cócteles de cepas que se emplearon posteriormente en la validación de parámetros típicos en la industria (600 MPa durante 3 min) para producir agua de coco segura durante su almacenamiento refrigerado.

Para evaluar el riesgo asociado a *C. botulinum* se procedió a inocular agua de coco verde (pH 5,2) con esporas de *C. botulinum* atoxigénico tipo E y *Clostridium* spp., y posteriormente se sometió a HPP (550 MPa durante 3 min). Se evaluaron rangos amplios de concentraciones de oxígeno disuelto (<0,5-11 mg/L) y temperaturas de incubación (4-20 °C) para establecer su efecto en el crecimiento de las esporas. Los resultados mostraron que las esporas no crecieron a lo largo de 61 días independientemente de la temperatura de incubación y de la concentración de oxígeno disuelto. Además, las bacterias responsables del deterioro que resistieron al proceso HPP crecieron en unos pocos días a 10 y 20 °C, lo que potencialmente puede inhibir el crecimiento de *C. botulinum* incluso si la concentración de oxígeno disuelto se reduce. Para determinar los parámetros que afectan a la germinación y el

crecimiento de las esporas, se procedió a modificar las propiedades de la bebida para crear un escenario favorable para el crecimiento de *C. botulinum*. Este escenario consistió en agua de coco esterilizada por microfiltración ajustada a pH 7. Se observó que los recuentos totales de *C. botulinum* y *Clostridium* spp. disminuyeron a lo largo 61 días de incubación anaerobia a 30 °C. Esto puso de manifiesto que una fracción de las esporas germinó, pero no pudo reanudar crecimiento vegetativo. De forma similar, la adición de germinantes y aminoácidos libres solo favoreció la germinación de una fracción de esporas. La suplementación de agua de coco con caldo de laboratorio a bajas concentraciones fue suficiente para permitir el crecimiento de *C. botulinum* y *Clostridium* spp. Esto evidenció que el contenido de nutrientes limitante en el agua de coco verde pudo prevenir el crecimiento de las esporas.

No obstante, preferiblemente al recuento en placa, una adecuada evaluación del riesgo requiere establecer la formación de toxina botulínica por cepas proteolíticas y no proteolíticas en múltiples variedades de agua de coco. En base a este criterio se evaluó la seguridad de ocho variedades de agua de coco verde procedentes de distintos orígenes geográficos utilizando cócteles de múltiples cepas de *C. botulinum* proteolítico y *C. botulinum* no proteolítico. En condiciones muy favorables (agua de coco estéril incubada durante 50 días en anaerobiosis a 30 °C), la formación de toxina por *C. botulinum* proteolítico se detectó en un solo tipo de agua de coco, mientras que ninguna de las ocho aguas de coco estudiadas permitió el crecimiento de *C. botulinum* no proteolítico. Experimentos posteriores demostraron que el aumento del pH del agua de coco y la adición de caldo de laboratorio o de mezclas de aminoácidos libres, vitaminas y minerales favorecieron el crecimiento de *C. botulinum* proteolítico y *C. botulinum* no proteolítico, pero siempre en las mismas condiciones favorables de incubación. La futura evaluación del riesgo de agua de coco verde (pH 4,7-5,3) procesada por alta presión hidrostática y comercializada en refrigeración debe considerar que la bebida es un sustrato pobre para el crecimiento y la formación de neurotoxina por *C. botulinum*.

Desde un punto de vista de prevalencia, los peligros biológicos que deben controlarse en el agua de coco verde son patógenos vegetativos como *E. coli* enterohemorrágica, *L. monocytogenes* y *Salmonella* spp. La optimización de los parámetros del proceso (presión y tiempo), y la validación del proceso (que es un requisito legal en algunos países como los Estados Unidos) precisan el uso de cepas representativas para la realización de “estudios de desafío”. No obstante, existe una falta de consenso con respecto a la selección de cepas que deben emplearse en los estudios de validación. Para abordar este problema, se evaluó la respuesta a la alta presión (500 MPa durante 1 min a 10 °C) de múltiples cepas de *E. coli* O157:H7, *L. monocytogenes* y *S. enterica* en soluciones modelo consistentes en caldo de soja tríptico + extracto de levadura ajustado a pH 4,5 y 6,0 con ácido cítrico. La resistencia a la

presión varió mucho entre las cepas de una misma especie. Los patógenos más resistentes fueron *L. monocytogenes* y *E. coli* O157:H7 en base a los recuentos mínimos y máximos después de HPP a pH 6,0. Por otro lado, *S. enterica* fue la especie menos resistente con más del 82 % de las cepas mostrando recuentos no detectables después de HPP. Curiosamente, más del 50 % de las cepas de *E. coli* O157:H7 analizadas sobrevivieron al proceso a pH 4,5; pero no consiguieron adaptarse y registraron recuentos no detectables en los siguientes días de análisis. Ninguna de las cepas de *L. monocytogenes* y *S. enterica* sobrevivió a la alta presión a este pH. La recuperación de los patógenos durante el almacenamiento a 12 °C también fue variable a pH 6,0; pero finalmente la mayoría de las cepas se recuperaron y alcanzaron la fase estacionaria entre los días 7 y 14. El análisis estadístico descriptivo multivariante de los datos identificó cepas de cada especie con un fenotipo de resistencia a la presión y capaces de recuperarse después de HPP. Los cócteles de cepas diseñados como resultado de esta caracterización se utilizaron para validar la idoneidad de parámetros HPP empleados comúnmente a nivel industrial (600 MPa durante 3 min a 10 °C) para garantizar la seguridad de cuatro variedades de agua de coco verde. En todas las aguas de coco se lograron reducciones superiores a 5 $\log_{10}$ UFC/mL para los tres patógenos inmediatamente después de HPP. Esta reducción se mantuvo durante 60 días a 4 °C, excepto para *L. monocytogenes* en una de las aguas de coco (pH 5,3) donde las células que sobrevivieron al proceso HPP lograron adaptarse y crecer a lo largo de la vida útil. La evaluación del crecimiento de *L. monocytogenes* en muestras inoculadas, pero no procesadas por HPP, evidenció que la misma variedad de agua de coco era la única que permitía el crecimiento del patógeno, mientras que en las otras variedades murió progresivamente a pesar de ser menos ácidas (pH 5,3-5,6). Esto sugiere que el agua de algunas variedades de coco verde es una fuente natural de compuestos antimicrobianos contra *L. monocytogenes*. La identificación de estos compuestos serviría para definir las condiciones de procesamiento con mayor precisión.

En conclusión, el crecimiento de *C. botulinum* es improbable en agua de coco verde HPP en condiciones comerciales (presencia de oxígeno disuelto, presencia de microbiota competitiva, pH 4,7-5,5) o incluso bajo abuso de temperatura. Por lo tanto, el patógeno no es pertinente en el agua de coco verde. Además, procesar la bebida a 600 MPa durante 3 min es eficaz para reducir patógenos vegetativos (>5-$\log_{10}$), aunque se requiere optimizar el proceso para asegurar que esta inactivación se mantiene durante todo el almacenamiento en condiciones de refrigeración y de abuso de temperatura. Entre los patógenos vegetativos, *S. enterica* mostró la menor resistencia a la presión y la menor capacidad de crecimiento en la bebida, lo que sugiere que no debe considerarse pertinente. Por otro lado, *E. coli* O157:H7 y *L. monocytogenes* pueden considerarse los patógenos pertinentes en agua de coco verde por su elevada resistencia a la alta presión o su capacidad de crecer a 4 °C en algunas variedades.

PREFACE

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TABLE OF CONTENTS

Chapter 1: Introduction 21
1. History and global perspective of coconuts 21
1.1. Origin, spread and varieties 21
1.2. Economic importance of coconut 24
2. Coconut fruit and coconut water 27
2.1. Coconut structure 27
2.2. Coconut water 28
2.2.1. Uses and composition 28
2.2.2. Nutraceutical and medicinal properties 31
2.3. Preservation of coconut water for shelf-life extension 32
2.3.1. Conventional thermal processing 32
2.3.2. Nonthermal processing 33
3. High pressure processing (HPP) for juices and beverages 38
3.1. Definition, evolution and current status 38
3.2. Benefits of HPP in the juice industry 40
3.3. Microbial inactivation by HPP in fruit juices 43
3.3.1. Mechanisms of inactivation and bacterial spores 43
3.3.2. Factors affecting microbial inactivation by HPP in juices 46
3.3.3. Control of pathogens and spoilage microorganisms in juices by HPP 50
4. Aspects to consider for HPP coconut water validation 56
4.1. Process validation and pertinent microorganisms in coconut water 56
4.2. Challenges for the validation of HPP coconut water 58
4.2.1. Strain variability in process validation 60
4.2.2. Clostridium botulinum as a microorganism of concern 60
5. Conclusions and research objectives 62
6. References 64

Chapter 2: Evaluation of factors influencing the growth of non-toxigenic *Clostridium botulinum* type E and *Clostridium* spp. in high-pressure processed and conditioned tender coconut water from Thailand 89
1. Introduction 90
2. Materials and methods 92
2.1. Bacterial strains, sporulation and purification of spore crops 92
2.2. Coconut water preparation and spore inoculation 93
2.3. High-pressure processed coconut water conditioning 94
2.3.1. Dissolved oxygen (DO_2) adjustment 94
2.3.2. Molecular methods for identification of microbial species in coconut water 94
2.4. Filter-sterilized (FS) coconut water conditioning 95
2.4.1. Supplementation with germinants, amino acids and TPGY broth 95
2.5. Microbial growth assessment during storage 96
2.6. Statistical analysis 96
3. Results and discussion 96
3.1. Effect of oxygen concentration and incubation temperature on *C. botulinum* and surviving microbiota growth in HPP treated tender coconut water 96

3.1.1. Refrigeration temperature (4 °C) 96
3.1.2. Moderate temperature abuse (10 °C) 98
3.1.3. Intense temperature abuse (20 °C) 100
3.2. Behavior of *C. botulinum* in microfiltered coconut water at pH 7.0 (FS) under strictly anoxic conditions at 30 ºC 101
3.3. Supplementation with germinants 103
3.4. Supplementation with amino acids 104
3.5. Supplementation with TPGY broth 106
4. Conclusions 108
5. References 109

Chapter 3: Influence of acidity and nutrient content on the growth and toxin formation by proteolytic *Clostridium botulinum* and nonproteolytic *Clostridium botulinum* in anoxic tender coconut water at 30 °C 119
1. Introduction 120
2. Materials and methods 122
2.1. *C. botulinum* strains and spore preparation 122
2.2. Preparation of sterile anoxic coconut water, inoculation with *C. botulinum* and evaluation of growth/toxin formation 122
2.3. Preparation of nutrient-supplemented anoxic coconut water, inoculation with *C. botulinum* and evaluation of growth/toxin formation. 124
2.4. Determination of physicochemical parameters 125
2.5. Multiplex PCR test for detection of *C. botulinum* neurotoxin genes (*bont*) 125
2.6. ELISA tests for detection of *C. botulinum* neurotoxin (BoNT) 125
3. Results and discussion 125
3.1. Limited growth of proteolytic *C. botulinum* and nonproteolytic *C. botulinum* in natural and pH-adjusted anoxic tender coconut water 126
3.2. Effect of pH on proteolytic *C. botulinum* and nonproteolytic *C. botulinum* growth in anoxic tender coconut water T2 128
3.3. Effect of nutrient supplementation on growth of proteolytic *C. botulinum* and nonproteolytic *C. botulinum* in anoxic tender coconut water T2 130
3.4. Effect of pH on growth and toxin formation by proteolytic *C. botulinum* and nonproteolytic *C. botulinum* in five representative anoxic tender coconut waters 132
4. Conclusions 134
5. References 136

Chapter 4: Assessing the pressure resistance of *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella enterica* to high pressure processing (HPP) in citric acid model solutions for process validation 143
1. Introduction 144
2. Materials and methods 146
2.1. Bacterial strains and growth conditions 146
2.2. Screening the pressure resistance of bacterial strains in model solutions 147
2.3. Microbiological analyses and incubation conditions 147
2.4. Statistical analysis 148
2.4.1. Hypothesis testing and data visualization 148
2.4.2. Multivariate descriptive analyses 148

3. Results and discussion 149
3.1. Assessing the pressure resistance of foodborne pathogens in model solutions 149
3.1.1. *Escherichia coli* O157:H7 149
3.1.2. *Listeria monocytogenes* 150
3.1.3. *Salmonella enterica* 152
3.2. Classification and identification of pressure resistant pathogen strains 153
4. Conclusions 158
5. References 159

Chapter 5: Assessing the potential of typical industrial high pressure processing (HPP) parameters for vegetative pathogen control and shelf-life extension of tender coconut water 167
1. Introduction 168
2. Materials and methods 170
2.1. Coconut water 170
2.2. Physicochemical characterization 170
2.3. Determination of phenolic content 170
2.4. Bacterial strains, acid habituation and cocktail preparation 171
2.5. Coconut water inoculation and HPP conditions 171
2.6. Microbial enumeration 172
2.7. Statistical analysis 172
3. Results and discussion 173
3.1. Physicochemical characterization of tender coconut water 173
3.2. HPP safety validation study 175
3.3. Survival of pathogens in coconut water 177
3.4. Shelf-life of HPP coconut water 178
4. Conclusions 180
5. References 182

General discussion and conclusion 189
1. Risk of *C. botulinum* growth in tender HPP coconut water 189
2. Assessing strain variability for process validation of HPP coconut water 193
3. References 199

Conclusions and future work 203

Appendix to Chapter 2 207

Appendix to Chapter 3 211

Appendix to Chapter 4 225

LIST OF TABLES

Table 1. Main differences in the characteristics of tall and dwarf coconuts.....24

Table 2. Cultivation area and production of coconuts, total oil seeds and soybean between 1968 and 2018. 24

Table 3. Composition and physicochemical properties range of coconut water from tender and mature coconuts.....29

Table 4. Effect of emerging nonthermal processing techniques on tender coconut water.....35

Table 5. Commercial examples and characteristics of HPP not-blended coconut waters.....37

Table 6. Examples comparing the effect of HPP and thermal pasteurization in fruit and vegetable juices 41

Table 7. Reduction of pathogens in selected fruit juices subjected to HPP.....53

Table 8. Characteristics of proteolytic *C. botulinum* (Group I) and nonproteolytic *C. botulinum* (Group II).....55

Table 9. Outbreaks of foodborne illness associated with the consumption of coconut-derived products 58

Table 10. Experimental study design with natural and conditioned coconut water.....93

Table 11. Concentration of essential free amino acids in 2 % casein hydrolysate and in a chemically defined medium (CDM) for type E *C. botulinum*. 106

Table 12. Vitamin content in chemically defined media (CDM) and coconut water. 107

Table 13. Details of the coconut waters used in the study. 123

Table 14. Growth of proteolytic *C. botulinum* and nonproteolytic *C. botulinum* cocktails in natural, pH-adjusted and PYGS-supplemented (+PYGS) coconut water from various geographical origins after 50 days of incubation at 30 °C. 127

Table 15. Growth of proteolytic *C. botulinum* and nonproteolytic *C. botulinum* in T2 coconut water, mixtures of T2 coconut water with PYGS broth (T2+PYGS) and PYGS broth adjusted to different pH values after 50 days of incubation at 30 °C. 128

Table 16. Growth and neurotoxin formation by proteolytic *C. botulinum* and nonproteolytic *C. botulinum* in coconut waters from various geographical origins adjusted to different pH values after 50 days of incubation at 30 °C. 133

Table 17. Principal component (PC) scaled loadings from principal component analysis of pathogen counts in model solutions at pH 4.5 and 6.0 processed with HPP (500 MPa for 1 min) and stored at 12 ° C. 154

Table 18. Statistical summary of median counts on day 1 after HPP (500 MPa for 1 min) and median growth rates for strains of each pathogen grouped into clusters by principal component and dendrogram analyses. 156

Table 19. Pathogens cocktails proposed for further validation of HPP juices 157

Table 20. Details of the coconut waters used in the study. 170

Table 21. pH, titratable acidity (TA), total soluble solids (TSS) and total phenolic content (TPC) before and after HPP (600 MPa for 3 min) for 60 days of storage at 4 °C in four different varieties of coconut water 173

Table 22. *E. coli* O157:H7, *L. monocytogenes* and *S. enterica* counts (log_{10} CFU/mL) before and after HPP (600 MPa for 3 min) and logarithmic reductions (log_{10} CFU/mL) for 60 days of storage at 4 °C in four different varieties of coconut water 176

Table 23. Total plate counts, lactic acid bacteria, molds & yeasts and *Enterobacteriaceae* counts before and after HPP (600 MPa for 3 min) for 60 days of storage at 4 °C of four different varieties of coconut water 179

Table 24. Summary of the characteristics of strains proposed for HPP validation 195

Table A1. Nutritional composition of raw Thai coconut water used in the study 207

Table A2. Individual nutrients used to supplement T2 coconut water and final concentration in the mixture 211

Table A3. Combinations of nutrient groups used to separately supplement samples of T2 coconut water.....212

Table A4. Proteolytic *C. botulinum* growth visualized as turbidity and/or gas production in various types of natural coconut water, coconut water adjusted to a higher pH and mixtures of coconut water with PYGS broth incubated anaerobically at 30 °C..213

Table A5. Nonproteolytic *C. botulinum* growth visualized as turbidity and/or gas production in various types of natural coconut water, coconut water adjusted to a higher pH and mixtures of coconut water with PYGS broth incubated anaerobically at 30 °C..215

Table A6. Proteolytic *C. botulinum* growth visualized as turbidity and/or gas production in T2 coconut water, mixtures of T2 coconut water with PYGS broth and PYGS broth adjusted to different pH values incubated anaerobically at 30 °C 217

Table A7. Nonproteolytic *C. botulinum* growth visualized as turbidity and/or gas production in T2 coconut water, mixtures of T2 coconut water with PYGS broth and PYGS broth adjusted to different pH values incubated anaerobically at 30 °C 219

Table A8. Proteolytic *C. botulinum* growth visualized as turbidity and/or gas production in various types of natural and pH-adjusted coconut water incubated anaerobically at 30 °C.....221

Table A9. Nonproteolytic *C. botulinum* growth visualized as turbidity and/or gas production in various types of natural and pH-adjusted coconut water incubated anaerobically at 30 °C.....223

Table A10. Details of the thirty-four *E. coli* strains used in the study 225

Table A11. Details of the forty-four *L. monocytogenes* strains used in the study.....226

Table A12. Details of the forty-five *S. enterica* strains used in the study 227

Table A13. *E. coli* O157:H7 counts in model solutions of TSBYE at pH 6.0 and 4.5 after HPP (500 MPa for 1 min) and during subsequent storage at 12 °C 228

Table A14. *L. monocytogenes* counts in model solutions of TSBYE at pH 6.0 and 4.5 after HPP (500 MPa for 1 min) and during subsequent storage at 12 °C.....230

Table A15. *S. enterica* counts in model solutions of TSBYE at pH 6.0 and 4.5 after HPP (500 MPa for 1 min) and during subsequent storage at 12 °C 232

LIST OF FIGURES

Figure 1. Map showing the chronological dispersal of Austronesian peoples across the Pacific and Indian Oceans 22

Figure 2. Global spread of coconut from original centers of diversity in the Pacific and Indian oceans. 23

Figure 3. Global cultivation area (bars) and production (line) of coconut palm between 1998 and 2018 25

Figure 4. Coconut cultivation area (left) and production (right) per country 26

Figure 5. Six-month tender coconut (A) and eleven-month mature coconut (B) structures 28

Figure 6. Estimation of global HPP food production in 2019 in different food sectors 39

Figure 7. Schematic diagram of a dormant spore structure 44

Figure 8. Events of nutrient-triggered spore germination 55

Figure 9. *C. botulinum* total counts, aerobic plate counts and dissolved oxygen concentration in high-pressure processed coconut water (550 MPa for 3 min) stored at 4 °C with initial different dissolved oxygen concentration levels: (A) low (LO_2; <0.5±0.1 mg/L); (B) unaltered (UO_2; 7.0±0.8 mg/L); (C) high (HO_2; 11.0±0.5 mg/L); and pH evolution of coconut water (D) in LO_2, UO_2, HO_2 97

Figure 10. *C. botulinum* total counts, aerobic plate counts and dissolved oxygen concentration in high-pressure processed coconut water (550 MPa for 3 min) stored at 10 °C with initial different dissolved oxygen concentration levels and pH evolution of coconut water 99

Figure 11. *C. botulinum* total counts, aerobic plate counts and dissolved oxygen concentration in high-pressure processed coconut water (550 MPa for 3 min) stored at 20 °C with initial different dissolved oxygen concentration levels and pH evolution of coconut water 102

Figure 12. *C. botulinum* total counts and aerobic plate counts in FS coconut water (pH 7.0) incubated at 30 °C under strict anaerobic conditions 103

Figure 13. *C. botulinum* total counts and spore counts in FS+G coconut water after incubation for 48 h at 30 °C under strict anoxic conditions 104

Figure 14. *C. botulinum* total counts and spore counts in FS+AA coconut water after incubation for 48 h at 30 °C under strict anoxic conditions 105

Figure 15. *C. botulinum* total counts in coconut water supplemented with TPGY broth at different concentrations after incubation for 48 h at 30 ºC under strictly anoxic conditions 106

Figure 16. Time to turbidity at 30 °C based on OD_{600} in mixtures of T2 coconut water and PYGS broth at different concentrations adjusted to pH 5.5 for the proteolytic *C. botulinum* cocktail and to pH 6.0 for the nonproteolytic *C. botulinum* cocktail 131

Figure 17. Violin plot combining box plot and density trace with cell counts distribution of *E. coli* O157:H7 strains in pH 6.0 (filled) and 4.5 (blank) model solutions processed at 500 MPa for 1 min and stored at 12 °C. 149

Figure 18. Violin plot combining box plot and density trace with cell counts distribution of *L. monocytogenes* strains in pH 6.0 model solution processed at 500 MPa for 1 min and stored at 12 °C. 151

Figure 19. Violin plot combining box plot and density trace with cell counts distribution of *S. enterica* strains at in pH 6.0 model solution processed at 500 MPa for 1 min and stored at 12 °C. 152

Figure 20. Dendrogram analysis for the classification of strains in clusters (A, B or C) 155

Figure 21. Growth curves at 4 °C of *E. coli* O157:H7 (A), *L. monocytogenes* (B) and *S. enterica* (C) in four different varieties of coconut water 177

Figure A1. Growth of background microbiota present in HPP coconut water (550 MPa for 3 min) after 20 days of incubation at 10 °C assessed in reinforced clostridial medium (RCM) and selective RCM_{CY} (Reinforced Clostridial Medium supplemented with 100 µg/mL of cycloserine) after 48 h at 30 °C in an anaerobic workstation.....208

Figure A2. Three-spore cocktail suspension counts assessed in reinforced clostridial medium (RCM) and selective RCM_{CY} (Reinforced Clostridial Medium supplemented with 100 µg/mL of cycloserine) after 24 h of incubation at 30 °C in an anaerobic workstation.....208

Figure A3. *C. botulinum* counts in coconut water with initial high oxygen concentration (HO_2, 11.0±0.5 mg/L O_2), low oxygen concentration (LO_2, <0.5±0.1 mg/L O_2) and unaltered (UO_2, 7.0±0.8 mg/L O_2) after 61 days of incubation at 4 °C 209

Figure A4. *C. botulinum* counts in coconut water with initial high oxygen concentration (HO_2, 11.0±0.5 mg/L O_2), low oxygen concentration (LO_2, <0.5±0.1 mg/L O_2) and unaltered (UO_2, 7.0±0.8 mg/L O_2) after 61 days of incubation at 10 °C 209

Figure A5. *C. botulinum* counts in coconut water with initial high oxygen concentration (HO_2, 11.0±0.5 mg/L O_2), low oxygen concentration (LO_2, <0.5±0.1 mg/L O_2) and unaltered (UO_2, 7.0±0.8 mg/L O_2) after 61 days of incubation at 20 °C 210

Chapter 1

Introduction

CHAPTER 1

INTRODUCTION

1. History and global perspective of coconuts

1.1. Origin, spread and varieties

The coconut palm (*Cocos nucifera* L.) is a monocotyledon member of the *Arecaceae* family and the only species of the genus *Cocos*. It has a pantropical distribution, growing at latitudes 20° north and south of the equator and at altitudes from sea level up to 1200 m (Perera, Perera, Bandaranayake, & Harries, 2009). Several theories have hypothesized the origin of coconuts as we know them nowadays. The first scientific models date from the late 19th century and postulate an American origin. However, advanced evaluation of historical information available at that time served to propose alternate theories and suggest an Asian origin (De Condolle, 1886). In spite of these progresses, renowned botanists during the first half of the 20th century rebutted the widely prevalent belief that the plant originated in Asia, and sustained the introduction of coconuts in Polynesia from South America during the post-Columbian period (Cook, 1901). The main arguments used to support this hypothesis were that: (i) coconuts assumed their distinctive characters on the American continent, (ii) the coconut is unable to spread and establish anywhere without human aid, and (iii) ocean currents are not efficient means of dispersal of the fruit. These arguments were soon countered insisting on an eastern Indian Ocean origin of the plant alleging that *Elaeis* and *Jubaeopsis* palm genera found in Madagascar and South Africa, respectively, are closely related to the *Cocos* genus, and that coconuts are very common in tiny Pacific Ocean islands and inhabitable atolls, which suggests ocean currents as a feasible means of propagation (Beccari, 1917). More precise research on the characteristics of multiple coconut varieties and taxonomic aspects shed light on the topic and positioned the primitive location in a region north of New Zealand, east of Australia, and south of New Guinea (Harries, 1990). Finally, the introduction of genetic analyses confirmed their putative origin in a wider region comprised between Southwest Asia and Melanesia (Baudouin & Lebrun, 2009; Perera et al., 2009). A genetic characterization of 1322 coconut palms around the world revealed two clearly differentiated subpopulations of coconuts: one originating in the Indian Ocean (around the periphery of India, the Maldives and Sri Lanka), and other group from the Pacific Ocean (Southeast Asia islands) (Gunn, Baudouin, & Olsen, 2011). Among subtypes, only the Pacific group displays domestication traits. This served to postulate that the global spread of coconuts is attributed to Austronesian peoples, who migrated from the current territory of Taiwan to the Philippines, and from there to other regions of the Indo-Pacific and eventually to South America (Figure 1).

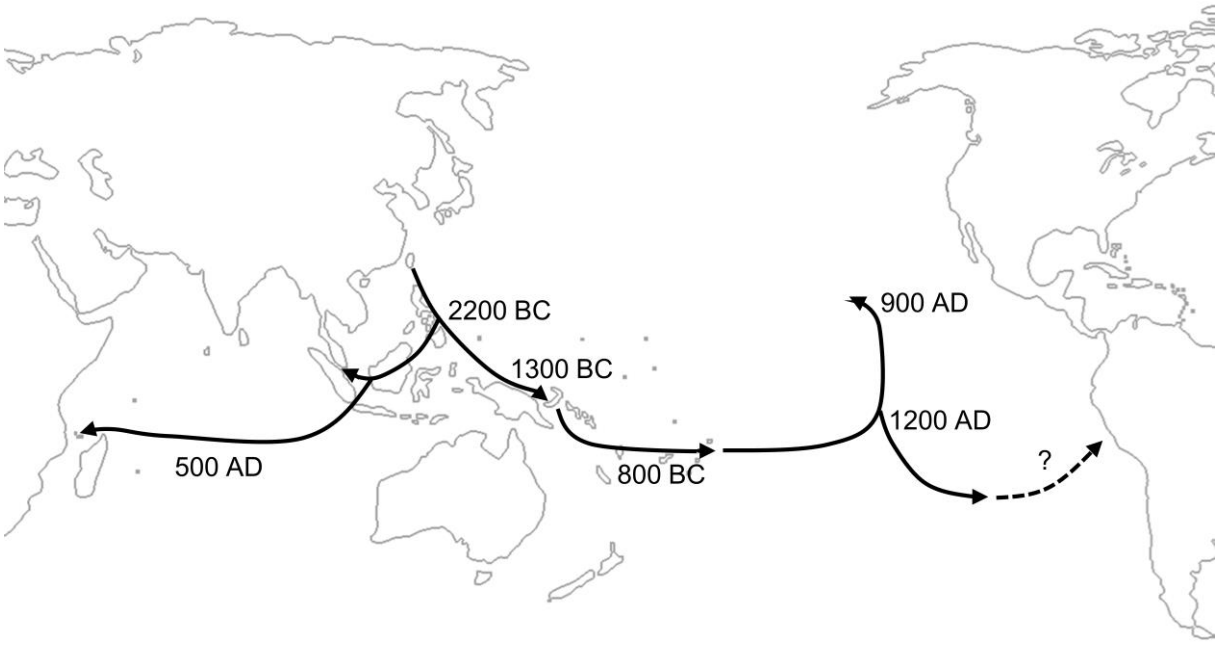

Figure 1. Map showing the chronological dispersal of Austronesian peoples across the Pacific and Indian Oceans (adapted from Benton et al., 2012).

Coconut was disseminated by ancient sea voyagers who carried them as a source of food and drink (Harries, 1978). Together with other plants (such as rice or bananas), coconuts are grouped by the name of “canoe plants” since they were indirectly introduced in the multiple islands in which Austronesians settled. Recent findings of pre-Columbian chicken bones in an archaeological site in Chile dated 1304-1424 AD suggest the introduction of the animal to the Americas by Austronesian voyagers (Storey et al., 2007). Since they often carried coconuts on their canoe trips, it is likely that the plant was introduced in the west coast of the American continent at that time. Nonetheless, short-distance dispersal and establishment of coconut has also been proved. Most symbolic example refers to the Krakatoa caldera. A series of volcanic eruptions between 1928 and 1932 originated new islands that later disappeared. The germination of many seeds (including coconuts) without human intervention was documented in the beach of one of these islands (van Leeuwen, 1933).

Domestication of coconuts took part merely in the Pacific Ocean. Austronesians selected round varieties with a larger endosperm-to-husk ratio because of their higher water content. Other phenotypic indicators suggesting their domestication include dwarf habit (i.e. coconuts were easier to collect) and self-pollination, which lead to a low genetic diversity (Gunn et al., 2011). Plants with these domestication traits are often referred as *niu vai*, and it is believed that they mutated from the tall variety under human pressure (Rivera et al., 1999). A more ancestral morphology (so called *niu kafa*) consists on elongated fruits with think husk.

This subtype was predominant in the Indian Ocean and was cross-pollinated with Pacific varieties when Austronesians travelled west and settled in Madagascar. Arab and Persian traders contributed in the spread of this particular morphology into the east coast of Africa. Indian Ocean coconuts were further introduced in coastal India, Sri Lanka, West Africa and the Caribbean by Portuguese merchants. On the other hand, Pacific Ocean coconuts were largely spread by Spanish galleons from the East Indies into the west coast of America (Gunn et al., 2011) (Figure 2). Endemic coconut populations in the Atlantic coasts of Africa, the Caribbean region and America are the same as the coconuts in India and east Africa, whereas coconuts in the Pacific coast of America are closely related to the Southeast Asian group. This explains the important role of humans in the dissemination of this crop.

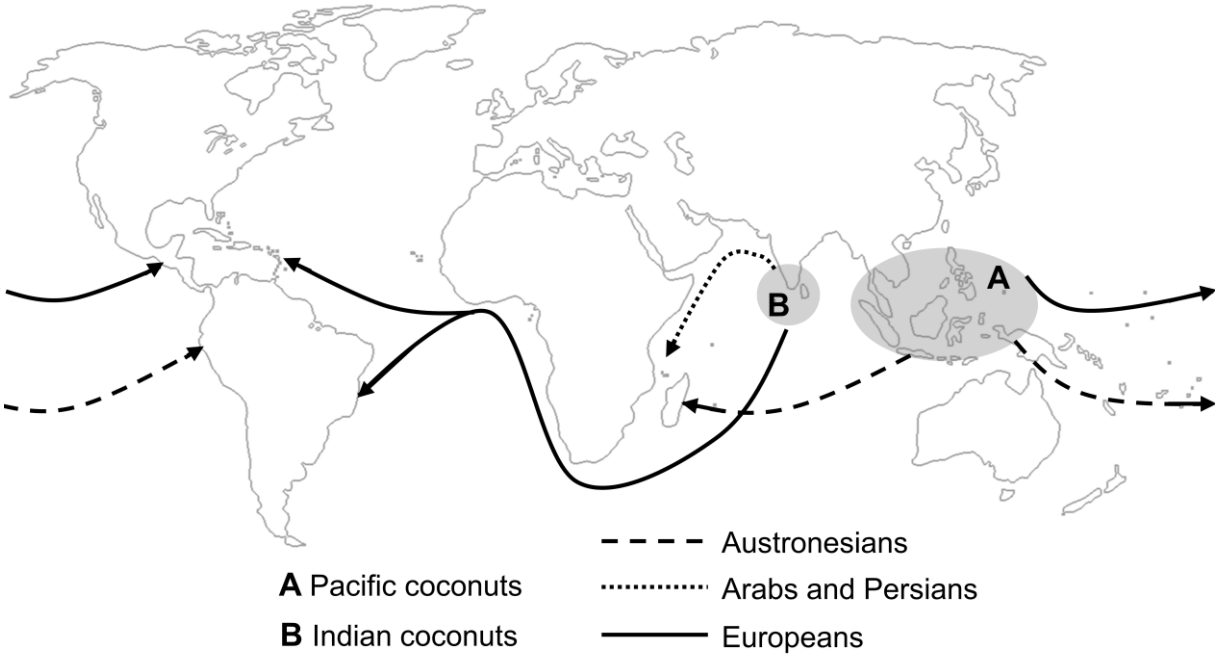

Figure 2. Global spread of coconut from original centers of diversity in the Pacific and Indian oceans (adapted from Gunn et al., 2011).

Despite morphological differences among coconuts, the classification of the species in smaller groups is highly non-standardized. Probably, the most common strategy is based on breeding behavior and structure of palms, which groups coconuts in two types: tall (or *typica*) and dwarf (or *nana*). The main characteristics of each group are summarized in Table 1. In addition to these groups, a few intermediate types (semi-dwarf or semi-tall) can also be found. King Coconut (or a*urantiaca*) in Sri Lanka is the most representative intermediate group. Cross-pollination gives more variability to tall coconuts, which are generally more adapted to climatic fluctuations. This, together with their non-seasonal breeding behavior, higher copra content and longer economic life span, make of tall coconuts the most widely cultivated type worldwide. Dwarf coconuts are differentiated in their larger endosperm-to-husk ratio and

characteristic aroma of the water. It is believed that domestication in the Pacific Ocean islands targeted these traits to make coconut more attractive for Austronesian voyagers in terms of water supply and taste (Nayar, 2017). As a consequence, the main economic interest of this variety nowadays is coconut water extraction from tender fruits (6 to 8 months) and copra production for oil extraction from mature fruits (11 to 13 months). Commercially available minimally processed tender coconut water is mostly extracted from dwarf coconut varieties such as Thailand Green Dwarf (including aromatic *Nam Hom*) or Brazilian Green Dwarf.

Table 1. Main differences in the characteristics of tall and dwarf coconuts (Perera et al., 2009).

Characteristic	Tall (*typica*)	Dwarf (*nana*)
Stature	About 20-30 m	About 10-15 m
Time until flowering	6-8 years	3-4 years
Economic lifespan	About 80-100 years	About 40 years
Bearing nature	Continuous	Seasonal
Nuts/palm/year	Average 40	Average 80-100
Copra amount	200 g/nut	80-100 g/nut
Breeding habit	Cross-pollination	Shelf-pollination

1.2. Economic importance of coconut

It was not until the mid-19th century when European colonial powers began setting extensive coconut plantations lead by the increasing demand of coconut oil for food and industrial purposes. This continued until the mid-20th century, when the oil extracted from the endosperm of the fruit became the most treated vegetable oil worldwide (Nayar, 2017). However, other vegetable oils began to displace coconut oil since then, even though in the 50 year period comprised between 1968 and 2018, the area dedicated to coconut palm cultivation and coconut production increased a 194 % and 237 %, respectively (Table 2).

Table 2. Cultivation area and production of coconuts, total oil seeds and soybean between 1968 and 2018.

Indicator	Crop	Year		Increase (%)
		1968	2018	
Area (10^6 ha)	Coconut	6	12	194
	Soybean[a]	29	125	433
	Oil seeds (total)[a]	90	263	294
Production (10^6 t)	Coconut	26	62	237
	Soybean	41	349	842
	Oil seeds (total)	141	754	534

[a]Soybean and total oil seeds data are included for comparison

Nonetheless, the contribution of coconut to overall oil seeds production decreased from 18 % in 1968 to 8 % in 2018 due to the dramatic increase of other crops, such as soybean or oil palm (*Elaeis guineensis*) whose production raised a 433 % and 1900 %, respectively, in the same time frame. Soybean represents now the major oil crop worldwide with more than 45 % of total oil seed production, whereas in 1968 it only represented 29 %. More meaningful is the case of palm oil, that nowadays is the second vegetable oil in terms of production (36 %) but in 1968 only represented 10 % (behind coconut production at that time) (FAO, 2020).

The significant economic value of coconut palm and the great number of uses (from cooking to applications in the pharmaceutic, cosmetic, construction or clothing industries) have made it the most widespread fruit plant on Earth (Lima et al., 2015). Currently, coconut palms are grown in 93 countries for which the Food and Agriculture Organization reports production and cultivation area figures (FAO, 2020). Global cultivation area and production has been increasing steadily but at low rates in the past 20 years (Figure 3).

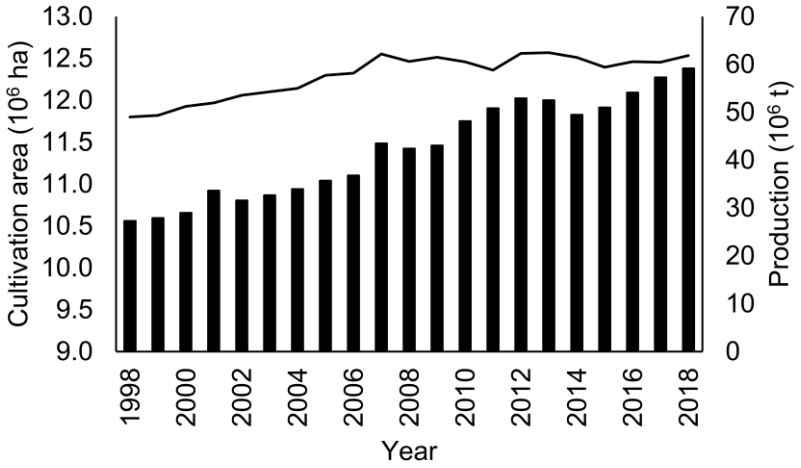

Figure 3. Global cultivation area (bars) and production (line) of coconut palm between 1998 and 2018.

This trend is mainly attributed to the following factors:

- The greater yield of other oil crops compared to coconut. For instance, at the end of 2018, the yield of oil palm fruit production was estimated in 14.4 t/ha, whereas that of coconut was around 4.9 t/ha (almost three times less) (FAO, 2020). Additionally, oil palm fruit gives the highest oil yield (22.5-25.5 %), which makes the oil palm the most efficient oil-producing crop with about 3.6-3.7 t/ha (Murphy, 2007). In the case of coconut, oil is only extracted from the dried endosperm of mature coconuts, which represents a small portion of the whole fruit. Based on global production data and total exports value, the price per kilogram of palm oil in 2018 was 0.628 US$/kg, almost half than the estimated for coconut oil (1.293 US$/kg) (FAO, 2020).

- Technological properties of palm oil are more diverse and suited for the food industry. It can be easily fractionated into stearins and oleins (such as palm olein) of different composition that fit with market demands. The oil is semi-solid at room temperature (around 28 °C) due to its low polyunsaturated fatty acid content, which drastically increases oxidative stability and reduces the need for hydrogenation (Wai Lin, 2011).
- The negative image associated to palm oils in recent years attributed to their high content on saturated fat may also play a role in the slowdown of the production growth since 2007, when it reached its maximum (Figure 3). Nonetheless, it is noteworthy to mention that coconut oil is rich on medium-chain saturated fatty acids, whose implication on cardiovascular disease remains to be established (Kris-Etherton & Fleming, 2015). Recent research suggests that when compared to animal fat, coconut oil is beneficial for cardiovascular health by raising HDL cholesterol and lowering LDL cholesterol. Additionally, it showed no significant effect on LDL cholesterol when compared to monounsaturated-rich plant oils, such as olive oil (Teng et al., 2020).

Despite these factors, coconut plays an important role in the economies of producing countries thanks to the added value of its products. Major coconut-growing countries are not necessarily large countries. In this regard, the Philippines, Indonesia, India, Tanzania and Sri Lanka have the maximum area under cultivation for this specific crop (Figure 4). These five countries together contribute to nearly 83 % of the global area under coconut cultivation. In terms of production, the leading nations contributing to 81 % of total coconut production are Indonesia, the Philippines, India, Sri Lanka and Brazil (FAO, 2020). Although the latter is not amongst the countries with more cultivation area, Brazil has developed an industry around this crop and is the world's fifth-largest producer of coconuts (Figure 4).

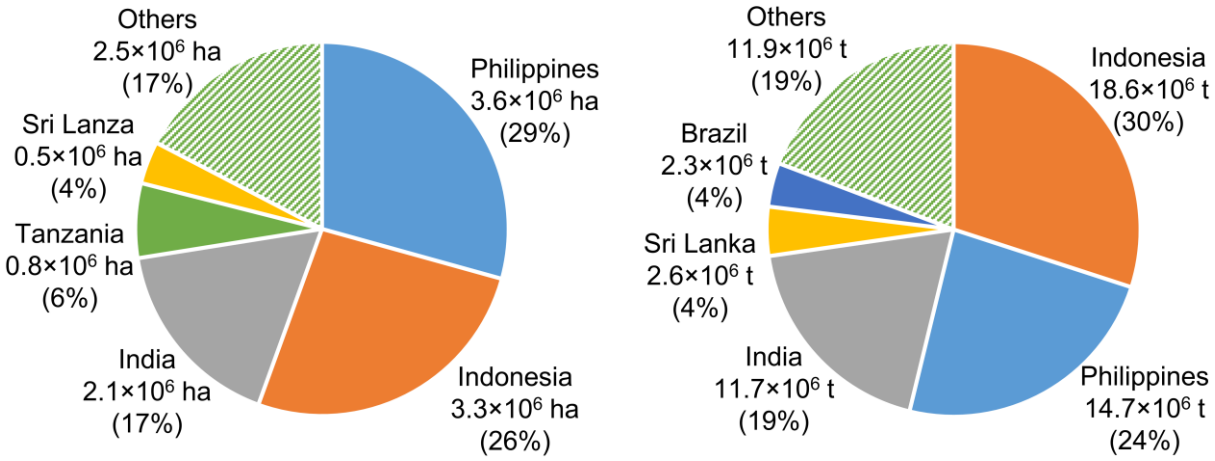

Figure 4. Coconut cultivation area (left) and production (right) per country (FAO, 2020).

Coconut industry in these countries traditionally relies on copra (*i.e.* desiccated coconut endosperm) for oil extraction, and coir from the fibrous husk. Being a versatile crop, other opportunities for diversification and value addition in multiple industrial sectors consist on desiccated coconut meat (chips, powder, roasted paste, coconut milk, milk powder, coconut powder), coconut water (tender nut water, spry dried water, wine, vinegar, *nata de coco*), husk and coconut shell (fiber, mats, ropes, mattresses, textiles, bags, compost, brushes, ornaments, charcoal, activated charcoal) and coconut leaves and stem (furniture, compost, construction material for doors, windows or floors) (Rethinam, 2018). Mature tall coconuts (>10 months) are preferred for oil production because of their longer lifespan, bigger copra content, increased versatility due to the genetic diversity that cross-pollination provides. On the other hand, tender nuts are appreciated for their higher water content and more aromatic flavor. Coconut water accounted in 2016 for 96 % of the volume share of all plant-based water, with more than 700 million liters sold and a market value of about US$ 2.2 billion. The United States is the major importer of coconut water as evidenced by the projected market size of US$ 1.98 billion by 2024 (Rethinam, 2018). Export price of the beverage has increased accordingly a 32 % in the 20-year period comprised between 1997 and 2017 from 0.807 US$/L to 1.07 US$/L (Salum, Foale, Biddle, Bazrafshan, & Adkins, 2020).

Natural and human dissemination of coconuts, agronomic practices and selection resulting from the crossing of different cultivars provides a great variability within the species *Cocos nucifera*. This facilitated the development of numerous commercial applications, including coconut water production. As a result, coconut water composition varies among cultivars and maturity stage of the fruit. Considering this variability is not only important for the identification of varieties with an attractive flavor profile, but also for the optimization of preservation processes that ensure food safety at the same time that maintain sensory quality.

2. Coconut fruit and coconut water

2.1. Coconut structure

Development of the fruit starts upon fertilization of the female flower of palm trees. Tall coconuts tend to cross-pollinate, whereas the dwarf variety self-pollinate. From a botanical point of view, the coconut fruit is not a true nut, but a drupe. The pericarp of coconuts has three differentiated structures: the exocarp, which is fibrous and varies in color depending on the variety or maturation stage of the fruit. The mesocarp, which is the husk portion immediately beneath the exocarp, is fibrous and dry (unlike in other drupes). The endocarp constitutes the remaining outer wall (shell) that protects the seed. The thickness of the endosperm varies depending on the maturity stage of the fruit. The solid endosperm (or kernel) creates a cavity

whose walls become thicker as the coconut matures. This cavity is filled with water (liquid endosperm). In immature fruits, the cavity is completely filled with water, but the quantity of liquid gradually decreases during development and on storage after harvesting (Niral & Jerard, 2018). Figure 5 shows the different structures of coconuts. Tender coconuts from dwarf varieties generally contain sweeter and more flavored water, whereas coconuts from tall varieties tend to have less sweet water with a more plain aromatic profile. Additionally, dwarf palms generally produce more coconuts per year than tall palms, and their nuts have larger endosperm-to-husk ratio, which results in an increased production yield.

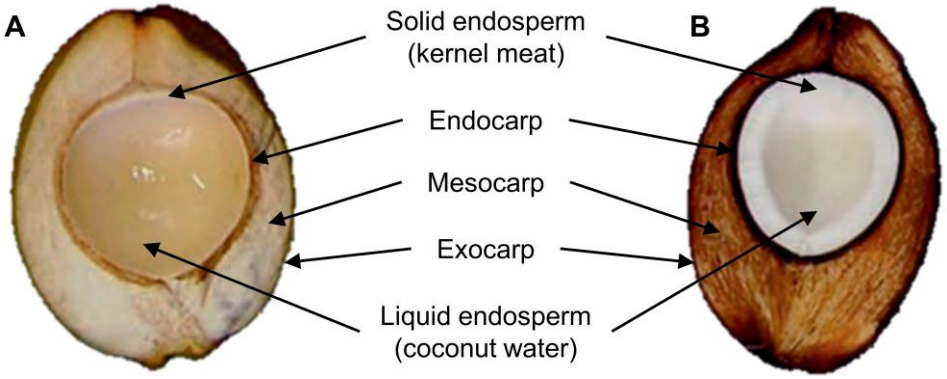

Figure 5. Six-month tender coconut (A) and eleven-month mature coconut (B) structures (adapted from Prades, Dornier, Diop, & Pain, 2012).

2.2. Coconut water

2.2.1. Uses and composition

The maximum content of water in coconuts occurs between 6 and 8 months after flowering (Jackson, Gordon, Wizzard, McCook, & Rolle, 2004). At this time, the fruit has already achieved full size but remains immature. Historically, coconut water from green fruits has been preferred because of its higher yield and delicate flavor profile. It is believed that beverage played an important role in the colonization of Oceania, where small islands and atolls often lack sources of freshwater (Niral & Jerard, 2018). Aside from water of the nuts, other derived products that have emerged in recent years due to the improvement of technological processes are coconut vinegar (*i.e.* concentered coconut water fermented by cultures of *Saccharomyces cerevisiae* and *Acetobacter* spp.), coconut honey (*i.e.* evaporated coconut water) or *nata de coco* (*i.e.* gelatinous substance formed on the surface of coconut water after fermentation by *Acetobacter* spp.). Additional miscellaneous uses given to coconut water include growth medium for microorganisms and edible mushrooms, source of growth-promoting factors for plants, or even blood plasma substitute under extreme circumstances (Campbell-Falck, Thomas, Falck, Tutuo, & Clem, 2000; Manikantan, Pandiselvam, Beegum, & Mathew, 2018; Quimio, 1986; M. E. Smith & Bull, 1976). Nonetheless, production of tender

coconut water is the application that generates more interest from a commercial point of view because of the high volume of water in tender nuts and the added value of the product. This is why the cultivation of coconut palms dedicated to the sole production of coconut water is gaining attention, especially in Thailand, the Philippines, Malaysia, Sri Lanka and Brazil, where suitable dwarf and tall varieties are grown (Niral & Jerard, 2018).

The main constituents of coconut water are soluble sugars, minerals and proteins. To a lesser extent the beverage is also source of lipids, vitamins and organic acids, which contribute to its low caloric content and health-related properties (Table 3). Composition of coconut water fluctuates broadly depending on the variety of coconut, the location and the soil condition; but more dramatic changes are observed over maturation of the fruit (Prades, Dornier, et al., 2012).

Table 3. Composition and physicochemical properties range of coconut water from tender and mature coconuts.

Parameters	Coconut maturity stage	
	Tender (6-8 months)	Mature (>9 months)
Sugar content (g/100 mL)	3.2-7.0	1.9-5.8
Fructose	1.1-4.3	1.4-2.6
Glucose	1.2-3.9	1.5-2.6
Sucrose	0.1-0.9	2.4-5.4
Minerals content (mg/100 mL)	280-860	260-1035
Potassium	205–310	35-310
Calcium	9-18	17-32
Magnesium	5-45	10-38
Sodium	1-37	16-105
Phosphorus	5-71	13-73
Total protein (g/L)	0.03-0.13	0.03-0.51
Total soluble solids (°Brix)	3.9-7.7	3.3-5.8
pH	4.6-5.8	5.2-6.1
Titratable acidity (% malic acid)	0.04-0.19	0.03-0.06
Water volume (mL/nut)	190-600	100-385

Data was retrieved from published literature (Jackson et al., 2004; Keng et al., 2017; Kwiatkowski & Clemente, 2008; Prades, Dornier, et al., 2012; Santoso, Kubo, Ota, Tadokoro, & Maekawa, 1996; Tan, Cheng, Bhat, Rusul, & Easa, 2014; Yong, Ge, Ng, & Tan, 2009).

Carbohydrates (mainly sugars) are the main fraction of soluble solids in coconut water (4 to 7 %). In the tender stage (6-8 months), coconut water reaches its higher sugar content with glucose and fructose as dominating monosaccharides, but in the mature stage (from 9 months onwards), total sugar concentration decreases as it is gradually used by the fruit to form the solid endosperm. The proportion of reducing sugars also decreases at the expense

of non-reducing sugars (mainly sucrose) (Jackson et al., 2004; Santoso et al., 1996). Total soluble solids are an indicator of the sweetness of coconut water and relates well with sugar content. This indicator decreases in mature fruits as a result of the conversion of fructose and glucose into sucrose (Keng et al., 2017; Kwiatkowski & Clemente, 2008; Tan et al., 2014). Other minor sugars present in mature coconut water are sorbitol, xylose and mannose. The second major constituent of the nut water in terms of quantity are minerals (0.4 to 1 %). Potassium is the most abundant electrolyte, followed by calcium, magnesium, sodium and phosphorus. Whereas K concentration slightly decreases or remains stable in the water of mature fruits, Ca, Mg, Na and P concentrations increase. Overall, mineral content of mature coconut water is higher in comparison with tender coconut water (Keng et al., 2017; Kwiatkowski & Clemente, 2008; Tan et al., 2014). Manganese and zinc are microelements also present but in smaller concentrations. Coconut water contains amino acids and proteins, but in a very small proportion compared with the rest of nutrients and to other foods (0.03 to 0.14 %). Total protein only represents between 2.1 and 9.4 % of the dry matter of coconut water depending on the maturity stage of the fruit (Santoso et al., 1996). From a compositional perspective, proteins in coconut water have a favorable amino acid profile and a high nutritional value with alanine, arginine, cysteine and serine contents proportionally higher than cow's milk (Woodroof, 1970). Protein concentration shows an increase along with maturation of coconuts, which may be associated with the endosperm development in the nut (Figure 5) because storage protein content increases as the endosperm hardens from a "jelly" stage to the kernel stage (Jackson et al., 2004).

Besides sugars, minerals and proteins, other minor constituents of coconut water are aromatic compounds. Nut water has a typical taste and aroma due to organic acids (mainly malic acid, but also succinic, citric, acetic and tartaric acids) (Santoso et al., 1996) and volatile compounds. A characterization of the volatile fraction of coconut water from tender dwarf coconuts identified 73 compounds (De Marchi et al., 2015). Ketones (δ-octalactone and nonalactone), aldehydes (acetaldehyde, benzaldehyde, 3,4-dimethyl benzaldehyde), alcohols (2-ethyl-1-hexanol, octanol), lactones (3-penten-2-one) and fatty acids (octanoic, decanoic, dodecanoic acids) mainly characterize the aromatic profile of the beverage, which greatly differs depending on the variety and the maturity stage of the fruit (Prades, Assa, Dornier, Pain, & Boulanger, 2012). Vitamins and phenolic compounds are also trace elements present in coconut water that contribute to its health-promoting attributes. Vitamin C is predominant in the beverage and confers certain oxidation stability. Other vitamins from the B group such as thiamin (B1), riboflavin (B2), nicotinamide (B3), pyridoxine (B6) or folic acid (B9) have been identified as well in concentrations ranging from 0.003 mg/L to 0.64 mg/L (Santoso et al., 1996; Yong et al., 2009). As with other constituents, the concentration and composition of phenolic

compounds varies with maturity stage and variety of coconut. Total phenolic content (TPC) is higher in the water of tender nuts (up to 99 mg/L) than in mature nuts (up to 46 mg/L) as a result of oxidation during ripening process (Tanqueco, Rodriguez, Laude, & Cueno, 2007). Specific phenolic compounds identified include phenolic acids (gallic acid, ferulic acid or syringic acid, among others) (Geetha, Mhavana, Chetana, Gopala Krishna, & Suresh Kumar, 2016), dimeric and trimeric procyanidins (including their monomers catechin and epicatechin) (Chang & Wu, 2011; Cunha et al., 2020) and caffeoylshikimic and dicaffeoyl quinic acids (derived from caffeic acid) (Cunha et al., 2020). Overall, concentration of phenolic compounds is lower in coconut water than in coconut me kernel because the solid endosperm contains more cellular tissue that encloses these molecules (Mahayothee et al., 2016).

2.2.2. Nutraceutical and medicinal properties

Traditionally, coconut water has been considered a natural medicinal remedy in various civilizations. Because of its mineral composition, the beverage has rehydrating properties and can be used to treat diarrhea or to alleviate the symptoms or other diseases such as dysentery (Adams & Bratt, 1992; DebMandal & Mandal, 2011). Several studies in animals attribute to coconut water anti-inflammatory, anti-diabetic, anti-thrombotic and cardioprotective properties (Anurag & Rajamohan, 2003; Bhagya, Prema, & Rajamohan, 2012; Loki & Rajamohan, 2013; Preetha, Devi, & Rajamohan, 2015). In this regard, recent research elucidated the mechanism by which coconut water suppresses hepatic inflammation and tissue damage (Lakshmanan et al., 2020). The identification in tender nut water of the host defense peptide *Cn*-AMP2 potentially provides anti-cancer activity to the beverage. Research showed for this peptide antiproliferative activity against human glioma cells with half maximal inhibitory concentration (IC_{50}) values between 1.25 and 1.85 mM (Prabhu et al., 2014). Similarly, coconut water is source of kinetin, a phytohormone from the group of cytokinin that shows anti-thrombotic activity and potential antiproliferative effect on mammalian cancer cells (Hsiao et al., 2003; Vermeulen et al., 2002). Typical phenolic compounds that are present in coconut water are responsible of its *in vitro* antioxidant activity. A 1:10 dilution of four different varieties of tender coconut water (green, yellow and red dwarfs and yellow Malaysian) scavenged between 3.6 % and 38.3 % the 2,2-diphenyl-1-picrilhidrazilo (DPPH) radical (Santos et al., 2013). Free radical scavenging capacity decreases with maturity of coconuts, which relates well with total phenolic content. A 1:10 dilution of tender coconut water (4 to 6 months) inhibited DPPH and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) activities in a 35.2 % and 32.3 %, respectively. On the other hand, mature coconut water (>9 months) diluted in the same proportion inhibited 7.8 % DPPH and 15.3 % ABTS radicals (Mantena et al., 2003).

2.3. Preservation of coconut water for shelf-life extension

Coconut water has been traditionally consumed locally in production areas and directly from the fruit. After collection from palm trees, tender coconuts can be kept without spoilage at ambient conditions between 6 and 15 days. Afterward, fermentation takes place and the liquid inside the nuts becomes unfit for consumption (Reddy, Das, & Das, 2005). Coconut water is sterile inside the fruit, but the conventional collection process requires cracking the endocarp to extract the water inside. This procedure exposes the liquid to environmental microbial contamination, which leads to spoilage within 4 to 6 days under refrigeration (Donsingha & Assatarakul, 2018; Raghubeer et al., 2020). Even if coconut water is aseptically extracted, exposure to air has a detrimental effect on nutritional and sensory quality due to oxidation. Nevertheless, some patent applications describe methods to aseptically extract and pack the liquid endosperm from tender coconuts in a way that this oxidation is prevented (Lam et al., 2015a, Lam et al., 2015b). Polyphenol oxidase (PPO) and peroxidase (POD) are enzymes naturally present in the liquid endosperm that catalyze the oxidation of phenolic compounds (Campos, Souza, Coelho, & Glória, 1996). In presence of oxygen, they are responsible of the characteristic pink discoloration observed in coconut water (Prades, Dornier, et al., 2012). Nonetheless, recent research suggests that other non-enzymatic reactions may also play a role in the pink color development, such as the accumulation of oligomeric procyanidins, which are precursors of anthocyanin pigments (Cunha et al., 2020). Due to these limitations, the implementation of a conservation process is required to extend commercial shelf-life of coconut water and to facilitate its distribution.

2.3.1. Conventional thermal processing

Heat pasteurization and sterilization are the most widely used methods to preserve coconut water at industrial level. Research has been conducted on novel microwave processing, ohmic heating and combinations of high pressure with high temperature (HPHT), but these techniques have not been implemented yet by the coconut water industry (Aniesrani Delfiya & Thangavel, 2016; Chourio, Salais-Fierro, Mehmood, Martinez-Monteagudo, & Saldaña, 2018; Matsui, Gut, de Oliveira, & Tadini, 2008). Dominating oxidative enzymes (PPO and POD) and spoilage microorganisms can be effectively inactivated with the right combination of temperature and time. However, nutritional and sensory qualities are often adversely affected because coconut water is source of heat-sensitive vitamins and aroma compounds. Butz et al. (1997) demonstrated that HPP had the potential to preserve antimutagenic activities of grapefruit, strawberry, carrot, cauliflower, kohlrabi, leek and spinach juices, whereas heat processing destroyed their protective attributes. Additionally, heat catalyzes Maillard reactions that induce changes in color and physicochemical composition

such as pH, reducing sugars, turbidity or titratable acidity (Adubofuor, Amoah, & Osei-Bonsu, 2016; De Marchi et al., 2015; Jayalekshmy & Mathew, 1990; Nasution, Jirapakkul, & Lorjaroenphon, 2019).

Subjecting coconut water to intense processing conditions (100 °C for 10 min) can preserve the beverage from a microbiological point of view up to six months at room temperature, but acidity slightly decreases over time (Chowdhury, Rahman, Islam, & Islam, 2009). Milder processing conditions (85 °C for 10 min) maintained microbiological quality of tender coconut water up to five weeks at 5 °C. Total aerobes, molds and yeasts were effectively inactivated, and *Escherichia coli* was not detected. However, sensory analysis by trained panelists revealed a loss of the desired sweet and sour flavors, and the development of astringent notes. Additionally, ascorbic acid was completely degraded after three weeks and total phenolic content was reduced a 43.5 % immediately after pasteurization (Rajashri, Roopa, Negi, & Rastogi, 2020). Other authors reported that a more optimized high temperature short time (HTST) treatment (72 °C, 15 s) also reduced microbial spoilage indicators to extend shelf-life up to 30 days at 4 °C. Nonetheless, aroma, flavor and overall acceptability achieved low ratings after sensory evaluation (Ma et al., 2019). Enzymatic inactivation gives color stability during storage. Processing tender coconut water at 95 °C for 5 min completely inactivated PPO, which is more heat-resistant than POD (Sanganamoni, Mallesh, Vandana, & Rao, 2017). Although color remains stable, intense heat treatments induce irreversible changes due to the formation of Maillard products as a result of the interaction between free amino acids and reducing sugars, which leads to yellow/brown discoloration and alteration of organoleptic qualities (Tan et al., 2014). Industrially, chemical additives such as citric acid, sodium citrate or sodium chloride are added to adjust acidity and minimize color changes due to intense heat processing (Manikantan et al., 2018). Coconut water can also be spray dried to ease logistics for distribution and increase stability during storage. In this case, maltodextrin (15-30 %) has to be dissolved in the liquid prior to drying to improve fluidity of the final product. An inlet temperature between 150 and 160 °C and an outlet temperature around 65 °C is required to efficiently remove the water and concentrate the powder (Jayasundera & Kulatunga, 2014; Rattanaburee, Amnuaikit, & Puripattanavong, 2017).

2.3.2. Nonthermal processing

Consumers demand nowadays more natural and healthier foods with fresh-like attributes. This trend is pushing the food industry to invest in preservation methods that minimally affect nutritional and sensory qualities of food products while ensuring safety. An increasing number of nonthermal processing technologies have emerged in recent years to

minimize the negative effects associated to intense heat treatments and to inactivate spoilage and pathogenic microorganisms (Barba, Koubaa, do Prado-Silva, Orlien, & Sant'Ana, 2017). Among these, the most widely implemented in the coconut water industry are high pressure processing (HPP) and membrane filtration, but research has been conducted at laboratory scale to evaluate the potential of other processing techniques. For instance, UV-based radiation is industrially implemented to preserve apple juice or cider mainly in the US, but it is not yet used by the coconut water industry. Similarly, high-pressure carbon dioxide (HPCD) is another nonthermal technology used in the past to process fruit juices, but not coconut water. Nevertheless, it is nowadays abandoned due to high operational costs, the need for a continuous supply of CO_2 and the need for aseptic filling stations.

UV-C radiation is available at industrial scale to extend shelf-life and guarantee food safety of liquid foods, such as juices or apple cider (Koutchma, 2008). Although not industrially applied to coconut water yet, potential of UV-C radiation to control pathogens in the beverage has been evaluated. For instance, a dose of 30 mJ/cm^2 from a 240 nm wavelength radiation achieved >5 $\log_{10}$ CFU/mL reduction of *Listeria monocytogenes*, *Salmonella* Typhimurium and *E. coli* (Bhullar et al., 2018). In addition to vegetative microorganisms, UV-C radiation can also be used to control spore forming bacteria in coconut water. In this case, UV dose required to achieve a 5-$\log_{10}$ reduction of *Bacillus cereus* and *Clostridium sporogenes* increased to 46 and 75 mJ/cm^2, respectively (Pendyala, Patras, Gopisetty, Sasges, & Balamurugan, 2019). Spoilage indicators such as total aerobes, molds and yeasts are also sensitive to UV radiation in coconut water. For instance, shelf-life of tender coconut water increased up to 18 days in UV treated samples compared to only 5 days of fresh samples stored at 4 °C (Donsingha & Assatarakul, 2018). Similarly to UV radiation, HPCD technology has also been developed to industrial scale to process juices and beverages, but its implementation has been abandoned and currently there are no commercial products in the market processed with this technology (Picart-Palmade et al., 2019). Research on coconut water revealed that subjecting the beverage to 120 bar, 40 °C and 30 min induced a 5 $\log_{10}$ reduction of total aerobes, lactic acid bacteria, molds and yeasts, and a 7 $\log_{10}$ reduction of coliforms. Additionally, no differences in the physicochemical characteristics of coconut water were detected between HPCD and fresh samples. Although HPCD resulted in reduction of volatile compounds (especially short and medium chain alcohols), a triangle sensory test showed no differences compared to unprocessed samples (Cappelletti et al., 2015; De Marchi et al., 2015). Similarly, processing coconut water at 34.5 MPa at 25 °C and 13 % CO_2 for 6 min reduced total aerobic counts and extended shelf-life of the beverage up to 9 weeks at 4 °C (Damar, Balaban, & Sims, 2009). The main results that can be drawn from research conducted with other incipient technologies such as cold plasma, ultrasound, pulsed light or ozone processing are summarized in Table 4.

Table 4. Effect of emerging nonthermal processing techniques on tender coconut water (adapted from Naik et al. 2020).

Technology	Conditions	Main observations	References
Ultrasound processing	286 W/L, 20 kHz	POD activity was reduced 27 % and became sensitized to heat	(Rojas, Trevilin, Funcia, Gut, & Augusto, 2017)
	655.8 W/L, 20 kHz	Complete inactivation of PPO and POD	(Ribeiro, Valdramidis, Nunes, & de Souza, 2017)
	60 % amplitude, 20 kHz, 6 min	A 2 $\log_{10}$ reduction of total aerobes was observed in samples with 5000 IU nisin/200 mL. No growth of molds, yeasts and *E. coli* took place	(Rajashri et al., 2020)
Cold plasma	18 and 28 kV, 0.84 to 2.53 min	POD is more resistant to cold plasma than PPO	(Chutia, Kalita, Mahanta, Ojah, & Choudhury, 2019)
	Input power of 450 and 650 W for 1.86 to 3.11 min	*S. enterica* is more resistant than *Staphylococcus aureus* and can be used as reference organism	(Gabriel et al., 2016)
	90 kV, 2 min	A 1.3 $\log_{10}$ reduction of *S. enterica* was observed. Addition of 400 ppm citric acid increased reduction to 5 $\log_{10}$. More than 80 % ascorbic acid was lost	(Mahnot, Mahanta, Keener, & Misra, 2019)
	15 to 20 kV, 0.2 to 0.73 kHz, 15 min	POD inactivation increased at higher frequencies, but total phenolic content decreased	(Porto et al., 2020)
Pulsed light	5 mm juice layer, 5 cm shelf distance from the lamp, 240 pulses	A 5.2 $\log_{10}$ reduction of *E. coli* was observed. Total soluble solids, pH and color were not affected	(Preetha, Prasath Venugopal, Varadharaju, & Kennedy, 2017)
Ozone processing	0.075 to 0.37 g O_3/L, 10-30 °C	Complete inactivation of POD	(Porto et al., 2020)
	0.02 g O_3/L with a flow rate of 1 L/min	Total aerobes and *E. coli* were reduced in samples containing 5000 IU nisin/200 mL up to 3 weeks at 4°C	(Rajashri et al., 2020)

Membrane filtration is a nonthermal processing technology industrially implemented and currently used to process coconut water (Harmless Harvest, 2020; Symbiosis, 2020). It is not considered a lethal intervention step as it solely relies on the separation of solutes from the fluid, including microorganisms, to guarantee food safety and shelf-life extension. Prefiltration or centrifugation are common operations applied before microfiltration or ultrafiltration to remove gross solid particles and delay cake formation, which is considered the most dominant membrane fouling mechanism (Lamdande, Mittal, & Raghavarao, 2020). Microfiltration typically uses cellulosic materials with a pore opening between 0.22 and 0.45 µm, and requires aseptic or close-to-aseptic filling of the filtered beverage to prevent cross contamination. Previous work showed that microfiltered coconut water remained sterile during the whole duration of the studies (between 1 and 6 months) (Mahnot, Gupta, & Mahanta, 2019; Reddy, Das, & Das, 2007). Despite microbial stability, overall sensory acceptability and composition of coconut water is affected. A two-stage filtration reduced flavor and overall acceptability by 9 and 11 %, respectively. After one month of storage, these indicators further decreased by 6 %. Additionally, fat, ash, total sugar, reducing sugar and protein decreased by 40.0, 43.9, 23.4, 29.2 and 13.3 %, respectively. Similarly, between 10.2 and 22.2 % of different minerals were removed. As a result, surface tension of coconut water increased and viscosity decreased (Reddy et al., 2007). On the other hand, the ultrafiltration process uses membranes differentiated on molecular weight cut-off, generally between 30 and 150 kDa. This allows enzyme removal from coconut water which results in a more stable product from a sensory perspective. For instance, PPO and POD activities were reduced a 95.1 % and 97.9 % in tender coconut water after ultrafiltration through a 30 kDa polyethersulfone membrane (Lamdande et al., 2020). Similarly, a 30 kDa cellulose membrane resulted in a 100 % enzymatic retention (Debien, Gomes, Ongaratto, & Viotto, 2013). Microbial analyses confirmed sterility of the beverage, and physicochemical properties can be maintained with an optimized configuration that includes a stirred cell and ultrasounds to minimize fouling effects. Sensory quality of membrane-processed coconut water was similar to that of fresh samples, and exhibited higher acceptability than thermally processed coconut water (Lamdande et al., 2020).

Among nonthermal preservation technologies, high pressure processing (HPP) is the most versatile and widely implemented in the food industry. Coconut water benefits from HPP as evidenced by the increasing number of companies that have adopted this innovative process to extend the commercial shelf-life pure tender coconut water (Table 5). A common practice in the HPP industry is to blend the beverage with more acidic juices such as lime or lemon juice. This gives rise to new flavor profiles and reduces the pH of the low-acid beverage, which increases microbiological stability and controls spore growth. Section 3 of the present chapter deals in depth with different aspects of HPP technology.

Table 5. Commercial examples and characteristics of HPP not-blended tender coconut waters.

Commercial brand	Company location	Variety of coconut	Sourcing country
Chi	United Kingdom	Thailand Green Dwarf	Thailand
Coco Wilson	Spain	Thailand Green Dwarf	Thailand
Coco Loco	Singapore	Thailand Green Dwarf	Thailand
Happy Coco	The Netherlands	King Coconut	Sri Lanka
Pure Brazilian	United States	Brazilian Green Dwarf	Brazil
Rebel Kitchen	United Kingdom	Pacific Tall	The Philippines
Romantics	Spain	Thailand Green Dwarf	Thailand

Despite the commercial success of HPP, research evaluating the effect of this technology on tender coconut water is scarce. Ma et al., (2019) described the potential of HPP (500 MPa for 5 min) to reduce total aerobic, molds and yeasts counts in coconut water (pH 5.5) below 2 and 1.5 $\log_{10}$ CFU/mL, respectively, for a period of 30 days at 4 °C. Additionally, HPP preserved amino acid and total phenolic content better than heat pasteurization (72 °C, 15 s). Consequently, antioxidant activity of HPP coconut water was higher than that of pasteurized samples. With regards to quality, instrumental color measurements revealed that coordinates L*a*b* remained more stable through storage in HPP samples than in heat treated ones. This was confirmed by sensory analysis where aroma, flavor, color and overall acceptability of HPP coconut water ranked closer to unprocessed samples and always higher than pasteurized samples during refrigerated storage. In addition to longer shelf-life than unprocessed samples and improved quality compared to heat-treated counterparts, HPP also inactivates foodborne pathogens to ensure safety. Processing two coconut water varieties from Brazil (pH 5.2) and Florida (pH 5.4) at 593 MPa for 3 min achieved a >5-$\log_{10}$ reduction of *E. coli* O157:H7, *L. monocytogenes* and *S. enterica* for 75 and 54 days of storage at 4 °C (Raghubeer et al., 2020). Additionally, same processing conditions maintained microbiological quality indicators (aerobic plate counts, molds, yeasts, coliforms and lactic acid bacteria) below 2 $\log_{10}$ CFU/mL for 120 days under refrigerated storage in both coconut water types. In accordance with these findings, other authors reported reductions greater than 5 $\log_{10}$ CFU/mL for the three pathogens immediately after processing coconut water from Florida (pH 5.5-6.1) at 500 or 600 MPa for 2 min (Lukas, 2013).

Until recently, the coconut water available on the market was preserved only by heat treatments. The emergence of HPP and the lack of research generate the need to assess the risks associated with this technology. Next section of this chapter will deal in detail with high pressure processing, its current status of implementation in the juice industry and the research challenges that require attention for adequate process validation of pure (not blended) coconut water, with a special emphasis on the control of vegetative pathogens and spores.

3. High pressure processing (HPP) for juices and beverages

3.1. Definition, evolution and current status

High pressure processing (HPP) is a nonthermal food preservation technology that uses hydrostatic pressure generated by water in the range of 400 to 600 MPa for a few minutes, to inactivate spoilage and pathogenic vegetative microorganisms at chilled or room temperature (González-Angulo, Serment-Moreno, Queirós, & Tonello-Samson, 2021). It is typically applied to high or medium moisture foods (a_w >0.8) already packaged in flexible and waterproof materials (in-pack HPP), although new equipment has been recently developed to process liquid foods in bulk before packaging (in-bulk HPP) (Tonello-Samson, Queirós, & González-Angulo, 2020). The isostatic principle ensures that pressure is homogenously distributed to all points of the product with the same intensity, independently of size and shape. Another governing principle is that of Le Chatelier's, which states that a system at equilibrium tends to minimize the effect of any external factor that is perturbed (Martínez-Monteagudo & Balasubramaniam, 2016). Molecular covalent bonds are not affected by pressure because their working distance cannot be reduced any further. Additionally, covalent bond breaking is associated with a volume increase, which according to Le Chatelier's principle is not favored by HPP (Mozhaev, Heremans, Frank, Masson, & Balny, 1994). On the contrary, weak bondns, which are distance-dependent interactions such as van der Waals forces, hydrogen bonds, hydrophobic or electrostatic interactions experience under pressure reversible or irreversible changes in the secondary and tertiary structures of macromolecules such as proteins, carbohydrates or lipids (Martinez-Monteagudo & Saldaña, 2014). The fact that HPP does not alter covalent bonds has been the central hypothesis behind functionality preservation of vitamins, antioxidants, other micronutrients, flavor and aroma compounds. These compounds are small molecules with no secondary or tertiary structures.

The ability of HPP to inactivate microorganisms was first established in 1892 by H. Roger, who already ascertained two important phenomena: (i) the different behavior between bacterial species under pressure (*S. aureus* was not inactivated at 300 MPa, whereas *Streptococcus* spp. was more sensitive), and (ii) the difference between physiological states of bacteria (*B. anthracis* spores were found to be more resistant to pressure than vegetative cells of the pathogen) (Roger, 1892, 1895). A few years later, B. H. Hite became a pioneer in the application of HPP to real food systems. It was observed that processing milk between 400 and 700 MPa was sufficient to delay microbiological spoilage for at least 24 h (Hite, 1898). Research continued during the 20th century focusing in diverse areas not only related to microbial inactivation, but also on protein structure, enzyme activity or even virus attenuation for the preparation of vaccines (Demazeau & Rivalain, 2011). It was a century later, in 1990,

when the first industrial HPP unit was installed in a food industry in Japan. Strawberry, apple and kiwi jams became the first HPP products commercially available worldwide (Hori et al., 1992). HPP fruit juices (grapefruit and mandarin) followed and were introduced in the market in Japan as well in 1991 (Tonello, 2011). Three years later, in 1997, the commercialization of HPP guacamole began in the US (Tonello, 2011). The partial inactivation of PPO, which is the enzyme responsible for avocado browning, has made HPP a standard operation in this industry (Jacobo-Velázquez & Hernández-Brenes, 2011). First HPP meat products (*i.e.* sliced cooked ham) were introduced in Spain in 1998 (Grèbol, 2002). During the 21st century, the number of applications and companies that adopted HPP technology increased exponentially (from 19 HPP units installed at the end of 2000 to 520 HPP units operating in the food industry at the end of 2019) (González-Angulo et al., 2021). Global HPP food production can be estimated in 1.8 billion kg, among which plant-based products represent a 60 % (including juices, other beverages, avocado products, salsas and wet salads). Protein-based products form animal origin (meat, dairy, seafood, ready-to-eat meals and pet food) represent the remaining 40 % (González-Angulo et al., 2021). An estimation of the distribution of the global production for each food application in 2019 can be found in Figure 6. As evidenced by these figures, juices & beverages are the fastest-growing segments among HPP food sectors, representing almost 30 % of total HPP food production. In the particular case of HPP coconut water, the first commercial product was launched in the US in 2011 (Zonis, 2011). Figures for HPP coconut water are not known, but it is estimated that in 2019 world coconut water market (including fresh and processed) was around 2.7 billion US$. The United States is the biggest market with 37 % of global share. In this country, pasteurized coconut water from Vita Coco accounts for 50 % of the share, and microfiltered coconut water from Harmless Harvest for 7 to 8 % of the US market (D. Lam, personal communication, 2020).

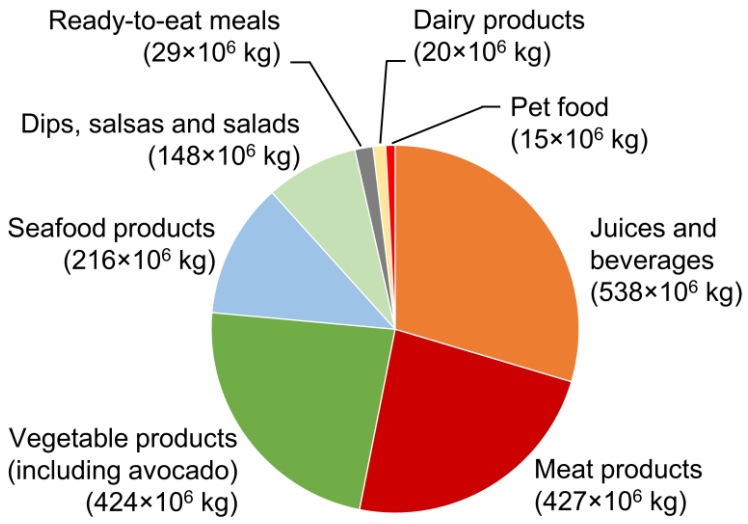

Figure 6. Estimation of global HPP food production in 2019 in different food sectors (adapted from González-Angulo et al., 2021).

3.2. Benefits of HPP in the juice industry

Consumers demand nowadays less processed food with a cleaner label and more natural quality attributes. Among alternate methods developed to overcome the limitations of conventional processing, HPP is the most widely implemented. Additionally, consumers perceive HPP more positively when compared to other cold preservation methods (Cardello, Schutz, & Lesher, 2007). The success of the technology in the juice industry relies on the retention of sensory attributes, which represents an advantage compared to traditional heat pasteurization. Color and appearance are the primary quality indicators perceived by consumers. Carotenoids, chlorophylls, anthocyanins and betacyanins are the main chemical structures that determine the color of juices and beverages (Rodriguez-Amaya, 2016). HPP has the ability to preserve them in the final product immediately after processing, or even to increase their concentration due to vegetable cell membrane disruption as a consequence of compression and decompression stresses (Serment-Moreno, Jacobo-Velázquez, Torres, & Welti-Chanes, 2017). In addition to color, flavor and aroma also play an important role in consumer willingness to acquire a food product. Some of the volatile compounds responsible for the aroma of fruit juices are heat-sensitive. Table 6 summarizes the effect of HPP in the concentration of the molecules responsible for these important sensory attributes, and compares it to thermal pasteurization in various fruits fruit juices.

From a nutritional standpoint, HPP also preserves low molecular weight essential compounds and other bioactive substances. For instance, processing at 600 MPa for 3 min apple juices from different apple varieties (Pink Lady, Granny Smith or Jonagold) did not cause significant changes in the concentration of glucose, fructose and sucrose. In addition, the concentrations of malic, citric and quinic acids were statistically the same before and after HPP (Yi et al., 2017). Other authors described that the concentrations of α- and β-carotene (provitamin A carotenoids) in carrot juice remained stable after HPP (600 MPa for 5 min) compared to unprocessed controls (Picouet et al., 2015). Similarly, Sánchez-Moreno et al., (2003) observed that the concentration of six different types of carotenoids was not affected by HPP (400 MPa for 1 min) in orange juice. Subjecting a smoothie containing twelve different fruits to 400 MPa for 5 min enhanced retention of δ-tocopherol (vitamin E) compared to heat pasteurization (90 °C, 30 s), but α- and γ-tocopherols were better preserved by heat. Nonetheless, the bioaccessibility of all tocopherols increased after HPP. In addition, HPP improved the bioaccessibility of carotenoids compared to the unprocessed controls and yielded a higher retention of vitamin C when soymilk was used in the elaboration of the smoothies (Cilla et al., 2012). In agreement with these findings, processing at 400 MPa for 5 min orange-based fruit smoothies containing kiwi, pineapple and mango resulted in a higher concentration

Table 6. Examples comparing the effect of HPP and thermal pasteurization in fruit and vegetable juices.

Juice	Attribute	HPP	Pasteurization	Main observations	Reference
Açaí	Antioxidants	500 MPa, 5 min	85 °C, 1 min	- HPP retained anthocyanins, whereas pasteurization caused a significant reduction. Concentration of non-anthocyanin phenolic compounds increased after HPP - Antioxidant activity was higher in the HPP juice	(da Silveira et al., 2019)
Carrot	Color, odor, flavor	600 MPa, 5 min	80 °C, 7 min	- Overall color, odor and flavor of HPP juice was similar to unprocessed controls and scored higher than pasteurized juice - HPP provides “fresh-like” juice with better sensory properties for 29 days in refrigerated storage	(Picouet, Sárraga, Cofán, Belletti, & Dolors Guàrdia, 2015)
Coconut water	Color, sensory quality	500 MPa, 5 min	72 °C, 15 s	- HPP showed more stable color attributes (L*a*b*) than pasteurized coconut water for 25 days at 4 °C - Color, aroma, flavor, and overall acceptability of HPP coconut water were closer to that of the fresh coconut water and scored higher than the HTST beverage	(Ma et al., 2019)
Grape	Antioxidants, sensory quality	300-600 MPa, 3 min	90 °C, 1 min	- Day 0: HPP juice had improved sensory attributes, and no differences were observed in phenolic content and anthocyanins - Day 20: HPP juice had higher antioxidant capacity than pasteurized juice, and sensory attributes were better preserved	(Chang, Wu, Chen, Huang, & Wang, 2017)
Kiwi	Aroma	500 MPa, 10 min	100 °C, 5 min	- Aroma characteristics of HPP juice were similar to these of fresh samples. More volatile compounds were retained after HPP than after heat pasteurization	(Zhao et al., 2020)
Mulberry	Antioxidants and flavor	550 MPa, 10 min	85 °C, 15 min	- HPP maintained significantly higher contents of total phenols, flavonoids and resveratrol than pasteurized samples. Antioxidant activity was also in HPP juice - HPP enhanced volatile compounds, whereas thermal processing reduced them in comparison to controls	(Wang, Du, Cui, Xu, & Li, 2017)

Table 6. Examples comparing the effect of HPP and thermal pasteurization in fruit and vegetable juices.

Juice	Attribute	HPP	Pasteurization	Main observations	Reference
Orange	Antioxidants	500 MPa, 70 s	70 °C, 30 s	- Day 0: HPP juice had higher antioxidant activity, better retention of total phenolic compounds, carotenoids - Day 35: HPP showed better retention of previously enlisted compounds, except for flavonoids	(Vieira et al., 2018)
Orange	Color, flavor, antioxidants	600 MPa, 4 min	80 °C, 1 min	- Shelf-life of HPP juice based on vitamin C concentration increased significantly compared to pasteurized juice. - Color and overall flavor were superior in HPP juice through storage	(Polydera, Stoforos, & Taoukis, 2005)
Papaya	Color, antioxidants, flavor	350-650 MPa, 5-10 min	110 °C, 8.6 s	- Day 0: thermal processing induced grater color changes, in addition to a loss of antioxidant capacity and total phenols - Day 40: HPP juice displayed higher antioxidant activity and better retention of color, total phenols and flavor	(Chen et al., 2015)
Pear	Antioxidants	500 MPa, 10 min	110 °C, 8.6 s	- The concentration of ascorbic acid and phenolic compounds was better retained in the HPP juice - HPP juice showed higher antioxidant capacity	(Liang Zhao, Wang, Hu, Sun, & Liao, 2016)
Pomegranate	Antioxidants and color	400 MPa, 5 min	110 °C, 8.6 s	- Color coordinates (L*a*b*) of HPP juice were more similar to unprocessed juice than pasteurized samples - Phenolic compounds and antioxidant activity of HPP juice were higher during the 90-day storage period	(Chen et al., 2013)
Prickly pear	Antioxidants and color	600 MPa, 5 min	95 °C, 3 min	- Pasteurization and HPP retained equal concentration of flavanols and betacyanins, but HPP yielded a better retention of ascorbic acid	(Moussa-Ayoub et al., 2017)
Wheatgrass	Antioxidants and color	500 MPa, 1 min	75 °C, 15 s	- HPP increased chlorophyll concentration, whereas pasteurization caused a 12.4 % decrease. Ascorbic acid and color remained stable after HPP, whereas heat pasteurization caused significant changes	(Ali, Popović, Koutchma, Warriner, & Zhu, 2020)

and bioavailability of carotenoids, total phenolic compounds and vitamin C compared to same smoothies subjected to heat pasteurization (90 °C for 1 min). Additionally, antioxidant activity was also enhanced by HPP (Rodríguez-Roque et al., 2015, 2016). Gupta et al., (2011) showed that processing tomato juice between 500 and 700 MPa for 10 min retained 75-93 % of β-carotene as compared to control samples, but its bioaccessibility was improved. Furthermore, the same study reported that the concentration of lycopene increased up to a 12 % after HPP, although this did not correlate with an increase in bioaccessibility. Recent research evaluating bioaccessibility and Caco-2 cell uptake of bioactive compounds from a kale-based juice revealed that HPP (500 MPa for 3 min) was the preferred method for total carotenoid delivery among other thermal and nonthermal technologies such as pasteurization, ohmic heating or pulsed electric fields (PEF). Additionally, the color was better preserved by HPP since not significantly changes were observed in the concentration of β-carotene, lutein and chlorophylls *a* + *b* (Zhong et al., 2019).

3.3. Microbial inactivation by HPP in fruit juices

3.3.1. Mechanisms of inactivation and bacterial spores

Shelf-life extension of HPP juices and beverages is not based solely on the maintenance of sensory attributes, but also on the inactivation of spoilage and pathogenic microorganisms. Although different species in varying food systems behave in a different way, the generic mechanisms leading to microbial inactivation are: *(i)* protein and enzyme unfolding, *(ii)* modification of cell membrane fluidity due to lipid phase transition, (iii) disintegration of ribosomes, and (iv) intracellular pH changes related to membrane damage and enzyme inactivation (Georget et al., 2015). The cell membrane is the first organelle to be damaged. Structural changes lead to functional alterations such as impaired nutrient absorption and waste elimination. Therefore, normal metabolic pathways are disrupted (Torres & Velazquez, 2005). In addition, HPP can also denature membrane proteins (such as ATPase) or other enzymes and organelles inside the cell (such as ribosomes or proteins regulating metabolism and DNA replication). This results in a reduction of intracellular pH due to limited proton flow, the dissociation of ribosomes for protein synthesis inhibition, the alteration of metabolic pathways and the inhibition of DNA replication due to the condensation of chromosomal DNA and nucleic acid enzymes (Dubins, Lee, Macgregor, & Chalikian, 2001; Kaletunç, Lee, Alpas, & Bozoglu, 2004; Simpson & Gilmour, 1997b; Tholozan, Ritz, Jugiau, Federighi, & Tissier, 2000). The abovementioned mechanisms leading to microbial inactivation refer mainly to vegetative cells of microorganisms. Spore forms are less susceptible to pressure-induced damage because of their particular physiology and structure (Figure 7).

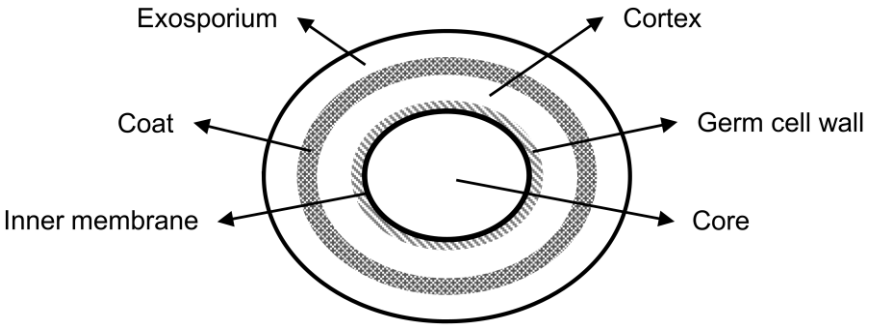

Figure 7. Schematic diagram of a dormant spore structure (adapted from Black et al., 2007).

The outermost layer of spores (exosporium) is mainly composed of carbohydrates and proteins. Its precise function is not clear, but it is not known to play any role in spore resistance properties (Setlow, 2006). The coat layer is located right below the exosporium. This layer is composed of proteins and provides protection against chemicals, perhaps by reacting and detoxifying these substances before they cause any damage. It also serves as a barrier against lytic enzymes (such as lysozyme) by restricting access to peptidoglycan (PG) within the spore (Black et al., 2007; Setlow, 2006). Resistance to high pressure has been observed in spores with defective coats, so it is believed that this structure is not important for pressure resistance (Paidhungat et al., 2002). The cortex is the next layer and is mainly composed of PG. It appears to be extremely important in maintaining spore dormancy by keeping the low water content in the central region of the spore (Paidhungat et al., 2002). Immediately beneath the cortex is the germ cell wall, which is composed of PG and has an identical structure to that of the growing spore, so it becomes the cell wall of the emerging cell (Popham, 2002). The inner membrane is the next spore layer. Although its phospholipid and fatty acid composition is very similar to that of the plasma membrane of growing cells, it has several unusual properties such as low permeability to charged and hydrophobic molecules, including water (Cortezzo, Koziol-Dube, Setlow, & Setlow, 2004; Cortezzo & Setlow, 2005). This membrane also appears to be compressed in dormant spores, as evidenced by a 2- to 3-fold increase in size after germination in the absence of ATP synthesis or new lipid production (Cowan et al., 2004). This particular configuration and its low permeability are important in restricting the access of damaging substances to the core, which is the inner layer of spores (Cortezzo & Setlow, 2005). The core contains enzymes, ribosomes and DNA. It has two important biochemical properties: (i) extremely low water content (ranging from 25 to 55 % of wet weight), and (ii) lower intracellular pH than growing cells (between 1.0 and 1.5 units lower). These factors are important for pressure resistance and for spore dormancy (Paidhungat et al., 2002; Setlow, 2006). Additionally, the core contains two unique molecules that contribute to spore resistance: (i) calcium-chelated dipicolinic acid (Ca-DPA), and (ii) α/β-type small acid-soluble proteins (SASP). Both molecules are expressed late in the sporulation stage of

developing spores. Ca-DPA represents about 20 % of core dry weight and is responsible of maintaining a low water content in the core. SASP proteins are associated with spore DNA to protect it from multiple environmental stresses (Gerhardt & Marquis, 1989; Paidhungat et al., 2002; Setlow, 2006).

These structural characteristics confer spores an extremely high resistance to environmental stresses, including high pressure. Early reports already showed that pressures up to 1200 MPa failed to inactivate spores of *B. subtilis* (Gerhardt & Marquis, 1989; Larson, Hartzell, & Harold, 1918). Reddy et al. (1999) described that pressures above 827 MPa for 5 min at 35 °C caused slight viability reductions in type E *Clostridium botulinum* spores (inactivation between 1 and 2 $\log_{10}$ CFU/mL). The combination of high pressure (500 to 900 MPa) with high temperatures (90 to 120 °C) is required to achieve an efficient spore inactivation in a process called high-pressure high-temperature (HPHT). However, this technology is not yet implemented at commercial scale (Mújica-Paz, Valdez-Fragoso, Samson, Welti-Chanes, & Torres, 2011). Industrial HPP equipment have a maximum operating pressure of 600 MPa with a temperature range between 4 and 25 °C (González-Angulo et al., 2021). Design limitations related to the materials used in the construction of such equipment, the difficulty to ensure a homogenous temperature distribution and operational costs are the main challenges that the development of this alternate method is facing. Another gentle strategy for the control of spores takes advantage of the increased sensitivity of germinated spores to milder processing conditions (Setlow, 2006). In the particular case of *Bacillus* spp., it has been observed that HPP can trigger spore germination (Gould & Sale, 1970; Reineke, Mathys, Heinz, & Knorr, 2013). This specific behavior served to postulate a “germination-inactivation” strategy that first artificially triggers spore germination by HPP, and then eliminates those spores which lost their extreme resistance during germination with second lethal step (e.g. UV radiation, heat treatment or a second exposure to HPP) (Wuytack, Boven, & Michiels, 1998). However, the germination behavior of spores is very heterogeneous. Whereas most spores rapidly germinate after exposure to germinant stimuli, a fraction remains dormant but viable (*i.e.* superdormant spores). This subpopulation represents the major limitation of germination-inactivation strategies because superdormant spores can potentially resume growth during storage of food products and become a hazard (Zhang & Mathys, 2019).

Current practice in the HPP juice industry to address the spore issue relies in a hurdle approach. Preventing spore germination and further vegetative growth ensures product safety. The control of intrinsic parameters (*i.e.* acidity) is the most straightforward strategy to avoid intense thermal processing or chemical preservatives and maintain a “clean label” in the final product. Temperature is an extrinsic parameter that plays a major role in spore growth

dynamics. Nonetheless, it cannot be used solely as a control since psychrotrophic pathogenic spores could still grow even under refrigeration, and because it is not easy to control through the whole distribution chain until the final consumer (temperature at retail level and at home refrigerators might reach up to 10 °C) (Lindström, Kiviniemi, & Korkeala, 2006). Section 3.3.3 of the present chapter tackles with the risks associated with spores and other foodborne pathogens in juices. Section 4 introduces research needs to fill knowledge gaps regarding adequate risk assessment of minimally processed juices.

3.3.2. Factors affecting microbial inactivation by HPP in juices

As previously discussed, typical processing conditions used by the juice industry (400 to 600 MPa for 1 to 5 min) induce many physiological and morphological changes in the cells of spoilage or pathogenic microorganisms. Nonetheless, the specific pressure resistance of microbial cells depends on factors like pressure intensity and holding time, temperature, compression and decompression rates, microbiota and intrinsic properties of the juice (Podolak, Whitman, & Black, 2020). Increasing pressure and holding time generally results in an increased inactivation of microorganisms, except in the cases where a first-order inactivation kinetics is not followed due to tailing effect (Alpas et al., 1999). This is evidenced by the calculated D-values of several published studies in which the lethal effect of increasing pressure intensities was evaluated. For instance, D-values for *E. coli* in apple juice processed at 150, 200, 250, 300 and 350 MPa were 55, 17.9, 9.22, 2.95 and 0.80 min, respectively (Ramaswamy, Riahi, & Idziak, 2003). A similar trend was observed for *L. monocytogenes* after processing peach juice (D-values of 6.17, 3.39 and 1.52 min) and orange juice (D-values of 2.87, 1.80 and 0.87 min) at 300, 400 and 600 MPa, respectively (Dogan & Erkmen, 2004). This study also concluded that *L. monocytogenes* was more sensitive to increased pressure than to increased holding time. Basak et al. (2002) described that spoilage microorganism *Leuconostoc mesenteroides* became more sensitive to HPP with increased pressure intensities since D-values at 200, 300 and 400 MPa were 26, 5.8 and 2.0 min, respectively. Different microbial species show different pressure resistance. Eukaryotic cells are generally more sensitive to HPP because they are more complex organisms than bacteria, so it is easier to cause damage in their structures. D-values for vegetative cells of the yeast *Saccharomyces cerevisiae* in orange juice processed at 350, 400, 450 and 500 MPa were 38, 7, 4 and 1 seconds, respectively, which evidences the increased sensitivity of eukaryotic cells compared to bacteria (Parish, 1998). Common processing parameters used by the food industry to process fruit juices (including coconut water) are 600 MPa for 3 min, as they offer a balance between profitability and food safety. However, these parameters have not been yet validated as “safe harbor” since the inactivation of pathogens depends on other factors detailed below.

Processing temperature is an important parameter for microbial inactivation during HPP when it is above room conditions. Values exceeding room temperature increase the inactivation rate of microbial cells due to the synergy between the two variables (Farkas & Hoover, 2000). For instance, temperatures >35 °C combined with pressures between 300 and 800 MPa lead to phase transition of membrane lipids and changes in their fluidity due to crystallization (Molina-Höppner, Doster, Vogel, & Gänzle, 2004). Linton et al. (1999) described that processing orange juice (pH 5) at 550 MPa for 5 min at 30 °C achieved a 6-$\log_{10}$ reduction of *E. coli* O157:H7, whereas at 20 °C it was necessary to increase the acidity of the juice (pH 3.4 to 4.5) to obtain the same inactivation of the pathogen. In a similar study, a reduction greater than 8 $\log_{10}$ CFU/mL was observed in orange juice for *E. coli* O157:H7 at lower pressure (350 MPa for 5 min), but at 40 °C (Bayındırlı, Alpas, Bozoğlu, & Hızal, 2006). Conversely, based on published results, this parameter is not critical for the inactivation of microorganisms when HPP is applied below room conditions. Buzrul et al. (2008) observed no differences in the inactivation of *E. coli* and *L. innocua* in pineapple juice processed at 350 MPa for 5 min and three different temperatures (-10, 0 and 20 °C). Adequate process design should consider that industrial HPP equipment typically operates between 4 and 25 °C. Current practice in the juice industry uses temperatures below 10 °C to maintain the cold chain during the whole process and preserve delicate organoleptic characteristics.

Pressurization and depressurization rates also have an influence in the inactivation of microorganisms by HPP, although results from published research are contradictory. Nonetheless, the number of reports evaluating the effects of these parameters on juices and beverages is very limited. Syed et al. (2013) studied the lethality of *E. coli* O157:H7 of different pressurization (1.3, 3.6 and 11.4 MPa/s) and depressurization rates (2.6, 6.0 and 12.9 MPa/s) in orange juice. The lowest inactivation at 600 MPa for 3 min (1.49 $\log_{10}$ CFU/mL) was obtained for the intermediate compression and decompression rates in orange juice. On the other hand, fast compression and slow decompression rates provided the highest inactivation. Other studies on buffer systems show opposite results. For instance, subjecting *S. enterica*, *E. coli*, and *Pseudomonas aeruginosa* to pressure levels from 70 to 400 MPa in 0.9 % NaCl solutions revealed that a rapid decompression (>400 MPa/s) had an increased lethal effect compared to a slow decompression (>13.3 MPa/s) (Noma, Shimoda, & Hayakawa, 2002). Conversely, *S. enterica* and *L. monocytogenes* showed increased tolerance to HPP (400 and 500 MPa) in phosphate buffered saline (PBS) with the slowest compression (1 MPa/s) and decompression (5 MPa/s) rates applied in the study (Chapleau et al., 2006). It is believed that slow pressurizations might induce stress response of bacterial cells and result in a lower microbial inactivation (Smelt, 1998). Although more research is needed to elucidate the specific mechanisms leading to increased or reduced sensitivity of microorganisms to varying

pressurization and depressurization rates, adequate process design should consider that common practice in the HPP juice industry tries to reduce compression and decompression times to increase production capacity and minimize total cycle time. Typical pressurization and depressurization rates range 1.5-5 MPa/s and 30-600 MPa/s, respectively.

In addition to process parameters, the intrinsic characteristics of juices and beverages can also affect the efficacy of HPP. The amount of free water and the acidity of juices are the main physicochemical critical factors for the inactivation of microorganisms under pressure. Water activity (a_w) is one of the most important parameters to define processing conditions. However, juices and beverages (including coconut water) generally have high a_w values (>0.98), so the efficiency of HPP is not negatively affected. It is expected that the addition of solutes like soluble carbohydrates (*i.e.* fructose or sucrose) could protect bacteria, molds and yeasts against HPP by lowering a_w. A higher solute content reduces the compressibility of beverages, a parameter that is defined by the free volume between water molecules and that correlates well with an increase in the pressure resistance of microorganisms (Min, Sastry, & Balasubramaniam, 2010). This might be due to (i) cell membrane thickening as a consequence of cell shrinkage, which reduces permeability and sensitizes microbial cells to HPP, and/or (ii) a decrease in the mechanical energy transferred during HPP as a result of lower compressibility (Fauzi, Farid, & Silva, 2017; Palou, López-Malo, Barbosa-Cánovas, Welti-Chanes, & Swanson, 1997). A clear example illustrating the enhanced resistance of microorganisms at lower a_w was conducted by Buerman et al. (2020) on apple juice (pH 4.6) adjusted to different a_w values (0.94, 0.96, 0.98 and 1.00). Samples inoculated with filamentous fungi (*Paecilomyces variotii*, *Penicillium* spp. and *Aspergillus niger*) were subjected to HPP (450 MPa for 1.5 min, pH 4.6). Reductions ranged from 2.5 to 4.9 $\log_{10}$ CFU/mL at a_w 0.94, from 3.3 to 5.5 $\log_{10}$ CFU/mL at a_w 0.96, and from 5.3 to 5.5 $\log_{10}$ CFU/mL at a_w 0.98 and 1.00. Similarly, apple juice concentrate (a_w 0.94) provided increased resistance to *P. variotii*, *Penicillium* spp. and *A. niger* when pH was adjusted to 7.0 (inactivation ranging 0.1 to 4.1 $\log_{10}$ CFU/mL) than at pH 4.6 (inactivation ranging 2.5 to 4.9 $\log_{10}$ CFU/mL) after processing at 450 MPa for 1.5 min (Buerman et al., 2020). It is important to highlight that a_w values of 0.96 and 0.94 in this particular study are equivalent to total soluble solids concentrations of 32 and 39 °Brix, respectively, so it differs considerably from typical parameters in juices. In addition to a_w, pH also determines pressure inactivation of microorganisms. It is generally accepted that vegetative microbial cells are more susceptible to HPP at lower pH values (Alpas, Kalchayanand, Bozoglu, & Ray, 2000). For example, processing orange juice (pH 3.4 to 4.5) at 500 MPa for 5 min produced an inactivation of *E. coli* O157:H7 greater than 5-$\log_{10}$. However, same processing conditions failed to achieve the 5-$\log_{10}$ reduction when orange juice was adjusted to pH 5.0 (Linton et al., 1999). Different fruit juices naturally have different acidity.

For instance, higher inactivation rates were observed for aerobic bacteria and *E. coli* in orange juice (pH 3.55) compared to peach juice (pH 5.21) processed from 200 to 600 MPa because of the difference in acidity between the two juices (Erkmen & Doğan, 2004).

Other intrinsic properties of juices and beverages such as the type of organic acid, protein, fat or mineral content might determine pressure resistance of microorganisms, their survival and growth during storage period after HPP. As an example, depending on the pH, the organic acids present in juices might be undissociated and enhance inactivation of microorganisms not only during HPP, but also inhibiting further outgrowth of sublethally injured cells. It is widely reported that pathogens may survive HPP, but will die off during refrigerated storage under acidic conditions. Nasiłowska et al. (2018) showed that processing beetroot juice (pH 4.0 to 4.2) at 300 MPa for 10 min achieved less than 1 $\log_{10}$ CFU/mL reduction for *L. innocua* and *E. coli*, whereas an additional 5.15 and 6.53 $\log_{10}$ CFU/mL reduction was achieved for each species, respectively, for 28 days of refrigerated storage. This goes in agreement with findings in other acidic fruit juices. For instance, processing kiwifruit juice (pH 3.32) with five consecutive HPP cycles at 350 MPa for 1 min achieved more than a 5-$\log_{10}$ reduction immediately after the treatment for *L. innocua* and *E. coli*, whereas only 2.5 and 3.5-$\log_{10}$ reductions were observed in pineapple juice (pH 3.77) for each bacteria, respectively. However, both species were reduced by more than 7-$\log_{10}$ CFU/mL after subsequent storage at 4 °C for three weeks, and no recovery was observed (Buzrul et al., 2008). Similar results were obtained for orange juice (pH 3.8) inoculated with *E. coli* O157 and subjected to HPP (500 MPa for 5 min). A 2-$\log_{10}$ reduction of the pathogen was obtained immediately after processing, but a greater reduction was reported when the juice was held at 4 °C for 3, 7 or 24 h (Jordan, Pascual, Bracey, & Mackey, 2001). Same authors observed that processing tomato juice (pH 4.1) at 450 MPa for 5 min achieved an immediate reduction of 0.66-$\log_{10}$ *E. coli* O157:H7, but storing processed samples at 4 °C for 24 h yielded an increased inactivation of 4.7 $\log_{10}$ CFU/mL. Whitney et al. (2007) also found similar results in apple juice (pH 3.7) inoculated with *E. coli* O157:H7 and *S. enterica* and processed at 550 MPa for 2 min. Immediate reductions achieved for each pathogen were 2.29 and 5.66 $\log_{10}$ CFU/mL, respectively. Further enumeration after 24 h of incubation at 6 °C revealed an increased inactivation that reached 4.28-$\log_{10}$ and 5.83-$\log_{10}$ reductions for *E. coli* O157:H7 and *S. enterica*, respectively. On the other hand, other studies showed that pathogens might recover and grow after HPP during shelf-life. This typically occurs in juices with favorable physicochemical properties. For instance, *L. innocua* survived HPP (500 MPa for 5 min) in carrot juice (pH 6.0 to 6.7) and managed to grow during 28 days of refrigerated storage (5 °C) (Nasiłowska et al., 2018). Teo et al. (2001) stated that pressure-injured *E. coli* O157:H7 and *S. enterica* survived for 7 days at 4 °C in acidic juices, although growth was not reported.

Juices and beverages are not a representative source of protein and fat. This might account for the lack of published research on the effects of proteins and lipids on microorganisms subjected to HPP. A study using a model system assessed the effect of varying HPP intensities (300, 350, 375, 400 and 450 MPa up to 30 min) on *L. monocytogenes* in a matrix with different concentrations of protein (1, 2, 5 and 5 % [w/v] bovine serum albumin) and lipids (30 % [v/v] olive oil) (Simpson & Gilmour, 1997a). In this study, authors showed that increasing the concentration of bovine serum albumin from 1 % to 8 % reduced the level of inactivation achieved for *L. monocytogenes* Scott A from 3 to <1-$\log_{10}$ reductions after processing at 375 MPa for 10 min. Similarly, the same strain exhibited increased resistance in 30 % (w/v) olive oil emulsions (<1-$\log_{10}$ reduction) than in the buffer matrix alone (2.5-$\log_{10}$ reduction) after processing at 375 MPa for 10 min. A possible explanation of this protective effect might be related to a transitory decrease of water activity during HPP. Protein denaturation under pressure causes breakage of hydrophobic and electrostatic interactions, but not covalent bonds (Mozhaev et al., 1994). This increase of free water during HPP will increase the hydration of denatured proteins around the newly exposed charged groups and would consequently reduce a_w (Masson, 1992). A similar approach can be used to explain the protective effect of lipid-rich media subjected to HPP. A reduction of free water is expected in the lipid-water interface of emulsions, especially in those with a high concentration of hydrophilic emulsifier groups (Podolak et al., 2020). Moreover, protein and other solutes such as carbohydrates can be used by damaged microorganisms as nutrient sources. This facilitates recovery and growth during storage after HPP, especially in low-acid juices as they represent a less adverse environment than high-acid juices. The use of ingredients such as whey powder, hemp protein or essential oils is relatively common in the food industry due to the innovative and pioneering spirit of HPP juice companies. Therefore, adequate risk assessment and process validation should be conducted to ensure that HPP can effectively reduce biological hazards to safe levels in formulas that include ingredients with a potential protective effect on microorganisms.

3.3.3. Control of pathogens and spoilage microorganisms in juices by HPP

Fresh juices and beverages may represent a vehicle for pathogens because fruits and vegetables are exposed to environmental contamination and human handling. Additionally, blends of raw ingredients from different sources possess different biological hazards based on agricultural and manufacturing practices or the specific pathogens that are representative of each component. The most characteristic microbial species responsible of outbreaks associated with raw juices are *Salmonella* spp., *E. coli* O157:H7 and *Cryptosporidium parvum* (Jackson-Davis et al., 2018). The latter is a protozoan parasite commonly associated with

diarrheal disease outbreaks from contaminated water and fruit (typically apple-derived products). Interestingly, HPP has shown the potential to reduce the infectivity and viability of *C. parvum* by more 4.1 $\log_{10}$ oocysts/mL after processing apple and orange juices at 552 MPa for >60 s (Slifko, Raghubeer, & Rose, 2000). This parasite has not been associated with coconut products, including coconut water.

Although *L. monocytogenes* is not commonly associated with outbreaks related to juices, it is an enteric pathogen linked to human diseases due to the consumption of raw fruits and vegetables (Beuchat, 1996; McCollum et al., 2013). Additionally, it is ubiquitous in the environment and food processing plants, so it is highly encouraged to consider it always in hazard analyses and risk assessment plans. It is a Gram-positive facultative anaerobe bacillus. Several species have been added to the genus in recent years: ten species were known until 1992, whereas twenty-one species had been identified by 2020 (Quereda et al., 2020). *L. monocytogenes* is typically considered the only pathogenic species to humans, although rare cases have been reported associated to *L. grayi* and *L. ivanovii* (Guillet et al., 2010; Todeschini et al., 1998). A total of 13 serotypes can be distinguished for *L. monocytogenes* based on somatic and flagellar antigens, but more than 90 % of the isolates responsible for human listeriosis belong to serotypes 1/2a, 1/2b and 4b. Particularly, serotype 4b accounts for all major foodborne outbreaks reported in Europe and North America since the 1980s (Ward et al., 2004). It is also possible to differentiate the pathogen by its evolutionary lineage (I to IV). Each lineage represents different genetic, phenotypic, and ecologic characteristics. For instance, lineage I gathers most of human isolates. On the other hand, lineage II strains represent the majority of animal cases and are more prevalent in food and environmental samples. Lineages III and IV are very rare and mainly isolated from animal sources (Orsi, Bakker, & Wiedmann, 2011). It is estimated listeriosis is responsible for more than 23,000 illnesses and 5,400 deaths around the world, being especially severe to the elderly and pregnant women (de Noordhout et al., 2014). Despite its low incidence, mortality reached a 15.6 % in the European Union in 2018 and 97 % of diseased people required hospitalization (EFSA, 2019).

E. coli is a Gram-negative facultative anaerobe bacillus. Although most strains occur naturally in the intestines of ruminants and are harmless, there are six pathotypes associated with human disease: enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC) and enterohemorrhagic *E. coli* (EHEC) (Palaniappan et al., 2006). The latter is the group that is most commonly related with foodborne illness. Among serotypes of this particular pathotype, O157:H7 strains are typically associated with outbreaks due to the consumption of juices and

beverages. It is estimated that EHEC causes 2,801,000 acute illnesses annually worldwide (Majowicz et al., 2014). In the case of juices and beverages, irrigation water contaminated by cattle manure is considered the major transmission route to fresh produce.

Salmonella spp. is another human pathogen sharing the same contamination route of fruits and vegetables. It is also a Gram-negative facultative anaerobe bacillus, which contains two species: *S. enterica* and *S. bongori*. The former includes six subspecies, each designated with Roman numerals: *enterica* (I), *salamae* (II), a*rizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV) and *indica* (VI); gathering together more than 2,600 serotypes. *S. enterica* subsp. *enterica* is the most common foodborne subspecies, and more than 1,400 serotypes have been identified only for this particular subspecies (Gal-Mor, Boyle, & Grassl, 2014). Important pathogenic serotypes are *S.* Enteritidis and *S.* Typhimurium, although consumption of juices has been associated with outbreaks due to additional serotypes, such as *S.* Gaminara, *S.* Hartford, *S.* Anatum, *S.* Muenchen or *S.* Saintpaul, among others (Jackson-Davis et al., 2018). Additionally, *Salmonella* spp. was the causative agent of most outbreaks related to coconut products (Strawn, Schneider, & Danyluk, 2011). Globally, the pathogen is considered a major worldwide public health concern, accounting for 93.8 million foodborne illnesses every year (Majowicz et al., 2010).

High pressure processing has the potential to control vegetative pathogens in high-acid and low-acid juices and deliver safe products with an extended shelf-life under refrigeration. Table 7 shows some examples of the reduction of pathogenic microorganisms in fruit juices subjected to HPP. Another major advantage of HPP is that spoilage microorganisms such as molds, yeasts, mesophilic or lactic acid bacteria can be effectively controlled. This extends commercial shelf-life of juices and beverages because quality-related aspects are preserved for more time. For instance, processing apple juice at 400 MPa for 3 min caused a reduction of 4.3 and 3.3 $\log_{10}$ CFU/mL of aerobic mesophilic bacteria and molds & yeasts which extended shelf-life at 4 °C up to 56 days (Lavinas, Miguel, Lopes, & Valente Mesquita, 2008). Zhao et al. (2013) reported a similar shelf-life for cucumber juice (50 days) after processing at 500 MPa for 2 min, which caused a reduction of molds & yeasts between 3 and 4 $\log_{10}$ CFU/mL. Keiskei juice (a traditional Japanese leafy green juice) also benefits from HPP, not only because color and flavor are better preserved, but also because microbial quality indicators are controlled. Processing the juice at 550 MPa for 1.5 min reduced counts of coliforms, molds & yeasts and *Pseudomonas* spp. by 6.1, 4.7 and 5.3 $\log_{10}$ CFU/mL (Chai, Lee, Lee, Na, & Park, 2014). Other authors reported reductions of molds & yeasts, lactic acid bacteria and *Enterobacteriaceae* of 3.6, 4.2 and 2.1 $\log_{10}$ CFU/mL after processing tomato juice between 300 and 500 MPa for 10 min. This served to extend shelf-life of the juice under refrigeration for

Table 7. Reduction of pathogens in selected fruit juices subjected to HPP.

Juice	HPP conditions	Reduction (log_{10} CFU/mL)			Reference
		E. coli O157:H7	*S. enterica*	*L. monocytogenes*	
Açai (pH 4.3)	≥400 MPa, 3 min, 5 °C	>6	>6	>6	(Gouvea et al., 2020)
Apple (pH 3.5)	500 MPa, 5 min, 20 °C	>5	NA	>5	(Jordan et al., 2001)
Apple (pH 4.1)	400 MPa, 2 min, 25 °C	4.2	NA	NA	(Lavinas et al., 2008)
Apple (pH 3.5)	>400 MPa, 181 s, 5 °C	>5	>5	>5	(Petrus, Churey, & Worobo, 2020b)
Apple (pH 3.7)	615 MPa, 2 min, 15 °C	0.4	5.5	NA	(Teo et al., 2001)
Apple (pH 3.8)	500 MPa, 1 min, 25 °C	NA	>7	4.9	(Shahbaz et al., 2016)
Apricot (pH 3.8)	350 MPa, 5 min, 30 °C	>7	>7	NA	(Bayındırlı et al., 2006)
Carrot (pH 6.2)	615 MPa, 2 min, 15 °C	6.4	6.5	NA	(Teo et al., 2001)
Coconut (pH 5.2)	586 MPa, 2 min, 4 °C	>5	>5	>5	(Raghubeer et al., 2020)
Cherry (pH 3.3)	350 MPa, 5 min, 30 °C	7.4	NA	NA	(Bayındırlı et al., 2006)
Grape (pH 3.4)	425 MPa, 2 min, 5 °C	>7	>8	NA	(Petrus, Churey, & Worobo, 2019)
Grapefruit (pH 3.0)	615 MPa, 2 min, 15 °C	>8	>8	NA	(Teo et al., 2001)
Mango (pH 4.5)	500 MPa, 1 min, 20 °C	>8	NA	NA	(Hiremath & Ramswamy, 2012)
Mango (pH 4.5)	400 MPa, 5 min, 20 °C	NA	NA	>6	(Hiremath & Ramswamy, 2012)
Orange (pH 3.8)	500 MPa, 5 min, 20 °C	>5	NA	>5	(Jordan et al., 2001)
Orange (pH 3.4)	550 MPa, 5 min, 20 °C	>6	NA	NA	(Linton et al., 1999)
Orange (pH 3.7)	≥400 MPa, ≥200 s, 5 °C	6.9	7.3	7.3	(Petrus, Churey, & Worobo, 2020a)
Orange (pH 3.7)	615 MPa, 2 min, 15 °C	2.2	>8	NA	(Teo et al., 2001)
Orange (pH 3.6)	400 MPa, 1 min, 25 °C	2.4	NA	NA	(Yoo et al., 2015)
Peach (pH 5.2)	600 MPa, 5 min, 25 °C	NA	NA	>7	(Erkmen & Doğan, 2004)
Tomato (pH 4.1)	500 MPa, 5 min, 20 °C	>5	NA	>5	(Jordan et al., 2001)

NA: not available.

twenty-eight days (Hsu, Tan, & Chi, 2008). This goes in agreement with findings by Hurtado et al. (2017) who described reductions between 1.8 and 2.5-$\log_{10}$ of mesophilic bacteria, psychrotrophic bacteria, molds & yeasts and *Enterobacteriacea*e after processing a multi-fruit smoothie (pH 3.8) at 350 MPa for 7 min. Shelf-life at 4 °C increased up to 28 days. It can be observed that molds & yeasts are generally very sensitive to HPP and completely inactivated at pressures above 400 MPa. Nevertheless, fungi spores (ascospores and conidiospores) exhibit more resistance (similar to vegetative bacteria) and require more intense processing conditions (up to 600 MPa) to be effectively controlled (Pinto et al., 2020).

As discussed in section 3.3.1 of this chapter, the control of bacterial spores represents a challenge for nonthermal technologies, including HPP. It is possible to achieve commercial sterility (*i.e.* inactivation of vegetative microorganisms and spores for preservation at room temperature) through the combination of high-pressure (600 MPa) and high-temperature (90 to 120 °C) for short periods of time (up to 5 min), although this process is not industrially implemented due to limitations related to the design of reliable equipment and operational costs, and would not be considered a nonthermal preservation technology (Mújica-Paz et al., 2011). Hence, because spores remain viable after HPP, the control of physicochemical characteristics is used as the primary strategy in juices to prevent spore outgrowth during storage. The most resistant spore former of public health significance is *C. botulinum*. This obligate anaerobe is defined by the ability to produce the botulinum neurotoxin, which is the most toxic natural agent known, and as little as 30 ng of neurotoxin is potentially fatal to humans (Peck et al., 2011). The toxin is susceptible to heat denaturation (85 °C for 5 min) so it is expected that normal cooking conditions inactivate it. The species comprises four phylogenetically and physiologically distinct groups (I-IV) of Gram positive bacilli. The distinction between these groups is strong enough to merit the creation of four different species, but the name of *C. botulinum* is retained to emphasize the common ability of neurotoxin production. Comparison of 16S *rrna* gene sequences revealed that in terms of genetic distance, the divergence between *C. botulinum* groups (I-IV) is greater than that found between different species such as *B. subtilis* and *S. aureus* (Collins et al., 1998). Moreover, DNA from non-proteolytic *C. botulinum* types B and E gave weak hybridization responses (Carter et al., 2009). Groups I and II of *C. botulinum* are the most relevant to the food industry as they are responsible for the majority of cases of human botulism. Group I strains (also known as proteolytic) are highly proteolytic and can use certain sugars as substrate for growth, such as glucose. These strains produce botulinum neurotoxin of types A, B and/or F. On the other hand, group II (also known as nonproteolytic) are saccharolytic bacteria capable of metabolizing a wide range of monosaccharides and disaccharides. Group II strains produce a single botulinum neurotoxin of type B, E or F (Peck, Stringer, & Carter, 2011). Spores of the

bacterium are not hazardous as long as they remain dormant. Control strategies are oriented to prevent their germination and growth, either by spore inactivation or by the modification of the intrinsic properties of the food (*e.g.* additives, modified atmosphere, pH) since the botulinum neurotoxin is produced in the late exponential/early stationary phase of growth (Peck et al., 2009). Other characteristics of these two physiologically distinct *C. botulinum* groups can be found in Table 8.

Table 8. Characteristics of proteolytic *C. botulinum* (Group I) and nonproteolytic *C. botulinum* (Group II) (adapted from Peck et al., 2011).

Characteristic	Proteolytic *C. botulinum* (Group I)	Nonproteolytic *C. botulinum* (Group II)
Neurotoxins formed	A, B, F	B, E, F
Minimum growth temperature	10-12 °C	2.5-3.0 °C
Optimum growth temperature	37 °C	25 °C
Minimum pH for growth	4.6	5.0
Minimum a_w for growth[a]	0.94	0.97

[a]Value obtained using NaCl as humectant.

Traditionally, the safety of low-acid (pH <4.6) shelf-stable foods with high water activity has relied on severe heat treatments (*i.e. retorting*). Alternatively, mild processing require a multi-hurdle approach (*i.e*. refrigeration, pH control, additives) to prevent spore germination, growth and toxin production throughout shelf-life. Although molecular details of signal transduction leading to germination are not fully understood, it is well established that *Clostridium* and *Bacillus* species are triggered to germinate in response to a range of nutrients or germinants (Peck, 2009) (Figure 8). These germinants react with cognate germinant receptors located in the spore inner membrane and start a cascade reaction involving preformed enzymes already present in the core of dormant spores (Peck et al., 2011).

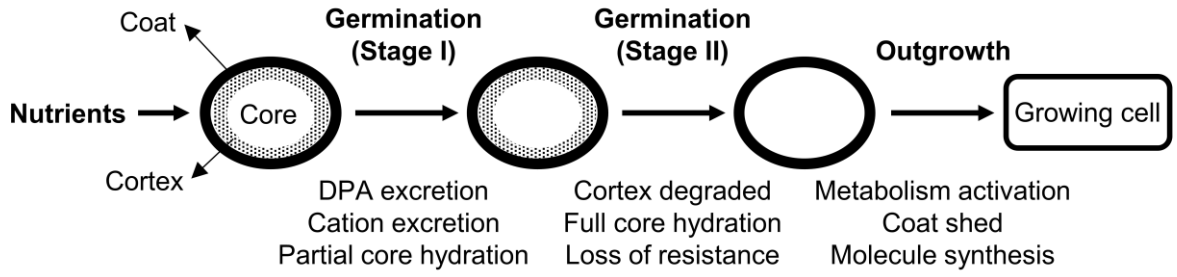

Figure 8. Events of nutrient-triggered spore germination (adapted from Black et al., 2007).

Proteolytic *C. botulinum* strains germinate in the presence of L-alanine, although a combination of the amino acid and L-lactate accelerates this process and facilitates

germination of a greater proportion of spores (Alberto, Broussolle, Mason, Carlin, & Peck, 2003; Broussolle et al., 2002; Sarathchandra, Wolf, & Barker, 1977). On the other hand, germination of nonproteolytic *C. botulinum* spores requires both, an amino acid (*e.g.* L-alanine, L-cysteine, L-serine) and L-lactate (Ando & Lida, 1970; Plowman & Peck, 2002). The interaction of these molecules with germinant receptors leads to the release of ions from the spore core (K^+, H^+ and Ca-DPA) and water fills the cavity created by the release of Ca-DPA (Figure 8). This triggers the next steps of the germination process, being the hydrolysis of the cortex the hallmark event. Core volume expands 2- to 3-fold, rising the core water content up to 80 % of wet weight. As a consequence, enzymes and other proteins in the core become mobile and begin to operate (Cowan, Koppel, Setlow, & Setlow, 2003). In addition to nutrients, other agents that induce spore germination are: (i) Ca-DPA, which activates cortex hydrolysis, (ii) lytic enzymes such as lysozyme, which leads to Ca-DPA release in coat-defective spores, and (iii) cationic surfactants such as dodecylamine, which interacts with in the Ca-DPA release channel (Peter Setlow, Wang, & Li, 2017). With the initiation of enzyme activity, spore germination is complete, and spores convert into growing cells. Outgrowth requires exogenous nutrient sources and favorable conditions to start the exponential growth phase and produce the botulinum neurotoxin. This opens the door to alternate control strategies in food products with specific intrinsic characteristics.

4. Aspects to consider for HPP coconut water validation

4.1. Process validation and pertinent microorganisms in coconut water

The successful implementation of HPP in the juice industry for the last 30 years has led to some food regulatory agencies to conclude that the process does not rise concerns related to food safety. The Canadian government communicated in 2015 that processing fruit and vegetable-based juices at 600 MPa between 2 and 9 min delivers safe products from a microbiological, chemical, nutritional and toxicological point of view (Health Canada, 2015). In addition, the Canadian Food Inspection Agency conducted an in-depth evaluation of commercially available HPP-treated juices and beverages to assess the safety and the hygiene of manufacturing practices. A total of 1216 retail samples (68 % fruit and vegetable juice blends, 30 % fruit juices and 2 % vegetable juices) processed at 600 MPa between 3 and 5 min were evaluated. Authorities reported that all HPP juices met microbiological quality indicators and highlighted that bacterial pathogens, virus and parasites were not detected (Canadian Food Inspection Agency, 2018). These facts served to adopt 600 MPa with a 3 min holding time as a “safe harbor” for HPP juices and beverages, which implies that processors meeting these parameters do not require further validation (Canadian Food Inspection Agency, 2018). A similar approach was followed by the European Union, where occasionally HPP

products fell under the “Novel Food” regulation (EU) 258/97, later modified by regulation (EU) 2015/2283, based on the definition of “food resulting from a production process not used for food production within the Union before 15 May 1997”. Nonetheless, processes that do not give rise to significant changes in the composition or structure of the food are excluded from this definition. Therefore, in 2018 the European Commission issued a guidance document on the implementation of certain provisions of regulation (EC) 825/2004, stating that it is not expected that a food will fall under the Novel Food regulation just because of the use of HPP. Conversely, other countries require the validation of processing conditions before commercialization. In the particular case of the United States, regulation 21 CFR part 120.24 mandates food processors to include in their Hazard Analysis and Critical Control Point (HACCP) plans control measures that will consistently achieve a 5-$\log_{10}$ reduction of the pertinent microorganisms for a period at least as long as the shelf-life of the product (U.S. Food and Drug Administration, 2001). In the context of this regulation, the pertinent microorganism is defined as the most resistant microorganism of public health significance that is likely to occur in the juice (U.S. Food and Drug Administration, 2001).

Coconut water has not been associated with foodborne outbreaks. This can be attributed to the lack of efficient outbreak surveillance mechanisms that establish the cause of foodborne illnesses in countries where the consumption of the beverage is very popular. Additionally, the liquid endosperm of coconut is sterile and stable inside the fruit, so the consumption of fresh coconut water rarely poses any risk. Moreover, microbiological stability of the beverage for distribution and commercialization has largely relied on standardized thermal processes. Nevertheless, the emergence of new nonthermal technologies such as HPP has raised questions about food safety of coconut water, especially in the US. Although no outbreaks have been confirmed, the intrinsic properties of the beverage make it a potential vector for infections. Based on physicochemical characteristics, coconut water is considered a low-acid beverage (pH >4.6). Therefore, since HPP does not inactivate bacterial spores, the US Food and Drug Administration (FDA) considers proteolytic *C. botulinum* and nonproteolytic *C. botulinum* the pertinent microorganisms in the tropical beverage because based on minimum pH requirements (Table 8) the pathogen can potentially grow during storage and produce the botulinum neurotoxin (U.S. Food and Drug Administration, 2007). The FDA’s position in this regard became clear back in 2015 when two companies producing high-pressure processed coconut water were issued with warning letters stating that their HACCP plans should include control measures to achieve a 5-$\log_{10}$ reduction of spores of *C. botulinum* (U.S. Food and Drug Administration, 2015a, 2015b). Since then, commercialization of refrigerated HPP tender coconut water has been restricted in the US. Food processors discontinued the production of high-pressure processed coconut water, switched to alternate

processing methods such as microfiltration (Harmless Harvest, 2020; Symbiosis, 2020), or increased acidity of the beverage with additives or by blending it with other fruits, such as lime or lemon. However, an interesting possibility that is not currently used commercially would be to high-pressure process coconut water to control vegetative pathogens, and freeze it to be distributed and sold in supermarkets.

Derived products from coconut have been implicated in foodborne outbreaks in the last century (Table 9). *Salmonella* spp. was the causative agent of most outbreaks related to coconut products, especially desiccated coconut. Hence, this vegetative pathogen may be considered of concern in coconut water as well. Additionally, it has been reported that the beverage can support growth of *Salmonella* spp. (Beristaín-Bauza et al., 2018; Lukas, 2013). Although not associated to outbreaks due to the consumption of coconut products, other vegetative pathogens able to grow in the liquid endosperm of the fruit are *E. coli* and *L. monocytogenes* (Lukas, 2013; Walter, Kabuki, Esper, Sant'Ana, & Kuaye, 2009). Global incidence and fatality rates related to infections caused by both pathogens suggest that they may represent a potential hazard in coconut water. Additionally, humans are potential carriers and they can be found in food-related industrial environments. For this reason, critical parameters of preservation processes should be validated to ensure that they are controlled.

Table 9. Outbreaks of foodborne illness associated with the consumption of coconut-derived products (adapted from Strawn, Schneider, & Danyluk, 2011).

Product	Pathogen	Year	Location
Desiccated coconut	*Salmonella* Typhi, *Salmonella* Senftenberg and others	1953	Australia
	Salmonella Paratyphi	1960	England
	Salmonella Java	1999	United Kingdom
Coconut milk	*Shigella* spp.	1991	Thailand
Shelled coconut	*Vibrio cholerae*	1991	United States
	Salmonella Paratyphi	1997	Singapore

4.2. Challenges for the validation of HPP coconut water

Validation is mandatory in the US for the commercialization of juices and beverages processed by alternative methods to heat pasteurization, such as HPP (21 CFR part 120.24). The US National Advisory Committee on Microbiological Criteria for Foods (NACMCF) defines validation studies as the artificial inoculation of a food with its pertinent pathogens to evaluate the ability of a particular process to achieve the desired level of inactivation followed by growth inhibition of survivors (NACMCF, 2010). In the particular case of juices (including coconut

water), the FDA requires a 5-$\log_{10}$ reduction of the appropriate pathogens throughout commercial shelf-life (21 CFR part 120.24). Companies must validate each product separately, although it is possible to group products with similar physicochemical and compositional characteristics under a single “umbrella study” with the aim of reducing costs (the price of a validation study ranges from 4000 to 6000 US$). A copy of the validation study signed by a process authority must be presented to third-party processors if the company that manufactures the product is not in charge of HPP.

Guidelines form the NACMCF recommend the use of three to five bacterial strains, either individually or in a cocktail. If there is little information known about growth of a certain microorganism in a food product (*e.g. C. botulinum*), as many as ten strains may be used. The use of stationary phase cells (18 to 24 h) is encouraged. Published work revealed that bacterial cells in this growth phase are more pressure resistant than exponential-phase cells. For instance, Dogan & Erkmen (2004) found that *L. monocytogenes* cells in the stationary phase were 1.40 times more resistant to HPP (300 MPa for up to 20 min) than mid- to-end exponential phase cells in orange and peach juices. It is also desirable to adapt the culture to specific conditions that characterize the juice (*i.e.* low pH). Although tender coconut water is mildly acidic (pH 4.6 to 5.5), nonselective media used for the growth of isolates typically have a higher pH (pH 7.0 to 7.2), so acid habituation would resemble more realistic conditions. Researchers compared the survival of a wild type and acid-adapted strain of *E. coli* in strawberry juice (pH 3.4) for 48 h. It was observed that the acid-adapted strain showed the highest resistance to the acidic medium. Hence, further inactivation experiments using different processing techniques (*i.e.* HPP, PEF, ultrasound and pasteurization) were conducted with acid-adapted cells (Yildiz, Pokhrel, Unluturk, & Barbosa-Cánovas, 2019). Other work demonstrated that acid-habituated *E. coli* O157:H7 strains were more resistant to HPP (450 MPa between 1 and 7 min at 4 °C) than non-habituated cells in blueberry juice (pH 3.2). Calculated D-values for habituated and non-habituated *E. coli* O157:H7 were 13.7 and 7.76 min, respectively (Kabir et al., 2019). Isolates should also appropriate for the food product (*i.e.* from the same food type, food processing environments or clinical isolates). Cells that are grown at higher temperature may be more pressure resistant. Research suggests that bacteria from animal or clinical sources exhibit higher tolerance to HPP than those of environmental origin due to the temperatures at which the cells grow. It is believed that lower growth temperatures induce the formation of shorter fatty acids in the cell membranes. This may increase sensitivity to HPP by reducing membrane flexibility. In addition to these considerations, validation studies should use strains that demonstrate tolerance to HPP (Hayman, Anantheswaran and Knabel, 2007). Since there is considerable variation between species or strains of the same species in response to HPP, isolates should be selected carefully (Podolak et al., 2020). Nevertheless,

there are few studies that identify strains suitable for validation studies. Current practice uses uncharacterized isolates, which may underestimate the risk.

4.2.1. Strain variability in process validation

It is widely established that different species of foodborne pathogens and different strains of the same species exhibit considerable variations in pressure resistance (Alpas et al., 1999; Benito, Ventoura, Casadei, Robinson, & Mackey, 1999; Robey et al., 2001; Van Boeijen, Moezelaar, Abee, & Zwietering, 2008). This may account for the variations in the levels of inactivation obtained using different strains of the same species (Table 7). As an example, strain C9490 exhibited the greatest pressure resistance among six different *E. coli* O157:H7 isolates with less than 1-$\log_{10}$ reduction in PBS after processing at 500 MPa for 5 min. On the other hand, the most pressure sensitive strain (NCTC 8003) showed a reduction greater than 6 $\log_{10}$ CFU/mL when exposed to the same processing conditions (Benito et al., 1999). Similar variability has been described for *L. monocytogenes* and *S. enterica*. A reduction lower than 2 $\log_{10}$ CFU/mL was observed for strain NCTC 11994 of *L. monocytogenes* when subjected to HPP (375 MPa for 15 min) in PBS, whereas same processing conditions achieved a >6-$\log_{10}$ reduction for strain 2433. In addition, *Salmonella* Typhimurium NCTC 74 was much more sensitive than *Salmonella* Enteritidis phage type 4 when tested under the same conditions (300 MPa for 15 min) (Patterson, Quinn, Simpson, & Gilmour, 1995). Alpas et al. (1999) reported viability losses between 0.92 and 3.53-$\log_{10}$ for nine strains of *L. monocytogenes*, between 2.80 and 5.64-$\log_{10}$ for six strains of *E. coli* O157:H7 and between 5.45 and 8.34-$\log_{10}$ for six strains of *S. enterica* after processing at 345 MPa for 5 min in peptone water.

For conducting validation studies, NACMCF guidelines do not specify any bacterial strain to be used. Since molecular mechanisms or measurable indicators predicting HPP resistance are not fully understood or remain unknown, adequate strain selection requires a comprehensive characterization. In this regard, FDA authorities communicated that the lack of consensus concerning the selection of specific pathogenic strains is one of the unresolved issues in process validation (Podolak et al., 2020).

4.2.2. Clostridium botulinum as a microorganism of concern

Few botulism outbreaks have been previously reported associated to the consumption of heat-pasteurized low-acid juices, such as carrot juice (Sheth et al., 2008). Presumptively, the juice was subjected to temperature abuse (>10 °C) by the final consumers. This fact promoted germination and growth of heat-resistant proteolytic *C. botulinum* spores that survived the pasteurization treatment. Since then, the implementation of control measures

to address the risk associated with psychrotrophic (nonproteolytic) *C. botulinum* and mesophilic (proteolytic) *C. botulinum* became mandatory in the US for low-acid juices (pH >4.6), such as coconut water (U.S. Food and Drug Administration, 2007). Coconut water or coconut-derived products have not been directly associated with foodborne botulism intoxications. However, in 1985, a rare botulism case was confirmed in a 37-year-old woman who suffered an intestinal infection due to *C. botulinum*, followed by secondary intoxication (as occurs in infants younger than one year of age). *C. botulinum* type A spores were found in a refrigerated jar of cream of coconut, although preformed toxin was not detected. It is noteworthy to mention that the contents of the jar had been transferred from the original can, and three unopened cans of the same product were negative for the toxin and the bacteria (Chia, Clark, Ryan, & Pollack, 1986). It could not be elucidated whether *C. botulinum* spores were originally present in coconut cream or if the product was cross-contaminated on transferring to a secondary container.

Due to the low acidity of coconut water (pH >4.6) and since bacterial spores are not inactivated by HPP, other control factors need to be considered in the beverage. Apart from acidity, several intrinsic and extrinsic factors could also play a role in restricting growth and toxin production, and should also be considered in evaluating the risk of foodborne botulism in minimally processed coconut water. For instance, Raghubeer et al. (2020) demonstrated that proteolytic *C. botulinum* types A, B, F, and nonproteolytic *C. botulinum* types B, E, F failed to produce the botulinum neurotoxin in two types of coconut water during 45 days of incubation at 10 °C. Nevertheless, the study did not evaluate the impact of critical parameters such as dissolved oxygen, redox potential or pH. It was suggested that this could be due to naturally-present inhibitory compounds or to the lack of specific nutrients required by *C. botulinum*, although no attempt was made to explore this hypothesis further. It is well known that HPP can preserve some of these heat-sensitive antimicrobial compounds that heat pasteurization typically destroys (Patterson, McKay, Connolly, & Linton, 2012). Additionally, minimally processed chilled foods typically have a shorter shelf-life because not all spoilage microorganisms are inactivated and may grow during storage. Growth of *C. botulinum* spores could be affected as substrate availability would be reduced, pH could decrease or antimicrobial substances may be released (Lyver, Smith, Austin, & Blanchfield, 1998; Ramaroson et al., 2018). Previous work also demonstrated that *C. botulinum* failed to grow in other products of vegetable origin such as garlic or Brussels sprouts purées (both pH >5) even under extremely favorable conditions (*i.e.* incubation at 30 °C for up to 60 days in an anaerobic atmosphere) (Carlin & Peck, 1995). Hence, adequate risk assessment of HPP coconut water should consider all intrinsic characteristics of the beverage in addition to acidity to determine whether *C. botulinum* should be considered a microorganism of concern.

5. Conclusions and research objectives

There are several factors affecting microbial inactivation during high pressure processing in juices and beverages, including coconut water. These factors include pressure and holding time, process temperature, compression and decompression rates, microbiota and intrinsic characteristics of the products. Some of these parameters are inherent in the process since they depend on the design of industrial HPP equipment: (i) process temperature is restricted to a narrow range between 4 and 25 °C (typically below 10 °C for quality reasons), (ii) pressurization rate depends on the number of high-pressure intensifiers, but it often ranges between 1.5 and 5.0 MPa/s, and (ii) depressurization from 600 MPa to ambient pressure is instantaneous (<1 s). For this reason, it is important to perform validations on industrial HPP equipment. Moreover, among the physicochemical characteristics of coconut water, a_w is not considered a critical parameter since it is higher than 0.98 and it is not expected to affect the efficiency of the process. Therefore, critical parameters for process validation of HPP coconut water are the variety of the fruit, the acidity of the beverage, the magnitude of pressure and holding time and the strains used in the bacterial cocktails of pertinent microorganisms.

Although extensive research has been carried out to understand microbial inactivation during HPP in juices and beverages, each study used different pathogenic strains with varying pressure resistance. Additionally, work on HPP coconut water is scarce. It also appears that there is no consensus concerning the selection of specific strains for process validation. Conducting robust validation studies is of great importance to ensure that process parameters can achieve the required pathogen inactivation. Furthermore, despite the lack of scientific and epidemiology evidence, no other parameters were considered apart from acidity to control the risk of growth and neurotoxin production by *C. botulinum* in coconut water. A more comprehensive evaluation of other intrinsic factors would serve to adequately establish whether *C. botulinum* should be considered a microorganism of concern in the tropical beverage. Therefore, the present work was designed to address the following objectives:

Objective 1: To determine if the intrinsic properties of coconut water support spore germination and growth of nonproteolytic *C. botulinum* and *Clostridium* spp. spores in the high-pressure processed beverage.

Objective 2: To evaluate growth potential and toxin production by proteolytic *C. botulinum* and nonproteolytic *C. botulinum* in multiple varieties of tender coconut water aiming to establish whether the pathogen should be considered of concern in the minimally processed tropical juice.

Objective 3: To characterize a wide range of *E. coli* O157:H7, *L. monocytogenes* and *S. enterica* strains based on their pressure resistance, adaptation and recovery phenotypes in model solutions for the identification of representative isolates with potential to be used in validation studies of HPP juices.

Objective 4: To assess the adequacy of typical processing parameters used in the juice industry to achieve a 5-$\log_{10}$ reduction of bacterial cocktails of pressure-resistant vegetative pathogens in multiple varieties of tender coconut water.

6. References

Adams, W., & Bratt, D. E. (1992). Young coconut water for home rehydration in children with mild gastroenteritis. *Tropical and Geographical Medicine*, *44*, 149–153.

Adubofuor, J., Amoah, I., & Osei-Bonsu, I. (2016). Sensory and physicochemical properties of pasteurized coconut water from two varieties of coconut. *Food Science and Quality Management*, *54*, 26–32.

Alberto, F., Broussolle, V., Mason, D. R., Carlin, F., & Peck, M. W. (2003). Variability in spore germination response by strains of proteolytic *Clostridium botulinum* types A, B and F. *Letters in Applied Microbiology*, *36*(1), 41–45. https://doi.org/10.1046/j.1472-765X.2003.01260.x

Ali, N., Popović, V., Koutchma, T., Warriner, K., & Zhu, Y. (2020). Effect of thermal, high hydrostatic pressure, and ultravioletC processing on the microbial inactivation, vitamins, chlorophyll, antioxidants, enzyme activity, and color of wheatgrass juice. *Journal of Food Process Engineering*, *43*(1). https://doi.org/10.1111/jfpe.13036

Alpas, H., Kalchayanand, N., Bozoglu, F., & Ray, B. (2000). Interactions of high hydrostatic pressure, pressurization temperature and pH on death and injury of pressure-resistant and pressure-sensitive strains of foodborne pathogens. *International Journal of Food Microbiology*, *60*, 33–42.

Alpas, H., Kalchayanand, N., Bozoglu, F., Sikes, A., Dunne, C. P., & Ray, A. B. (1999). Variation in Resistance to Hydrostatic Pressure among Strains of Food-Borne Pathogens. *Applied and Environmental Microbiology*, *65*(9), 4248–4251.

Ando, Y., & Lida, H. (1970). Factors Affecting the Germination of Spores of *Clostridium botulinum* Type E. *Japanese Journal of Microbiology*, *14*(5), 361–370.

Aniesrani Delfiya, D. S., & Thangavel, K. (2016). Effect of ohmic heating on polyphenol oxidase activity, electrical and physicochemical properties of fresh tender coconut water. *International Journal of Food Engineering*, *12*(7), 691–700. https://doi.org/10.1515/ijfe-2015-0329

Anurag, P., & Rajamohan, T. (2003). Cardioprotective effect of tender coconut water in experimental myocardial infarction. *Plant Foods for Human Nutrition*, *58*(3), 1–12. https://doi.org/10.1023/B:QUAL.0000040363.64356.05

Barba, F. J., Koubaa, M., do Prado-Silva, L., Orlien, V., & Sant'Ana, A. de S. (2017). Mild processing applied to the inactivation of the main foodborne bacterial pathogens: A review. *Trends in Food Science and Technology*, *66*, 20–35. https://doi.org/10.1016/j.tifs.2017.05.011

Basak, S., Ramaswamy, H. S., & Piette, J. P. G. (2002). High pressure destruction kinetics of *Leuconostoc mesenteroides* and *Saccharomyces cerevisiae* in single strength and concentrated orange juice. *Innovative Food Science and Emerging Technologies*, *3*(3), 223–231. https://doi.org/10.1016/S1466-8564(02)00008-5

Baudouin, L., & Lebrun, P. (2009). Coconut (*Cocos nucifera* L.) DNA studies support the hypothesis of an ancient Austronesian migration from Southeast Asia to America. *Genetic Resources and Crop Evolution*, *56*(2), 257–262. https://doi.org/10.1007/s10722-008-9362-6

Bayındırlı, A., Alpas, H., Bozoğlu, F., & Hızal, M. (2006). Efficiency of high pressure treatment on inactivation of pathogenic microorganisms and enzymes in apple, orange, apricot and sour cherry juices. *Food Control*, *17*(1), 52–58. https://doi.org/10.1016/j.foodcont.2004.09.002

Beccari, U. (1917). The origin and dispersal of *Cocos nucifera*. *Philippine Journal of Science*, *12*, 27–43.

Benito, A., Ventoura, G., Casadei, M., Robinson, T., & Mackey, B. (1999). Variation in resistance of natural isolates of *Escherichia coli* O157 to high hydrostatic pressure, mild heat, and other stresses. *Applied and Environmental Microbiology*, *65*(4), 1564–1569. https://doi.org/10.1128/aem.65.4.1564-1569.1999

Benton, M., Macartney-Coxson, D., Eccles, D., Griffiths, L., Chambers, G., & Lea, R. (2012). Complete Mitochondrial Genome Sequencing Reveals Novel Haplotypes in a Polynesian Population. *PLoS ONE*, *7*(4), e35026. https://doi.org/10.1371/journal.pone.0035026

Beristaín-Bauza, S., Martínez-Niño, A., Ramírez-González, A. P., Ávila-Sosa, R., Ruíz-Espinosa, H., Ruiz-López, I. I., & Ochoa-Velasco, C. E. (2018). Inhibition of *Salmonella* Typhimurium growth in coconut (*Cocos nucifera* L.) water by hurdle technology. *Food Control*, *92*, 312–318. https://doi.org/10.1016/j.foodcont.2018.05.010

Beuchat, L. R. (1996). *Listeria monocytogenes*: Incidence on vegetables. *Food Control*, *7*(4–5), 223–228. https://doi.org/10.1016/s0956-7135(96)00039-4

Bhagya, D., Prema, L., & Rajamohan, T. (2012). Therapeutic effects of tender coconut water on oxidative stress in fructose fed insulin resistant hypertensive rats. *Asian Pacific Journal of Tropical Medicine*, *5*(4), 270–276. https://doi.org/10.1016/S1995-7645(12)60038-8

Bhullar, M. S., Patras, A., Kilanzo-Nthenge, A., Pokharel, B., Yannam, S. K., Rakariyatham, K., … Sasges, M. (2018). Microbial inactivation and cytotoxicity evaluation of UV irradiated coconut water in a novel continuous flow spiral reactor. *Food Research International*, *103*, 59–67. https://doi.org/10.1016/j.foodres.2017.10.004

Black, E. P., Setlow, P., Hocking, A. D., Stewart, C. M., Kelly, A. L., & Hoover, D. G. (2007). Response of Spores to High-Pressure Processing. *Comprehensive Reviews in Food Science and Food Safety*, *6*(4), 103–119. https://doi.org/10.1111/j.1541-4337.2007.00021.x

Broussolle, V., Alberto, F., Shearman, C. A., Mason, D. R., Botella, L., Nguyen-The, C., … Carlin, F. (2002). Molecular and physiological characterisation of spore germination in *Clostridium botulinum* and *Clostsridium sporogenes*. *Anaerobe*, *8*(3), 89–100. https://doi.org/10.1006/anae.2002.0424

Buerman, E. C., Worobo, R. W., & Padilla-Zakour, O. I. (2020). High pressure processing of spoilage fungi as affected by water activity in a diluted apple juice concentrate. *Food Control*, *107*, 106779. https://doi.org/10.1016/J.FOODCONT.2019.106779

Butz, P., Edenharder, R., Fister, H., & Tauscher, B. (1997). The influence of high pressure processing on antimutagenic activities of fruit and vegetable juices. *Food Research International*, *30*(3-4), 287-291.

Buzrul, S., Alpas, H., Largeteau, A., & Demazeau, G. (2008). Inactivation of *Escherichia coli* and *Listeria innocua* in kiwifruit and pineapple juices by high hydrostatic pressure. *International Journal of Food Microbiology*, *124*(3), 275–278. https://doi.org/10.1016/j.ijfoodmicro.2008.03.015

Campbell-Falck, D., Thomas, T., Falck, T. M., Tutuo, N., & Clem, K. (2000). The intravenous use of coconut water. *American Journal of Emergency Medicine*, *18*(1), 108–111. https://doi.org/10.1016/S0735-6757(00)90062-7

Campos, C. F., Souza, P. E. A., Coelho, J. V., & Glória, M. B. A. (1996). Chemical composition, enzyme activity and effect of enzyme inactivation on flavor quality of green coconut water. *Journal of Food Processing and Preservation*, *20*(6), 487–500. https://doi.org/10.1111/j.1745-4549.1996.tb00761.x

Canadian Food Inspection Agency. (2018). Novel Food Information: High Pressure Processing (HPP)-Treated Fruit and Vegetable-Based Juices. Retrieved October 5, 2020, from https://www.canada.ca/en/health-canada/services/food-nutrition/genetically-modified-foods-other-novel-foods/approved-products/novel-food-information-high-pressure-processing-treated-fruit-vegetable-based-juices.html

Cappelletti, M., Ferrentino, G., Endrizzi, I., Aprea, E., Betta, E., Corollaro, M. L., … Spilimbergo, S. (2015). High Pressure Carbon Dioxide pasteurization of coconut water: A sport drink with high nutritional and sensory quality. *Journal of Food Engineering*, *145*, 73–81. https://doi.org/10.1016/j.jfoodeng.2014.08.012

Cardello, A. V., Schutz, H. G., & Lesher, L. L. (2007). Consumer perceptions of foods processed by innovative and emerging technologies: A conjoint analytic study. *Innovative Food Science and Emerging Technologies*, *8*(1), 73–83. https://doi.org/10.1016/j.ifset.2006.07.002

Carlin, F., & Peck, M. W. (1995). Growth and toxin production by nonproteolytic and proteolytic *Clostridium botulinum* in cooked vegetables. *Letters in Applied Microbiology*, *20*(3), 152–156. https://doi.org/10.1111/j.1472-765X.1995.tb00414.x

Carter, A. T., Paul, C. J., Mason, D. R., Twine, S. M., Alston, M. J., Logan, S. M., ... & Peck, M. W. (2009). Independent evolution of neurotoxin and flagellar genetic loci in proteolytic *Clostridium botulinum*. *BMC genomics*, *10*(1), 115.

Chai, C., Lee, J., Lee, Y., Na, S., & Park, J. (2014). A combination of TiO_2-UV photocatalysis and high hydrostatic pressure to inactivate *Bacillus cereus* in freshly squeezed *Angelica keiskei* juice. *LWT - Food Science and Technology*, *55*(1), 104–109. https://doi.org/10.1016/j.lwt.2013.08.015

Chang, C.-L., & Wu, R.-T. (2011). Quantification of (+)-catechin and (−)-epicatechin in coconut water by LC–MS. *Food Chemistry*, *126*(2), 710–717. https://doi.org/10.1016/j.foodchem.2010.11.034

Chang, Y. H., Wu, S. J., Chen, B. Y., Huang, H. W., & Wang, C. Y. (2017). Effect of high-pressure processing and thermal pasteurization on overall quality parameters of white grape juice. *Journal of the Science of Food and Agriculture*, *97*(10), 3166–3172. https://doi.org/10.1002/jsfa.8160

Chapleau, N., Ritz, M., Delépine, S., Jugiau, F., Federighi, M., & De Lamballerie, M. (2006). Influence of kinetic parameters of high pressure processing on bacterial inactivation in a buffer system. *International Journal of Food Microbiology*, *106*(3), 324–330. https://doi.org/10.1016/j.ijfoodmicro.2005.09.009

Chen, D., Pang, X., Zhao, J., Gao, L., Liao, X., Wu, J., & Li, Q. (2015). Comparing the effects of high hydrostatic pressure and high temperature short time on papaya beverage. *Innovative Food Science & Emerging Technologies*, *32*, 16–28. https://doi.org/10.1016/J.IFSET.2015.09.018

Chen, D., Xi, H., Guo, X., Qin, Z., Pang, X., Hu, X., … Wu, J. (2013). Comparative study of quality of cloudy pomegranate juice treated by high hydrostatic pressure and high temperature short time. *Innovative Food Science and Emerging Technologies*, *19*, 85–94. https://doi.org/10.1016/j.ifset.2013.03.003

Chia, J. K., Clark, J. B., Ryan, C. A., & Pollack, M. (1986). Botulism in an Adult Associated with Food-Borne Intestinal Infection with *Clostridium botulinum*. *New England Journal of Medicine*, *315*(4), 239–241. https://doi.org/10.1056/nejm198607243150407

Chourio, A. M., Salais-Fierro, F., Mehmood, Z., Martinez-Monteagudo, S. I., & Saldaña, M. D. A. (2018). Inactivation of peroxidase and polyphenoloxidase in coconut water using pressure-assisted thermal processing. *Innovative Food Science and Emerging Technologies*, *49*, 41–50. https://doi.org/10.1016/j.ifset.2018.07.014

Chowdhury, M. G. F., Rahman, M. M., Islam, A. F. M. T., & Islam, M. S. (2009). Processing and preservation of green coconut wawter. *Journal of Innovation & Development Strategy*, *3*(1), 1–5.

Chutia, H., Kalita, D., Mahanta, C. L., Ojah, N., & Choudhury, A. J. (2019). Kinetics of inactivation of peroxidase and polyphenol oxidase in tender coconut water by dielectric barrier discharge plasma. *LWT*, *101*, 625–629. https://doi.org/10.1016/j.lwt.2018.11.071

Cilla, A., Alegría, A., de Ancos, B., Sánchez-Moreno, C., Cano, M. P., Plaza, L., … Barberá, R. (2012). Bioaccessibility of Tocopherols, Carotenoids, and Ascorbic Acid from Milk- and Soy-Based Fruit Beverages: Influence of Food Matrix and Processing. *Journal of Agricultural and Food Chemistry*, *60*(29), 7282–7290. https://doi.org/10.1021/jf301165

Collins, M., & East, A. K. (1998). Phylogeny and taxonomy of the food-borne pathogen *Clostridium botulinum* and its neurotoxins. *Journal of applied microbiology*, *84*(1), 5-17.

Cook, O. F. (1901). Origin and distribution of the cocoa palm. *US Government Printing Office*.

Cortezzo, D. E., Koziol-Dube, K., Setlow, B., & Setlow, P. (2004). Treatment with oxidizing agents damages the inner membrane of spores of *Bacillus subtilis* and sensitizes spores to subsequent stress. *Journal of Applied Microbiology*, *97*(4), 838–852. https://doi.org/10.1111/j.1365-2672.2004.02370.x

Cortezzo, D. E., & Setlow, P. (2005). Analysis of factors that influence the sensitivity of spores of *Bacillus subtilis* to DNA damaging chemicals. *Journal of Applied Microbiology*, *98*(3), 606–617. https://doi.org/10.1111/j.1365-2672.2004.02495.x

Cowan, A. E., Koppel, D. E., Setlow, B., & Setlow, P. (2003). A soluble protein is immobile in dormant spores of *Bacillus subtilis* but is mobile in germinated spores: Implications for spore dormancy. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(7), 4209–4214. https://doi.org/10.1073/pnas.0636762100

Cowan, A. E., Olivastro, E. M., Koppel, D. E., Loshon, C. A., Setlow, B., & Setlow, P. (2004). Lipids in the inner membrane of dormant spores of *Bacillus species* are largely immobile. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(20), 7733–7738. https://doi.org/10.1073/pnas.0306859101

Cunha, A. G., Alves Filho, E. G., Silva, L. M. A., Ribeiro, P. R. V., Rodrigues, T. H. S., de Brito, E. S., & de Miranda, M. R. A. (2020). Chemical composition of thermally processed coconut water evaluated by GC–MS, UPLC-HRMS, and NMR. *Food Chemistry*, *324*, 126874. https://doi.org/10.1016/j.foodchem.2020.126874

da Silveira, T. F. F., Cristianini, M., Kuhnle, G. G., Ribeiro, A. B., Filho, J. T., & Godoy, H. T. (2019). Anthocyanins, non-anthocyanin phenolics, tocopherols and antioxidant capacity of açaí juice (*Euterpe oleracea*) as affected by high pressure processing and thermal pasteurization. *Innovative Food Science and Emerging Technologies*, *55*, 88–96. https://doi.org/10.1016/j.ifset.2019.05.001

Damar, S., Balaban, M. O., & Sims, C. A. (2009). Continuous dense-phase CO 2 processing of a coconut water beverage. *International Journal of Food Science and Technology*, *44*(4), 666–673. https://doi.org/10.1111/j.1365-2621.2008.01784.x

De Condolle, A. (1886). *Origin of cultivated plants* (2nd ed.). New York: Hafner Publishing

Company.

De Marchi, F., Aprea, E., Endrizzi, I., Charles, M., Betta, E., Corollaro, M. L., … Gasperi, F. (2015). Effects of Pasteurization on Volatile Compounds and Sensory Properties of Coconut (*Cocos nucifera* L.) Water: Thermal vs. High-Pressure Carbon Dioxide Pasteurization. *Food and Bioprocess Technology*, *8*(7), 1393–1404. https://doi.org/10.1007/s11947-015-1501-4

de Noordhout, C. M., Devleesschauwer, B., Angulo, F. J., Verbeke, G., Haagsma, J., Kirk, M., … Speybroeck, N. (2014). The global burden of listeriosis: A systematic review and meta-analysis. *The Lancet Infectious Diseases*, *14*(11), 1073–1082. https://doi.org/10.1016/S1473-3099(14)70870-9

Debien, I. C. N., Gomes, M. T. M. S., Ongaratto, R. S., & Viotto, L. A. (2013). Ultrafiltration performance of PVDF, PES, and cellulose membranes for the treatment of coconut water (*Cocos nucifera* L.). *Food Science and Technology*, *33*(4), 676–684.

DebMandal, M., & Mandal, S. (2011). Coconut (*Cocos nucifera* L.: *Arecaceae*): In health promotion and disease prevention. *Asian Pacific Journal of Tropical Medicine*, *4*(3), 241–247. https://doi.org/10.1016/S1995-7645(11)60078-3

Demazeau, G., & Rivalain, N. (2011). High hydrostatic pressure and biology: A brief history. *Applied Microbiology and Biotechnology*, *89*(5), 1305–1314. https://doi.org/10.1007/s00253-010-3070-9

Dogan, C., & Erkmen, O. (2004). High pressure inactivation kinetics of *Listeria monocytogenes* inactivation in broth, milk, and peach and orange juices. *Journal of Food Engineering*, *62*(1), 47–52. https://doi.org/10.1016/S0260-8774(03)00170-5

Donsingha, S., & Assatarakul, K. (2018). Kinetics model of microbial degradation by UV radiation and shelf-life of coconut water. *Food Control*, *92*, 162–168. https://doi.org/10.1016/j.foodcont.2018.04.030

Dubins, D. N., Lee, A., Macgregor, R. B., & Chalikian, T. V. (2001). On the stability of double stranded nucleic acids. *Journal of the American Chemical Society*, *123*(38), 9254–9259. https://doi.org/10.1021/ja004309u

EFSA. (2019). The European Union One Health 2018 Zoonoses Report. *EFSA Journal*, *17*(12). https://doi.org/10.2903/j.efsa.2019.5926

Erkmen, O., & Doğan, C. (2004). Kinetic analysis of *Escherichia coli* inactivation by high hydrostatic pressure in broth and foods. *Food Microbiology*, *21*(2), 181–185. https://doi.org/10.1016/S0740-0020(03)00055-8

FAO. (2020). FAOSTAT. Crops and Livestock Products. Retrieved August 23, 2020, from http://www.fao.org/faostat/en/

Farkas, D. F., & Hoover, D. G. (2000). High Pressure Processing. *Journal of Food Science*, *65*(8),

47–64. https://doi.org/10.1111/j.1750-3841.2000.tb00618.x

Fauzi, N. A., Farid, M. M., & Silva, F. (2017). An insight on the relationship between food compressibility and microbial inactivation during high pressure processing. *Journal of Food Science and Technology*, *54*(3), 802–809. https://doi.org/10.1007/s13197-017-2526-7

Gabriel, A. A., Aba, R. P. M., Tayamora, D. J. L., Colambo, J. C. R., Siringan, M. A. T., Rosario, L. M. D., … Ramos, H. J. (2016). Reference organism selection for microwave atmospheric pressure plasma jet treatment of young coconut liquid endosperm. *Food Control*, *69*, 74–82. https://doi.org/10.1016/j.foodcont.2016.04.034

Gal-Mor, O., Boyle, E. C., & Grassl, G. A. (2014). Same species, different diseases: How and why typhoidal and non-typhoidal Salmonella enterica serovars differ. *Frontiers in Microbiology*. Frontiers Research Foundation. https://doi.org/10.3389/fmicb.2014.00391

Geetha, V., Mhavana, K. P., Chetana, R., Gopala Krishna, A. G., & Suresh Kumar, G. (2016). Studies on the Composition and In-Vitro Antioxidant Activities of Concentrates from Coconut Testa and Tender Coconut Water. *Journal of Food Processing & Technology*, *7*(5), 1–5. https://doi.org/10.4172/2157-7110.1000588

Georget, E., Sevenich, R., Reineke, K., Mathys, A., Heinz, V., Callanan, M., … Knorr, D. (2015). Inactivation of microorganisms by high isostatic pressure processing in complex matrices: A review. *Innovative Food Science & Emerging Technologies*, *27*, 1–14. https://doi.org/10.1016/j.ifset.2014.10.015

Gerhardt, P., & Marquis, R. E. (1989). Spore thermoresistance mechanisms. In I. Smith, R. A. Slepecky, & P. Setlow (Eds.), *Regulation of prokaryotic development* (pp. 43–63). Washington DC: American Society for Microbiology.

González-Angulo, M., Serment-Moreno, V., Queirós, R. P., & Tonello-Samson, C. (2021). Food and Beverage Commercial Applications of High Pressure Processing. In K. Muthukumarappan & K. Knoerzer (Eds.), *Innovative Food Processing Technologies* (pp. 39–73). Amsterdam: Elsevier. https://doi.org/10.1016/b978-0-12-815781-7.00009-3

Gould, G. W., & Sale, A. J. (1970). Initiation of germination of bacterial spores by hydrostatic pressure. *Journal of General Microbiology*, *60*(3), 335–346. https://doi.org/10.1099/00221287-60-3-335

Gouvea, F. S., Padilla-Zakour, O. I., Worobo, R. W., Xavier, B. M., Walter, E. H. M., & Rosenthal, A. (2020). Effect of high-pressure processing on bacterial inactivation in açaí juices with varying pH and soluble solids content. *Innovative Food Science and Emerging Technologies*, *66*, 102490. https://doi.org/10.1016/j.ifset.2020.102490

Grèbol, N. (2002). Commercial use of High Hydrostatic Pressure in Sliced Cooked Ham in Spain. In *Trends in High Pressure Bioscience and Biotechnology* (pp. 385–388).

Guillet, C., Join-Lambert, O., Le Monnier, A., Leclercq, A., Mechaï, F., Mamzer-Bruneel, M. F., … Lecuit, M. (2010). Human listeriosis caused by *Listeria ivanovii*. *Emerging Infectious Diseases*, *16*(1), 136–138. https://doi.org/10.3201/eid1601.091155

Gunn, B. F., Baudouin, L., & Olsen, K. M. (2011). Independent origins of cultivated coconut (*Cocos nucifera* L.) in the old world tropics. *PLoS ONE*, *6*(6), 21143. https://doi.org/10.1371/journal.pone.0021143

Gupta, R., Kopec, R. E., Schwartz, S. J., & Balasubramaniam, V. M. (2011). Combined Pressure-Temperature Effects on Carotenoid Retention and Bioaccessibility in Tomato Juice. *J. Agric. Food Chem*, *59*, 7808–7817. https://doi.org/10.1021/jf200575t

Harmless Harvest. (2020). Original coconut water. Retrieved October 26, 2020, from https://harmlessharvest.com/

Harries, H. (1978). The evolution, dissemination, and classification of *Cocos nucifera* L. *Botanical Review*, *44*(3), 265–320.

Harries, H. (1990). Malesian origin for a domestic *Cocos nucifera*. In *The plant diversity of Malesia* (pp. 351–357). Dordrecht: Springer.

Hayman, M. M., Anantheswaran, R. C., & Knabel, S. J. (2007). The effects of growth temperature and growth phase on the inactivation of *Listeria monocytogenes* in whole milk subject to high pressure processing. *International journal of food microbiology*, *115*(2), 220-226.

Health Canada. (2015). Novel Food Information - High Pressure Processing (HPP)-Treated Fruit and Vegetable-Based Juices. Retrieved December 3, 2020, from https://www.canada.ca/en/health-canada/services/food-nutrition/genetically-modified-foods-other-novel-foods/approved-products/novel-food-information-high-pressure-processing-treated-fruit-vegetable-based-juices.html

Hiremath, N. D., & Ramswamy, H. S. (2012). High-pressure destruction kinetics of spoilage and pathogenic microorganisms in mango juice. *Journal of Food Processing and Preservation*, *36*(2), 113–125. https://doi.org/10.1111/j.1745-4549.2011.00559.x

Hite, B. H. (1898). The effect of pressure in the preservation of milk: a preliminary report. *West Virginia Agricultural Experiment Station, Bulletin*, *58*, 15–35.

Hori, K., Manbe, Y., Kaneko, M., Sekimoto, T., Sugimoto, Y., & Yamane, T. (1992). Development of high pressure processor for food industries. *High Pressure and Biotechnology*, *224*, 499–507.

Hsiao, G., Shen, M. Y., Lin, K. H., Chou, C. Y., Tzu, N. H., Lin, C. H., … Sheu, J. R. (2003). Inhibitory activity of kinetin on free radical formation of activated platelets in vitro and on thrombus formation in vivo. *European Journal of Pharmacology*, *465*(3), 281–287. https://doi.org/10.1016/S0014-2999(03)01528-0

Hsu, K. C., Tan, F. J., & Chi, H. Y. (2008). Evaluation of microbial inactivation and physicochemical properties of pressurized tomato juice during refrigerated storage. *LWT - Food Science and Technology*, *41*(3), 367–375. https://doi.org/10.1016/j.lwt.2007.03.030

Hurtado, A., Guàrdia, M. D., Picouet, P., Jofré, A., Ros, J. M., & Bañón, S. (2017). Stabilization of red fruit-based smoothies by high-pressure processing. Part A. Effects on microbial growth, enzyme activity, antioxidant capacity and physical stability. *Journal of the Science of Food and Agriculture*, *97*(3), 770–776. https://doi.org/10.1002/jsfa.7796

Jackson-Davis, A., Mendonca, A., Hale, S., Jackson, J., King, A., & Jackson, J. (2018). Microbiological Safety of Unpasteurized Fruit and Vegetable Juices Sold in Juice Bars and Small Retail Outlets. In S. Ricke, G. Atungulu, C. Rainwater, & S. Park (Eds.), *Food and Feed Safety Systems and Analysis* (pp. 213–225). London: Academic Press. https://doi.org/10.1016/B978-0-12-811835-1.00012-9

Jackson, J. C., Gordon, A., Wizzard, G., McCook, K., & Rolle, R. (2004). Changes in chemical composition of coconut (*Cocos nucifera*) water during maturation of the fruit. *Journal of the Science of Food and Agriculture*, *84*(9), 1049–1052. https://doi.org/10.1002/jsfa.1783

Jacobo-Velázquez, D. A., & Hernández-Brenes, C. (2011). Sensory shelf-life limiting factor of high hydrostatic pressure processed avocado paste. *Journal of Food Science*, *76*(6). https://doi.org/10.1111/j.1750-3841.2011.02259.x

Jayalekshmy, A., & Mathew, A. G. (1990). Changes in the carbohydrates and proteins of coconut during roasting. *Food Chemistry*, *37*(2), 123–134. https://doi.org/10.1016/0308-8146(90)90086-J

Jayasundera, J. M. M. A., & Kulatunga, A. R. (2014). Spray-drying of coconut treacle into an amorphous powder. *Emirates Journal of Food and Agriculture*, *26*(8), 672–678. https://doi.org/10.9755/ejfa.v26i8.17581

Jordan, S. L., Pascual, C., Bracey, E., & Mackey, B. M. (2001). Inactivation and injury of pressure-resistant strains of *Escherichia coli* O157 and *Listeria monocytogenes* in fruit juices. *Journal of Applied Microbiology*, *91*(3), 463–469. https://doi.org/10.1046/j.1365-2672.2001.01402.x

Kabir, M. N., Aras, S., Allison, A., Adhikari, J., Chowdhury, S., & Fouladkhah, A. (2019). Interactions of Carvacrol, Caprylic Acid, Habituation, and Mild Heat for Pressure-Based Inactivation of O157 and Non-O157 Serogroups of Shiga Toxin-Producing *Escherichia coli* in Acidic Environment. *Microorganisms*, *7*(5). https://doi.org/10.3390/microorganisms7050145

Kaletunç, G., Lee, J., Alpas, H., & Bozoglu, F. (2004). Evaluation of Structural Changes Induced by High Hydrostatic Pressure in *Leuconostoc mesenteroides*. *Applied and Environmental Microbiology*, *70*(2), 1116–1122. https://doi.org/10.1128/AEM.70.2.1116-1122.2004

Keng, S. E., Mat Easa, A., Munir, A., Muhamed, C., Ooi, C.-H., & Chew, T. T. (2017). Composition

and Physicochemical Properties of Fresh and Freeze-Concentrated Coconut (*Cocos nucifera*) Water. *Journal of Agrobiotechnology*, *8*(1), 13–24.

Koutchma, T. (2008). UV light for processing foods. In *Ozone: Science and Engineering* (Vol. 30, pp. 93–98). Taylor & Francis Group . https://doi.org/10.1080/01919510701816346

Kris-Etherton, P. M., & Fleming, J. A. (2015). Emerging Nutrition Science on Fatty Acids and Cardiovascular Disease: Nutritionists' Perspectives. *Advances in Nutrition*, *6*(3), 326S-337S. https://doi.org/10.3945/an.114.006981

Kwiatkowski, A., & Clemente, E. (2008). Quality of coconut water "in natura" belonging to Green Dwarf fruit variety in different stages of development, in plantation on the northwest area of Paraná, Brazil. *Journal of Food, Agriculture & Environment*, *6*(1), 102–105.

Lakshmanan, J., Zhang, B., Wright, K., Motameni, A. T., Jaganathan, V. L., Schultz, D. J., … Harbrecht, B. G. (2020). Tender coconut water suppresses hepatic inflammation by activating AKT and JNK signaling pathways in an in vitro model of sepsis. *Journal of Functional Foods*, *64*, 103637. https://doi.org/10.1016/j.jff.2019.103637

Lam, D., Montcoudiol, H., Herve, L., Falque, S. (2015a). Process for extracting coco water and corresponding system. Patent application, FR3042688A1

Lam, D., Montcoudiol, H., Herve, L., Falque, S. (2015b). Multilayer flexible material, packaging device made of same with the material, and method of using the device. Pattent application, FR3042733A1

Lamdande, A. G., Mittal, R., & K.S.M.S., R. (2020). Flux evaluation based on fouling mechanism in acoustic field-assisted ultrafiltration for cold sterilization of tender coconut water. *Innovative Food Science and Emerging Technologies*, *61*, 102312. https://doi.org/10.1016/j.ifset.2020.102312

Larson, W. P., Hartzell, T. B., & Harold, S. D. (1918). The Effect of High Pressures on Bacteria. *The Journal of Infectious Diseases1*, *22*(3), 271–279.

Lavinas, F. C., Miguel, M. A. L., Lopes, M. L. M., & Valente Mesquita, V. L. (2008). Effect of High Hydrostatic Pressure on Cashew Apple (*Anacardium occidentale* L.) Juice Preservation. *Journal of Food Science*, *73*(6), 273–277. https://doi.org/10.1111/j.1750-3841.2008.00791.x

Lindström, M., Kiviniemi, K., & Korkeala, H. (2006). Hazard and control of group II (non-proteolytic) *Clostridium botulinum* in modern food processing. *International Journal of Food Microbiology*, *108*(1), 92–104. https://doi.org/10.1016/j.ijfoodmicro.2005.11.003

Linton, M., McClements, J. M. J., & Patterson, M. F. (1999). Inactivation of *Escherichia coli* O157:H7 in orange juice using a combination of high pressure and mild heat. *Journal of Food Protection*, *62*(3), 277–279. https://doi.org/10.4315/0362-028X-62.3.277

Loki, A. L., & Rajamohan, T. (2013). Hepatoprotective and antioxidant effect of tender coconut

water on carbon tetrachloride induced liver injury in rats. *Indian Journal of Biochemistry & Biophysics*, *40*(5), 354–357.

Lukas, A. R. (2013). *Use of High Pressure Processing to Reduce Foodborne Pathogens in Coconut Water*. Masther thesis. University of Virginia.

Lyver, A., Smith, J. P., Austin, J., & Blanchfield, B. (1998). Competitive inhibition of *Clostridium botulinum* type E by *Bacillus species* in a value-added seafood product packaged under a modified atmosphere. *Food Research International*, *31*(4), 311–319. https://doi.org/10.1016/S0963-9969(98)00097-0

Ma, Y., Xu, L., Wang, S., Xu, Z., Liao, X., & Cheng, Y. (2019). Comparison of the quality attributes of coconut waters by high-pressure processing and high-temperature short time during the refrigerated storage. *Food Science & Nutrition*, *7*(4), 1512–1519. https://doi.org/10.1002/fsn3.997

Mahayothee, B., Koomyart, I., Khuwijitjaru, P., Siriwongwilaichat, P., Nagle, M., & Müller, J. (2016). Phenolic Compounds, Antioxidant Activity, and Medium Chain Fatty Acids Profiles of Coconut Water and Meat at Different Maturity Stages. *International Journal of Food Properties*, *19*(9), 2041–2051. https://doi.org/10.1080/10942912.2015.1099042

Mahnot, N. K., Gupta, K., & Mahanta, C. L. (2019). Shelf-life enhancement and associated quality and sensory changes on refrigerated storage of tender coconut water subjected to non-thermal microfiltration and treated with additives. *Journal of Food Science and Technology*, *56*(7), 3408–3421. https://doi.org/10.1007/s13197-019-03825-3

Mahnot, N. K., Mahanta, C. L., Keener, K. M., & Misra, N. N. (2019). Strategy to achieve a 5-log *Salmonella* inactivation in tender coconut water using high voltage atmospheric cold plasma (HVACP). *Food Chemistry*, *284*, 303–311. https://doi.org/10.1016/j.foodchem.2019.01.084

Majowicz, S. E., Musto, J., Scallan, E., Angulo, F. J., Kirk, M., O'Brien, S. J., … Hoekstra, R. M. (2010). The global burden of nontyphoidal salmonella gastroenteritis. *Clinical Infectious Diseases*. Clin Infect Dis. https://doi.org/10.1086/650733

Majowicz, S. E., Scallan, E., Jones-Bitton, A., Sargeant, J. M., Stapleton, J., Angulo, F. J., … Kirk, M. D. (2014). Global incidence of human shiga toxin-producing *Escherichia coli* infections and deaths: A systematic review and knowledge synthesis. *Foodborne Pathogens and Disease*. Mary Ann Liebert Inc. https://doi.org/10.1089/fpd.2013.1704

Manikantan, M. R., Pandiselvam, R., Beegum, S., & Mathew, A. C. (2018). Harvest and Postharvest technology. In K. U. K. Nampoothiri, V. Krishnakumar, M. Achuthan Nair, & P. K. Thampan (Eds.), *The Coconut Palm (Cocos nucifera L.) - Research and Development Perspectives* (pp. 635–722). Singapore: Springer.

Mantena, S. K., Jagadish, Badduri, S. R., Siripurapu, K. B., & Unnikrishnan, M. K. (2003). In vitro

evaluation of antioxidant properties of *Cocos nucifera* Linn. water. *Molecular Nutrition & Food Research*, *47*(2), 126–131. https://doi.org/10.1002/food.200390023

Martínez-Monteagudo, S. I., & Balasubramaniam, V. M. (2016). Fundamentals and Applications of High-Pressure Processing Technology. In *High Pressure Processing of Food: Principles, Technology and Applications* (pp. 3–17). New York: Springer. https://doi.org/10.1007/978-1-4939-3234-4_1

Martinez-Monteagudo, S. I., & Saldaña, M. D. A. (2014). Chemical Reactions in Food Systems at High Hydrostatic Pressure. *Food Engineering Reviews*, *6*(4), 105–127. https://doi.org/10.1007/s12393-014-9087-6

Masson, P. (1992). Pressure denaturation of proteins. In C. Balny, R. Hayashi, K. Heremans, & P. Masson (Eds.), *High Pressure and Biotechnology* (Vol. 224, pp. 89–99). Montrogue: John Libbey and Co., Ltd.

Matsui, K. N., Gut, J. A. W., de Oliveira, P. V., & Tadini, C. C. (2008). Inactivation kinetics of polyphenol oxidase and peroxidase in green coconut water by microwave processing. *Journal of Food Engineering*, *88*(2), 169–176. https://doi.org/10.1016/j.jfoodeng.2008.02.003

McCollum, J. T., Cronquist, A. B., Silk, B. J., Jackson, K. A., O'Connor, K. A., Cosgrove, S., … Mahon, B. E. (2013). Multistate Outbreak of Listeriosis Associated with Cantaloupe. *New England Journal of Medicine*, *369*(10), 944–953. https://doi.org/10.1056/NEJMoa1215837

Min, S., Sastry, S. K., & Balasubramaniam, V. M. (2010). Compressibility and density of select liquid and solid foods under pressures up to 700 MPa. *Journal of Food Engineering*, *96*(4), 568–574. https://doi.org/10.1016/j.jfoodeng.2009.09.003

Molina-Höppner, A., Doster, W., Vogel, R. F., & Gänzle, M. G. (2004). Protective Effect of Sucrose and Sodium Chloride for *Lactococcus lactis* during Sublethal and Lethal High-Pressure Treatments. *Applied and Environmental Microbiology*, *70*(4), 2013–2020. https://doi.org/10.1128/AEM.70.4.2013-2020.2004

Moussa-Ayoub, T. E., Jäger, H., Knorr, D., El-Samahy, S. K., Kroh, L. W., & Rohn, S. (2017). Impact of pulsed electric fields, high hydrostatic pressure, and thermal pasteurization on selected characteristics of *Opuntia dillenii* cactus juice. *LWT - Food Science and Technology*, *79*, 534–542. https://doi.org/10.1016/J.LWT.2016.10.061

Mozhaev, V. V., Heremans, K., Frank, J., Masson, P., & Balny, C. (1994). Exploiting the effects of high hydrostatic pressure in biotechnological applications. *Trends in Biotechnology*, *12*(12), 493–501. https://doi.org/10.1016/0167-7799(94)90057-4

Mújica-Paz, H., Valdez-Fragoso, A., Samson, C. T., Welti-Chanes, J., & Torres, J. A. (2011). High-Pressure Processing Technologies for the Pasteurization and Sterilization of Foods. *Food and Bioprocess Technology*, *4*(6), 969–985. https://doi.org/10.1007/s11947-011-0543-5

Murphy, D. J. (2007). Future prospects for oil palm in the 21st century: Biological and related challenges. *European Journal of Lipid Science and Technology*, *109*(4), 296–306. https://doi.org/10.1002/ejlt.200600229

NACMCF. (2010). Parameters for determining inoculated pack/challenge study protocols. *Journal of Food Protection*, *73*(1), 140–202. https://doi.org/10.4315/0362-028X-73.1.140

Naik, M., K, S. C., & Rawson, A. (2020). Tender Coconut Water: A Review on Recent Advances in Processing and Preservation. *Food Reviews International*, 1–22. https://doi.org/10.1080/87559129.2020.1785489

Nasiłowska, J., Sokołowska, B., & Fonberg-Broczek, M. (2018). Long-Term Storage of Vegetable Juices Treated by High Hydrostatic Pressure: Assurance of the Microbial Safety. *BioMed Research International*, *2018*. https://doi.org/10.1155/2018/7389381

Nasution, Z., Jirapakkul, W., & Lorjaroenphon, Y. (2019). Aroma compound profile of mature coconut water from tall variety through thermal treatment. *Journal of Food Measurement and Characterization*, *13*(1), 277–286. https://doi.org/10.1007/s11694-018-9942-x

Nayar, N. M. (2017). *The Coconut: Phylogeny, Origins and Spread*. Amsterdam: Elsevier.

Niral, V., & Jerard, B. A. (2018). Botany, Origin and Genetic Resources of Coconut. In K. U. K. Nampoothiri, V. Krishnakumar, M. Achuthan Nair, & P. K. Thampan (Eds.), *The Coconut Palm (Cocos nucifera L.) - Research and Development Perspectives* (pp. 57–112). Singapore: Springer.

Noma, S., Shimoda, M., & Hayakawa, I. (2002). Inactivation of Vegetative Bacteria by Rapid Decompression Treatment. *Journal of Food Science*, *67*(9), 3408–3411. https://doi.org/10.1111/j.1365-2621.2002.tb09598.x

Orsi, R. H., Bakker, H. C. de., & Wiedmann, M. (2011). *Listeria monocytogenes* lineages: Genomics, evolution, ecology, and phenotypic characteristics. *International Journal of Medical Microbiology*. Urban & Fischer. https://doi.org/10.1016/j.ijmm.2010.05.002

Paidhungat, M., Setlow, B., Daniels, W. B., Hoover, D., Papafragkou, E., & Setlow, P. (2002). Mechanisms of induction of germination of *Bacillus subtilis* spores by high pressure. *Applied and Environmental Microbiology*, *68*(6), 3172–3175. https://doi.org/10.1128/AEM.68.6.3172-3175.2002

Palaniappan, R. U. M., Zhang, Y., Chiu, D., Torres, A., DebRoy, C., Whittam, T. S., & Chang, Y. F. (2006). Differentiation of *Escherichia coli* pathotypes by oligonucleotide spotted array. *Journal of Clinical Microbiology*, *44*(4), 1495–1501. https://doi.org/10.1128/JCM.44.4.1495-1501.2006

Palou, E., López-Malo, A., Barbosa-Cánovas, G. V., Welti-Chanes, J., & Swanson, B. (1997). Effect of water activity on high hydrostatic pressure inhibition of *Zygosaccharomyces bailii*. *Letters*

in Applied Microbiology, *24*(5), 417–420.

Parish, M. E. (1998). High pressure inactivation of *Saccharomyces cerevisiae*, endogenous microflora and pectinmethylesterase in orange juice. *Journal of Food Safety*, *18*(1), 57–65. https://doi.org/10.1111/j.1745-4565.1998.tb00202.x

Patterson, M. F., McKay, A. M., Connolly, M., & Linton, M. (2012). The effect of high hydrostatic pressure on the microbiological quality and safety of carrot juice during refrigerated storage. *Food Microbiology*, *30*(1), 205–212. https://doi.org/10.1016/j.fm.2011.09.013

Patterson, M. F., Quinn, M., Simpson, R., & Gilmour, A. (1995). Sensitivity of vegetative pathogens to high hydrostatic pressure treatment in phosphate-buffered saline and foods. *Journal of Food Protection*, *58*(5), 524–529. https://doi.org/10.4315/0362-028X-58.5.524

Peck, M. W. (2009). Biology and Genomic Analysis of *Clostridium botulinum*. *Advances in Microbial Physiology*, *55*, 183–320. https://doi.org/10.1016/S0065-2911(09)05503-9

Peck, M. W., Stringer, S. C., & Carter, A. T. (2011). *Clostridium botulinum* in the post-genomic era. *Food Microbiology*, *28*(2), 183–191. https://doi.org/10.1016/j.fm.2010.03.005

Pendyala, B., Patras, A., Gopisetty, V. V. S., Sasges, M., & Balamurugan, S. (2019). Inactivation of *Bacillus* and *Clostridium* Spores in Coconut Water by Ultraviolet Light. *Foodborne Pathogens and Disease*, *16*(10), 704–711. https://doi.org/10.1089/fpd.2019.2623

Perera, L., Perera, S., Bandaranayake, C. K., & Harries, H. C. (2009). Coconut. In J. Vollmann & I. Rajcan (Eds.), *Handbook of Plant Breeding. Oil Crops* (pp. 369–396). New York: Springer.

Petrus, R., Churey, J., & Worobo, R. (2019). Searching for high pressure processing parameters for *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* reduction in Concord grape juice. *British Food Journal*, *122*(1), 170–180. https://doi.org/10.1108/BFJ-06-2019-0446

Petrus, R., Churey, J., & Worobo, R. (2020a). Challenging a range of high pressure processing parameters to inactivate pathogens in orange juice. *High Pressure Research*, *40*(4), 537–542. https://doi.org/10.1080/08957959.2020.1830081

Petrus, R., Churey, J., & Worobo, R. (2020b). High-pressure processing of apple juice: the most effective parameters to inactivate pathogens of reference. *British Food Journal*, *122*(12), 3969–3979. https://doi.org/10.1108/BFJ-03-2020-0178

Picart-Palmade, L., Cunault, C., Chevalier-Lucia, D., Belleville, M. P., & Marchesseau, S. (2019). Potentialities and limits of some non-thermal technologies to improve sustainability of food processing. *Frontiers in Nutrition*, *5*, 130. https://doi.org/10.3389/fnut.2018.00130

Picouet, P. A., Sárraga, C., Cofán, S., Belletti, N., & Dolors Guàrdia, M. (2015). Effects of thermal and high-pressure treatments on the carotene content, microbiological safety and sensory properties of acidified and of non-acidified carrot juice. *LWT - Food Science and Technology*,

62(1), 920–926. https://doi.org/10.1016/J.LWT.2014.07.027

Pinto, C. A., Moreira, S. A., Fidalgo, L. G., Inácio, R. S., Barba, F. J., & Saraiva, J. A. (2020). Effects of high-pressure processing on fungi spores: Factors affecting spore germination and inactivation and impact on ultrastructure. *Comprehensive Reviews in Food Science and Food Safety*, *19*(2), 553-573.

Plowman, J., & Peck, M. W. (2002). Use a novel method to characterize the response of spores of non-proteolytic *Clostridium botulinum* types B E and F to a wide range of germinants and conditions. *Journal of Applied Microbiology*, *92*, 681–694.

Podolak, R., Whitman, D., & Black, D. G. (2020). Factors Affecting Microbial Inactivation during High Pressure Processing in Juices and Beverages: A Review. *Journal of Food Protection*, *83*(9), 1561–1575. https://doi.org/10.4315/JFP-20-096

Polydera, A. C., Stoforos, N. G., & Taoukis, P. S. (2005). Quality degradation kinetics of pasteurised and high pressure processed fresh Navel orange juice: Nutritional parameters and shelf-life. *Innovative Food Science and Emerging Technologies*, *6*(1), 1–9. https://doi.org/10.1016/j.ifset.2004.10.004

Popham, D. L. (2002). Specialized peptidoglycan of the bacterial endospore: The inner wall of the lockbox. *Cellular and Molecular Life Sciences*. Springer. https://doi.org/10.1007/s00018-002-8435-5

Porto, E., Alves Filho, E. G., Silva, L. M. A., Fonteles, T. V., do Nascimento, R. B. R., Fernandes, F. A. N., … Rodrigues, S. (2020). Ozone and plasma processing effect on green coconut water. *Food Research International*, *131*, 109000. https://doi.org/10.1016/j.foodres.2020.109000

Prabhu, S., Dennison, S. R., Mura, M., Lea, R. W., Snape, T. J., & Harris, F. (2014). *Cn*-AMP2 from green coconut water is an anionic anticancer peptide. *Journal of Peptide Science*, *20*(12), 909–915. https://doi.org/10.1002/psc.2684

Prades, A., Assa, R. R. A., Dornier, M., Pain, J.-P., & Boulanger, R. (2012). Characterisation of the volatile profile of coconut water from five varieties using an optimised HS-SPME-GC analysis. *Journal of the Science of Food and Agriculture*, *92*(12), 2471–2478. https://doi.org/10.1002/jsfa.5655

Prades, A., Dornier, M., Diop, N., & Pain, J. P. (2012). Coconut water uses, composition and properties: A review. *Fruits*, *67*(2), 87–107. https://doi.org/10.1051/fruits/2012002

Preetha, P. P., Devi, V. G., & Rajamohan, T. (2015). Mature coconut water exhibits antidiabetic and antithrombotic potential via L-arginine-nitric oxide pathway in alloxan induced diabetic rats. *Journal of Basic and Clinical Physiology and Pharmacology*, *26*(6), 575–583. https://doi.org/10.1515/jbcpp-2014-0126

Preetha, P., Prasath Venugopal, A., Varadharaju, N., & Kennedy, Z. J. (2017). Inactivation of *Escherichia coli* in Tender Coconut (*Cocos Nucifera* L.) Water by Pulsed Light Treatment. *International Journal of Current Microbiology and Applied Sciences*, *6*(7), 1453–1461. https://doi.org/10.20546/ijcmas.2017.607.174

Quereda, J. J., Leclercq, A., Moura, A., Vales, G., Gómez-Martín, Á., García-Muñoz, Á., … Lecuit, M. (2020). *Listeria valentina* sp. nov., isolated from a water trough and the faeces of healthy sheep. *International Journal of Systematic and Evolutionary Microbiology*. https://doi.org/10.1099/ijsem.0.004494

Quimio, T. H. (1986). Coconut water as a growth medium for edible and plant pathogenic fungi. *Plant Pathology*, *67*(1), 1–8.

Raghubeer, E. V., Phan, B. N., Onuoha, E., Diggins, S., Aguilar, V., Swanson, S., & Lee, A. (2020). The use of High-Pressure Processing (HPP) to improve the safety and quality of raw coconut (*Cocos nucifera* L) water. *International Journal of Food Microbiology*, *331*, 108697. https://doi.org/10.1016/j.ijfoodmicro.2020.108697

Rajashri, K., Roopa, B. S., Negi, P. S., & Rastogi, N. K. (2020). Effect of ozone and ultrasound treatments on polyphenol content, browning enzyme activities, and shelf-life of tender coconut water. *Journal of Food Processing and Preservation*, *44*(3). https://doi.org/10.1111/jfpp.14363

Ramaroson, M., Guillou, S., Rossero, A., Rezé, S., Anthoine, V., Moriceau, N., … Zagorec, M. (2018). Selection procedure of bioprotective cultures for their combined use with High Pressure Processing to control spore-forming bacteria in cooked ham. *International Journal of Food Microbiology*, *276*, 28–38. https://doi.org/10.1016/j.ijfoodmicro.2018.04.010

Ramaswamy, H. S., Riahi, E., & Idziak, E. (2003). High-Pressure Destruction Kinetics of *E. coli* (29055) in Apple Juice. *Journal of Food Science*, *68*(5), 1750–1756. https://doi.org/10.1111/j.1365-2621.2003.tb12323.x

Rattanaburee, P., Amnuaikit, T., & Puripattanavong, J. (2017). Physicochemical Properties of Spray-Dried Young Coconut Juice. *International Journal of Pharma Medicine and Biological Sciences*, *6*(2), 43–47. https://doi.org/10.18178/ijpmbs.6.2.43-47

Reddy, K. V., Das, M., & Das, S. K. (2007). Nonthermal sterilization of green coconut water for packaging. *Journal of Food Quality*, *30*(4), 466–480. https://doi.org/10.1111/j.1745-4557.2007.00136.x

Reddy, N. R., Solomon, H. M., Fingerhut, G. A., Rhodehamel, E. J., Balasubramaniam, V. M., & Palaniappan, S. (1999). Inactivation of *Clostridium botulinum* type E spores by high pressure processing. *Journal of Food Safety*, *19*(4), 277–288. https://doi.org/10.1111/j.1745-4565.1999.tb00252.x

Reddy, K. V., Das, M., & Das, S. K. (2005). Filtration resistances in non-thermal sterilization of

green coconut water. *Journal of Food Engineering*, *69*(3), 381–385. https://doi.org/10.1016/j.jfoodeng.2004.08.029

Reineke, K., Mathys, A., Heinz, V., & Knorr, D. (2013). Mechanisms of endospore inactivation under high pressure. *Trends in Microbiology*. Elsevier Current Trends. https://doi.org/10.1016/j.tim.2013.03.001

Rethinam, P. (2018). International Scenario of Coconut Sector. In K. U. K. Nampoothiri, V. Krishnakumar, M. Achuthan Nair, & P. K. Thampan (Eds.), *The Coconut Palm (Cocos nucifera L.) - Research and Development Perspectives* (pp. 21–56). Singapore: Springer.

Ribeiro, M. de M., Valdramidis, V. P., Nunes, C. A., & de Souza, V. R. (2017). Synergistic effect of thermosonication to reduce enzymatic activity in coconut water. *Innovative Food Science and Emerging Technologies*, *41*, 404–410. https://doi.org/10.1016/j.ifset.2017.04.013

Rivera, R., Edwards, K. J., Barker, J., Arnold, G. M., Ayad, G., Hodgkin, T., & Karp, A. (1999). Isolation and characterization of polymorphic microsatellites in *Cocos nucifera* L. *Genome*, *42*(4), 668–675. https://doi.org/10.1139/g98-170

Robey, M., Benito, A., Hutson, R. H., Pascual, C., Park, S. F., & Mackey, B. M. (2001). Variation in Resistance to High Hydrostatic Pressure and *rpoS* Heterogeneity in Natural Isolates of *Escherichia coli* O157:H7. *Applied and Environmental Microbiology*, *67*(10), 4901–4907. https://doi.org/10.1128/AEM.67.10.4901-4907.2001

Rodriguez-Amaya, D. B. (2016). Natural Food Pigments and Colorants. *Current Opinion in Food Science*, *7*, 20–26. https://doi.org/10.1007/978-3-319-78030-6_12

Rodríguez-Roque, M. J., de Ancos, B., Sánchez-Moreno, C., Cano, M. P., Elez-Martínez, P., & Martín-Belloso, O. (2015). Impact of food matrix and processing on the in vitro bioaccessibility of vitamin C, phenolic compounds, and hydrophilic antioxidant activity from fruit juice-based beverages. *Journal of Functional Foods*, *14*, 33–43. https://doi.org/10.1016/j.jff.2015.01.020

Rodríguez-Roque, M. J., De Ancos, B., Sánchez-Vega, R., Sánchez-Moreno, C., Cano, M. P., Elez-Martínez, P., & Martín-Belloso, O. (2016). Food matrix and processing influence on carotenoid bioaccessibility and lipophilic antioxidant activity of fruit juice-based beverages. *Food and Function*, *7*(1), 380–389. https://doi.org/10.1039/c5fo01060h

Roger, H. (1892). Action des hautes pressions sur quelques bactéries. *Comptes Rendus de l'Académie Des Sciences*, *114*.

Roger, H. (1895). Action des hautes pressions sur quelques bactéries. *Arch Physiol Norm Path*, *7*, 12–17.

Rojas, M. L., Trevilin, J. H., Funcia, E. dos S., Gut, J. A. W., & Augusto, P. E. D. (2017). Using ultrasound technology for the inactivation and thermal sensitization of peroxidase in green coconut water. *Ultrasonics Sonochemistry*, *36*, 173–181.

https://doi.org/10.1016/j.ultsonch.2016.11.028

Salum, U., Foale, M., Biddle, J., Bazrafshan, A., & Adkins, S. (2020). Towards the sustainability of the “Tree of life”: An introduction. In *Coconut Biotechnology: Towards the Sustainability of the “Tree of Life”* (pp. 1–15). Springer International Publishing. https://doi.org/10.1007/978-3-030-44988-9_1

Sánchez-Moreno, C., Plaza, L., de Ancos, B., & Cano, M. P. (2003). Vitamin C, Provitamin A Carotenoids, and Other Carotenoids in High-Pressurized Orange Juice during Refrigerated Storage. *Journal of Agricultural and Food Chemistry*, *51*(3), 647–653. https://doi.org/10.1021/jf020795o

Sanganamoni, S., Mallesh, S., Vandana, K., & Rao, P. S. (2017). Thermal Treatment of Tender Coconut Water-Enzyme Inactivation and Biochemical Characterization. *International Journal of Current Microbiology and Applied Science*, *6*(5), 2919–2931. https://doi.org/10.20546/ijcmas.2017.605.331

Santos, J. LA, Bispo, V. S., Filho, A. B., Pinto, I. F., Dantas, L. S., Vasconcelos, D. F., … Matos, H. R. (2013). Evaluation of Chemical Constituents and Antioxidant Activity of Coconut Water (*Cocus nucifera* L.) and Caffeic Acid in Cell Culture. *Annals of the Brazilian Academy of Sciences*, *85*(4), 1235–1246. https://doi.org/10.1590/0001-37652013105312

Santoso, U., Kubo, K., Ota, T., Tadokoro, T., & Maekawa, A. (1996). Nutrient composition of *kopyor* coconuts (*Cocos nucifera* L.). *Food Chemistry*, *57*(2), 299–304. https://doi.org/10.1016/0308-8146(95)00237-5

Sarathchandra, S. U., Wolf, J., & Barker, A. N. (1977). Germination responses in three *Clostridium* species. In A. N. Barker, L. J. Wolf, D. J. Ellar, G. J. Dring, & G. W. Gould (Eds.), *Spore research* (pp. 721–734). Academic Press.

Serment-Moreno, V., Jacobo-Velázquez, D. A., Torres, J. A., & Welti-Chanes, J. (2017). Microstructural and Physiological Changes in Plant Cell Induced by Pressure: Their Role on the Availability and Pressure-Temperature Stability of Phytochemicals. *Food Engineering Reviews*, *9*(4), 314–334. https://doi.org/10.1007/s12393-017-9158-6

Setlow, P. (2006). Spores of *Bacillus subtilis*: Their resistance to and killing by radiation, heat and chemicals. In *Journal of Applied Microbiology* (Vol. 101, pp. 514–525). John Wiley & Sons, Ltd. https://doi.org/10.1111/j.1365-2672.2005.02736.x

Setlow, Peter, Wang, S., & Li, Y.-Q. (2017). Germination of Spores of the Orders *Bacillales* and *Clostridiales*. *Annual Review of Microbiology Annu. Rev. Microbiol*, *71*, 459–477. https://doi.org/10.1146/annurev-micro-090816

Shahbaz, H. M., Yoo, S., Seo, B., Ghafoor, K., Kim, J. U., Lee, D. U., & Park, J. (2016). Combination of TiO_2-UV Photocatalysis and High Hydrostatic Pressure to Inactivate Bacterial Pathogens

and Yeast in Commercial Apple Juice. *Food and Bioprocess Technology*, *9*(1), 182–190. https://doi.org/10.1007/s11947-015-1614-9

Sheth, A. N., Wiersma, P., Atrubin, D., Dubey, V., Zink, D., Skinner, G., … Sobel, J. (2008). International Outbreak of Severe Botulism with Prolonged Toxemia Caused by Commercial Carrot Juice. *Clinical Infectious Diseases*, *47*(10), 1245–1251. https://doi.org/10.1086/592574

Simpson, R. K., & Gilmour, A. (1997a). The effect of high hydrostatic pressure on *Listeria monocytogenes* in phosphate-buffered saline and model food systems. *Journal of Applied Microbiology*, *83*(2), 181–188. https://doi.org/10.1046/j.1365-2672.1997.00215.x

Simpson, R. K., & Gilmour, A. (1997b). The effect of high hydrostatic pressure on the activity of intracellular enzymes of *Listeria monocytogenes*. *Letters in Applied Microbiology*, *25*(1), 48–53. https://doi.org/10.1046/j.1472-765X.1997.00168.x

Slifko, T. R., Raghubeer, E., & Rose, J. B. (2000). Effect of high hydrostatic pressure on *Cryptosporidium parvum* infectivity. *Journal of Food Protection*, *63*(9), 1262–1267. https://doi.org/10.4315/0362-028X-63.9.1262

Smelt, J. P. P. . (1998). Recent advances in the microbiology of high pressure processing. *Trends in Food Science & Technology*, *9*(4), 152–158. https://doi.org/10.1016/S0924-2244(98)00030-2

Smith, M. E., & Bull, A. T. (1976). Studies of the Utilization of Coconut Water Waste for the Production of the Food Yeast *Saccharomyces fragilis*. *Journal of Applied Bacteriology*, *41*(1), 81–95. https://doi.org/10.1111/j.1365-2672.1976.tb00608.x

Storey, A. A., Ramírez, J. M., Quiroz, D., Burley, D. V., Addison, D. J., Walter, R., … Matisoo-Smith, E. A. (2007). Radiocarbon and DNA evidence for a pre-Columbian introduction of Polynesian chickens to Chile. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(25), 10335–10339. https://doi.org/10.1073/pnas.0703993104

Strawn, L. K., Schneider, K. R., & Danyluk, M. D. (2011). Microbial Safety of Tropical Fruits. *Critical Reviews in Food Science and Nutrition*, *51*(2), 132–145. https://doi.org/10.1080/10408390903502864

Syed, Q. A., Buffa, M., Guamis, B., & Saldo, J. (2013). Lethality and injuring the effect of compression and decompression rates of high hydrostatic pressure on *Escherichia coli* O157:H7 in different matrices. *High Pressure Research*, *33*(1), 64–72. https://doi.org/10.1080/08957959.2013.767898

Symbiosis. (2020). Truly Raw Thai Coconut Water. Retrieved October 26, 2020, from https://cocosymbiosis.com/

Tan, T. C., Cheng, L. H., Bhat, R., Rusul, G., & Easa, A. M. (2014). Composition, physicochemical properties and thermal inactivation kinetics of polyphenol oxidase and peroxidase from

coconut (*Cocos nucifera*) water obtained from immature, mature and overly-mature coconut. *Food Chemistry*, *142*, 121–128. https://doi.org/10.1016/j.foodchem.2013.07.040

Tanqueco, R. E., Rodriguez, F. M., Laude, R. P., & Cueno, M. E. (2007). Total Free Sugars , Oil and Total Phenolics Content of Stored Coconut (*Cocos nucifera* L .) Water. *Philippine Journal of Science*, *136*(2), 103–108.

Teng, M., Zhao, Y. J., Khoo, A. L., Yeo, T. C., Yong, Q. W., & Lim, B. P. (2020). Impact of coconut oil consumption on cardiovascular health: A systematic review and meta-Analysis. *Nutrition Reviews*, *78*(3), 249–259. https://doi.org/10.1093/nutrit/nuz074

Teo, A. Y., Ravishankar, S., & Sizer, C. E. (2001). Effect of low-temperature, high-pressure treatment on the survival of *Escherichia coli* O157:H7 and *Salmonella* in unpasteurized fruit juices. *Journal of Food Protection*, *64*(8), 1122–1127.

Tholozan, J. L., Ritz, M., Jugiau, F., Federighi, M., & Tissier, J. P. (2000). Physiological effects of high hydrostatic pressure treatments on *Listeria monocytogenes* and *Salmonella* typhimurium. *Journal of Applied Microbiology*, *88*(2), 202–212. https://doi.org/10.1046/j.1365-2672.2000.00917.x

Todeschini, G., Friso, S., Lombardi, S., Casaril, M., Fontana, R., & Corrocher, R. (1998). A case of listeria murray/grayi bacteremia in a patient with advanced Hodgkin's disease. *European Journal of Clinical Microbiology and Infectious Diseases*, *17*(11), 808–810. https://doi.org/10.1007/s100960050193

Tonello-Samson, C., Queirós, R. P., & González-Angulo, M. (2020). Advances in high-pressure processing in-pack and in-bulk commercial equipment. In *Present and Future of High Pressure Processing* (pp. 297–316). Elsevier. https://doi.org/10.1016/b978-0-12-816405-1.00013-3

Tonello, C. (2011). Case Studies on High-Pressure Processing of Foods. In *Nonthermal Processing Technologies for Food* (pp. 36–50). Oxford, UK: Wiley-Blackwell. https://doi.org/10.1002/9780470958360.ch4

Torres, J. A., & Velazquez, G. (2005). Commercial opportunities and research challenges in the high pressure processing of foods. *Journal of Food Engineering*, *67*(1–2), 95–112. https://doi.org/10.1016/j.jfoodeng.2004.05.066

U.S. Food and Drug Administration. Department of Health and Human Services. (2007). Guidance for Industry: Refrigerated Carrot Juice and Other Refrigerated Low-Acid Juices. Retrieved May 17, 2020, from https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-refrigerated-carrot-juice-and-other-refrigerated-low-acid-juices

U.S. Food and Drug Administration. Public Health Service. (2015a). Warning Letter. Harmless Harvest Thailand. Retrieved May 17, 2020, from https://www.fda.gov/inspections-compliance-

enforcement-and-criminal-investigations/warning-letters/harmless-harvest-thailand-11132015

U.S. Food and Drug Administration. Public Health Service. (2015b). Warning Letter. Vital Juice Company, Inc. Retrieved May 17, 2020, from https://www.fda.gov/inspections-compliance-enforcement-and-criminal-investigations/warning-letters/vital-juice-company-inc-05042015

U.S. Food and Drug Administration. (2001). Hazard analysis and critical control point (HACCP); procedures for the safe and sanitary processing and importing of juice; final rule, 21 CFR Part 120. *Federal Register*, *66*, 6137–6202.

Van Boeijen, I. K. H., Moezelaar, R. O. Y., Abee, T., & Zwietering, M. H. (2008). Inactivation kinetics of three *Listeria monocytogenes* strains under high hydrostatic pressure. *Journal of Food Protection*, *71*(10), 2007–2013. https://doi.org/10.4315/0362-028X-71.10.2007

van Leeuwen, W. D. (1933). Germinating coconuts on a new volcanic island, Krakatoa. *Nature*. Nature Publishing Group. https://doi.org/10.1038/132674b0

Vermeulen, K., Strnad, M., Kryštof, V., Havlí;ček, L., Van der Aa, A., Lenjou, M., … Berneman, Z. N. (2002). Antiproliferative effect of plant cytokinin analogues with an inhibitory activity on cyclin-dependent kinases. *Leukemia*, *16*(3), 299–305. https://doi.org/10.1038/sj.leu.2402378

Vieira, F. N., Lourenço, S., Fidalgo, L. G., Santos, S. A. O., Silvestre, A. J. D., Jerónimo, E., & Saraiva, J. A. (2018). Long-Term Effect on Bioactive Components and Antioxidant Activity of Thermal and High-Pressure Pasteurization of Orange Juice. *Molecules*, *23*(10), 2076–2091. https://doi.org/10.3390/MOLECULES23102706

Wai Lin, S. (2011). Palm Oil. In F. D. Gunstone (Ed.), *Vegetable Oils in Food Technology: Composition, Properties and Uses*. Wiley-Blackwell.

Walter, E. H. M., Kabuki, D. Y., Esper, L. M. R., Sant'Ana, A. S., & Kuaye, A. Y. (2009). Modelling the growth of *Listeria monocytogenes* in fresh green coconut (*Cocos nucifera* L.) water. *Food Microbiology*, *26*(6), 653–657. https://doi.org/10.1016/j.fm.2009.04.003

Wang, F., Du, B. L., Cui, Z. W., Xu, L. P., & Li, C. Y. (2017). Effects of high hydrostatic pressure and thermal processing on bioactive compounds, antioxidant activity, and volatile profile of mulberry juice. *Food Science and Technology International*, *23*(2), 119–127. https://doi.org/10.1177/1082013216659610

Ward, T. J., Gorski, L., Borucki, M. K., Mandrell, R. E., Hutchins, J., & Pupedis, K. (2004). Intraspecific phylogeny and lineage group identification based on the *prfA* virulence gene cluster of *Listeria monocytogenes*. *Journal of Bacteriology*, *186*(15), 4994–5002. https://doi.org/10.1128/JB.186.15.4994-5002.2004

Whitney, B. M., Williams, R. C., Eifert, J., & Marcy, J. (2007). High-Pressure Resistance Variation of *Escherichia coli* O157:H7 Strains and *Salmonella* Serovars in Tryptic Soy Broth, Distilled

Water, and Fruit Juice. *Journal of Food Protection*, *70*(9), 2078–2083. https://doi.org/10.4315/0362-028X-70.9.2078

Woodroof, J. G. (1970). Coconut storage and processing, and minor uses of products of the coconut tree. In J. G. Woodroof (Ed.), *Coconuts: Production, Processing, Products*. Westport.

Wuytack, E. Y., Boven, S., & Michiels, C. W. (1998). Comparative study of pressure-induced germination of *Bacillus subtilis* spores at low and high pressures. *Applied and Environmental Microbiology*, *64*(9), 3220–3224. https://doi.org/10.1128/aem.64.9.3220-3224.1998

Yi, J., Kebede, B. T., Hai Dang, D. N., Buvé, C., Grauwet, T., Van Loey, A., … Hendrickx, M. (2017). Quality change during high pressure processing and thermal processing of cloudy apple juice. *LWT - Food Science and Technology*, *75*, 85–92. https://doi.org/10.1016/j.lwt.2016.08.041

Yildiz, S., Pokhrel, P. R., Unluturk, S., & Barbosa-Cánovas, G. V. (2019). Identification of equivalent processing conditions for pasteurization of strawberry juice by high pressure, ultrasound, and pulsed electric fields processing. *Innovative Food Science & Emerging Technologies*, *57*, 102195. https://doi.org/10.1016/J.IFSET.2019.102195

Yong, J. W. H., Ge, L., Ng, Y. F., & Tan, S. N. (2009). The chemical composition and biological properties of coconut (*Cocos Nucifera* L.) water. *Molecules*, *14*(12), 5144–5164. https://doi.org/10.3390/molecules14125144

Yoo, S., Ghafoor, K., Kim, J. U., Kim, S., Jung, B., Lee, D. U., & Park, J. (2015). Inactivation of *Escherichia coli* O157:H7 on orange fruit surfaces and in juice using photocatalysis and high hydrostatic pressure. *Journal of Food Protection*, *78*(6), 1098–1105. https://doi.org/10.4315/0362-028X.JFP-14-522

Zhang, Y., & Mathys, A. (2019). Superdormant spores as a hurdle for gentle germination-inactivation based spore control strategies. *Frontiers in Microbiology*, *10*, 3163. https://doi.org/10.3389/fmicb.2018.03163

Zhao, L., Wang, S., Liu, F., Dong, P., Huang, W., Xiong, L., & Liao, X. (2013). Comparing the effects of high hydrostatic pressure and thermal pasteurization combined with nisin on the quality of cucumber juice drinks. *Innovative Food Science & Emerging Technologies*, *17*, 27–36. https://doi.org/10.1016/J.IFSET.2012.10.004

Zhao, Liang, Wang, Y., Hu, X., Sun, Z., & Liao, X. (2016). Korla pear juice treated by ultrafiltration followed by high pressure processing or high temperature short time. *LWT - Food Science and Technology*, *65*, 283–289. https://doi.org/10.1016/J.LWT.2015.08.011

Zhao, Y., Wang, P., Zhan, P., Tian, H., Lu, C., & Tian, P. (2020). Aroma Characteristics of Cloudy Kiwifruit Juices Treated with High Hydrostatic Pressure and Representative Thermal Processes. *Food Research International*, 109841. https://doi.org/10.1016/j.foodres.2020.109841

Zhong, S., Vendrell-Pacheco, M., Heskitt, B., Chitchumroonchokchai, C., Failla, M., Sastry, S. K., … Kopec, R. E. (2019). Novel Processing Technologies as Compared to Thermal Treatment on the Bioaccessibility and *Caco*-2 Cell Uptake of Carotenoids from Tomato and Kale-Based Juices. *Journal of Agricultural and Food Chemistry*, *67*(36), 10185–10194. https://doi.org/10.1021/acs.jafc.9b03666

Zonis, S. (2011). Harmless Harvest - 100 % Organic & Raw Coconut Water. Retrieved November 4, 2020, from http://www.goodlifer.com/2011/08/harmless-harvest-100-organic-raw-coconut-water/comment-page-1/

Chapter 2

Evaluation of factors influencing the growth of non-toxigenic *Clostridium botulinum* type E and *Clostridium* spp. in high-pressure processed and conditioned tender coconut water from Thailand

CHAPTER 2

EVALUATION OF FACTORS INFLUENCING THE GROWTH OF NON-TOXIGENIC *CLOSTRIDIUM BOTULINUM* TYPE E AND *CLOSTRIDIUM* SPP. IN HIGH-PRESSURE PROCESSED AND CONDITIONED TENDER COCONUT WATER FROM THAILAND

Bacterial spores survive high pressure processing (HPP). Group II *Clostridium botulinum* is an obligate anaerobe spore-forming pathogen that can produce the botulinum neurotoxin under refrigeration (>3.3 °C). This study assessed nontoxigenic type E *C. botulinum* and *Clostridium* spp. closely related to group II *C. botulinum* growth in raw and HPP (550 MPa for 3 min at 10 °C) Thai coconut water (pH 5.2). No spore germination or growth occurred in HPP coconut water inoculated with 10^5 spores/mL after 61 days regardless of oxygen concentration (<0.5-11 mg/L) at 4 and 10 ºC, but germination was observed at 20 °C. Total *C. botulinum* and *Clostridium* spp. counts decreased by 3.0 $\log_{10}$ CFU/mL in a worst-case scenario consisting of non-HPP filter-sterilized coconut water (pH 7.0) under anoxic incubation at 30 ºC for 61 days, suggesting spore germination followed by cellular death. Supplementing filter-sterilized coconut water (pH 7.0) with selected germinants and free amino acids did not support spore growth, but the addition of nutrient-rich laboratory media (TPGY broth) at low concentrations (6.25 %) promoted outgrowth, suggesting that a lack of nutrients prevented *C. botulinum* and *Clostridium* spp. growth in tender Thai coconut water. Further risk assessment will require the evaluation of other coconut water varieties and toxin production.

1. Introduction

Coconut water is a traditional tropical beverage gaining popularity in western society, as it enjoys a healthy image associated with its natural hydrating qualities, health properties such as antioxidant, cardioprotective, anticancer, antidiabetic and hepatoprotective effects, and it represents a source of functional compounds (Da Fonseca et al., 2009; DebMandal & Mandal, 2011; Mantena, Jagadish, Badduri, Siripurapu, & Unnikrishnan, 2003). Nonetheless, conventional heat preservation methods traditionally applied for the safe commercialization of the beverage adversely affect its sensory and nutritional qualities (Awua, Doe, & Agyare, 2011). The implementation of high pressure processing (HPP) at industrial scale since the 1990's allowed the commercialization of minimally processed juices and beverages with improved organoleptic and nutritional properties when compared to their heat pasteurized homologues (Chen et al., 2013; Liu, Zhao, Zou, & Hu, 2013; Picouet, Sárraga, Cofán, Belletti, & Dolors Guàrdia, 2015). Food safety is guaranteed by the inactivation of foodborne pathogens (Jordan, Pascual, Bracey, & Mackey, 2001; Whitney, Williams, Eifert, & Marcy, 2007). For instance, processing coconut water (pH 5.50 to 6.10) at 600 MPa for 2 min at 4 °C yielded an inactivation greater than 5 $\log_{10}$ CFU/mL of *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella* Typhimurium (Lukas, 2013). This pressure/time/temperature combination is commonly used by food manufacturers at industrial level to process a wide range of products and results in a cost-effective intervention for HPP businesses (Jung & Tonello-Samson, 2018). Nevertheless, these industrial parameters cannot inactivate bacterial spores, which remain viable after processing and have the potential to grow during cold storage if conditions are favorable (Linton, Connolly, Houston, & Patterson, 2014).

Pure (not blended) HPP coconut water is commercialized worldwide, with Europe and Asia leading the market. Typically, the beverage has a retail shelf-life of 40 to 60 days under chilled conditions. Distribution and commercialization of HPP products is always done under refrigeration to delay the growth of pressure-resistant spoilage microorganisms that might survive the process (Jung & Tonello-Samson, 2018). In the United States, imports of coconuts increased a 165 % between 2007 and 2017 for a value of more than $47 million (FAOSTAT, 2017). Nonetheless, sales of HPP coconut water are currently restricted in this country due to warning letters issued by the Food and Drug Administration (FDA) to various producers alleging the noncompliance of the title 21 part 120.24 of the US Code of Federal Regulations (U.S. Food and Drug Administration. Public Health Service, 2015a, 2015b). According to this regulation, processing technologies must guarantee, at a minimum, a 5-$\log_{10}$ reduction of the "pertinent microorganism" (*i.e.* the most resistant microorganism of public health significance that is likely to be present in the food). FDA considers the spore-former *C. botulinum* the

pathogen of concern in pasteurized and HPP low-acid beverages (pH >4.6) distributed under refrigeration, such as tender coconut water (U.S. Food and Drug Administration, 2001). Depending on the cultivar, coconut water has a pH in the range of 4.8 to 5.4 when the fruit is harvested after 6 to 8 months of maturation in palm trees (Chidambaram, Singaraja, Prasanna, Ganesan, & Sundararajan, 2013; Jackson, Gordon, Wizzard, McCook, & Rolle, 2004). Non-proteolytic *C. botulinum* (group II) represents a major concern in refrigerated ready-to-drink low-acid beverages. This organism is psychrotrophic and its spores can germinate, grow and produce toxin even under chilled storage (3.3 ºC) and pH 5.0 or above, whereas proteolytic *C. botulinum* (group I) can only grow under temperature abuse (>10 ºC) and pH >4.6 (Graham, Mason, Maxwell, & Peck, 1997; Graham, Mason, & Peck, 1996; Lindström, Kiviniemi, & Korkeala, 2006).

Few botulism outbreaks linked to the consumption of heat pasteurized low-acid juices have been reported (Jackson-Davis et al., 2018), but none associated to HPP treated beverages, including coconut water. In 2006, six people were intoxicated by the intake of commercial heat-pasteurized carrot juice contaminated with botulinum neurotoxin type A (Sheth et al., 2008). Additionally, different laboratory studies also showed the potential of botulinum toxin production under refrigeration and temperature abuse in various heat pasteurized vegetable purées and juices inoculated with non-proteolytic spores of the bacterium (Carlin & Peck, 1996; Stringer, Haque, & Peck, 1999). Traditional heat pasteurization processes reduce competitive microbiota and deplete dissolved oxygen concentration, which may facilitate *C. botulinum* spore outgrowth and toxin release. Although spores are not inactivated in HPP coconut water, their germination and growth potential depends on multiple interrelated intrinsic and extrinsic factors. Dissolved oxygen (DO_2) plays a major role in the growth dynamics of the bacterium, which is an obligate anaerobe. The addition of oxygen to the final product has been proven a valid complementary strategy to control *C. botulinum* in some situations (Linton et al., 2014). Background microbiota could have an impact on spore outgrowth and toxin production as well, causing nutrient depletion or directly inhibiting growth by the modification of physicochemical properties of the media (pH decrease) or the production of substances with antimicrobial activity which would inhibit *C. botulinum* growth (Lyver, Smith, Austin, & Blanchfield, 1998; Rodgers, Kailasapathy, Cox, & Peiris, 2004; Skinner, Solomon, & Fingerhut, 1999). However, growth of other microorganisms (excluding lactic acid bacteria) could decrease oxygen concentration, increase pH or make nutrients more accessible, which potentially facilitates spore outgrowth (Hotchkiss et al., 2016; Kasai et al., 2016; Odlaug & Pflug, 1979).

Despite lack of scientific and epidemiology evidence, no other parameters apart from coconut water acidity were considered to assess botulism risk in the warning letters issued by FDA to HPP coconut water producers. In light of concerns raised about the pathogen in low-acid HPP beverages, this preliminary study aims to give a better understanding on the behavior of non-proteolytic *C. botulinum* type E spores (NCTC 8266 and NCTC 11219) and *Clostridium* spp. spores closely related to type E *C. botulinum* (DSM 1985) in raw HPP tender coconut water, taking into account different factors that could determine spore germination and outgrowth in the beverage.

2. Materials and methods

2.1. Bacterial strains, sporulation and purification of spore crops

Non-toxigenic mutants of *C. botulinum* type E NCTC 11219 and NCTC 8266 constructed by Clauwers *et al.* (Clauwers, Vanoirbeek, Delbrassinne, & Michiels, 2016), and strain DSM 1985 (obtained from the German Collection of Microorganisms and Cell Cultures) were combined in a non-toxigenic three-strain cocktail referred hereafter as *C. botulinum*. NCTC strains were genetically modified by deletion of the botulinum toxin gene (*bont*/E), whereas *Clostridium* spp. DSM 1985 is a natural nontoxigenic strain genetically related to type E *C. botulinum* based on 16S rDNA sequence and was previously used as surrogate in food challenge tests (Di Gioia et al., 2016).

Spore stock suspensions were prepared using a two-phase sporulation medium as described in (Peck, Fairbairn, & Lund, 1992) with minor adjustments. Single colonies of each strain were grown in reinforced clostridial medium at 30 °C for 24 h (RCM [Merck, Germany]; 37 g/L RCM with 15 g/L agar). Grown single colonies of the strains were inoculated in 10 ml Trypticase peptone glucose yeast extract broth (TPGY; 50 g/L Trypticase [Becton Dickinson, Belgium], 5 g/L bacteriological peptone [Oxoid, England], 20 g/L yeast extract [Oxoid, England], 4 g/L glucose [Merck, Germany], 1 g/L sodium thioglycolate [Merck, Germany]) and incubated at 30 °C. After 24 h, the culture was added to a two-phase medium consisting of 40 ml distilled deoxygenated water over solid sporulation medium (30 g cooked meat medium, CMM [Oxoid, England], 0.3 g glucose, 4.5 g agar in 300 ml water). Spores were harvested from the liquid phase after 6 days of incubation at 30 ºC in a Whitley DG250 anaerobic workstation (flushed with 80 % N_2, 10 % CO_2 and 10 % H_2) by centrifugation (3,400 × g, 4 ºC, 15 min). The resulting pellet was washed four times with 0.85 % sterile NaCl solution by centrifugation, concentrated 5-fold, and stored at 4 ºC outside the anaerobic workstation. Purity of the spore stocks was determined as the difference between unheated and heated (65 °C, 10 min) aliquots, yielding more than 99 % for the three strains used.

Quality of the spore crops was periodically assessed by means of phase-contrast microscopy, where mature phase-bright cells corresponded to dormant spores. Sporulation yield was determined as the spore count difference between untreated and heated (65 ºC, 10 min) spore suspensions. Final concentration of spores obtained for strains NCTC 11219, NCTC 8266 and DSM 1985 was around 10^8 CFU/mL. The three-strain cocktail was prepared by decimally diluting aliquots of each spore stock in peptone water to obtain a concentration of 10^7 CFU/mL for each strain. Diluted aliquots were mixed to create the final cocktail.

2.2. Coconut water preparation and spore inoculation

Unprocessed tender (6 to 8 months ripened before harvesting) Thai coconut (*Cocos nucifera*) water (pH of 5.2) provided by a manufacturer in the United States was frozen (-18 ºC) in sterile containers and shipped to Spain, and from there to Belgium. A certified laboratory (Agrolab Ibérica, Spain) analyzed the nutritional composition of coconut water and the report can be found in Table A1 of the Appendix to Chapter 1. In order to evaluate the effect of multiple intrinsic factors on *C. botulinum* growth, the dissolved oxygen concentration, pH, background microbiota and nutrient profile of coconut water were modified for various experiments as summarized in Table 10 and described over the next lines.

Table 10. Experimental study design with natural and conditioned coconut water.

Code	DO_2 (mg/L)	pH	T (°C)	HPP[a]
LO_2	<0.5	5.2	4, 10, 20	Yes
UO_2	~ 7.0	5.2	4, 10, 20	Yes
HO_2	~ 11.0	5.2	4, 10, 20	Yes
FS[b]	<0.5	7.0	30	No
FS+G[c]	<0.5	7.0	30	No
FS+AA[d]	<0.5	7.0	30	No
FS+TPGY[e]	<0.5	7.0	30	No

[a] HPP conditions used were 550 MPa for 3 min at 10 °C.
[b] Filter-sterilized coconut water.
[c] Germinant mixture.
[d] Amino acid mixture (from 2 % casein hydrolysate + 0.1 g/L tryptophan).
[e] TPGY broth.

After thawing coconut water overnight at 4 ºC, a volume of 29.7 mL was aseptically bottled in sterile polyethylene terephthalate (PET) 33 mL bottles, leaving a 9.1 % headspace. Samples were inoculated with 300 µL of the three-strain cocktail to obtain a final spore concentration of 10^5 CFU/mL.

2.3. High-pressure processed coconut water conditioning

HPP conditions applied were 550 MPa for 3 min with an initial temperature of pressurizing fluid of 10 °C. These conditions are typically used in the HPP juice industry to achieve a 5-$\log_{10}$ reduction of pertinent vegetative pathogens, such as *L. monocytogenes*, *E. coli* O157:H7 or *Salmonella* spp. (Jung & Tonello-Samson, 2018) and comply with FDA's requirements (U.S. Food and Drug Administration, 2001). HPP cycles were performed with a HPIU-10000, 95/1994 unit (Resato, Roden, The Netherlands) using ethylene glycol as the hydrostatic medium. The HPP unit consists of a vertically oriented vessel (2.5 L, 10 cm inner diameter), in which pressure build-up rate was 30 MPa/s and decompression was instantaneous. Adiabatic heat increase of ethylene glycol at an initial temperature of 10 °C is estimated in 4 °C/100 MPa (Buzrul, Alpas, Largeteau, Bozoglu, & Demazeau, 2008), so maximum temperature reached during pressure holding time was around 32 °C.

2.3.1. Dissolved oxygen (DO_2) adjustment

Dissolved oxygen concentration was adjusted immediately after HPP to assess any impact of initial dissolved gas composition on spore outgrowth. A hypodermic needle (0.7 x 30 mm) pierced through the bottle lid was used to inject sterile gas mixtures (Air Liquide, Germany) at 10 L/h flow rate for 6 min. A second needle allowed gas purging during insufflation. All lids had an attached septum to prevent gas exchange with the surroundings (Dansensor, Spain). Oxygen and nitrogen gas cylinders were connected to rotameters and a gas mixing panel using tubing of the same diameter inner diameter for the bifurcations. Rotameters were used to adjust the inlet flow of each gas to deliver the mixture with the desired gas proportion at 10 L/h. Natural, unaltered coconut water samples (UO_2) contained a 7.0 mg/L oxygen concentration. The injection of 100 % N_2 yielded the lowest oxygen concentration used in this study (LO_2; <0.5 mg/L), whereas a 35 % O_2 + 65 % N_2 mixture yielded the highest (HO_2; 11.0 mg/L). Non-invasive O_2 measurements were performed by attaching a gas permeable hydrophobic polymer dot with a metal organic fluorescent dye (O2xyDOT®, OxySense, United States) to the inner part of the bottles, and measuring with an optical oxygen analyzer (OxySense 5250i, OxySense, United States). Samples of natural coconut water were incubated at 4, 10 and 20 °C.

2.3.2. Molecular methods for identification of microbial species in coconut water

16S rDNA PCR was conducted to identify bacteria isolated from coconut water during storage. DNA was extracted from colonies grown in PCA plates using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Germany). Two colonies were isolated for each DO_2 and incubation temperature level displaying growth in PCA plates from HPP samples of

coconut water. Colonies were picked from the lower dilution plated to take into account greater diversity. rDNA fragments were amplified with B27F forward primer (5'-AGAGTTTG ATCMTGGCTCAG-3') and U1492R reverse primer (5'- GGTTACCTTGTTACGACTT-3') with the Phusion High-Fidelity polymerase (Thermo Fisher Scientific, Germany) following manufacturer specifications. The PCR program consisted of an initial denaturation of 3 min at 95 °C followed by 35 amplification cycles of 30 s at 95 °C, 30 s at 50 °C and 1 min at 72 °C, and a final extension of 1 min at 72 °C. PCR products obtained were purified with the GeneJET PCR Purification Kit (Thermo Fisher Scientific, Germany) and sequenced at Macrogen Europe (Amsterdam, The Netherlands). Sequences obtained were blasted against the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and species with similarity scores greater than 99 % were considered as identical (Janda & Abbott, 2007).

2.4. Filter-sterilized (FS) coconut water conditioning

To remove pressure-resistant microorganisms that could compete with *C. botulinum* and assess their impact on spore development, fresh coconut water was sterilized by centrifugation (3,400 × g, 4 ºC, 15 min) and subsequent microfiltration (pore size, 0.22 µm). In order to provide optimum conditions for *C. botulinum* germination and simulate a worst-case scenario from a food safety perspective, FS coconut water samples were adjusted to strict anoxic conditions (LO_2; section 2.3.1), and adjusted to pH 7.0 with sterile 1 N NaOH (Ando & Lida, 1970; Strasdine, 1967).

2.4.1. Supplementation with germinants, amino acids and TPGY broth

FS coconut water was supplemented with germinants (FS+G), amino acids (FS+AA), and TPGY broth (FS+TPGY) to evaluate the impact of their addition on spore growth dynamics. The effect of L-alanine, L-lactate sodium salt and $NaHCO_3$, which is a well-established germinant mixture for type E *C. botulinum* (Ando, 1971; Plowman & Peck, 2002), was evaluated. Germinant agent solutions (Sigma Aldrich, Germany) were prepared in 0.1 M Tris-HCl (pH 7.40) to target a 10-fold concentration. Germinant solutions were decimally diluted in coconut water to achieve final concentrations of L-alanine, L-lactate sodium salt and $NaHCO_3$ of 100 mM, 50 mM and 50 mM, respectively. Supplementation with free amino acids was done by the addition of filter sterilized 2 % acid hydrolyzed casein (Lab M, Belgium) and 0.1 g/L tryptophan (Sigma Aldrich, Germany) to coconut water. TPGY supplementation was carried out by serially diluting (1:1) coconut water with TPGY broth.

2.5. Microbial growth assessment during storage

C. botulinum counts were determined by plate counting on RCM supplemented with 100 µg/mL cycloserine (RCM_{CY}) to suppress background microbiota growth in the agar plates (24 h incubation, 30 ºC, anaerobic workstation). This concentration of cycloserine effectively inhibited the growth or spoilage microorganisms in agar plates but did not have an effect on the three-spore cocktail counts (Figure A1 and Figure A2 of the Appendix to Chapter 1). During storage, 500 µL aliquots were periodically extracted with sterile syringes through the bottle lid septum to enumerate *C. botulinum* total counts on RCM_{CY}. In the last testing point after 61 days of incubation, germination yield and growth were determined as the *C. botulinum* count difference between unheated aliquots (spores + germinated spores and/or vegetative cells) and heated aliquots (65 ºC, 10 min), which only accounts for spores. Total aerobic counts were determined in plate count agar (PCA; 17.5 g/L [Oxoid]) by incubating plates at 30 ºC outside the anaerobic workstation. Additionally, pH was routinely assessed.

2.6. Statistical analysis

Three independent replicate experiments were carried out for each scenario using spores from the same batch. One-way ANOVA was used to statistically assess the extent of germination or growth of *C. botulinum* spores in coconut water supplemented with germinants, casein hydrolysate + 0.1 g/L tryptophan and TPGY broth ($p = 0.05$) using Statgraphics Centurion version 16.1.15.

3. Results and discussion

3.1. Effect of oxygen concentration and incubation temperature on *C. botulinum* and surviving microbiota growth in HPP treated tender coconut water

3.1.1. Refrigeration temperature (4 °C)

Processing coconut water at 550 MPa for 3 min at 10 °C did not significantly reduce *C. botulinum* spore counts compared to the untreated control samples ($p > 0.05$). Bacterial spores are highly resistant to pressure. It has been reported that a HPP treatment of 827 MPa/5 min applied at ambient temperature (<35 °C) had no effect on the viability of non-proteolytic *C. botulinum* spores (Reddy et al., 1999). In the present work, total counts of *C. botulinum* remained constant after HPP during the 61 days of storage at 4 °C regardless of the initial dissolved oxygen (DO_2) content (Figure 9). Counts from heated aliquots (65 °C, 10 min) from the last testing point after 61 days of incubation revealed absence of germinated spores or vegetative C. botulinum cells (Figure A3 of the Appendix to Chapter 2).

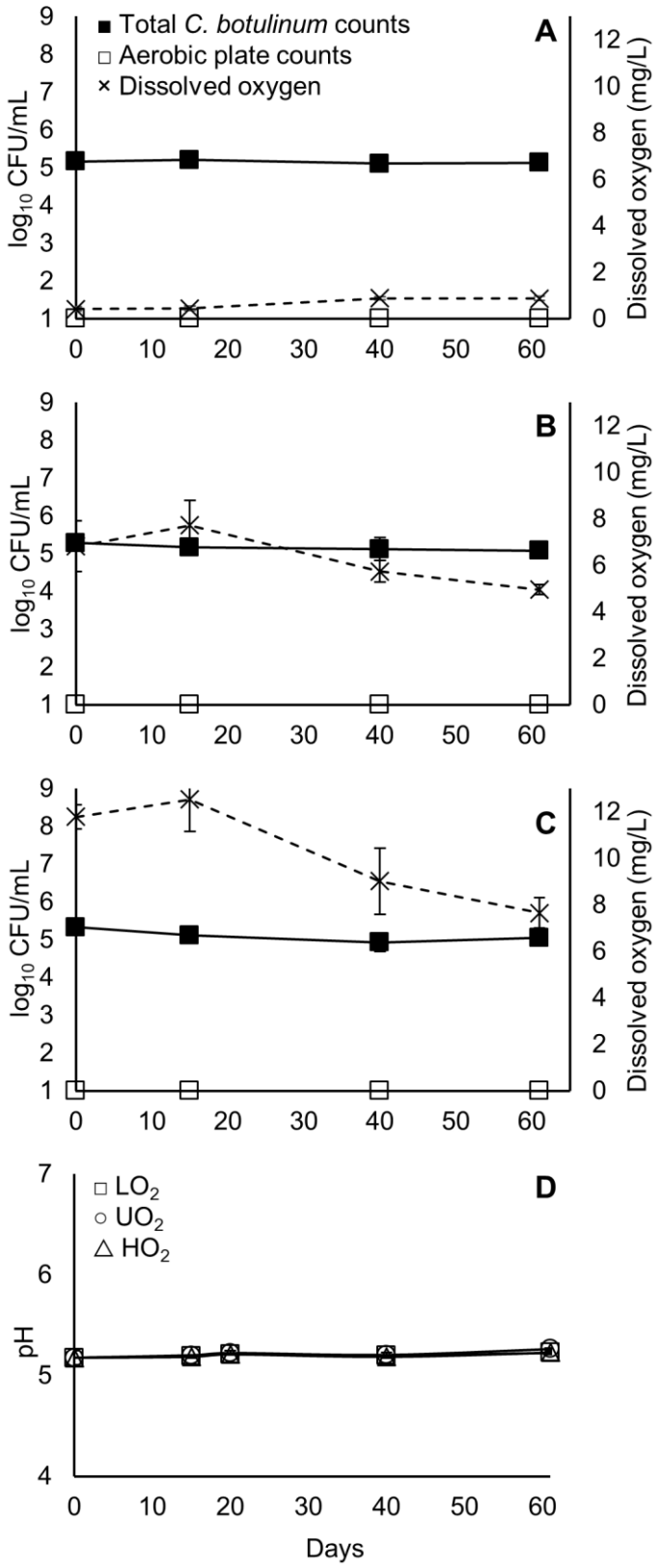

Figure 9. *C. botulinum* total counts, aerobic plate counts and dissolved oxygen concentration in high-pressure processed coconut water (550 MPa for 3 min) stored at 4 °C with initial different dissolved oxygen concentration levels: (A) low (LO_2; <0.5±0.1 mg/L); (B) unaltered (UO_2; 7.0±0.8 mg/L); (C) high (HO_2; 11.0±0.5 mg/L); and pH evolution of coconut water (D) in LO_2, UO_2, HO_2.

Although growth of type E *C. botulinum* has been reported at temperature levels below 4 °C in laboratory media (Doyle, 1989), its growth in food products depends on several interrelated factors like the presence of proper nutrients or natural antimicrobials, pH of the food matrix, and oxygen concentration. Total aerobes displayed counts below the detection limit (<10 CFU/mL) in all conditions throughout incubation (Figure 9A-C). During the 61 days of incubation, DO_2 concentration decreased by 1.86 mg/L in UO_2 coconut water (Figure 9B) and 4.13 mg/L in HO_2 (Figure 9C). This may be attributed to gas diffusion through the PET bottles used for this experiment or enzymatic oxidation by polyphenol oxidase (PPO), a pressure-resistant enzyme (Chakraborty, Baier, Knorr, & Mishra, 2015; Jayachandran, Chakraborty, & Rao, 2016; Terefe, Delon, Buckow, & Versteeg, 2015) that is responsible for the pink discoloration in coconut water (Prades, Dornier, Diop, & Pain, 2012). This phenomenon also occurs in fresh coconut water that remains unprocessed, so it is generally perceived as natural by frequent consumers of the beverage. Additionally, commercial labeling of HPP coconut water often indicates that pink discoloration can potentially occur (Happy Coco, 2019). In the case of LO_2 coconut water, dissolved oxygen remained between 0.1-0.45 mg/L after 40 days (Figure 9A).

3.1.2. Moderate temperature abuse (10 °C)

C. botulinum total counts followed a similar trend after HPP to those of samples stored at 4 °C with no changes over incubation period (Figure 10). Spore concentration determined by plate counting of heated aliquots (65 ºC, 10 min) after 61 days was constant, suggesting that spores remained dormant despite temperature abuse, independently of oxygen concentration (Figure A4 of the Appendix to Chapter 1). This can be attributed to the naturally low pH of coconut water used in this study (pH 5.2), which is close to the minimum pH documented for the germination of non-proteolytic *C. botulinum* spores (pH 5.0) (Plowman & Peck, 2002). However, total aerobic counts started to increase after 10 to 15 days of incubation evidencing that HPP did not inactivate all spoilage microorganisms. The highest concentration of total aerobes was found after 40 days, with values ranging from 6.4 to 8.2 $\log_{10}$ CFU/mL depending on initial oxygen concentration (Figure 10A-C). Growth of spoilage microorganisms recovered after HPP caused severe degradation in the aspect of coconut water.

The identification of bacterial species growing on PCA plates by means of 16S rDNA PCR showed only two species with similarity scores higher than 99 % independently of DO_2 and incubation temperature: *Tatumella ptyseos* (99 % identity) and *Xanthomonas* spp. (100 % identity). Both are facultative anaerobes associated to plant degradation and are cause of plant diseases (Bull et al., 2014; Marín-Cevada et al., 2010).

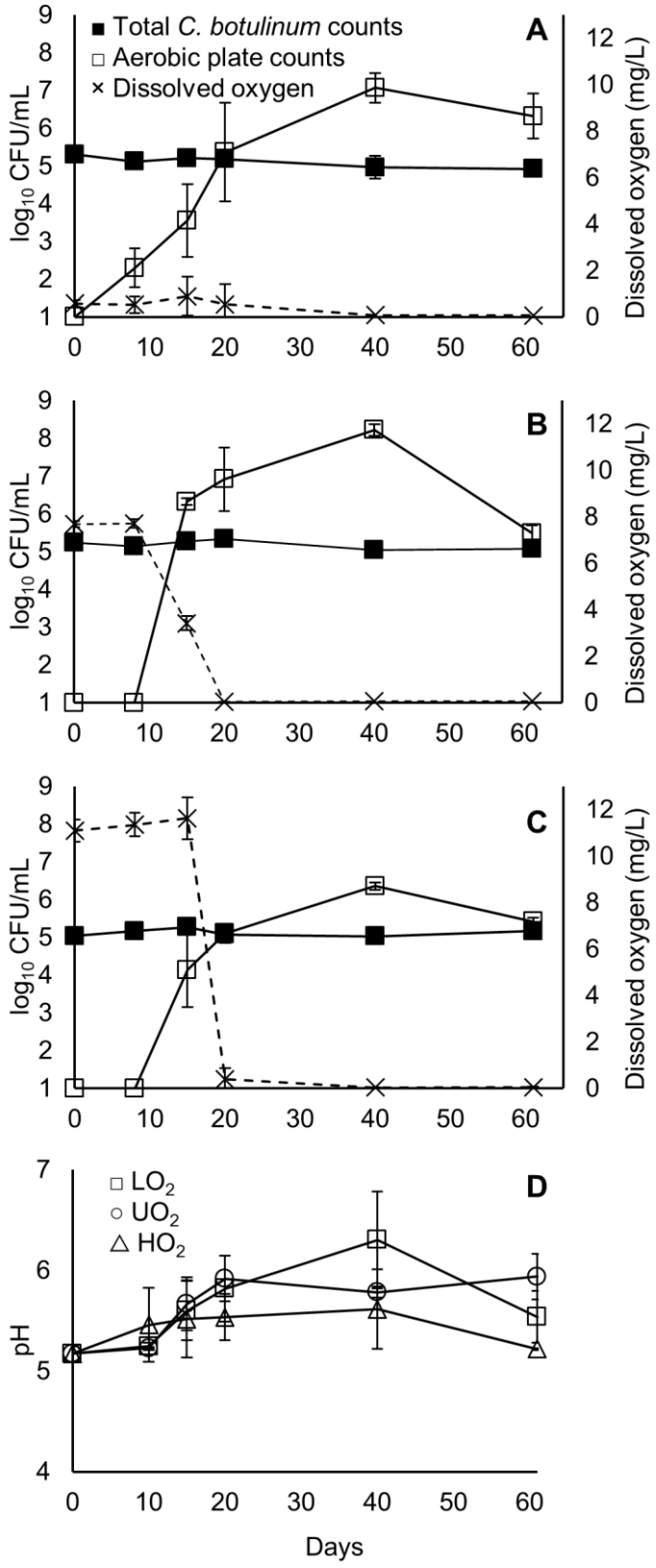

Figure 10. *C. botulinum* total counts, aerobic plate counts and dissolved oxygen concentration in high-pressure processed coconut water (550 MPa for 3 min) stored at 10 °C with initial different dissolved oxygen concentration levels: (A) low (LO_2; <0.5±0.1 mg/L); (B) unaltered (UO_2; 7.0±0.8 mg/L); (C) high (HO_2; 11.0±0.5 mg/L); and pH evolution of coconut water (D) in LO_2, UO_2, HO_2.

Their presence might be a consequence of cross contamination during coconut water extraction. *T. ptyseos* is a polyamine producer, with putrescine and diaminopropane as the most representative metabolites of this category (Hamana, 1996). Its growth may explain the pH increase by 0.7 to 1.1 units observed over storage (Figure 10D). Good agricultural and manufacturing practices during harvesting and coconut water extraction combined with more intense processing conditions (up to 600 MPa for several minutes) should be implemented to minimize cross contamination and increase the inactivation rate of spoilage microorganisms. Nevertheless, other microorganisms present in a food product might compete with *C. botulinum* and prevent its growth by depleting essential nutrients or antagonism (Kostrzynska & Bachand, 2006; Lyver et al., 1998; Rodgers et al., 2004; Skinner et al., 1999). Reduction of key nutrients required for spore germination might explain why spores remained dormant despite slight pH increase over incubation period.

DO_2 content of UO_2 and HO_2 samples showed a sharp decrease between days 15 and 20 that can be attributed to the microbial growth observed (Figure 10B-C). By the end of incubation period, DO_2 concentration of all samples remained below 0.5 mg/L. Oxygen uptake rate (OUR) increases during lag phase and exponential phase of microbial growth, meaning that consumption of oxygen increases as biomass is formed (Garcia-Ochoa, Gomez, Santos, & Merchuk, 2010). Oxygen demand might be so high that DO_2 concentration decreases until it approaches zero, as observed in this work. Other studies report that *Xanthomonas campestris* has very high OUR and is able to reduce oxygen saturation of broth to 5 % after only 10 h of growth (Garcia-Ochoa, Castro, & Santos, 2000). Although *C. botulinum* failed to grow in coconut water stored at these conditions, other authors showed that non-proteolytic strains of the bacterium could grow in vegetable products with residual O_2 concentrations. Growth and toxin production were observed in broccoli florets after 9 days of incubation at 12 ºC (Larson, Johnson, Barmore, & Hughes, 1997). However, other vacuum-packed vegetables, such as celery, did not support growth of proteolytic and non-proteolytic strains of *C. botulinum* incubated at 7 ºC for 56 days (Johnson, 1979). This evidences that *C. botulinum* growth is very dependent on the food matrix.

3.1.3. Intense temperature abuse (20 °C)

Total *C. botulinum* counts remained constant after HPP during the first 4 days of incubation in LO_2, UO_2 and HO_2 (Figure 11). However, a decrease of 1.7 $\log_{10}$ CFU/mL occurred in LO_2 coconut water after 61 days (Figure 11A). Similarly, spore counts slightly decreased between 0.8 and 1.1 $\log_{10}$ CFU/mL in UO_2 and HO_2 coconut water after 20 days of incubation, respectively (Figure 11B-C). This was observed right after DO_2 depleted below

0.05 mg/L, most likely due to background microbiota growth. Total aerobes recovered from HPP and reached a concentration between 6.0 and 7.3 CFU/mL within 4 days, causing severe spoilage as observed by turbidity change even in LO_2 samples where initial DO_2 concentration was already low. Additionally, pH increased between 0.4-1.8 units (Figure 11D). This proves that dissolved oxygen varies depending on temperature and presence of spoilage microorganisms, so it should never be considered alone as a valid strategy to control *C. botulinum* in liquid foods. The observed progressive reduction in spore counts might be attributed to a fraction that germinated when conditions turned adequate but failed to resume vegetative growth. Nonetheless, at the end of the experiment (day 61), no vegetative cells of *C. botulinum* nor germinated spores were detected in any sample (Figure A5 of the Appendix to Chapter 1).

Other authors have reported that type E spores of *C. botulinum* germinate and grow within 3 days at 20 °C in laboratory media with a concentration of DO_2 adjusted to 0.013 atm O_2 (~0.58 mg/L O_2) (Lund, Knox, & Sims, 1984), whereas in the present study the bacterium failed to grow even at lower concentrations (<0.50 mg/L O_2). This can be attributed to: *i)* low incubation temperature (in samples kept at 4 °C), *ii)* growth of spoilage microorganisms competing with *C. botulinum* at 10 and 20 °C, *iii)* high DO_2 in some scenarios, *iv)* pH 5.2 being too low to support growth in coconut water, *v)* presence of natural growth inhibitors in the raw beverage, *vi)* absence of key nutrients in coconut water required by the bacterium to grow, *vii)* impact of high pressure processing on germination and growth, or a combination of factors.

3.2. Behavior of *C. botulinum* in microfiltered coconut water at pH 7.0 (FS) under strictly anoxic conditions at 30 ºC

Since *C. botulinum* did not grow in HPP tender coconut water with different initial oxygen concentrations, other key parameters were modified to assess whether germination and growth can take place in conditions favorable for the bacterium, resembling a “worst-case scenario” from a food safety perspective. During the first 10 days of storage of FS coconut water (pH 7.0) under strictly anaerobic conditions at 30 °C, concentration of *C. botulinum* counts decreased by 1.9 $\log_{10}$ CFU/mL (Figure 12). Furthermore, *C. botulinum* counts steadily continued to decrease during the incubation period, reaching a final concentration of 2.3 $\log_{10}$ CFU/mL after 61 days with no vegetative cells detected (Figure 12). This reduction was slightly greater than that observed in LO_2 coconut water incubated at 20 °C (1.7 $\log_{10}$ CFU/mL) after 20 days of incubation (Figure 11A). Optimum growth conditions for *C. botulinum* in coconut water (pH 7.0, strictly anoxic atmosphere, 30 ºC and absence of background microbiota) did apparently favor spore germination followed by subsequent germinated spores’ death.

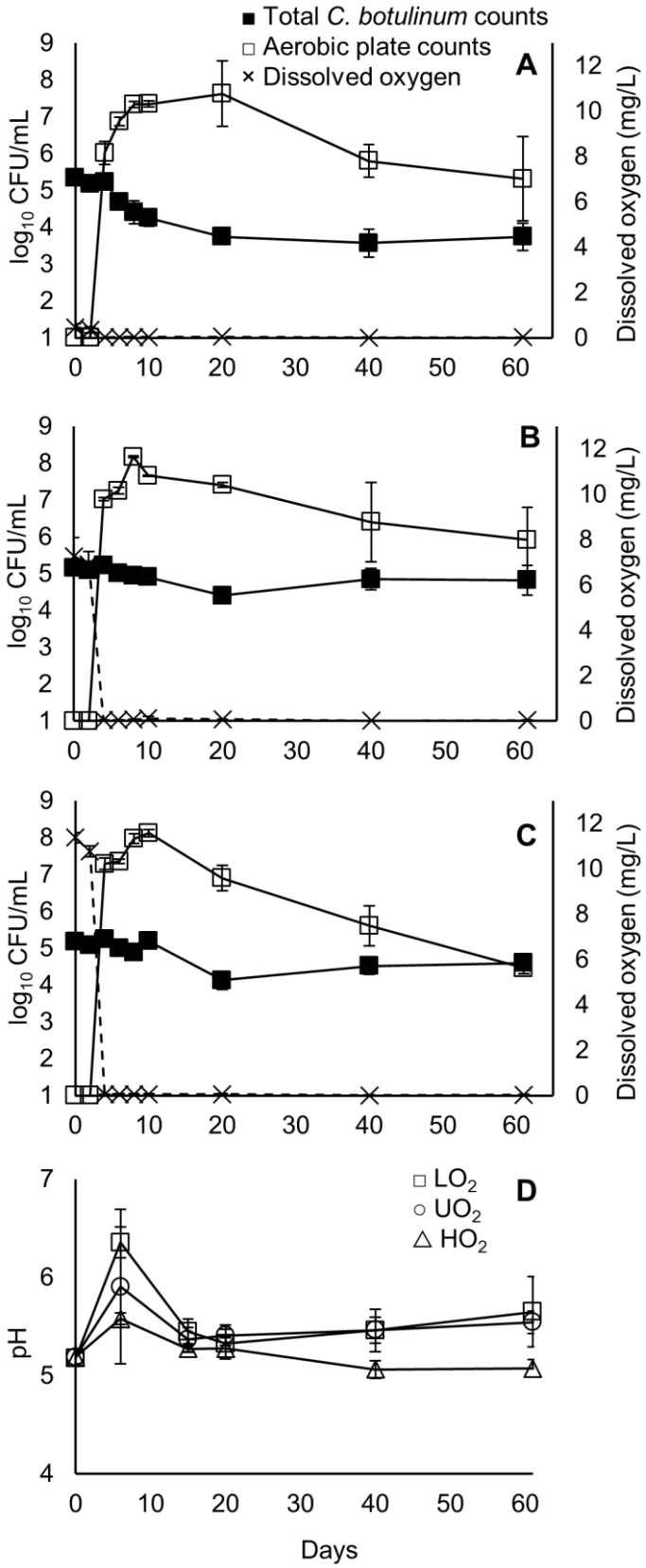

Figure 11. *C. botulinum* total counts, aerobic plate counts and dissolved oxygen concentration in high-pressure processed coconut water (550 MPa for 3 min) stored at 20 °C with initial different dissolved oxygen concentration levels: (A) low (LO_2; <0.5±0.1 mg/L); (B) unaltered (UO_2; 7.0±0.8 mg/L); (C) high (HO_2; 11.0±0.5 mg/L); and pH evolution of coconut water (D) in LO_2, UO_2, HO_2.

Optimum growth conditions for *C. botulinum* in coconut water (pH 7.0, strictly anoxic atmosphere, 30 ºC and absence of background microbiota) did apparently favor spore germination followed by subsequent germinated spores' death since no vegetative cells were identified. It is therefore likely that the beverage lacks some essential nutrients that the bacterium requires to resume growth or that growth is inhibited by naturally-occurring substances present in coconut water. Some vegetables are source of phytochemicals with the ability to inhibit *Clostridium* spp. germination and growth when present at sufficient concentration (Bowles & Miller, 1994; Juneja, Bari, Inatsu, Kawamoto, & Friedman, 2007; Pacheco et al., 2017). Both approaches were furtherly studied in the present work.

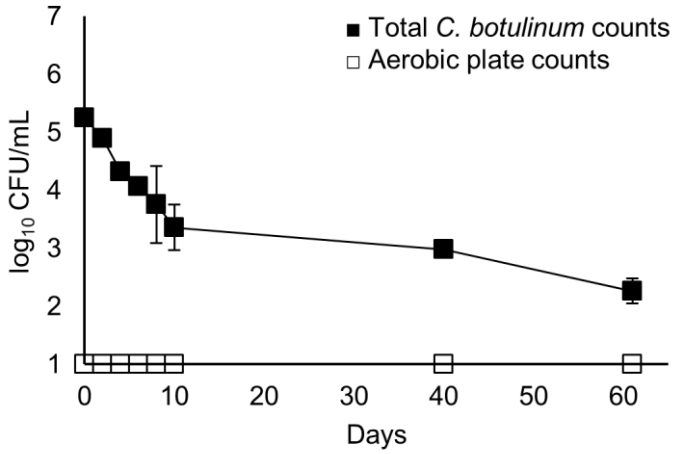

Figure 12. *C. botulinum* total counts and aerobic plate counts in FS coconut water (pH 7.0) incubated at 30 °C under strict anaerobic conditions.

3.3. Supplementation with germinants

Heating FS coconut water aliquots supplemented with germinants (FS+G) at 65 ºC for 10 min reduced the concentration of *C. botulinum* by 1.6 $\log_{10}$ CFU/mL after 1 h of incubation (Figure 13). This difference with respect to the initial spore count (5.36 $\log_{10}$ CFU/mL) was significant ($p < 0.05$; Figure 13), which indicates that a fraction of spores germinated and lost their heat resistance. *C. botulinum* total counts further decreased to reach 3.5 and 2.9 $\log_{10}$ CFU/mL, after 24 and 48 h, respectively. Heating at 65 ºC for 10 min reduced counts below the detection limit (2.3 $\log_{10}$ CFU/mL), which suggests that >1.9 $\log_{10}$ CFU/mL and >0.6 $\log_{10}$ CFU/mL of the remaining total cells were germinated spores after 24 and 48 h, respectively. Supplementing coconut water with selected germinants accelerated the germination process of *C. botulinum*. As reported in previous experiments, it took 61 days to reduce from 5.25 to 2.26 $\log_{10}$ CFU/mL the concentration of spores without the addition of germinants (Figure 12), whereas it only took 48 h to achieve a similar spore concentration reduction under the same conditions in germinant-supplemented coconut water (Figure 13).

Apparently, addition of selected germinant agents enhanced spore germination, but germinated spores failed to resume vegetative growth and progressively died.

Previous reports in the literature show that spore concentration of the pathogen decreased 3 $\log_{10}$ CFU/mL in black and oolong teas incubated at 30 °C under strict anaerobic conditions for 3 months (Hara-Kudo, Watanabe, & Sakaguchi, 1989). Other authors reported reductions in the spore concentration to undetectable levels (<10 CFU/mL) after 12 weeks of incubation at 15 ºC in extracts of green tea (Hara-Kudo et al., 2005). In both cases, reduction in the counts of *C. botulinum* was associated to a possible sporicidal effect of the catechins present in the tea. Chang *et al.* (Chang & Wu, 2011) found that coconut water is a source of catechins and epicatechins at concentrations ranging 0.26 to 0.36 µg/mL. However, concentrations reported in coconut water are much lower than those reported in green tea (10-418 µg/mL) (Arts, van de Putte, & Hollman, 2000; Bronner & Beecher, 1998). Other authors showed that germination efficiency of *Bacillus subtilis* spores was not affected in the presence of catechins and epicatechins extracted from green tea, but growth of vegetative cells did not take place at certain concentrations of these compounds which exerted a sporostatic activity (Pandey et al., 2015).

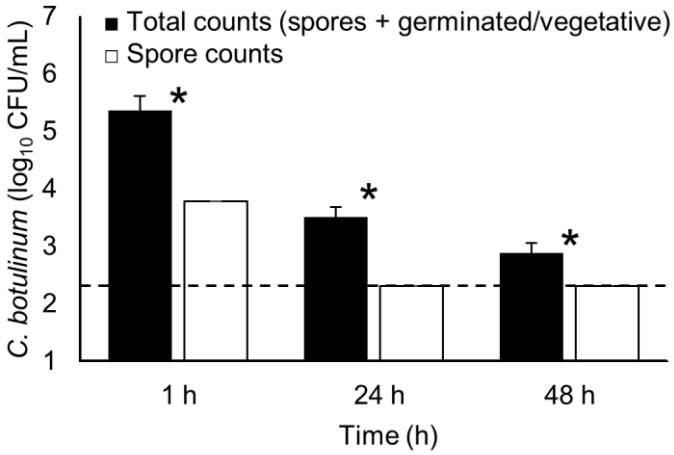

Figure 13. *C. botulinum* total counts and spore counts in FS+G coconut water after incubation for 48 h at 30 °C under strict anoxic conditions. *Significant difference ($p < 0.05$) (dashed line: detection limit).

3.4. Supplementation with amino acids

Amino acids are a key element for non-proteolytic *C. botulinum* growth and toxin production (Perkins & Tsuji, 1962; Strasdine & Melville, 1968; Whitmer & Johnson, 1988). Based on compositional analysis performed on the coconut water used in this study (Table A1 of the Appendix to Chapter 1), concentration of free amino acids in the beverage is very poor

(<0.2 to <0.1 g/L). To evaluate if the lack of certain essential amino acids hindered *C. botulinum* vegetative growth, 2 % casein hydrolysate and 0.1 g/L tryptophan were added to FS coconut water (FS+AA) to ensure that all essential amino acids were present in a free form at sufficient concentration. Results indicated that this addition had a limited impact on germination of spores inoculated in coconut water, resulting in the germination of ~1.0 $\log_{10}$ CFU/mL of spores (Figure 14). Total *C. botulinum* counts decreased in 48 h from 5.2 to 4.1 $\log_{10}$ CFU/mL, as previously observed during the first 48 h in FS coconut water (Figure 12). Heating aliquots at 65 ºC for 10 min did not reveal a significant reduction of the spore concentration in coconut water after 1 or 24 h. The difference in the counts between heated and unheated aliquots after 48 h was significant (0.56 $\log_{10}$ CFU/mL, $p < 0.05$) but similar to that observed in FS+G coconut water (0.88 $\log_{10}$ CFU/mL, $p > 0.05$) (Figure 13).

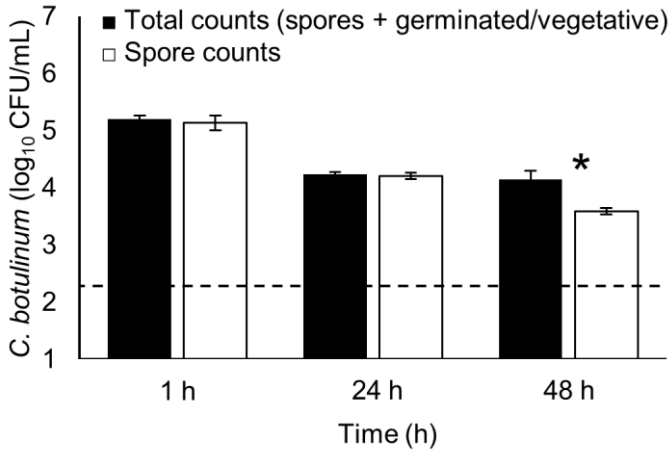

Figure 14. *C. botulinum* total counts and spore counts in FS+AA coconut water after incubation for 48 h at 30 °C under strict anoxic conditions. *Significant difference ($p < 0.05$) (dashed line: detection limit).

Type E *C. botulinum* requirements for specific amino acids are not well established. However, several authors agree to include a group of seven amino acids in the design of different chemically defined media (CDM) to evaluate the germination, growth and toxin production of the bacterium (Strasdine & Melville, 1968; Ward & Carroll, 1966; Whitmer & Johnson, 1988). These include histidine, isoleucine, leucine, serine, tryptophan, tyrosine and valine. The casein hydrolysate and tryptophan at the concentrations used in this study (Table 11) provided to coconut water all the above-mentioned amino acids at a concentration higher than the established in chemically defined media for type E *C. botulinum* growth (Whitmer & Johnson, 1988). These findings suggest that free amino acids alone are not sufficient to promote growth of the bacterium in coconut water. Other compounds usually added to CDM are vitamins, such as biotin, thiamine (B1), pyridoxine (B6), nicotinamide (B3), folate (B9) and

choline (or their vitamers) (Strasdine & Melville, 1968; Ward & Carroll, 1966; Whitmer & Johnson, 1988). Although coconut water is a natural source of most of these nutrients, the concentration at which they are present is lower than that added in CDM to promote growth and toxin production of *C. botulinum* (Table 12).

Table 11. Concentration of essential free amino acids in 2 % casein hydrolysate and in a chemically defined medium (CDM) for type E *C. botulinum*.

Amino acid	2 % casein hydrolysate (g/L)	CDM[a] (g/L)
Histidine	0.37	0.10
Isoleucine	0.56	0.10
Leucine	0.62	0.10
Tryptophan	-[b]	0.10
Tyrosine	0.25	0.05
Valine	0.70	0.10
Serine	0.60	0.10

[a] Concentration of amino acids in chemically defined medium (CDM) (Whitmer & Johnson, 1988).
[b] 0.1 g/L tryptophan was separately added to complement 2 % casein hydrolysate.

3.5. Supplementation with TPGY broth

To evaluate if coconut water is a source of antimicrobial compounds with activity against *C. botulinum*, coconut water was supplemented with Tryptone peptone glucose yeast extract (TPGY) broth, a well-described medium widely used to grow the bacterium (Figure 15).

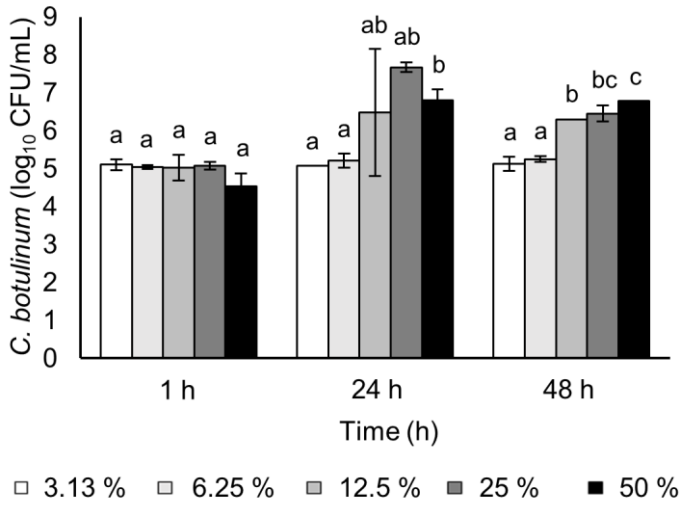

Figure 15. *C. botulinum* total counts in coconut water supplemented with TPGY broth at different concentrations after incubation for 48 h at 30 ºC under strictly anoxic conditions. Different letters indicate significant differences ($p < 0.05$).

Table 12. Vitamin content in chemically defined media (CDM) and coconut water.

Vitamin	Concentration reported in CDM for *C. botulinum* growth	Concentration reported in coconut water
Biotin	0.01 mg/L (Strasdine & Melville, 1968) 0.2 mg/L (Whitmer & Johnson, 1988) 1 mg/L (Ward & Carroll, 1966)	0.016 mg/L (Hoppner, Lampi, & O'Grady, 1994) 0.02 mg/L (Gopikrishna, Thomas, & Kandaswamy, 2008)
Thiamine	0.4 mg/L (Strasdine & Melville, 1968) 0.4 mg/L (Whitmer & Johnson, 1988) 1mg/L (Ward & Carroll, 1966)	0.1-0.4 mg/L (Santoso, Kubo, Ota, Tadokoro, & Maekawa, 1996) 0.3 mg/L (Yong, Ge, Ng, & Tan, 2009)
Pyridoxine	1 mg/L (Strasdine & Melville, 1968) 1 mg/L (Ward & Carroll, 1966) 1 mg/L (Whitmer & Johnson, 1988)	0.32 mg/L (Yong et al., 2009)
Nicotinamide	1 mg/L (Strasdine & Melville, 1968) 1 mg/L (Ward & Carroll, 1966) 1 mg/L (Whitmer & Johnson, 1988)	0.64 mg/L (Gopikrishna et al., 2008) 0.8 mg/L (Yong et al., 2009)
Folate	1 mg/L (Strasdine & Melville, 1968) 0.25 mg/L (Whitmer & Johnson, 1988) 0.5 mg/L (Ward & Carroll, 1966)	0.003 mg/L (Gopikrishna et al., 2008) 0.03 mg/L (Yong et al., 2009)
Choline	10 mg/L (Strasdine & Melville, 1968) 50 mg/L (Whitmer & Johnson, 1988)	NA[a]

[a] Not available.

Serial dilutions of the beverage (FS+TPGY) with the laboratory broth revealed that between 6.25 to 12.5 % TPGY was sufficient to stimulate outgrowth from 5 $\log_{10}$ CFU/mL of spores to a cell concentration of 6.5 $\log_{10}$ CFU/mL in 24 h. A lower concentration of 3.13 % TPGY, in contrast, did not support spore outgrowth. Final cell concentrations further increased when increasing TPGY broth concentration up to 50 % (Figure 15). Since growth already occurred at low TPGY concentrations, it can be presumptively discarded that the raw coconut water used in this study contains inhibitory substances at a sufficient concentration to prevent *C. botulinum* spore germination and growth. These results suggest that the beverage lacks certain essential, but not yet identified, nutrients required by the bacterium.

4. Conclusions

HPP Thai raw coconut water (pH 5.2) used in this study did not support vegetative growth of *C. botulinum* after 61 days of storage at refrigeration and moderate temperature abuse (4-10 °C), and only germinated after 61 days of continuous incubation at 20 °C. Similarly, the three-strain spore cocktail failed to grow in coconut water incubated at 30 °C under strict anoxic conditions, even when competitive microorganisms were not present and pH was favorable (7.0), but spore germination took place.

Supplementation of coconut water with selected germinants under the same optimum conditions accelerated germination, but still did not induce growth. Mixtures of amino acids did not promote germination nor vegetative growth of the bacterium, but nutrient-rich laboratory media (TPGY broth) mixed at low concentrations (6.25 %) with coconut water allowed growth of the three-strain cocktail, suggesting that coconut water lacks of some essential nutrients required by *C. botulinum* for vegetative growth.

Further work is needed to accurately establish the intrinsic and extrinsic factors that prevent *C. botulinum* growth in coconut water, aiming to use these as control critical points in HACCP plans for the safe production of different varieties of non-thermally treated coconut water. The use of toxigenic *C. botulinum* strains (types I and II) and botulinum neurotoxin determination rather than plate counts would assess the risk more precisely.

5. References

Ando, Y. (1971). The Germination Requirements of Spores of *Clostridium botulinum* Type E. *Japanese Journal of Microbiology*, *15*(6), 515–525.

Ando, Y., & Lida, H. (1970). Factors Affecting the Germination of Spores of *Clostridium botulinum* Type E. *Japanese Journal of Microbiology*, *14*(5), 361–370.

Arts, I. C. W., van de Putte, B., & Hollman, P. C. H. (2000). Catechin Contents of Foods Commonly Consumed in The Netherlands: Tea, Wine, Fruit Juices, and Chocolate Milk. *Journal of Agricultural and Food Chemistry*, *48*(5), 1752–1757. https://doi.org/10.1021/JF000026+

Awua, A. K., Doe, E. D., & Agyare, R. (2011). Exploring the influence of sterilisation and storage on some physicochemical properties of coconut (*Cocos nucifera* L.) water. *BMC Research Notes*, *4*. https://doi.org/10.1186/1756-0500-4-451

Bowles, B. L., & Miller, A. J. (1994). Caffeic Acid Activity Against *Clostridium botulinum* Spores. *Journal of Food Science*, *59*(4), 905–908. https://doi.org/10.1111/j.1365-2621.1994.tb08154.x

Bronner, W. E., & Beecher, G. R. (1998). Method for determining the content of catechins in tea infusions by high-performance liquid chromatography. *Journal of Chromatography A*, *805*(1), 137–142. https://doi.org/10.1016/S0021-9673(98)00040-5

Bull, C. T., Coutinho, T. A., Denny, T. P., Firrao, G., Saux, M. F.-L., Li, X., … Takikawa, Y. (2014). List of new names of plant pathogenic bacteria (2011-2012). *Journal of Plant Pathology*, *96*(2), 223–226. https://doi.org/10.4454/JPP.V96I2.026

Buzrul, S., Alpas, H., Largeteau, A., Bozoglu, F., & Demazeau, G. (2008). Compression heating of selected pressure transmitting fluids and liquid foods during high hydrostatic pressure treatment. *Journal of Food Engineering*, *85*(3), 466–472. https://doi.org/10.1016/j.jfoodeng.2007.08.014

Carlin, F., & Peck, M. W. (1996). Growth and toxin production by nonproteolytic *Clostridium botulinum* in cooked puréed vegetables at refrigeration temperatures. *Applied and Environmental Microbiology*, *62*(8), 3069–3072.

Chakraborty, S., Baier, D., Knorr, D., & Mishra, H. N. (2015). High pressure inactivation of polygalacturonase, pectinmethylesterase and polyphenoloxidase in strawberry purée mixed with sugar. *Food and Bioproducts Processing*, *95*, 281–291. https://doi.org/10.1016/j.fbp.2014.10.016

Chang, C.-L., & Wu, R.-T. (2011). Quantification of (+)-catechin and (−)-epicatechin in coconut water by LC–MS. *Food Chemistry*, *126*(2), 710–717. https://doi.org/10.1016/j.foodchem.2010.11.034

Chen, D., Xi, H., Guo, X., Qin, Z., Pang, X., Hu, X., … Wu, J. (2013). Comparative study of quality of cloudy pomegranate juice treated by high hydrostatic pressure and high temperature short time. *Innovative Food Science and Emerging Technologies*, *19*, 85–94. https://doi.org/10.1016/j.ifset.2013.03.003

Chidambaram, S., Singaraja, C., Prasanna, M. V., Ganesan, M., & Sundararajan, M. (2013). Chemistry of Tender Coconut Water from the Cuddalore Coastal Region in Tamil Nadu, India. *Natural Resources Research*, *22*(2), 91–101. https://doi.org/10.1007/s11053-013-9203-y

Clauwers, C., Vanoirbeek, K., Delbrassinne, L., & Michiels, C. W. (2016). Construction of nontoxigenic mutants of nonproteolytic *Clostridium botulinum* NCTC 11219 by insertional mutagenesis and gene replacement. *Applied and Environmental Microbiology*, *82*(10), 3100–3108. https://doi.org/10.1128/AEM.03703-15

Da Fonseca, A. M., Bizerra, A. M. C., Sammy, J., De Souza, N., Monte, F. J. Q., Da Conceição, M., … Lemos, T. L. G. (2009). Constituents and antioxidant activity of two varieties of coconut water *(Cocos nucifera* L.*)*. *Revista Brasileira de Farmacognosia Brazilian Journal of Pharmacognosy, 19*(1), 193-198. https://doi.org/10.1590/S0102-695X2009000200002

DebMandal, M., & Mandal, S. (2011). Coconut (*Cocos nucifera* L.: *Arecaceae*): In health promotion and disease prevention. *Asian Pacific Journal of Tropical Medicine*, *4*(3), 241–247. https://doi.org/10.1016/S1995-7645(11)60078-3

Di Gioia, D., Mazzola, G., Nikodinoska, I., Aloisio, I., Langerholc, T., Rossi, M., … Rovira, J. (2016). Lactic acid bacteria as protective cultures in fermented pork meat to prevent *Clostridium* spp. growth. *International Journal of Food Microbiology*, *235*, 53–59. https://doi.org/10.1016/j.ijfoodmicro.2016.06.019

Doyle, M. P. (1989). *Foodborne Bacterial Pathogens*. New York: Marcel Dekker, Inc.

FAOSTAT. (2017). Crops and livestock products. Retrieved December 15, 2019, from http://www.fao.org/faostat/en/

Garcia-Ochoa, F., Castro, E. G., & Santos, V. E. (2000). Oxygen transfer and uptake rates during xanthan gum production. *Enzyme and Microbial Technology*, *27*(9), 680–690.

Garcia-Ochoa, F., Gomez, E., Santos, V. E., & Merchuk, J. C. (2010). Oxygen uptake rate in microbial processes: An overview. *Biochemical Engineering Journal*, *49*, 289–307. https://doi.org/10.1016/j.bej.2010.01.011

Gopikrishna, V., Thomas, T., & Kandaswamy, D. (2008). A quantitative analysis of coconut water: a new storage media for avulsed teeth. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics*, *105*(2), e61-5. https://doi.org/10.1016/j.tripleo.2007.08.003

Graham, A. F., Mason, D. R., Maxwell, F. J., & Peck, M. W. (1997). Effect of pH and NaCl on growth from spores of non-proteolytic *Clostridium botulinum* at chill temperature. *Letters in Applied*

Microbiology, *24*(2), 95–100.

Graham, A. F., Mason, D. R., & Peck, M. W. (1996). Predictive model of the effect of temperature, pH and sodium chloride on growth from spores of non-proteolytic *Clostridium botulinum*. *International Journal of Food Microbiology*, *31*(1), 69–85. https://doi.org/10.1016/0168-1605(96)00965-8

Hamana, K. (1996). Distribution of diaminopropane and acetylspermidine in *Enterobacteriaceae*. *Canadian Journal of Microbiology*, *42*(2), 107–114.

Happy Coco. (2019). Raw coconut water. Retrieved December 15, 2019, from http://happycoco.com/en/coconut-water/

Hara-Kudo, Y., Watanabe, M., & Sakaguchi, G. (1989). The Fate of *Clostridium botulinum* Spores Inoculated into Tea Drinks. *Nippon Shokuhin Kogyo Gakkaishi*, *36*(5), 375–379.

Hara-Kudo, Y., Yamasaki, A., Sasaki, M., Okubo, T., Minai, Y., Haga, M., … Sugita-Konishi, Y. (2005). Antibacterial action on pathogenic bacterial spore by green tea catechins. *Journal of the Science of Food and Agriculture*, *85*(14), 2354–2361. https://doi.org/10.1002/jsfa.2259

Hoppner, K., Lampi, B., & O'Grady, E. (1994). Biotin content in vegetables and nuts available on the Canadian market. *Food Research International*, *27*(5), 495–497. https://doi.org/10.1016/0963-9969(94)90245-3

Hotchkiss, J. H., Banco, M. J., Busta, F. F., Genigeorgis, C. A., Kociba, R., Rheaume, L., … Sugiyama, H. (2016). The Relationship Between Botulinal Toxin Production and Spoilage of Fresh Tomatoes Held at 13 and 23°C Under Passively Modified and Controlled Atmospheres and Air. *Journal of Food Protection*, *55*(7), 522–527. https://doi.org/10.4315/0362-028x-55.7.522

Jackson-Davis, A., Mendonca, A., Hale, S., Jackson, J., King, A., & Jackson, J. (2018). Microbiological Safety of Unpasteurized Fruit and Vegetable Juices Sold in Juice Bars and Small Retail Outlets. In S. Ricke, G. Atungulu, C. Rainwater, & S. Park (Eds.), *Food and Feed Safety Systems and Analysis* (pp. 213–225). London: Academic Press. https://doi.org/10.1016/B978-0-12-811835-1.00012-9

Jackson, J. C., Gordon, A., Wizzard, G., McCook, K., & Rolle, R. (2004). Changes in chemical composition of coconut (*Cocos nucifera*) water during maturation of the fruit. *Journal of the Science of Food and Agriculture*, *84*(9), 1049–1052. https://doi.org/10.1002/jsfa.1783

Janda, J. M., & Abbott, S. L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *Journal of Clinical Microbiology*, *45*(9), 2761–2764. https://doi.org/10.1128/JCM.01228-07

Jayachandran, L. E., Chakraborty, S., & Rao, P. S. (2016). Inactivation Kinetics of the Most Baro-Resistant Enzyme in High Pressure Processed Litchi-Based Mixed Fruit Beverage. *Food and*

Bioprocess Technology, *9*(7), 1135–1147. https://doi.org/10.1007/s11947-016-1702-5

Johnson, C. E. (1979). Behavior of *Clostridium botulinum* in vacuum-packed fresh celery. *Journal of Food Protection*, *423*(1), 49–50.

Jordan, S. L., Pascual, C., Bracey, E., & Mackey, B. M. (2001). Inactivation and injury of pressure-resistant strains of *Escherichia coli* O157 and *Listeria monocytogenes* in fruit juices. *Journal of Applied Microbiology*, *91*(3), 463–469. https://doi.org/10.1046/j.1365-2672.2001.01402.x

Juneja, V. K., Bari, M. L., Inatsu, Y., Kawamoto, S., & Friedman, M. (2007). Control of *Clostridium perfringens* spores by green tea leaf extracts during cooling of cooked ground beef, chicken, and pork. *Journal of Food Protection*, *70*(6), 1429–1433. https://doi.org/10.4315/0362-028X-70.6.1429

Jung, S., & Tonello-Samson, C. (2018). Chapter 7: High Hydrostatic Pressure Food Processing: Potential and Limitations. *RSC Green Chemistry*, *2018*(53), 251–315. https://doi.org/10.1039/9781782626596-00251

Kasai, Y., Kimura, B., Kawasaki, S., Fukaya, T., Skuma, K., & Fujii, T. (2016). Growth and Toxin Production by *Clostridium botulinum* in Steamed Rice Aseptically Packed under Modified Atmosphere. *Journal of Food Protection*, *68*(5), 1005–1011. https://doi.org/10.4315/0362-028x-68.5.1005

Kostrzynska, M., & Bachand, A. (2006). Use of microbial antagonism to reduce pathogen levels on produce and meat products: a review. *Canadian Journal of Microbiology*, *52*(11), 1017–1026. https://doi.org/10.1139/w06-058

Larson, A. E., Johnson, E. A., Barmore, C. R., & Hughes, M. D. (1997). Evaluation of the botulism hazard from vegetables in modified atmosphere packaging. *Journal of Food Protection*, *60*(10), 1208–1214.

Lima, E. B. C., Sousa, C. N. S., Meneses, L. N., Ximenes, N. C., Santos Júnior, M. A., Vasconcelos, G. S., … Vasconcelos, S. M. M. (2015). *Cocos nucifera* (L.) (*Arecaceae*): A phytochemical and pharmacological review. *Brazilian Journal of Medical and Biological Research*. Associacao Brasileira de Divulgacao Cientifica. https://doi.org/10.1590/1414-431X20154773

Lindström, M., Kiviniemi, K., & Korkeala, H. (2006). Hazard and control of group II (non-proteolytic) *Clostridium botulinum* in modern food processing. *International Journal of Food Microbiology*, *108*(1), 92–104. https://doi.org/10.1016/j.ijfoodmicro.2005.11.003

Linton, M., Connolly, M., Houston, L., & Patterson, M. F. (2014). The control of *Clostridium botulinum* during extended storage of pressure-treated, cooked chicken. *Food Control*, *37*(1), 104–108. https://doi.org/10.1016/j.foodcont.2013.09.042

Liu, Y., Zhao, X. Y., Zou, L., & Hu, X. S. (2013). Effect of high hydrostatic pressure on overall quality parameters of watermelon juice. *Food Science and Technology International*, *19*(3), 197–207.

https://doi.org/10.1177/1082013212442194

Lukas, A. R. (2013). *Use of High Pressure Processing to Reduce Foodborne Pathogens in Coconut Water*. University of Virginia, Blacksburg.

Lund, B. M., Knox, M. R., & Sims, A. P. (1984). The effect of oxygen and redox potential on growth of *Clostridium botulinum* type E from a spore inoculum. *Food Microbiology*, *1*(4), 277–287. https://doi.org/10.1016/0740-0020(84)90062-5

Lyver, A., Smith, J. P., Austin, J., & Blanchfield, B. (1998). Competitive inhibition of *Clostridium botulinum* type E by *Bacillus* species in a value-added seafood product packaged under a modified atmosphere. *Food Research International*, *31*(4), 311–319. https://doi.org/10.1016/S0963-9969(98)00097-0

Mantena, S. K., Jagadish, Badduri, S. R., Siripurapu, K. B., & Unnikrishnan, M. K. (2003). In vitro evaluation of antioxidant properties of *Cocos nucifera* Linn. water. *Molecular Nutrition & Food Research*, *47*(2), 126–131. https://doi.org/10.1002/food.200390023

Marín-Cevada, V., Caballero-Mellado, J., Bustillos-Cristales, R., Muñoz-Rojas, J., Mascarúa-Esparza, M. A., Castañeda-Lucio, M., … Fuentes-Ramírez, L. E. (2010). *Tatumella ptyseos*, an Unrevealed Causative Agent of Pink Disease in Pineapple. *Journal of Phytopathology*, *158*(2), 93–99. https://doi.org/10.1111/j.1439-0434.2009.01575.x

Odlaug, T. E., & Pflug, I. J. (1979). *Clostridium botulinum* growth and toxin production in tomato juice containing *Aspergillus gracilis*. *Applied and Environmental Microbiology*, *37*(3), 496–504.

Pacheco, A., Rodríguez-Sánchez, D. G., Villarreal-Lara, R., Navarro-Silva, J. M., Senés-Guerrero, C., & Hernández-Brenes, C. (2017). Stability of the antimicrobial activity of acetogenins from avocado seed, under common food processing conditions, against *Clostridium sporogenes* vegetative cell growth and endospore germination. *International Journal of Food Science and Technology*, *52*(11), 2311–2323. https://doi.org/10.1111/ijfs.13513

Pandey, R., Ter Beek, A., Vischer, N. O. E., Smelt, J. P. P. M., Kemperman, R., Manders, E. M. M., & Brul, S. (2015). Quantitative analysis of the effect of specific tea compounds on germination and outgrowth of *Bacillus subtilis* spores at single cell resolution. *Food Microbiology*, *45*, 63–70. https://doi.org/10.1016/j.fm.2014.03.006

Peck, M. W., Fairbairn, D. A., & Lund, B. M. (1992). The effect of recovery medium on the estimated heat-inactivation of spores of non-proteolytic *Clostridium botulinum*. *Letters in Applied Microbiology*, *15*(4), 146–151. https://doi.org/10.1111/j.1472-765X.1992.tb00749.x

Perkins, W. E., & Tsuji, K. (1962). Sporulation of *Clostridium botulinum*. II. Effect of arginine and its degradation products on sporulation in a synthetic medium. *Journal of Bacteriology*, *84*, 86–94.

Picouet, P. A., Sárraga, C., Cofán, S., Belletti, N., & Dolors Guàrdia, M. (2015). Effects of thermal

and high-pressure treatments on the carotene content, microbiological safety and sensory properties of acidified and of non-acidified carrot juice. *LWT - Food Science and Technology*, *62*(1), 920–926. https://doi.org/10.1016/j.lwt.2014.07.027

Plowman, J., & Peck, M. W. (2002). Use a novel method to characterize the response of spores of non-proteolytic *Clostridium botulinum* types B E and F to a wide range of germinants and conditions. *Journal of Applied Microbiology*, *92*, 681–694.

Prades, A., Dornier, M., Diop, N., & Pain, J.-P. (2012). Coconut water preservation and processing: a review. *Fruits*, *67*(3), 157–171. https://doi.org/10.1051/fruits/2012009

Reddy, N. R., Solomon, H. M., Fingerhut, G. A., Rhodehamel, E. J., Balasubramaniam, V. M., & Palaniappan, S. (1999). Inactivation of *Clostridium botulinum* type E spores by high pressure processing. *Journal of Food Safety*, *19*(4), 277–288. https://doi.org/10.1111/j.1745-4565.1999.tb00252.x

Rodgers, S., Kailasapathy, K., Cox, J., & Peiris, P. (2004). Co-incubation of *Clostridium botulinum* with protective cultures. *Food Research International*, *37*(7), 659–666. https://doi.org/10.1016/j.foodres.2004.02.007

Santoso, U., Kubo, K., Ota, T., Tadokoro, T., & Maekawa, A. (1996). Nutrient composition of *kopyor* coconuts (*Cocos nucifera* L.). *Food Chemistry*, *57*(2), 299–304. https://doi.org/10.1016/0308-8146(95)00237-5

Sheth, A. N., Wiersma, P., Atrubin, D., Dubey, V., Zink, D., Skinner, G., … Sobel, J. (2008). International Outbreak of Severe Botulism with Prolonged Toxemia Caused by Commercial Carrot Juice. *Clinical Infectious Diseases*, *47*(10), 1245–1251. https://doi.org/10.1086/592574

Skinner, G. E., Solomon, H. M., & Fingerhut, G. A. (1999). Prevention of *Clostridium botulinum* Type A, Proteolytic B and E Toxin Formation in Refrigerated Pea Soup by Lactobacillus plantarum ATCC 8014. *Journal of Food Science*, *64*(4), 724–727. https://doi.org/10.1111/j.1365-2621.1999.tb15119.x

Strasdine, G. A. (1967). Rapid germination of *Clostridium botulinum* type E spores. *Journal of the Fisheries Research Board of Canada*, *24*(3), 595–606.

Strasdine, G. A., & Melville, J. (1968). Growth and Spore Production of *Clostridium botulinum* Type E in Chemically Defined Media. *Journal of the Fisheries Research Board of Canada*, *25*(3), 547–553. https://doi.org/10.1139/f68-046

Stringer, S. C., Haque, N., & Peck, M. W. (1999). Growth from spores of nonproteolytic *Clostridium botulinum* in heat-treated vegetable juice. *Applied and Environmental Microbiology*, *65*(5), 2136–2142.

Terefe, N. S., Delon, A., Buckow, R., & Versteeg, C. (2015). Blueberry polyphenol oxidase: Characterization and the kinetics of thermal and high pressure activation and inactivation.

Food Chemistry, *188*, 193–200. https://doi.org/10.1016/j.foodchem.2015.04.040

U.S. Food and Drug Administration. Public Health Service. (2015a). Warning Letter. Harmless Harvest Thailand. Retrieved May 17, 2020, from https://www.fda.gov/inspections-compliance-enforcement-and-criminal-investigations/warning-letters/harmless-harvest-thailand-11132015

U.S. Food and Drug Administration. Public Health Service. (2015b). Warning Letter. Vital Juice Company, Inc. Retrieved May 17, 2020, from https://www.fda.gov/inspections-compliance-enforcement-and-criminal-investigations/warning-letters/vital-juice-company-inc-05042015

U.S. Food and Drug Administration. (2001). Hazard analysis and critical control point (HACCP); procedures for the safe and sanitary processing and importing of juice; final rule, 21 CFR Part 120. *Federal Register*, *66*, 6137–6202.

Ward, B. Q., & Carroll, B. J. (1966). Spore germination and vegetative growth of *Clostridium botulinum* type E in synthetic media. *Canadian Journal of Microbiology*, *12*, 1145–1156.

Whitmer, M. E., & Johnson, E. A. (1988). Development of improved defined media for *Clostridium botulinum* serotypes A, B, and E. *Applied and Environmental Microbiology*, *54*(3), 753–759.

Whitney, B. M., Williams, R. C., Eifert, J., & Marcy, J. (2007). High-Pressure Resistance Variation of *Escherichia coli* O157:H7 Strains and *Salmonella* Serovars in Tryptic Soy Broth, Distilled Water, and Fruit Juice. *Journal of Food Protection*, *70*(9), 2078–2083. https://doi.org/10.4315/0362-028X-70.9.2078

Yi, J., Kebede, B. T., Hai Dang, D. N., Buvé, C., Grauwet, T., Van Loey, A., … Hendrickx, M. (2017). Quality change during high pressure processing and thermal processing of cloudy apple juice. *LWT - Food Science and Technology*, *75*, 85–92. https://doi.org/10.1016/j.lwt.2016.08.041

Yong, J. W. H., Ge, L., Ng, Y. F., & Tan, S. N. (2009). The chemical composition and biological properties of coconut (*Cocos Nucifera* L.) water. *Molecules*, *14*(12), 5144–5164. https://doi.org/10.3390/molecules14125144

Chapter 3

Influence of acidity and nutrient content on the growth and toxin formation by proteolytic *Clostridium botulinum* and nonproteolytic *Clostridium botulinum* in anoxic tender coconut water at 30 °C

CHAPTER 3

INFLUENCE OF ACIDITY AND NUTRIENT CONTENT ON THE GROWTH AND TOXIN FORMATION BY PROTEOLYTIC *CLOSTRIDIUM BOTULINUM* AND NONPROTEOLYTIC *CLOSTRIDIUM BOTULINUM* IN ANOXIC TENDER COCONUT WATER AT 30 °C

Recent decades have seen an increase in consumer demand for mildly processed chilled foods with high organoleptic and nutritional properties, lower preservative content, and an extended shelf-life. These foods often receive a mild thermal or nonthermal process. Tender coconut water is a delicate tropical beverage that requires a mild process to retain its desirable properties. This makes the control of spore-forming pathogens challenging. Proteolytic *Clostridium botulinum* and nonproteolytic *Clostridium botulinum* are dangerous spore-forming bacteria that produce the highly potent botulinum neurotoxin, and are responsible for foodborne botulism, a severe and deadly intoxication. The purpose of this study was to evaluate the safety of eight tender coconut waters (from different geographical areas and varieties) with respect to proteolytic *C. botulinum* and nonproteolytic *C. botulinum*, and to characterize factors that control growth and toxin formation. Natural tender coconut water (pH 4.7-5.3) was shown to be a poor substrate for growth/neurotoxin formation. Under highly favorable conditions (anoxic sterile coconut water incubated for 50 days at 30 °C), growth/toxin formation by proteolytic *C. botulinum* was detected in only one type of coconut water, while none of the eight coconut waters tested supported growth/toxin formation by nonproteolytic *C. botulinum*. Additionally, it was demonstrated that poor growth/toxin formation in natural tender coconut water was associated with a combination of low pH and nutrient limitation (but not anti-clostridial compounds) for both proteolytic *C. botulinum* and nonproteolytic *C. botulinum*. Future risk assessments of chilled mildly processed tender coconut water should consider these results together with previously published work showing that the pathogen failed to grow in the unmodified beverage under refrigeration and temperature abuse conditions. Poor growth and toxin production observed in this study in anoxic and sterile coconut water incubated for 50 days at 30 °C confirms that the beverage (pH 4.7-5.3) is a poor substrate for growth/neurotoxin formation by proteolytic *C. botulinum* and nonproteolytic *C. botulinum* and ensures safety under commercial conditions.

1. Introduction

Increasing consumer demand for minimally processed foods with improved organoleptic and nutritional profiles, an extended shelf-life and reduced preservative content is driving the food industry to adopt alternate processing methods to ensure microbiological safety without compromising quality attributes. To avoid intensive heat processing, the use of nonthermal technologies such as UV-based irradiation, pulsed electric fields and high pressure processing (HPP) have emerged in recent decades (Barba, Koubaa, do Prado-Silva, Orlien, & Sant'Ana, 2017). Coconut water is a delicate beverage that benefits from milder processing since it contains heat sensitive bioactive and flavor constituents and is susceptible to color changes caused by traditional heat treatments (Awua, Doe, & Agyare, 2011; Ma et al., 2019; Yannam et al., 2020). Vegetative pathogens such as *Escherichia coli*, *Listeria monocytogenes* and *Salmonella* spp. can be effectively controlled in this tropical beverage by means of nonthermal technologies (Bhullar et al., 2018; Lukas, 2013). However, the control of spore-forming bacteria is challenging. HPP for instance did not significantly reduce the *Clostridium botulinum* spore concentration in coconut water when applied at typical industrial conditions (González-Angulo et al., 2020), and more than twice the UV-C dose required to achieve a 5-$\log_{10}$ reduction of vegetative pathogens was needed to obtain an equivalent inactivation of *Bacillus cereus* and *C. sporogenes* spores (Bhullar et al., 2018; Pendyala, Patras, Gopisetty, Sasges, & Balamurugan, 2019). This represents a potential limitation for food products that might potentially support spore germination, cell multiplication and toxin formation by *C. botulinum* during subsequent refrigerated storage.

Clostridium botulinum is an anaerobic spore-forming pathogen present in many different environments. When conditions become favorable, it can multiply in food and produce the botulinum neurotoxin that is responsible for botulism, a deadly disease. Proteolytic (Group I) *C. botulinum* and nonproteolytic (Group II) *C. botulinum* are physiologically and genetically different, and almost exclusively implicated in foodborne botulism (Peck, 2006, 2009). The former does not grow below 10 °C and pH <4.6, whereas the latter can grow and produce toxin at temperatures as low as 3.0 °C and pH >5.0 (Graham, Mason, Maxwell, & Peck, 1997). The control of nonproteolytic *C. botulinum* through refrigerated storage can be considered a valid approach to reduce the risk in minimally processed chilled juices. However, botulism outbreaks from temperature-abused and pasteurized commercial chilled products (*e.g.* carrot juice, Sheth et al., 2008) have prompted the Food and Drug Administration (FDA) to require food processors to implement additional control measures for both *C. botulinum* groups in refrigerated juices with pH >4.6 (U.S. Food and Drug Administration, 2007). Based on this, the FDA issued warning letters to companies using HPP to process tender coconut water stating

that their HACCP plans should include control measures to consistently achieve a 5-$\log_{10}$ reduction of *C. botulinum* spores (both proteolytic *C. botulinum* and nonproteolytic *C. botulinum*), which was considered the pertinent pathogen in the low-acid juice since HPP at the conditions used was not sufficient to deliver the required level of control (U.S. Food and Drug Administration, 2015a, 2015b).

If the beverage is not processed to eliminate or prevent the growth from *C. botulinum* spores, then other control factors need to be considered. The high water activity of coconut water (>0.99) is unlikely to prevent growth and neurotoxin formation. On the other hand, pH is a potential control factor. Tender coconut water typically has a pH in the range of 4.7 to 5.6 when the fruit is harvested after 6 to 8 months of maturation in palm trees (Chidambaram, Singaraja, Prasanna, Ganesan, & Sundararajan, 2013; Jackson, Gordon, Wizzard, McCook, & Rolle, 2004). It is a common practice to blend coconut water with more acidic juices. However, other intrinsic and extrinsic factors could also play a role in restricting growth and toxin formation, and should also be considered in evaluating the risk of foodborne botulism in minimally processed coconut water. González-Angulo et al. (2020) demonstrated that viable counts of nonproteolytic *C. botulinum* type E and *Clostridium* spp. did not increase in HPP tender coconut water even when the beverage was modified to optimal growth conditions (*i.e.* pH 7.0, incubation temperature of 30 °C under anoxic atmosphere and removal of competing microorganisms). The authors proposed that this could be due to a lack of specific nutrients required by *C. botulinum*. Additionally, fruits and vegetables can contain a wide range of antimicrobial agents (Davidson, Branen, & Sofos, 2005). Traditional heat pasteurization processes may destroy heat sensitive compounds, whereas nonthermal processing technologies tend to preserve them in the final product. Patterson et al. (2012) reported that after HPP (600 MPa for 1 min) carrot juice retained its anti-listerial activity (Beuchat & Brackett, 1990; Nguyen-The & Lund, 1991), whereas a heat treatment (92 °C for 20 s) eliminated anti-listerial activity. Minimally processed chilled foods often have a shorter commercial shelf-life because not all spoilage microorganisms are inactivated and may multiply during subsequent refrigerated storage. This could impact on growth from spores of *C. botulinum*, as substrate availability would be reduced, the pH could decrease or antimicrobial substances (*e.g.* bacteriocins) may be released (Lyver, Smith, Austin, & Blanchfield, 1998; Ramaroson et al., 2018; Skinner et al., 2015).

The present study aimed to establish whether *C. botulinum* should be considered the pathogen of concern in minimally processed chilled tender coconut water. To this end, growth and toxin production from spores of proteolytic *C. botulinum* and nonproteolytic *C. botulinum* was assessed in a range of tender coconut waters from various geographical origins and

varieties. Intrinsic characteristics of the tropical beverage were modified for the purpose of characterizing factors that control spore germination, cell multiplication and formation of botulinum neurotoxin.

2. Materials and methods

2.1. *C. botulinum* strains and spore preparation

Two spore cocktails were prepared containing an equal concentration of spores of either nine proteolytic *C. botulinum* (*C. botulinum* Group I) strains or nine nonproteolytic *C. botulinum* (*C. botulinum* Group II) strains. Proteolytic *C. botulinum* strains (toxin subtype) used in this study were 62A (A1), ATCC 3502 (A1), NCTC 9837 (A2), NCTC 2012 (A3), NCTC 7273 (B1), 213B (B2), 2045/98 (B2), NCTC 3807 (B7), and Langeland (F1). Nonproteolytic *C. botulinum* strains (toxin subtype) were Eklund 17B (B4), Eklund 2B (B4), CDC 5900 (B4), Kapchunka B9 (B4), Beluga (E1), CDC 7854 (E1), Alaska (E3), CB-K-36E (E6) and Craig 610 (F6). Further strain details have been given previously (Brunt et al., 2020a; Brunt et al., 2020b).

Spores of *C. botulinum* were produced in Robertson's cooked meat medium (Southern Group Laboratory, Corby, UK) incubated for 7-14 days at 30 °C. Spores were separated from meat particles using 20 µm Steriflip centrifuge tube filter units (Millipore, Watford, UK) followed by centrifugation at 4000×g and 2 °C for 20 min, washed five times in 20 mL of sterile 0.85 % (w/v) saline, resuspended in sterile saline and stored at 2 °C. Spore suspensions were enumerated on peptone, yeast extract, glucose, starch (PYGS) agar (Stringer, Haque, & Peck, 1999), incubated for 48 h at 30 °C under a headspace of 10 % CO_2/ 90 % H_2. Proteolytic activity was determined on Reinforced Clostridial Medium agar (Oxoid, Basingstoke, UK) containing 5 % (w/v) skim milk incubated under the same conditions. The absence of aerobic contamination was established by plating onto PYGS agar, incubated aerobically at 30 °C for 48h. Washed spores were stored at 2 °C prior to use.

2.2. Preparation of sterile anoxic coconut water, inoculation with *C. botulinum* and evaluation of growth/toxin formation

Eight different coconut waters extracted from coconuts harvested at the tender stage (6-8 months) were used in this study. They were selected to ensure a wide geographical spread in the origin of the raw materials (Table 13). Thai coconut waters (T1 to T4) were from different commercial brands and each was provided by a different supplier. Prior to inoculation, any microbiota naturally present in the coconut waters was removed by irradiating at 25 kGy (Aragogamma, Barcelona, Spain). For certain experiments, the pH of coconut water was adjusted by the addition of sterile 1 M KOH solution. The original redox potential of the coconut

waters was approximately +300 mV. Natural and pH-adjusted coconut waters were aseptically transferred to sterile 500 mL Duran bottles, deoxygenated by bubbling with anaerobic gas mixture (10 % H_2/90 % N_2) for 1 h at room temperature, and transferred to an anaerobic cabinet under an atmosphere of 10 % CO_2/10 % H_2/80 % N_2.

Table 13. Details of the coconut waters used in the study.

Code	Geographical origin	Cultivar
BE	Belize	Pacific Tall × Yellow Dwarf
BR	Brazil	Brazilian Green Dwarf
SL	Sri Lanka	King Coconut (*aurantiaca*)
T1	Thailand	Thailand Green Dwarf (*Nam Hom*)
T2	Thailand	Thailand Green Dwarf (*Nam Hom*)
T3	Thailand	Thailand Green Dwarf (*Nam Hom*)
T4	Thailand	Thailand Green Dwarf (*Nam Hom*)
TP	The Philippines	Pacific Tall

Anaerobic PYGS broth was prepared as described elsewhere (Stringer et al., 1999) at its natural pH (7.2) or adjusted with sterile 1 M HCl. For some experiments coconut water was supplemented with PYGS broth. Aliquots (10 or 20 mL) of natural or pH-adjusted coconut water, 1:1 mixtures of coconut water with anaerobic PYGS broth or PYGS broth were transferred to sterile 30 mL glass universal tubes or 16×125 mm Hungate tubes, containing an inverted Durham tube, to detect gas production, and stored at 2 °C until inoculation. Time to gas formation was periodically monitored and observations are shown in Table A4 to Table A9 of the Appendix to Chapter 3.

The ability of both proteolytic *C. botulinum* and nonproteolytic *C. botulinum* to multiply and form botulinum neurotoxin in the various media at different pH values was assessed. A minimum volume of each spore cocktail was added to samples in glass universal bottles (in triplicate) or Hungate tubes (five replicates) to ensure that all inoculated samples contained approximately 20,000 spores. Cultures were incubated for 50 days at 30 °C and inspected for evidence of growth (gas production and/or turbidity), then stored at -20 °C before testing for presence of botulinum neurotoxin genes (*bont*) and botulinum neurotoxin (BoNT) as described below. Proteolytic *C. botulinum* and nonproteolytic *C. botulinum* type F strains were excluded from the spore cocktails in the last experiment where the effect of pH on *C. botulinum* growth and toxin formation in representative types of coconut water was assessed because it is an uncommon cause of human disease.

2.3. Preparation of nutrient-supplemented anoxic coconut water, inoculation with *C. botulinum* and evaluation of growth/toxin formation.

To evaluate the presence of potential inhibitory compounds naturally present in coconut water and to assess the impact of specific groups of nutrients, samples of T2 coconut water were supplemented with PYGS broth at decreasing concentrations (*i.e.* 50, 25, 12.5, 6.25, 3.125 and 1.56 %) and combinations of amino acids, minerals, and vitamins (additional information on final nutrient concentrations and different combinations studied can be found in Table A2 and Table A3 of the Appendix to Chapter 3). Individual nutrients (all from Sigma, Poole, UK) were selected based on the defined medium described by Whitmer and Johnson (Whitmer & Johnson, 1988). Resazurin, cysteine hydrochloride, glucose, adenine and sodium acetate were not included as they were considered not essential. L-alanine and sodium L-lactate were incorporated in the amino acid and mineral nutrient groups, respectively, as germinant compounds (Brunt et al., 2018) (Table A2 of the Appendix to Chapter 3). Coconut water T2 and PYGS broth were both adjusted with sterile 1 M KOH or 1 M HCl to pH 5.5 and 6.0. Then, samples were inoculated with the proteolytic *C. botulinum* or nonproteolytic *C. botulinum* spore cocktails targeting a final concentration of 1000 spores/mL. The time to turbidity was determined using a Bioscreen C reader (Lab Systems, Finland) installed in an anaerobic cabinet. Appropriate volumes of anaerobic T2 coconut water and PYGS broth prepared as previously described were dispensed into each well of a Bioscreen plate to the desired concentrations. A total of four-well replicates for each PYGS concentration and *C. botulinum* group were dispensed and individually inoculated to a final volume of 400 µL and a final concentration of 1000 spores/mL. Filled plates were placed into the Bioscreen C reader and incubated at 30 °C for 14 days. Optical density at 600 nm (OD_{600}) was measured at 1 h intervals. The time for detectable turbidity was defined as the time taken for measured OD_{600} to reach 0.14 units (Stringer, Webb, George, Pin, & Peck, 2005). Control medium had an OD_{600} of 0.09 units. Additionally, the effect of different groups of defined nutrients on *C. botulinum* growth was evaluated. Amino acid, mineral and vitamin were dissolved in distilled water at 10X, 20X and 100X the final concentration (see Table A2 of the Appendix to Chapter 3), respectively, and filter sterilized. Aliquots of each nutrient group and all possible combinations (see Table A3 of the Appendix to Chapter 3) were added to T2 coconut water and adjusted to pH 5.5 or 6.0 before oxygen removal. One-milliliter aliquots were dispensed in triplicate in 1.5 mL sterile microcentrifuge tubes in an anaerobic cabinet and inoculated with the proteolytic *C. botulinum* or nonproteolytic *C. botulinum* spore cocktails to a final concentration of 1000 spores/mL. Samples were incubated at 30 °C for 7 days and stored at -20 °C before testing for presence of *bont* genes by multiplex PCR.

2.4. Determination of physicochemical parameters

The pH of deoxygenated coconut water was determined in duplicate tubes. Redox potentials were measured in duplicate tubes in an anaerobic cabinet using a Mettler Toledo Pt4805-DPAS-SC-K8S redox probe connected to a pH211 meter (Hanna Instruments, Leighton Buzzard, UK). Standard redox potential (E_h) was calculated as $E_h = E + E_{ref}$ where E is the observed redox potential and E_{ref} is the temperature dependent redox potential of the internal electrolyte (3 M KCl silver/silver chloride) of the electrode.

2.5. Multiplex PCR test for detection of *C. botulinum* neurotoxin genes (*bont*)

Samples frozen at the end of incubation were thawed at 4 °C and DNA extracted from 1 mL aliquots of each sample using PrepMan Ultra reagent (Applied Biosystems, Warrington, UK) according to manufacture instructions. The presence of *C. botulinum* neurotoxin genes (*bont*) was determined using a multiplex PCR test as described previously (Lindström et al., 2001; Peck et al., 2010).

2.6. ELISA tests for detection of *C. botulinum* neurotoxin (BoNT)

Enzyme-linked immunosorbent assays (ELISAs) for *C. botulinum* neurotoxins were used to detect toxin production in samples of natural and pH-adjusted coconut water. Five replicate samples for each condition tested were pooled together at the end of incubation and tested for botulinum neurotoxins A, B and E by ELISA as described previously (Peck, Webb, & Goodburn, 2020). Uninoculated samples for each condition were used as negative controls, and PYGS broth inoculated with proteolytic *C. botulinum* or nonproteolytic *C. botulinum* spore cocktails and incubated at 30 °C served as positive controls. Samples were considered positive for botulinum neurotoxins A, B and E if their mean absorbance was greater than the absorbance given by a neurotoxin standard containing 20 pg toxin/mL that was prepared in an extract of uninoculated coconut water. This detection limit is comparable to the mouse bioassay and several orders of magnitude lower than the human lethal oral dose (Anonymous, 2018).

3. Results and discussion

All experiments were conducted on a worst-case scenario consisting of anoxic and sterile coconut water (from -139 to -383 mV) incubated at 30 °C. Low redox potentials not only ensure absence of dissolved oxygen in the beverage, but also facilitate growth of obligate anaerobes. Cold sterilization by gamma-irradiation maintained the composition of the coconut waters unaffected and prevented interference from endogenous microflora. Moreover,

independent nine-strain cocktails of proteolytic *C. botulinum* and nonproteolytic *C. botulinum*, respectively, were used to cover wide variability range. A high spore inoculum was considered (1000 spores/mL) to increase the probability of germination and subsequent vegetative growth of a fraction of spores. Finally, pH of tender coconut water was modified and adjusted more favorable values (from 5.5 to 6.7) in some experiments. The aim of this scenario was to assess growth potential of *C. botulinum* in the beverage, and to identify the intrinsic parameters that influence growth of the pathogen.

3.1. Limited growth of proteolytic *C. botulinum* and nonproteolytic *C. botulinum* in natural and pH-adjusted anoxic tender coconut water

Growth from proteolytic *C. botulinum* and nonproteolytic *C. botulinum* spore cocktails was assessed in natural (pH 4.7-5.3) and pH-adjusted (5.5-5.8) anoxic coconut water from eight different geographical origins. Additionally, growth was also determined in 1:1 mixtures of each natural coconut water with PYGS broth (Table 14). There was no evidence of growth (gas formation or *bont* gene detection) in any of the natural coconut water samples. Similarly, all pH-adjusted samples of coconut water except one (T2) yielded negative results for gas production and *bont* gene detection. Gas formation was detected within 34 days of incubation in one of the three replicates of pH-adjusted T2 coconut water (pH 5.6) inoculated with the proteolytic *C. botulinum* spore cocktail. Multiplex PCR confirmed presence of *bont/A* and *bont/B* at the end of the incubation period. All PYGS-supplemented coconut water samples were positive for growth (gas formation and *bont* gene detection) of proteolytic *C. botulinum* and nonproteolytic *C. botulinum* in the 50-day incubation period (Table 14). Despite an optimum temperature (30 °C) (Graham, Mason, & Peck, 1996), redox potential (-146 to -272 mV) (Lund, 1993) and the absence of competing microorganisms, none of the natural and pH-adjusted coconut water samples supported growth of nonproteolytic *C. botulinum*. This is probably attributed to the naturally low pH of the beverage, which is slightly above or even below the minimum value reported for growth of nonproteolytic *C. botulinum* (pH 5.0) (Graham et al., 1997). Despite more favorable conditions in pH-adjusted samples, a lack of growth may be due to additional hurdles such as suboptimal nutritional conditions restricting spore germination and/or cell multiplication, or a combination of intrinsic factors (Stringer et al., 1999). Although growth of proteolytic *C. botulinum* is well documented above pH 4.6 (Wong, Young-Perkins, & Merson, 1988), a combination of the above mentioned intrinsic factors may account for the lack of growth in natural and, except for one replicate sample of T2, all pH-adjusted coconut waters. Since supplementation of coconut water with PYGS broth supported vigorous growth from both proteolytic *C. botulinum* and nonproteolytic *C. botulinum* spores, it is unlikely that there are natural antimicrobial compounds present in the beverage that inhibit

spore germination and/or cell multiplication. The higher pH resulting from supplementing coconut water with PYGS (between 5.7 and 6.1), the additional nutrients provided by the laboratory medium, or a combination of these factors play an important role in promoting growth in the tropical beverage.

Table 14. Growth of proteolytic *C. botulinum* and nonproteolytic *C. botulinum* cocktails (determined as gas production and botulinum neurotoxin gene detection by multiplex PCR) in natural, pH-adjusted and PYGS-supplemented (+PYGS) coconut water from various geographical origins after 50 days of incubation at 30 °C.

Sample type		pH[a]	E_h (mV)[a]	Proteolytic *C. botulinum*		Nonproteolytic *C. botulinum*	
				Gas	PCR	Gas	PCR
BE	Natural pH	5.0	-164	–[b]	–	–	–
	pH-adjusted	5.8	-190	–	–	–	–
	+PYGS	6.1	-230	+[c]	A, B	+	B
BR	Natural pH	4.7	-146	–	–	–	–
	pH-adjusted	5.7	-263	–	–	–	–
	+PYGS	5.7	-200	+	A, B	+	B, E
SL	Natural pH	4.7	-219	–	–	–	–
	pH-adjusted	5.6	-270	–	–	–	–
	+PYGS	5.7	-235	+	A, B	+	B, E, F
T1	Natural pH	5.3	-243	–	–	–	–
	pH-adjusted	5.5	-251	–	–	–	–
	+PYGS	6.1	-230	+	A, B	+	B
T2	Natural pH	5.2	-237	–	–	–	–
	pH-adjusted	5.6	-243	+	A, B	–	–
	+PYGS	6.1	-240	+	A, B	+	B, E, F
T3	Natural pH	5.2	-256	–	–	–	–
	pH-adjusted	5.6	-272	–	–	–	–
	+PYGS	6.1	-263	+	A, B	+	B, E
T4	Natural pH	5.2	-246	–	–	–	–
	pH-adjusted	5.6	-269	–	–	–	–
	+PYGS	6.1	-250	+	A, B	+	B, E, F
TP	Natural pH	5.2	-253	–	–	–	–
	pH-adjusted	5.6	-255	–	–	–	–
	+PYGS	6.1	-212	+	A, B	+	B, E, F

[a]Mean value (n = 2).

[b]Gas production and/or botulinum neurotoxin genes not detected in any of the five replicates.

[c]Gas production observed in at least one of the five replicates.

3.2. Effect of pH on proteolytic *C. botulinum* and nonproteolytic *C. botulinum* growth in anoxic tender coconut water T2

Coconut water T2 was chosen for further study as it was the only coconut water tested in which growth was recorded; albeit, in only one replicate tube inoculated with proteolytic *C. botulinum* and the coconut water adjusted to a higher pH (5.6). This suggests that the intrinsic characteristics of this particular coconut water may be more favorable to *C. botulinum* growth than others and, therefore, best suited to investigate the factors that control growth in coconut water. Growth was assessed (gas production and *bont* gene detection) during 50 days of incubation at 30 °C for separate proteolytic *C. botulinum* and nonproteolytic *C. botulinum* spore cocktails in T2 coconut water, a 1:1 mixture of T2 coconut water with PYGS broth and PYGS broth, all at four different pH values (Table 15).

Table 15. Growth of proteolytic *C. botulinum* and nonproteolytic *C. botulinum* (determined as gas production and botulinum neurotoxin gene detection by multiplex PCR) in T2 coconut water, mixtures of T2 coconut water with PYGS broth (T2+PYGS) and PYGS broth adjusted to different pH values after 50 days of incubation at 30 °C.

Sample type	pH[a]	Eh (mV)[a]	Proteolytic *C. botulinum*		Nonproteolytic *C. botulinum*	
			Gas	PCR	Gas	PCR
T2	5.2	-226	–[b]	–	–	–
	5.5	-261	–	–	–	–
	6.0	-296	+[c]	B	–	–
	6.4	-286	+	A, B	+	B
T2+PYGS	5.2	-223	+	A	+	–[d]
	5.5	-198	+	A, B	+	B, E, F
	6.3	-263	+	A, B	+	B, E
	6.7	-241	+	A, B	+	B, E, F
PYGS	5.3	-178	+	A, B	+	–[d]
	5.5	-139	+	A, B	+	B, E
	6.2	-231	+	A, B	+	B, E
	6.9	-223	+	A, B, F	+	B, E

[a]Mean value (n = 2).
[b]Gas production and/or botulinum neurotoxin genes not detected in any of the five replicates.
[c]Gas production observed in at least one of the five replicates.
[d]Botulinum neurotoxin genes were not detected despite gas production being observed.

Gas production was observed within the first 24 to 48 h of incubation, regardless of pH, in all samples of PYGS broth and mixtures of coconut water with PYGS broth inoculated with the proteolytic *C. botulinum* and nonproteolytic *C. botulinum* spore cocktails. The detection of botulinum neurotoxin gene (*bont*) by PCR after the 50-day incubation period confirmed

growth of both *C. botulinum* groups in all samples of PYGS broth and coconut water supplemented with PYGS broth (except in pH 5.2 samples inoculated with nonproteolytic *C. botulinum*, where gas formation was observed but neurotoxin genes were not detected). A positive PCR result requires spores to germinate and cells to multiply to a concentration that exceeds the detection limit of the PCR assay. The mean detection limit in PYGS medium was as 2×10^6 CFU/mL and 5×10^6 CFU/mL for proteolytic *C. botulinum* and nonproteolytic *C. botulinum*, respectively. Thus, it is possible that nonproteolytic *C. botulinum* did not reach this cell concentration at pH 5.2, which is close to the growth limit (Graham et al., 1997). Growth and toxin formation by both *C. botulinum* groups is widely reported at the pH values used in the present study (pH 5.3-6.9) (Graham et al., 1996; Lund, Graham, & Franklin, 1987). Growth in 1:1 mixtures of T2 coconut water and PYGS broth align with and extend previous observations (Table 14), with growth now reported at lower pH values (pH 5.2 and 5.5). These observations further support the hypothesis that coconut water does not contain anti-clostridial compounds that inhibit spore germination and/or cell multiplication, since vigorous growth was observed at suboptimal pH even in half-strength PYGS broth when coconut water was used as diluent. None of the five replicates of natural T2 coconut water (pH 5.2) or coconut water at pH 5.5 inoculated with spores of proteolytic *C. botulinum* or nonproteolytic *C. botulinum* showed evidence of growth (gas production or *bont* gene detection; Table 15). This is consistent with previous observations, although interestingly one of the three replicates of T2 coconut water previously adjusted to pH 5.6 tested positive for proteolytic *C. botulinum* growth (Table 14). This suggests that pH 5.5-5.6 may be borderline for proteolytic *C. botulinum* growth in T2 coconut water.

Higher pH values supported growth of proteolytic *C. botulinum* and nonproteolytic *C. botulinum* in at least one of the five replicate tubes of T2 coconut water (Table 15). Gas production was observed after 23 and 9 days of incubation in samples adjusted to pH 6.0 and 6.4, respectively, inoculated with proteolytic *C. botulinum*. Samples inoculated with nonproteolytic *C. botulinum* only showed gas production at pH 6.4 after 14 days of incubation. Multiplex PCR confirmed the presence of *bont* genes after the 50-day incubation at 30 °C in samples where gas production was observed (Table 15). Other vegetable products have also been reported to support growth and toxin production of both *C. botulinum* groups at lower pH values at 30 °C, such as cooked purées of spinach, potato, bean sprouts, cauliflower, asparagus, sweet corn, courgette, broccoli, sweet potato, white cabbage, leek and kale (all with pH between 5.0 and 5.8) (Carlin & Peck, 1995). The nonproteolytic *C. botulinum* type B strain Eklund 17B (which was included in the nonproteolytic *C. botulinum* spore cocktail in the present study) was able to multiply and produce botulinum neurotoxin in broccoli, potato and turnip juices (pH between 6.1 and 5.5) within 4, 11 and 14 days of anoxic incubation at 30 °C,

respectively (Stringer et al., 1999). Moreover, some nonproteolytic *C. botulinum* strains also showed the ability to multiply and produce toxin at a much lower temperature (10 °C) in cauliflower, broccoli, asparagus and kale cooked purées (Carlin & Peck, 1996). Another study reported that broccoli (pH 5.9) and potato purées (pH 5.8) also supported growth of four proteolytic *C. botulinum* strains (including 62A, 213B and NCTC 7273 were also used in the current study). Time for cell populations to increase 1,000-fold in both purées varied between 25 h and 5 days, depending on the strain and when samples were incubated under anoxic conditions at 30 °C (Braconnier, Broussolle, Dargaignaratz, Nguyen-The, & Carlin, 2003). Examples of commercially available juices and beverages that supported growth of proteolytic *C. botulinum* and led to botulism outbreaks include pasteurized carrot juice (pH between 6.0 and 7.0) (Sheth et al., 2008) and herbal tea infusions (pH between 5.1 and 7.6) (Kim et al., 2019). A very high concentration of botulinum neurotoxin type A was detected in the carrot juice. The inability of proteolytic *C. botulinum* and nonproteolytic *C. botulinum* to grow in T2 coconut water at pH 5.5 and 6.0, respectively, but with abundant growth visualized in nutrient-rich PYGS broth and mixtures of T2 coconut water with PYGS below these suboptimal pH values suggests that other intrinsic factors besides acidity may prevent spore germination and/or cell multiplication in natural coconut water. This is supported by other work in which botulinum neurotoxin production did not always correlate with the pH of the tested food (Carlin & Peck, 1995). Growth observed in T2 coconut water at pH 6.0 and 6.4 for proteolytic *C. botulinum* and nonproteolytic *C. botulinum*, respectively, confirmed that increased acidity combined with other yet unidentified factors represented a hurdle that prevented spore germination and/or cell multiplication at a lower pH. Combination of different hurdles has been previously postulated as a strategy to reduce the risk of growth and neurotoxin formation compared with the risk at optimal conditions (Graham & Lund, 1987).

3.3. Effect of nutrient supplementation on growth of proteolytic *C. botulinum* and nonproteolytic *C. botulinum* in anoxic tender coconut water T2

Aiming to establish the intrinsic factors in coconut water that in combination with pH may limit growth of *C. botulinum*, T2 coconut water was adjusted to the highest pH value at which growth was not previously observed (*i.e.* pH 5.5 for proteolytic *C. botulinum* and pH 6.0 for nonproteolytic *C. botulinum*) and supplemented with (i) PYGS broth at decreasing concentrations adjusted to the same pH values, and (ii) different groups of nutrients according to the minimal medium developed by Whitmer and Johnson (Whitmer & Johnson, 1988) (see Figure A2 and Figure A3 of the Appendix to Chapter 3). Optical density (600 nm) measurements were used to assess growth from spores in PYGS-supplemented samples (Figure 16) and *bont* gene detection to determine growth in nutrient-supplemented samples

incubated at 30 °C under strict anaerobic conditions. Turbidity was detected for both *C. botulinum* groups within the first 24 h of incubation in 100 % PYGS control samples and in T2 coconut water samples supplemented with 50 % and 25 % PYGS broth (Figure 16), which is in agreement with previous observations in 1:1 mixtures of coconut water with PYGS broth medium at pH 5.5 and 6.0 (Table 15). Decreased concentrations of PYGS broth also supported growth from spores, but times to turbidity increased to 24±1 h and 165±74 h for proteolytic *C. botulinum* and to 52±49 h and 264±46 h for nonproteolytic *C. botulinum* in samples supplemented with 12.5 % and 6.25 % PYGS broth, respectively. Interestingly, a PYGS concentration of 3.125 % was sufficient to stimulate growth of proteolytic *C. botulinum* (turbidity detected after 325±22 h), but not nonproteolytic *C. botulinum* (turbidity not detected during the 14-day incubation period, Figure 16).

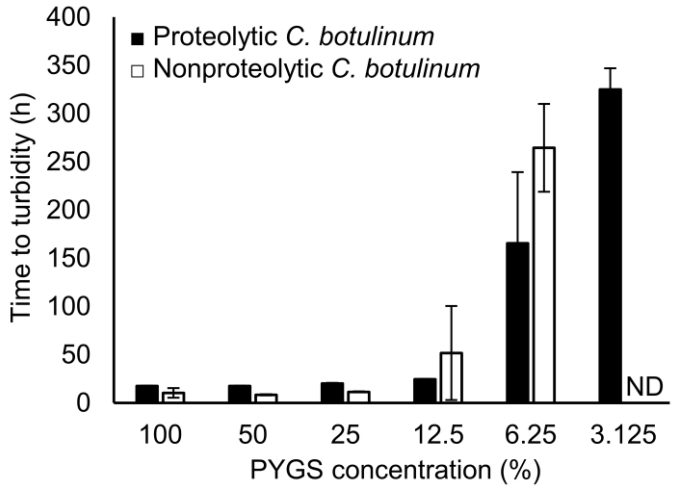

Figure 16. Time to turbidity at 30 °C based on OD_{600} in mixtures of T2 coconut water and PYGS broth at different concentrations adjusted to pH 5.5 for the proteolytic *C. botulinum* cocktail and to pH 6.0 for the nonproteolytic *C. botulinum* cocktail (ND: turbidity not detected after 14 days of incubation).

It was previously reported that supplementing tender coconut water T2 with 12.5 % tryptone-peptone-glucose-yeast extract (TPGY) broth medium at pH 7.0 also supported growth of nonproteolytic *C. botulinum* and *Clostridium* spp. within 24 h under the same incubation conditions used in this study, while lower concentrations were ineffective (González-Angulo et al., 2020). The observations of turbidity even when 3.125 to 6.25 % PYGS broth was used to supplement T2 coconut water (Figure 16) strongly supports the hypothesis of an absence of natural anti-clostridial substances in coconut water (at least at a sufficient concentration to inhibit *C. botulinum* spore germination and cell multiplication). When combinations of different groups of nutrients were added to T2 coconut water adjusted to pH 5.5 (proteolytic *C. botulinum*) or pH 6.0 (nonproteolytic *C. botulinum*), multiplex PCR detected *bont/B* gene after

7 days of incubation at 30 °C in samples inoculated with proteolytic *C. botulinum* and supplemented with thirteen free amino acids (Table A2 of the Appendix to Chapter 3). Other *bont* genes were not detected even when combinations of all groups of nutrients (amino acids + minerals + vitamins) were used to supplement coconut water. The detection of neurotoxin genes in samples inoculated with the nonproteolytic *C. botulinum* spore cocktail required the supplementation of T2 coconut water with the nutrient sets of amino acids + minerals + vitamins. Type F *bont* gene was detected after 7 days of incubation at 30 °C, but not *bont/B* or *bont/E*. Previous published work revealed that supplementing tender T2 coconut water adjusted to pH 7.0 with 2 % casein hydrolysate (free amino acid source) did not stimulate growth of nonproteolytic *C. botulinum* type E (González-Angulo et al., 2020). Although the final concentration of all sets of nutrients in T2 coconut water corresponded to that described in defined media developed to support growth of proteolytic *C. botulinum* and nonproteolytic *C. botulinum* (Ward & Carroll, 1966; Whitmer & Johnson, 1988), the failure of strains of some toxin types to grow could be attributed to the lower pH of coconut water samples (5.5 and 6.0) compared to that of the defined media (between 7.0 and 7.4), or different nutrient requirements among *C. botulinum* groups or even individual strains within the same group (Gullmar & Molin, 1967; Hawirko, Naccarato, Lee, & Maeba, 1979). This hampers identification of the specific nutrients that are limiting in coconut water which may limit *C. botulinum* growth in the beverage.

3.4. Effect of pH on growth and toxin formation by proteolytic *C. botulinum* and nonproteolytic *C. botulinum* in five representative anoxic tender coconut waters

Growth of *C. botulinum* (based on gas production and toxin gene detection) and formation of botulinum neurotoxin (based on detection by ELISA) was determined in natural and pH adjusted coconut waters (Table 16). After incubation at 30 °C for 50 days, growth/toxin formation were not detected in any of the coconut waters tested at their natural pH (4.7 - 5.2) except for proteolytic *C. botulinum* in coconut water T2, where the concentration of botulinum toxin type B exceeded the detection limit of 20 pg/mL of coconut water (Table 16). Growth of proteolytic *C. botulinum* in natural coconut water T2 (pH 5.2) was weak (and not detected as gas formation or *bont* gene, Table 14 to Table 16), and led to the formation of a small quantity of botulinum toxin (79 pg/mL, which equals to 10 MLD_{50}/mL, defined as fifty percent median mouse lethal doses per milliliter), substantially less than that in coconut water T2 (pH 6.8) and PYGS positive control. This contrasts with a botulinum toxin concentration more than four orders of magnitude higher (660,000 MLD_{50}/mL) reported in carrot juice associated with a severe outbreak of foodborne botulism (Sheth et al., 2008). It is estimated that approximately 30,000 pg or 3,000 MLD_{50} of botulinum toxin is sufficient to induce botulism in human adults (Peck et al., 2006).

The absorbance in the type B ELISA for natural coconut water T1 (pH 5.2) was marginally above that of background samples but below the detection limit of 20 pg/mL of coconut water. Growth/toxin formation were also not detected in any of the coconut waters tested at pH 5.5 except for proteolytic *C. botulinum* in coconut waters T1 and T2 (Table 16). Growth (gas formation and *bont* gene detection) were previously reported for proteolytic *C. botulinum* in coconut water T2 at pH 5.5 (Table 14), and now formation of botulinum neurotoxin has been confirmed (Table 16). However, neurotoxin formation by proteolytic *C. botulinum* was also detected in natural coconut water T2 (pH 5.2), and in pH adjusted (pH 5.5) coconut water T1, although gas formation and *bont* genes were not detected (Table 16).

Table 16. Growth (determined as gas production and botulinum neurotoxin gene detection) and neurotoxin formation (detected in ELISA) by proteolytic *C. botulinum* and nonproteolytic *C. botulinum* in coconut waters from various geographical origins adjusted to different pH values after 50 days of incubation at 30 °C.

Coconut water	pH[a]	E_h (mV)[a]	Proteolytic *C. botulinum*			Nonproteolytic *C. botulinum*		
			Gas	PCR	ELISA	Gas	PCR	ELISA
BE	5.0	-252	–[b]	–	–	–	–	–
	5.5	-314	–	–	–	–	–	–
	6.7	-383	+[c]	–[d]	B	+	–[d]	–[e]
SL	4.7	-263	–	–	–	–	–	–
	5.5	-310	–	–	–	–	–	–
	6.2	-350	–	–	–	–	–	–
T1	5.2	-296	–	–	–	–	–	–
	5.5	-309	–	–	B	–	–	–
	6.5	-363	+	A, B	B	–	–	–
T2	5.2	-279	–	–	B	–	–	–
	5.5	-309	+	B	B	–	–	–
	6.2	-349	+	B	A, B	+	–[d]	–[e]
TP	5.2	-293	–	–	–	–	–	–
	5.5	-306	–	–	–	–	–	–
	6.3	-350	+	–[d]	B	+	–[d]	–[e]

[a]Mean value (n = 2).

[b]Gas production, botulinum neurotoxin genes and/or botulinum neurotoxin not detected in any of the five replicates.

[c]Gas production observed in at least one of the five replicates.

[d]Botulinum neurotoxin genes not detected by multiplex PCR despite gas production and/or botulinum neurotoxin detection by ELISA.

[e]Botulinum neurotoxin not detected by ELISA despite gas production being observed.

This may indicate boundary conditions for growth/toxin formation. Furthermore, in previous studies the detection of botulinum toxin has been reported without other signs of growth, such as an increase in viable count (Hyytiä, Hielm, Mokkila, Kinnunen, & Korkeala, 1999; Keto-Timonen, Lindström, Puolanne, Niemistö, & Korkeala, 2012). Growth/toxin formation by nonproteolytic *C. botulinum* was not detected in any coconut water samples at their natural pH (4.7-5.3) or pH 5.5 (Table 16). Growth/toxin formation by proteolytic *C. botulinum* was detected in all samples adjusted to the highest pH (6.2-6.7), except coconut water SL (Table 16). For coconut waters T1 and T2, at the highest pH tested, all three methods of assessing growth/toxin formation (gas production, multiplex PCR and ELISA) were positive. This aligns with previous results with coconut water T2, where gas formation, *bont/A* and *bont/B* toxin genes were detected at pH 6.4 (Table 15). Similarly, coconut waters BE and TP tested positive for gas formation and BoNT/B, but not for the presence of the *bont* gene (Table 16). Growth/toxin formation by nonproteolytic *C. botulinum* was detected in all samples adjusted to the highest pH (6.2-6.7), except coconut waters SL and T1 (Table 16). Gas formation was observed for non-proteolytic *C. botulinum*, but the toxin gene and neurotoxin were not detected. Although gas formation could potentially result from abiotic reactions, similar gas formation was not recorded in non-inoculated samples (data not shown) and therefore it was assumed that nonproteolytic *C. botulinum* had multiplied and formed gas. Growth of nonproteolytic *C. botulinum* was previously also detected (gas formation and *bont/B* gene) at pH 6.8 (Table 15).

4. Conclusions

This study showed that anoxic natural tender coconut water (pH 4.7-5.3) supported very limited growth/toxin formation by proteolytic *C. botulinum*. Under highly favorable growth conditions (anoxic sterile coconut water incubated for 50 days at 30 °C), growth/ toxin formation by proteolytic *C. botulinum* was only reported in one type of coconut water (T2), while none of the eight coconut waters supported growth/toxin formation by nonproteolytic *C. botulinum*. It was established that the reasons for poor growth/toxin formation by proteolytic *C. botulinum* and nonproteolytic *C. botulinum* in natural coconut waters include a low pH and nutrient limitation, but not anti-clostridial compounds. Growth/toxin formation could be achieved in most coconut waters by raising the pH and/or nutrient supplementation.

This work explains results obtained in Chapter 2 and in other published studies, where proteolytic *C. botulinum* and nonproteolytic *C. botulinum* failed to grow and produce the neurotoxin in different varieties of natural tender coconut water when stored under refrigeration or temperature abuse conditions (4-10 °C) (Raghubeer et al., 2020). The highly favorable

scenario assessed in this study (anoxic sterile coconut water incubated for 50 days at 30 °C) does not resemble commercial storage conditions nor realistic abuse conditions required for nonthermal processed products. Therefore, it can be considered that C. botulinum is not the pertinent microorganism in tender coconut water (pH 4.7-5.3) since the beverage is a poor substrate for growth and neurotoxin formation by the pathogen.

5. References

Anonymous. (2018). Guidelines for Setting Shelf-life of Chilled Foods in Relation to Non-proteolytic *Clostridium Botulinum*, first ed. Available at: Retrieved October 21, 2020, from https://www.chilledfood.org/new-publication-guidelines-for-setting-shelf-life-of-chilled-foods-in-relation-to-non-proteolytic-clostridium-botulinum/

Awua, A. K., Doe, E. D., & Agyare, R. (2011). Exploring the influence of sterilisation and storage on some physicochemical properties of coconut (*Cocos nucifera* L.) water. *BMC Research Notes*, *4*. https://doi.org/10.1186/1756-0500-4-451

Barba, F. J., Koubaa, M., do Prado-Silva, L., Orlien, V., & Sant'Ana, A. de S. (2017). Mild processing applied to the inactivation of the main foodborne bacterial pathogens: A review. *Trends in Food Science and Technology*, *66*, 20–35. https://doi.org/10.1016/j.tifs.2017.05.011

Beuchat, L. R., & Brackett, R. E. (1990). Inhibitory effects of raw carrots on *Listeria monocytogenes*. *Applied and Environmental Microbiology*, *56*(6), 1734–1742.

Bhullar, M. S., Patras, A., Kilanzo-Nthenge, A., Pokharel, B., Yannam, S. K., Rakariyatham, K., … Sasges, M. (2018). Microbial inactivation and cytotoxicity evaluation of UV irradiated coconut water in a novel continuous flow spiral reactor. *Food Research International*, *103*, 59–67. https://doi.org/10.1016/j.foodres.2017.10.004

Braconnier, A., Broussolle, V., Dargaignaratz, C., Nguyen-The, C., & Carlin, F. (2003). Growth and germination of proteolytic *Clostridium botulinum* in vegetable-based media. *Journal of Food Protection*, *66*(5), 833–839. https://doi.org/10.4315/0362-028X-66.5.833

Brunt, J., Carter, A. T., Pye, H. V., & Peck, M. W. (2018). The orphan germinant receptor protein *GerXAO* (but not *GerX3b*) is essential for L-alanine induced germination in *Clostridium botulinum* Group II. *Scientific reports*, *8*(1), 1-10. https://doi.org/10.1038/s41598-018-25411-x

Brunt, J., van Vliet, A. H., Stringer, S. C., Carter, A. T., Lindström, M., & Peck, M. W. (2020a). Pan-Genomic Analysis of *Clostridium botulinum* Group II (Non-Proteolytic *C. botulinum*) Associated with Foodborne Botulism and Isolated from the Environment. *Toxins*, *12*(5), 306. https://www.mdpi.com/2072-6651/12/5/306

Brunt, J., van Vliet, A. H., Carter, A. T., Stringer, S. C., Amar, C., Grant, K. A., ... & Peck, M. W. (2020b). Diversity of the Genomes and Neurotoxins of Strains of *Clostridium botulinum* Group I and *Clostridium sporogenes* Associated with Foodborne, Infant and Wound Botulism. *Toxins*, *12*(9), 586. https://www.mdpi.com/2072-6651/12/9/586

Carlin, F., & Peck, M. W. (1995). Growth and toxin production by non-proteolytic and proteolytic *Clostridium botulinum* in cooked vegetables. *Letters in Applied Microbiology*, *20*(3), 152–156. https://doi.org/10.1111/j.1472-765X.1995.tb00414.x

Carlin, Frédéric, & Peck, M. W. (1996). Growth of and toxin production by nonproteolytic *Clostridium botulinum* in cooked puréed vegetables at refrigeration temperatures. *Applied and Environmental Microbiology*, *62*(8), 3069–3072. https://doi.org/10.1128/aem.62.8.3069-3072.1996

Chidambaram, S., Singaraja, C., Prasanna, M. V., Ganesan, M., & Sundararajan, M. (2013). Chemistry of Tender Coconut Water from the Cuddalore Coastal Region in Tamil Nadu, India. *Natural Resources Research*, *22*(2), 91–101. https://doi.org/10.1007/s11053-013-9203-y

Davidson, P. M., Branen, A. L., & Sofos, J. N. (2005). Naturally occurring compounds. In *Antimicrobials in foods* (pp. 441–468). New York: Marcel Dekker Inc. https://doi.org/10.1002/1521-3773(20010316)40:6

González-Angulo, M., Clauwers, C., Harastani, R., Tonello, C., Jaime, I., Rovira, J., & Michiels, C. W. (2020). Evaluation of factors influencing the growth of non-toxigenic *Clostridium botulinum* type E and *Clostridium* spp. in high-pressure processed and conditioned tender coconut water from Thailand. *Food Research International*, *134*, 109278. https://doi.org/10.1016/j.foodres.2020.109278

Graham, A. F., & Lund, B. M. (1987). The combined effect of sub-optimal temperature and sub-optimal pH on growth and toxin formation from spores of *Clostridium botulinum*. *Journal of Applied Bacteriology*, *63*(5), 387–393. https://doi.org/10.1111/j.1365-2672.1987.tb04859.x

Graham, A. F., Mason, D. R., Maxwell, F. J., & Peck, M. W. (1997). Effect of pH and NaCl on growth from spores of non-proteolytic *Clostridium botulinum* at chill temperature. *Letters in Applied Microbiology*, *24*(2), 95–100.

Graham, A. F., Mason, D. R., & Peck, M. W. (1996). Predictive model of the effect of temperature, pH and sodium chloride on growth from spores of non-proteolytic *Clostridium botulinum*. *International Journal of Food Microbiology*, *31*(1–3), 69–85. https://doi.org/10.1016/0168-1605(96)00965-8

Gullmar, B., & Molin, N. (1967). Effect of Nutrients on Physiological Properties of *Clostridium botulinum* Type E. *Journal of Bacteriology*, *94*(6), 1924–1929.

Hawirko, R. Z., Naccarato, C. A., Lee, R. P. W., & Maeba, P. Y. (1979). Outgrowth and sporulation studies on *Clostridium botulinum* type E: influence of isoleucine. *Canadian Journal of Microbiology*, *25*(4), 522–527. https://doi.org/10.1139/m79-076

Hyytiä, E., Hielm, S., Mokkila, M., Kinnunen, A., & Korkeala, H. (1999). Predicted and observed growth and toxigenesis by *Clostridium botulinum* type E in vacuum-packaged fishery product challenge tests. *International Journal of Food Microbiology*, *47*(3), 161–169. https://doi.org/10.1016/S0168-1605(98)00173-1

Jackson, J. C., Gordon, A., Wizzard, G., McCook, K., & Rolle, R. (2004). Changes in chemical

composition of coconut (*Cocos nucifera*) water during maturation of the fruit. *Journal of the Science of Food and Agriculture*, *84*(9), 1049–1052. https://doi.org/10.1002/jsfa.1783

Keto-Timonen, R., Lindström, M., Puolanne, E., Niemistö, M., & Korkeala, H. (2012). Inhibition of toxigenesis of group II (nonproteolytic) *Clostridium botulinum* type B in meat products by using a reduced level of nitrite. *Journal of Food Protection*, *75*(7), 1346–1349. https://doi.org/10.4315/0362-028X.JFP-12-056

Kim, M., Zahn, M., Reporter, R., Askar, Z., Green, N., Needham, M., … Terashita, D. (2019). Outbreak of foodborne botulism associated with prepackaged pouches of liquid herbal tea. *Open Forum Infectious Diseases*, *6*(2), 3–7. https://doi.org/10.1093/ofid/ofz014

Lindström, M., Keto, R., Markkula, A., Nevas, M., Hielm, S., & Korkeala, H. (2001). Multiplex PCR Assay for Detection and Identification of *Clostridium botulinum* Types A, B, E, and F in Food and Fecal Material. *Applied and Environmental Microbiology*, *67*(12), 5694–5699. https://doi.org/10.1128/AEM.67.12.5694-5699.2001

Lukas, A. R. (2013). *Use of High Pressure Processing to Reduce Foodborne Pathogens in Coconut Water*. Masther thesis. University of Virginia.

Lund, B. M. (1993). Quantification of factors affecting the probability of development of pathogenic bacteria, in particular *Clostridium botulinum*, in foods. *Journal of Industrial Microbiology*, *12*(3–5), 144–155. https://doi.org/10.1007/BF01584183

Lund, B. M., Graham, A. F., & Franklin, J. G. (1987). The effect of acid pH on the probability of growth of proteolytic strains of *Clostridium botulinum*. *International Journal of Food Microbiology*, *4*(3), 215–226. https://doi.org/10.1016/0168-1605(87)90039-0

Lyver, A., Smith, J. P., Austin, J., & Blanchfield, B. (1998). Competitive inhibition of *Clostridium botulinum* type E by *Bacillus* species in a value-added seafood product packaged under a modified atmosphere. *Food Research International*, *31*(4), 311–319. https://doi.org/10.1016/S0963-9969(98)00097-0

Ma, Y., Xu, L., Wang, S., Xu, Z., Liao, X., & Cheng, Y. (2019). Comparison of the quality attributes of coconut waters by high-pressure processing and high-temperature short time during the refrigerated storage. *Food Science & Nutrition*, *7*(4), 1512–1519. https://doi.org/10.1002/fsn3.997

Nguyen-The, C., & Lund, B. M. (1991). The lethal effect of carrot on *Listeria* species. *Journal of Applied Bacteriology*, *70*(6), 479–488. https://doi.org/10.1111/j.1365-2672.1991.tb02744.x

Patterson, M. F., McKay, A. M., Connolly, M., & Linton, M. (2012). The effect of high hydrostatic pressure on the microbiological quality and safety of carrot juice during refrigerated storage. *Food Microbiology*, *30*(1), 205–212. https://doi.org/10.1016/j.fm.2011.09.013

Peck, M.W. (2006). *Clostridium botulinum* and the safety of minimally heated, chilled foods: an

emerging issue? *Journal of Applied Microbiology*, *101*(3), 556–570. https://doi.org/10.1111/j.1365-2672.2006.02987.x

Peck, M.W. (2009). Biology and Genomic Analysis of *Clostridium botulinum*. *Advances in Microbial Physiology*, *55*, 183–320. https://doi.org/10.1016/S0065-2911(09)05503-9

Peck, Michael W., Webb, M. D., & Goodburn, K. E. (2020). Assessment of the risk of botulism from chilled, vacuum/modified atmosphere packed fresh beef, lamb and pork held at 3 °C–8 °C. *Food Microbiology*, 103544. https://doi.org/10.1016/j.fm.2020.103544

Peck, Michael W, Plowman, J., Aldus, C. F., Wyatt, G. M., Penaloza Izurieta, W., Stringer, S. C., & Barker, G. C. (2010). Development and Application of a New Method for Specific and Sensitive Enumeration of Spores of Nonproteolytic *Clostridium botulinum* Types B, E, and F in Foods and Food Materials. *Applied and Environmental Microbiology*, *76*(19), 6607–6614. https://doi.org/10.1128/AEM.01007-10

Pendyala, B., Patras, A., Gopisetty, V. V. S., Sasges, M., & Balamurugan, S. (2019). Inactivation of *Bacillus* and *Clostridium* Spores in Coconut Water by Ultraviolet Light. *Foodborne Pathogens and Disease*, *16*(10), 704–711. https://doi.org/10.1089/fpd.2019.2623

Raghubeer, E. V., Phan, B. N., Onuoha, E., Diggins, S., Aguilar, V., Swanson, S., & Lee, A. (2020). The use of High-Pressure Processing (HPP) to improve the safety and quality of raw coconut (*Cocos nucifera* L) water. *International Journal of Food Microbiology*, 108697.

Ramaroson, M., Guillou, S., Rossero, A., Rezé, S., Anthoine, V., Moriceau, N., … Zagorec, M. (2018). Selection procedure of bioprotective cultures for their combined use with High Pressure Processing to control spore-forming bacteria in cooked ham. *International Journal of Food Microbiology*, *276*, 28–38. https://doi.org/10.1016/j.ijfoodmicro.2018.04.010

Sheth, A. N., Wiersma, P., Atrubin, D., Dubey, V., Zink, D., Skinner, G., … Sobel, J. (2008). International Outbreak of Severe Botulism with Prolonged Toxemia Caused by Commercial Carrot Juice. *Clinical Infectious Diseases*, *47*(10), 1245–1251. https://doi.org/10.1086/592574

Skinner, G. E., Fleischman, G. J., Balster, F., Reineke, K., Reddy, N. R., & Larkin, J. W. (2015). Effect of fill temperature on *Clostridium botulinum* type a toxin activity during the hot filling of juice bottles. *Journal of Food Protection*, *78*(8), 1506–1511. https://doi.org/10.4315/0362-028X.JFP-14-378

Stringer, S C, Haque, N., & Peck, M. W. (1999). Growth from spores of nonproteolytic *Clostridium botulinum* in heat-treated vegetable juice. *Applied and Environmental Microbiology*, *65*(5), 2136–2142.

Stringer, Sandra C, Webb, M. D., George, S. M., Pin, C., & Peck, M. W. (2005). Heterogeneity of Times Required for Germination and Outgrowth from Single Spores of Nonproteolytic *Clostridium botulinum*. *Applied and Environmental Microbiology*, *71*(9), 4998–5003.

https://doi.org/10.1128/AEM.71.9.4998-5003.2005

U.S. Food and Drug Administration. Department of Health and Human Services. (2007). Guidance for Industry: Refrigerated Carrot Juice and Other Refrigerated Low-Acid Juices. Retrieved May 17, 2020, from https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-refrigerated-carrot-juice-and-other-refrigerated-low-acid-juices

U.S. Food and Drug Administration. Public Health Service. (2015a). Warning Letter. Harmless Harvest Thailand. Retrieved May 17, 2020, from https://www.fda.gov/inspections-compliance-enforcement-and-criminal-investigations/warning-letters/harmless-harvest-thailand-11132015

U.S. Food and Drug Administration. Public Health Service. (2015b). Warning Letter. Vital Juice Company, Inc. Retrieved May 17, 2020, from https://www.fda.gov/inspections-compliance-enforcement-and-criminal-investigations/warning-letters/vital-juice-company-inc-05042015

Ward, B. Q., & Carroll, B. J. (1966). Spore germination and vegetative growth of *Clostridium botulinum* type E in synthetic media. *Canadian Journal of Microbiology*, *12*, 1145–1156.

Whitmer, M. E., & Johnson, E. A. (1988). Development of improved defined media for *Clostridium botulinum* serotypes A, B, and E. *Applied and Environmental Microbiology*, *54*(3), 753–759.

Wong, D. M., Young-Perkins, K. E., & Merson, R. L. (1988). Factors Influencing *Clostridium botulinum* Spore Germination, Outgrowth, and Toxin Formation in Acidified Media. *Applied and Environmental Microbiology*, *54*(6), 1446–1450.

Yannam, S. K., Patras, A., Pendyala, B., Vergne, M., Ravi, R., Gopisetty, V. V. S., & Sasges, M. (2020). Effect of UV-C irradiation on the inactivation kinetics of oxidative enzymes, essential amino acids and sensory properties of coconut water. *Journal of Food Science and Technology*. https://doi.org/10.1007/s13197-020-04388-4

Chapter 4

Assessing the pressure resistance of *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella enterica* to high pressure processing (HPP) in citric acid model solutions for process validation

CHAPTER 4

ASSESSING THE PRESSURE RESISTANCE OF *ESCHERICHIA COLI* O157:H7, *LISTERIA MONOCYTOGENES* AND *SALMONELLA ENTERICA* TO HIGH PRESSURE PROCESSING (HPP) IN CITRIC ACID MODEL SOLUTIONS FOR PROCESS VALIDATION

Despite the commercial success of high pressure processing (HPP) in the juice industry and the fact that no foodborne outbreaks have been reported so far, some regulatory agencies still require process validation. However, there is a lack of consensus on various aspects regarding validation protocols, including the selection of representative strains to be used in challenge tests. This study characterized the variable response of *Escherichia coli* O157:H7 (34 strains), *Listeria monocytogenes* (44 strains) and *Salmonella enterica* (45 strains) to HPP, and identified potential candidates to use in process validation. Stationary phase cells were submitted to 500 MPa for 1 min at 10 °C in model solutions consisting of tryptic soy broth + 0.6 % yeast extract (TSBYE) adjusted to pH 4.5 and 6.0 with citric acid. At pH 6.0, pressure resistance widely varied between species and within strains of the same species. *E. coli* O157:H7 and *L. monocytogenes* were the most pressure resistant and showed high variability at strain level, as the total count range given by minimum and maximum counts spread between 2.0-6.5 $\log_{10}$ CFU/mL. *S. enterica* was the least resistant pathogen with more than 82 % of the isolates displaying non-detectable counts after HPP. Recovery through storage at 12 °C was also variable for all pathogens, but eventually most strains recovered with median counts on day 14 between 8.3 and 8.9 $\log_{10}$ CFU/mL. For pH 4.5 solutions, 26 *E. coli* O157:H7 strains displayed survivors after HPP but did not adapt, registering non-detectable counts in the next sampling dates. None of the *L. monocytogenes* and *S. enterica* strains survived HPP or incubation (<2.0 $\log_{10}$ CFU/mL) at pH 4.5, suggesting that citric acid at 4.16 g/L is a safe barrier for pathogen control under moderate HPP conditions (500 MPa for 1 min). Principal component and cluster analyses served to propose strain cocktails for each species based on their pressure resistant and adaptation phenotypes. Additionally, *S. enterica* was identified as less pressure resistant and less prone to recover following HPP than *E. coli* O157:H7 and *L. monocytogenes*, so its relevance in process validation for juices should be questioned. Future work will validate the proposed strain cocktails on real food systems.

1. Introduction

Demand for minimally processed food with clean label and improved organoleptic properties is increasing (Asioli et al., 2017). Numerous nonthermal food processing techniques emerged in the last decades to minimize changes in food quality attributed to heat preservation methods (Barba, Koubaa, do Prado-Silva, Orlien, & Sant'Ana, 2017). Among these, high pressure processing (HPP) is the most developed and industrially implemented nonthermal technology to ensure food safety and extend the shelf-life of a wide variety of products (González-Angulo, Serment-Moreno, Queirós, & Tonello-Samson, 2021). Juices and beverages have very delicate organoleptic characteristics and represent an excellent source of nutritional and functional compounds such as vitamins and antioxidants. Some of these molecules are heat sensitive, thus HPP becomes a suitable alternative to minimize nutritional and quality losses (Deliza, Rosenthal, Abadio, Silva, & Castillo, 2005). Industry experts estimate that 24 % of global HPP units are installed in juice processing facilities and produce around 540 million liters of juice per year (González-Angulo et al., 2021).

HPP conditions typically used by the industry (600 MPa for 3 to 5 min) consistently achieve full pathogen inactivation in juices and other products. The Canadian government collected 1,216 retail samples of commercial HPP juices extracted from fruits (30 % of samples), vegetables (2 %), or fruit/vegetable blends (68 %) during April 2016 to August 2017 (Canadian Food Inspection Agency, 2017). Authorities reported that HPP juices met microbial quality indicators (generic *Escherichia coli*, ≤100 CFU or MPN/mL), and highlighted that bacterial pathogens (*Salmonella* spp., *Shigella* spp., *E. coli* O157:H7), virus (Hepatitis A, Norovirus GI & GII) and parasites (*Cyclospora cayetanensis*, *Cryptosporidium* spp., *Toxoplasma gondii*, *Giardia* spp.) were not detected in any HPP juice samples (Canadian Food Inspection Agency, 2017). These facts led the Canadian government to adopt 600 MPa with 3 min holding time as a safe harbor for HPP juices and other products, implying that processors meeting the HPP parameters do not require further validation (Canadian Food Inspection Agency, 2017). Conversely, other countries legally require HPP validation of juices before commercialization. In the United States, the largest market for HPP foods, juice regulations mandate to demonstrate that processing achieves 5-$\log_{10}$ reductions of pertinent pathogens throughout the shelf-life (U.S. Food and Drug Administration, 2001). Based on foodborne illness outbreaks related to fresh juice consumption, enterohemorrhagic (EHEC) *E. coli*, *Listeria monocytogenes* and/or *Salmonella* spp. are typically considered as the pertinent pathogens in high-acid (pH <4.6) and low-acid (pH >4.6) juices. The ability of *E. coli* O157:H7 to adapt and survive in acidic environments raises significant concerns about this pathogen, as clearly evidenced by outbreaks associated to fresh juice consumption (Jackson-Davis et

al., 2018). *Salmonella* spp. has been linked to several outbreaks with hundreds of cases associated to citrus fruit juices (Jackson-Davis et al., 2018). The ubiquitous nature of *L. monocytogenes* makes this pathogen of special relevance in any food processing environment, and has been associated with mildly-acidic fruits outbreaks such as cantaloupe (McCollum et al., 2013).

Guidelines for process validation recommend the use of cocktails consisting of 5-10 representative strains of each species that demonstrate tolerance to the food system and high pressure (NACMCF, 2010). It is well established that different species of pathogens and different strains within the same species exhibit substantial variation in pressure resistance (Alpas et al., 1999). In this regard, FDA authorities communicated through an extensive review that the lack of consensus concerning the selection of specific pathogenic strains is one of the unresolved issues in process validation (Podolak, Whitman, & Black, 2020). Adequate strain selection requires a comprehensive characterization, but the variable response to high pressure among isolates within the same species makes this challenging. Molecular mechanisms leading to this phenotype are complex to elucidate or remain unknown. The RpoS sigma factor directs expression of the general stress response in *E. coli* and has proven to be key for its HPP resistance (Robey et al., 2001). Some of the genetic pathways leading to pressure inactivation of *E. coli* involve attenuating the regulation of the RpoS-independent cAMP/CRP homeostasis (Vanlint et al., 2013), compromising the succinyl-CoA synthetase enzyme of the tricarboxylic acid cycle (Gayán, Rutten, Van Impe, Michiels, & Aertsen, 2019) or affecting the upregulation of the heat shock response (Robey et al., 2001). In line with these findings, recent research suggests that downregulating the activity of CyaA, Cra and/or AceA proteins, or increasing RpoH and/or RpoS activity, may account for the increased HPP resistance of *E. coli* (Gayán, Van den Bergh, Michiels, Michiels, & Aertsen, 2020). Likewise, the RpoS activity also plays an important role in the pressure resistance of *S. enterica*, but research is scarce and other independent mechanisms also seem to confer increased resistance in certain serotypes (Tamber, 2018). In the case of *L. monocytogenes*, basal activity of σ^B factor encoding the general stress response (Wells-Bennik, Karatzas, Moezelaar, & Abee, 2014; Wemekamp-Kamphuis et al., 2004), the modulation of genes regulating the transcription of heat shock proteins (Liu, Ream, Joerger, Liu, & Wang, 2011; Van Boeijen, Moezelaar, Abee, & Zwietering, 2008), or the presence of certain prophage genes in the genome of *L. monocytogenes* (Duru et al., 2020) have been associated with the pressure resistance of the pathogen.

The unpredictable response of single isolates to HPP based on detectable genetic markers makes difficult the selection of specific strains for process validation. Even if the

genetic markers for pressure resistance are identified, strain survival also depends on the response and adaptation to the surrounding media. In example, González-Angulo (2015) and Wilches-Pérez (2015) observed that the pressure resistance of the same *L. monocytogenes* strains varied among buffer media, sliced deli meats, or cured meats. Same behavior has been reported for *E. coli* O157:H7 and *S. enterica* strains in fruit juices and buffer systems (Erkmen & Dogan, 2004; Jordan, Pascual, Bracey, & Mackey, 2001; Teo, Ravishankar, & Sizer, 2001; Whitney, Williams, Eifert, & Marcy, 2007). Consequently, screening pathogen strain resistance is required for appropriate strain selection for HPP since validation remains an active research area. Process validation not only requires the use of pressure resistant strains, but also needs to contemplate adaptation and recovery phenotypes to resemble worst-case scenarios from a food safety perspective. Most of published studies considered the immediate effect of HPP but usually did not assess adaptation and subsequent growth of the pathogens after the process (Bruschi et al., 2017; Liu, Gill, McMullen, & Gänzle, 2015; Tamber, 2018). The characterization of the variable responses at strain and species levels would help to elucidate which pathogenic species have the potential to be used in the validation of HPP juices. The present work is the starting point of a series of studies aiming to assess the pressure resistance and adaptation phenotypes of multiple strains of *E. coli* O157:H7, *L. monocytogenes* and *S. enterica* in different media. As a preliminary step for the design of more robust validation studies, this work screens the resistance of pathogens in model solutions of citric acid, one of the predominant organic acids in fruit juices.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A total of 34 strains of *E. coli* O157:H7 (Pasteur Institute of Paris CIP, France; LREC, University of Santiago de Compostela, Spain), 44 strains of *L. monocytogenes* (FSL, Cornell University, United States; ITACyL, Instituto Tecnológico Agrario de Castilla y León, Spain) and 45 strains of *S. enterica* (FSL, Cornell University, United States; HUBU, Hospital Universitario de Burgos, Spain) were used in this study (details in Table A10 to Table A12 of the Appendix to Chapter 4). All strains were isolated from various sources including food, environment, animals or humans. The criteria considered for strain selection were outbreak history, serotype diversity and origin, with a special focus on strains obtained from humans.

Strains stored in frozen glycerol stocks (-80 °C) were cultured on tryptic soy agar plates with 0.6 % yeast extract (TSAYE) (Oxoid, UK) at 37 °C for 24 h. Isolated colonies were streaked again on fresh TSAYE plates to obtain fully active cultures. Furthermore, single colonies were inoculated into 10 ml of tryptic soy broth (Merck, Germany) with 0.6 % yeast

extract (TSBYE) and incubated at 37 °C for 24 h. Overnight grown cultures were decimally diluted in 9 mL of pH-adjusted TSBYE to obtain a cell concentration around 10^8 CFU/mL.

2.2. Screening the pressure resistance of bacterial strains in model solutions

Sterile 30 mL PET bottles (Sunbox, Spain) were aseptically filled with 27 mL of the TSBYE model solution adjusted to acid (pH 4.5) and low-acid (pH 6.0) conditions by adding 4.16 and 1.47 g/L of citric acid (Prolabo, Belgium), respectively. Three milliliters of the strain culture were added to the model TSBYE solutions to reach 10^7 CFU/mL. Model solution samples were processed at sublethal conditions (500 MPa for 1 min) within an hour following inoculation in an industrial HPP equipment (Hiperbaric 135, Hiperbaric, Spain). Water at 10 °C was used as pressurizing fluid and the temperature increase due adiabatic heating during compression was estimated at about 3 °C/100 MPa. Pressurization rate was 221 MPa/min and pressure release after the 1 min holding time took less than 1 second. Samples were stored in ice until microbiological analysis (day 0) or transferred to incubation (days 1-14). Non-pressure treated samples were kept as control. All experiments were performed in three independent replicates.

2.3. Microbiological analyses and incubation conditions

Control and pressure-treated samples were serially diluted in quarter strength Ringer's solution (Oxoid, UK) and 10 µL were plated in TSAYE by the drop-plate method to assess total viable counts (Miles, Misra, & Irwin, 1938) which gave a detection limit of 2 $\log_{10}$ CFU/mL (a summary of the counts is included in Table A13 to Table A15 of the Appendix to Chapter 4). To account for non-injured cells, 10 µL aliquots were plated in TSAYE supplemented with 3 % NaCl (Merck, Germany) for *S. enterica*, or 4 % NaCl for *E. coli* O157:H7 and *L. monocytogenes*. The fraction of sublethally injured cells was determined as the count difference between cells grown in TSAYE and TSAYE+NaCl. This procedure was carried out in all samples immediately after HPP (day 0) and after 24 h at 12 °C (day 1). Plates were incubated at 30 °C for 24-48 h to facilitate recovery of injured cells (Koseki & Yamamoto, 2006). Enumeration of microbial counts on days 7 and 14 was only conducted on TSAYE to assess total viable counts through storage at 12 °C to facilitate recovery at suboptimal temperature.

Purity checks of samples were performed by streaking random colonies grown in TSAYE and TSAYE+NaCl on selective agar plates for each species: Sorbitol MacConkey Agar with BCIG (Oxoid, UK) for *E. coli* O157:H7, Chromogenic Listeria Agar (Scharlau, Spain) for *L. monocytogenes* and Xylose-Lysine-Deoxycholate Agar (Biolife, Italy) for *S. enterica*. In all cases, plates were incubated at 37 °C for 24 h.

2.4. Statistical analysis

2.4.1. Hypothesis testing and data visualization

Escherichia coli O157:H7, *L. monocytogenes* and *S. enterica* total counts were tested for significant differences between species on each incubation date (0, 1, 7 or 14 days) using the Kruskal-Wallis test ($p < 0.05$), since data did not follow a normal distribution based on the Shapiro-Wilk W statistic ($p < 0.05$). The response of each strain to media acidity was evaluated by comparing counts at pH 4.5 and 6.0 with one-way ANOVA ($p < 0.05$).

Violin plots are a visual method of plotting numeric data that combine the elements of a box plot and kernel density plot. They were used to summarize the microbial counts of each species over the incubation period. The vertical line inside the box of box plots denotes the median or 50^{th} percentile, whereas the boundaries of the box indicate the 25^{th} and 75^{th} percentiles. Crosses within each box represent mean counts of each individual strain. The horizontal bars extending up and down the box indicate the 5^{th} and 95^{th} percentiles, respectively. Count range was defined as the difference between the maximum and minimum observations. One of the main advantages of violin plots is that in addition to the median, interquartile range and total range, they also show the entire distribution of the data, which is of special interest when dealing with multimodal (non-normal) data series. The density trace serves as an estimator to the histograms of total counts data sets and gives a smoother indication of frequencies.

2.4.2. Multivariate descriptive analyses

Principal component (PC) analysis was used to determine the variables that allow to categorize strains of each species into different groups based on their response to experimental variables. The number of PC was selected to account for greater than 85 % of the variance. Absolute values of factor loadings were considered to depict the variance explained by the variable on that particular factor.

Clustering analysis by Euclidean distance following the Ward's method was used to classify strains of each species based on their response to pressure resistance in model solutions (day 0), adaptation to the medium (day 1), and cell recovery (days 7 and 14). This specific analysis has been previously conducted to classify multiple isolates based on their ability to tolerate different environmental conditions (Begot, Lebert, & Lebert, 1997; Van Boeijen et al., 2010; van der Veen, Moezelaar, Abee, & Wells-Bennik, 2008), or to group strains based on their response to various food processing interventions, including HPP (Sherry, Patterson, & Madden, 2004). The Kruskal-Wallis hypothesis test ($p < 0.05$) assessed statistical

differences between median growth rates of the clusters formed for each species. Growth rates were calculated as the slope between two successive sampling points. All statistical tests abovementioned were performed by using Statgraphics Centurion XVIII (StatPoint Technologies, USA).

3. Results and discussion

3.1. Assessing the pressure resistance of foodborne pathogens in model solutions

3.1.1. Escherichia coli O157:H7

Inactivation ranged between 0.7 and >5.0 $\log_{10}$ CFU/mL with a median reduction of 4.0 $\log_{10}$ CFU/mL for pH 6.0 (Figure 17), matching other studies that used similar processing conditions (500 MPa, between 1 and 20 min) in TSB (Benito, Ventoura, Casadei, Robinson, & Mackey, 1999; Usajewicz & Nalepa, 2006; Whitney et al., 2007). Likewise, Liu et al., reported 1.1-5.5 $\log_{10}$ CFU/mL reductions for 112 *E. coli* strains (including 10 strains with the O157:H7 serotype), after applying 600 MPa for 3 min in phosphate buffered saline (PBS) (Liu et al., 2015). In the present study, 9 % of the strains presented counts below the detection limit on day 0, the lowest proportion among the three pathogen species. After 24 h, 44 % of the strains showed non-detectable levels and 91 % of the cells presented sublethal injury. After 24 h, only 48 % of the cells presented sublethal injury, indicating that damaged isolates progressively adapted to the medium and eventually recovered during storage (Figure 17).

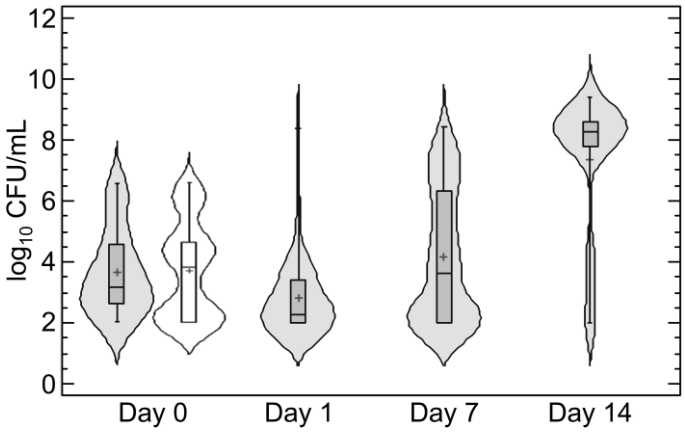

Figure 17. Violin plot combining box plot and density trace with cell counts distribution of *E. coli* O157:H7 strains in pH 6.0 (filled) and 4.5 (blank) model solutions processed at 500 MPa for 1 min and stored at 12 °C.

By day 7 of storage, the median cell concentration for all *E. coli* O157:H7 isolates was 3.6 $\log_{10}$ CFU/mL and likely reached the stationary phase by day 14 (median 8.3 $\log_{10}$ CFU/mL; Figure 17). Recovery of individual isolates varied as indicated by the count spread on day 7 and the bimodality of the density trace in the violin plot (Figure 17). Some strains exhibited a

faster recovery and reached a high cell concentration (around 6 $\log_{10}$ CFU/mL), whereas the majority remained around 1-3 $\log_{10}$ CFU/mL. Nonetheless, the count range given by the 5 % and 95 % quantiles narrowed to 0.8 $\log_{10}$ CFU/mL after 14 days of incubation with most strains growing above 8.0 $\log_{10}$ CFU/mL, except for some outliers that did not recover after HPP (Figure 17).

In pH 4.5 model solutions, 24 % of *E. coli* O157:H7 strains displayed counts below the detection limit immediately after HPP (day 0). The median viable cell concentration for all strains was 3.8 $\log_{10}$ CFU/mL, but the response widely varied as revealed by counts spreading along 4.7 $\log_{10}$ units (Figure 17). Parallel enumeration in TSAYE+4 % NaCl evidenced that all survivors sustained sublethal injury. This accounts for the drastic drop in cell viability on the following day, where 88 % of strains exhibited counts below the detection limit (day 1) and no survivors were detected on subsequent sampling dates. Isolates CIP 105231 and CIP 105212 displayed less than 1.0 $\log_{10}$ CFU/mL reductions at pH 4.5, but were not found amongst the most pressure resistant strains at pH 6.0. Other authors found an analogous behavior on a specific non-pathogenic *E. coli* strain (Reineke et al., 2015) and attributed this to changes in cell membrane composition induced by increased acidity (Yuk & Marshall, 2004).

Our findings suggest that inactivation of *E. coli* O157:H7 after HPP on day 0 was limited, but sublethal injury of survivors increased the susceptibility of the pathogen to acidic environments. Pathogen cells might slowly die during prolonged exposure to acid media like citrus juices. Some authors have reported that HPP sensitizes microbial cells and considerably reduces the storage time required eliminate pathogens (Jordan et al., 2001; Linton, Mcclements, & Patterson, 1999; Noma, Tomita, Shimoda, & Hayakawa, 2004). Processing orange and apple juices around 550-600 MPa for 2-3 min achieved viability losses of *E. coli* O15:H7 between 1.5 and 4.4 $\log_{10}$ CFU/mL, but further enumeration after 24 h at 4 °C evidenced enhanced inactivation (3.1-5.6 $\log_{10}$ CFU/mL) due to sublethal injury (Syed, Buffa, Guamis, & Saldo, 2013; Whitney et al., 2007). The variable response and the ability to survive HPP in acidic environments is of great importance in the selection of representative EHEC *E. coli* strains to use in process validation, along with characteristics of the food matrix that may influence microbial resistance (Gänzle & Liu, 2015).

3.1.2. Listeria monocytogenes

Pathogen *L. monocytogenes* also showed moderate to high pressure resistance at pH 6.0, with only 34 % of strains displaying counts below detection limit immediately after HPP and median viable cell counts of 2.8 $\log_{10}$ CFU/mL. Differences were not statistically significant

(p >0.05) from *E. coli* O157:H7 counts on day 0, although *L. monocytogenes* exhibited greater variability at this point as represented by the interquartile range (3.2 $\log_{10}$ CFU/mL) and bimodal count dispersion (Figure 18). Despite 100 % of sublethal injury among survivors on day 0, all strains adapted to the medium and showed median counts of 3.5 CFU/mL after 24 h (day 1; Figure 17). Similarly, other studies have reported 1-7 $\log_{10}$ reductions of different *L. monocytogenes* strains in the 400-500 MPa for 2-5 min holding time range (Bruschi et al., 2017; Chen, Neetoo, Ye, & Joerger, 2009).

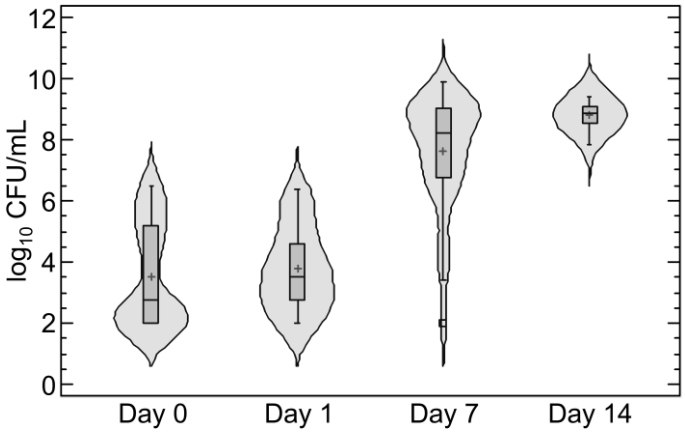

Figure 18. Violin plot combining box plot and density trace with cell counts distribution of *L. monocytogenes* strains in pH 6.0 model solution processed at 500 MPa for 1 min and stored at 12 °C.

During storage, all individual strains of *L. monocytogenes* adapted and grew to a 7.9 $\log_{10}$ CFU/mL median cell concentration on day 7, the highest among the three species studied (p <0.05; Figure 18). This can be attributed to the psychrotrophic nature of the microorganism, which is able to recover from sublethal damage and proliferate at low temperatures and slightly acidic conditions during extended storage (González-Tejedor, Garre, Esnoz, Artés-Hernández, & Fernández, 2018). At the end of the 14-day incubation at 12 °C, all strains recovered as evidenced by the average and median counts (8.8 and 8.9 $\log_{10}$ CFU/mL, respectively), and count data followed a normal distribution (Figure 18). Dispersion at this point was the narrowest of the three pathogens studied with a count range of 1.6 $\log_{10}$ CFU/mL. Similarly, Patterson et al., (2012) reported 1.7 $\log_{10}$ reductions of *L. monocytogenes* in TSBYE after HPP (500 MPa for 1 min) followed by steady growth up to 7.0 (day 7) and 9.0 $\log_{10}$ CFU/mL (day 14) during incubation at 12 °C. Nakaura et al., (2019) reported 100 % of sublethally injured cells in PBS (pH 7.2) after processing at 500 MPa for 10 min. Transferring aliquots of pressurized PBS samples into TSB enhanced recovery during storage at 5-15 °C, reaching a cell concentration above 8.0 $\log_{10}$ CFU/mL within 5 days.

Processing pH 4.5 model solutions at 500 MPa for 1 min induced greater than 5.0 $\log_{10}$ CFU/mL reductions for all 43 strains of *L. monocytogenes* and no recovery after 14 days of incubation, with counts remaining below the detection limit (<2.0 $\log_{10}$ CFU/mL). These findings are in agreement with those reported in other high-acid media (pH 3.4-4.5) processed at 345-500 MPa for 1-2 min, such as grape juice (Petrus, Churey, & Worobo, 2019), apple juice (Shahbaz et al., 2016) and acidified peptone solution (Alpas, Kalchayanand, Bozoglu, & Ray, 2000).

3.1.3. Salmonella enterica

Salmonella enterica exhibited the lowest pressure tolerance at pH 6.0 with 82 % of the strains displaying counts below the detection limit on day 0 and surviving counts ranging 2.0 to 3.1 $\log_{10}$ CFU/mL (Figure 19). Additionally, all survivors (100 %) presented sublethal injury on day 0 that slightly decreased to 83.5 % after 24 h, the greatest among the three species studied. Most *S. enterica* strains adapted to the low-acid solution (pH 6.0) and grew during storage at 12 °C. Recovery among isolates was very variable as evidenced by total count range on day 7 (7.0 $\log_{10}$ CFU/mL) and the bimodal data spread (Figure 19). Median cell concentration at this point was 5.2 $\log_{10}$ CFU/mL and not statistically different from that of *E. coli* O157:H7 ($p > 0.05$; Figure 17), but lower than the counts recorded for *L. monocytogenes* (8.2 $\log_{10}$ CFU/mL; Figure 18). This can be attributed to the mesophilic nature of both *Enterobacteriaceae* species and suboptimal incubation temperature of 12 °C. On day 14, median counts reached 8.9 $\log_{10}$ CFU/mL, which were higher than *E. coli* O157:H7 ($p < 0.05$), but not different from *L. monocytogenes* counts ($p > 0.05$). The interquartile count range narrowed to 1.3 $\log_{10}$ CFU/mL, but *S. enterica* presented the largest spread of the three species studied with a 7.9 $\log_{10}$ CFU/mL range, indicating that some strains failed to adapt and recover (Figure 19).

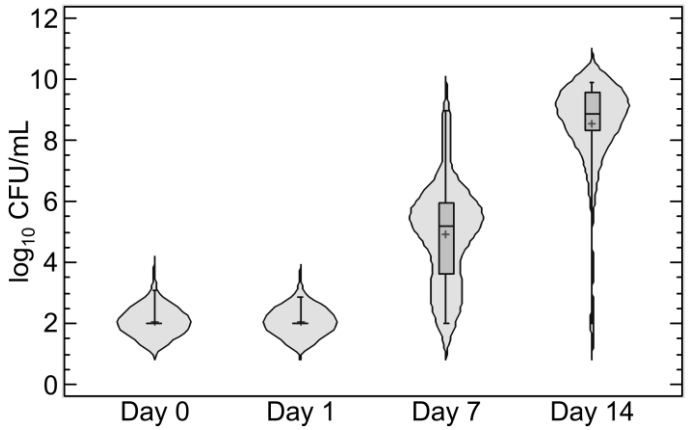

Figure 19. Violin plot combining box plot and density trace with counts distribution of *S. enterica* strains at in pH 6.0 model solution processed at 500 MPa for 1 min and stored at 12 °C.

Conversely, Tamber (2018) observed that 30 % of 99 strains displayed counts above 2.0 $\log_{10}$ CFU/mL in PBS (pH 7.2) after processing at 600 MPa with 3 min holding time. In the present study, the pH of TSBYE was adjusted with citric acid, which accounts for the greater sensitivity of *S. enterica* to milder processing conditions. Arvizu-Medrano & Escartín (2005), reported reductions between 3.5 and 4.5 $\log_{10}$ CFU/mL of *Salmonella* spp. after 1 h of acid challenge in TSB adjusted to pH 3.0 with citric acid. Additionally, buffering capacity of PBS under pressure is expected to be higher than that of TSBYE. Overall, our findings evidence that *S. enterica* is more susceptible to HPP than *E. coli* O157:H7 or *L. monocytogenes,* which goes in agreement with previous reports on fruit juices. Processing five strains of *S. enterica* at 500 MPa for 2 min in TSB (pH 7.2) yielded 4.6 $\log_{10}$ CFU/mL reductions, which increased to 5.7 $\log_{10}$ CFU/mL when the pathogen was inoculated in apple juice. On the other hand, six *E. coli* O157:H7 strains subjected to the same HPP conditions showed 1.5 and 2.3 $\log_{10}$ CFU/mL reductions in TSB and apple juice, respectively (Whitney et al., 2007). Similarly, processing at 345 MPa for 5 min in peptone water acidified with citric acid (pH 4.5) reduced *L. monocytogenes* by 5 $\log_{10}$ CFU/mL, whereas *S. enterica* suffered 7.3 $\log_{10}$ CFU/mL reductions (Alpas et al., 2000).

In the acidic model solution used in this study (pH 4.5), none of the 44 strains of *S. enterica* displayed counts above the detection limit (2.0 $\log_{10}$ CFU/mL) after HPP (500 MPa for 1 min), and did not recover during incubation. This goes in agreement with various strains of this pathogen in acidified synthetic media (pH 4.5 and 5.5) after processing at 345 MPa for 5 min (Alpas et al., 2000) or in apple and orange juices (both with pH 3.7), where processing between 550 and 615 MPa for 1 or 2 min, exceeded 5.0 $\log_{10}$ CFU/mL reductions (Teo et al., 2001; Whitney et al., 2007).

3.2. Classification and identification of pressure resistant pathogen strains

Principal component (PC) analysis classified strains of each species by grouping isolates with similar response to experimental variables, where Table 17 reports factor loadings (FL) of the constructed PC. In the case of *E. coli* O157:H7, PC1 is mainly described by strain capacity to adapt and grow at pH 6.0 after 7 and 14 days following HPP (FL = 0.654 and 0.571, respectively). Based on absolute variable loadings, PC2 differentiates strains according to their pressure resistance (FL = 0.425) and cell concentration at the end of the 14-day incubation period (FL = 0.769). The third component (PC3) explained the pressure resistance of *E. coli* O157:H7 in acid media as suggested by FL = 0.732. *L. monocytogenes* strains were differentiated in PC1 by their pressure resistance on day 0 (FL = 0.731), and by their ability to recover and grow at pH 6.0 after 7 days of incubation with PC2 (FL = 0.906). As discussed

above, all strains reached a similar cell concentration after 14 days, which explains the minor contribution of this variable in the construction of the principal components. The high pressure sensitivity of *S. enterica* accounts for the low impact of this variable in PC1 and PC2, registering FL close to 0 for both components (Table 17). In this case, *S. enterica* strains were mainly described by their adaptability and recovery after HPP in pH 6.0 as noted by FL = 0.810 and 0.809, for days 7 and 14, respectively.

Table 17. Principal component (PC) scaled loadings from principal component analysis of pathogen counts in model solutions at pH 4.5 and 6.0 processed by HPP (500 MPa for 1 min) and stored at 12 ° C.

Scaled loadings	Variable	PC1	PC2	PC3
E. coli O157:H7	Day 0 (pH 6.0)	0.260	-0.425	0.073
	Day 1 (pH 6.0)	0.253	-0.019	-0.497
	Day 7 (pH 6.0)	0.654	-0.305	-0.406
	Day 14 (pH 6.0)	0.571	0.769	0.218
	Day 0 (pH 4.5)	0.339	-0.368	0.732
L. monocytogenes	Day 0 (pH 6.0)	0.731	-0.413	
	Day 1 (pH 6.0)	0.561	-0.086	
	Day 7 (pH 6.0)	0.385	0.906	
	Day 14 (pH 6.0)	0.054	0.022	
S. enterica	Day 0 (pH 6.0)	0.008	-0.043	
	Day 1 (pH 6.0)	0.010	0.012	
	Day 7 (pH 6.0)	0.810	-0.586	
	Day 14 (pH 6.0)	0.586	0.809	

Clusters for each species were constructed with the data from the variables that PC analysis identified more relevant to explain the pressure resistance and ability of the strains to adapt to the medium (Figure 20). Cluster A grouped the most pressure resistant isolates with the highest median counts on day 0, registering 4.6 and 5.6 $\log_{10}$ CFU/mL for *E. coli* O157:H7 and *L. monocytogenes,* respectively (Table 18). Cluster A also incorporated strains with the greatest capacity to adapt and recover through incubation at 12 °C based on growth rates between days 1 and 7, which were the highest among clusters with the exception of *L. monocytogenes* (Table 18). For this pathogen, strains of cluster A averaged 5.6 $\log_{10}$ CFU/mL at day 1 and reached 8.2 $\log_{10}$ CFU/mL for day 14, which likely sets the maximum growth at some point in between. Cluster B gathered strains with lower pressure resistance (median counts for the three species between 2.0 and 3.8 $\log_{10}$ CFU/mL on day 0), but differentiated in their ability to adapt and recover following HPP as shown by growth rates, and the counts also averaged 8.2 $\log_{10}$ CFU/mL on day 14 (Table 18).

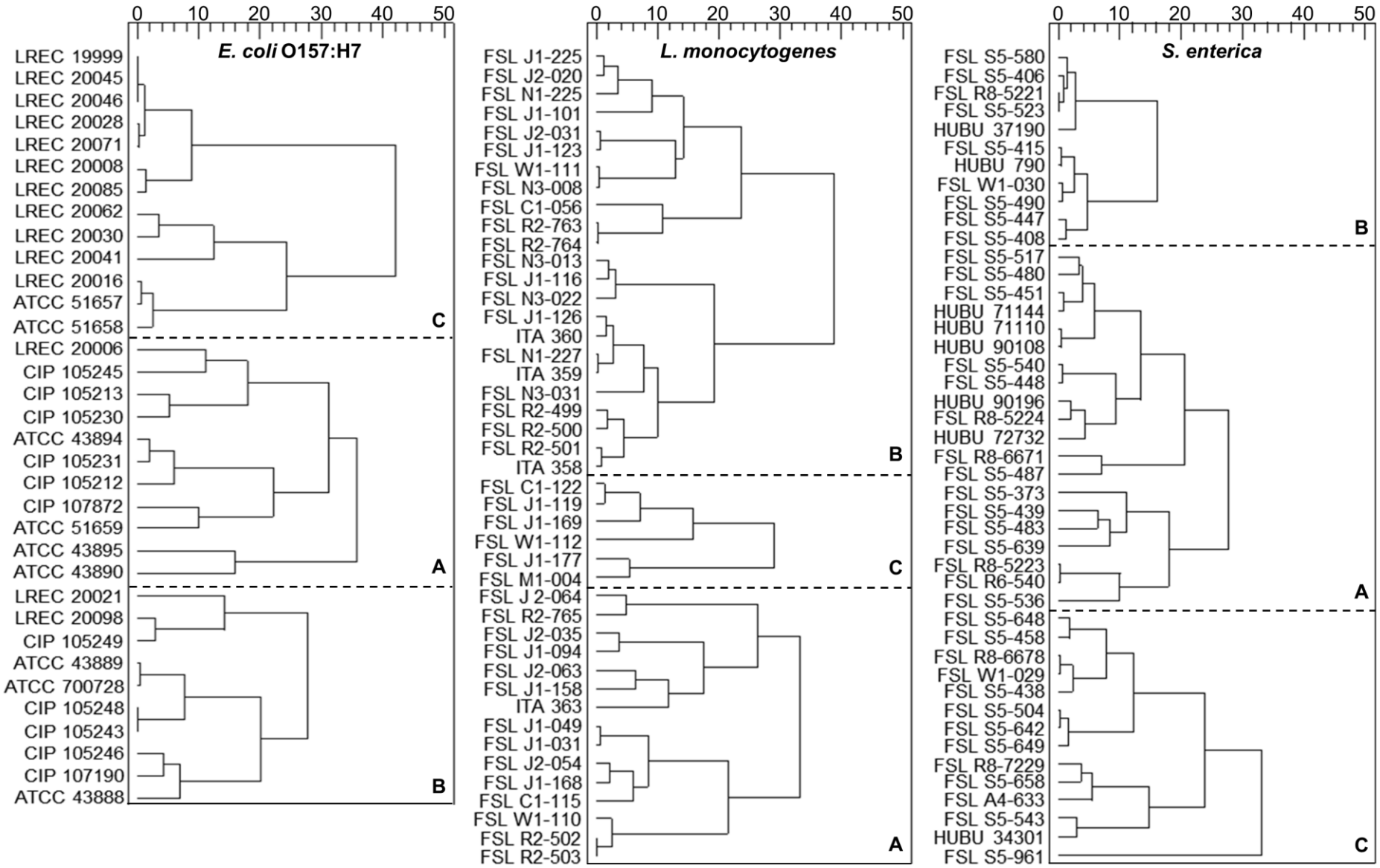

Figure 20. Dendrogram analysis for the classification of strains in clusters (A, B or C). Dashed lines separate clusters for each species.

Finally, cluster C included the most pressure sensitive and less prone to recover isolates after HPP. Higher growth rates for all species between days 7-14 compared to days 1-7 suggest that strains belonging to this group required more time to adapt and recover.

Table 18. Statistical summary of median counts on day 1 after HPP (500 MPa for 1 min) and median growth rates for strains of each pathogen grouped into clusters by principal component and dendrogram analyses.

Species	Cluster	Counts on day 1 (log_{10} CFU/mL)	Growth rates ($log_{10} N_t$ - $log_{10} N_o$)/day	
			From day 1 to 7	From day 7 to 14
E. coli O157:H7	A	4.6±1.6[a]	0.48±0.35[a]	0.24±0.21[c]
	B	3.8±1.0[a]	0.00±0.14[b]	0.81±0.26[a]
	C	2.5±0.5[b]	0.00±0.30[b]	0.46±0.34[b]
L. monocytogenes	A	5.6±0.7[a]	0.55±0.34[b]	0.05±0.23[b]
	B	2.0±0.3[b]	0.86±0.21[a]	0.07±0.16[b]
	C	3.4±1.1[a]	0.26±0.26[c]	0.59±0.21[a]
S. enterica	A	2.0±0.3[a]	0.63±0.21[a]	0.36±0.18[b]
	B	2.0±0.0[a]	0.50±0.13[b]	0.62±0.09[a]
	C	2.0±0.1[a]	0.18±0.18[c]	0.75±0.26[a]

Different columns with different letters within species are significantly different ($p < 0.05$).

Strains in cluster A are representative isolates for the validation of HPP parameters. In addition to PC and dendogram evaluation, an individualized analysis of the genotypic characteristics and isolation source provided by the supplier of each strain were considered for the pathogen cocktails proposed in Table 19. All constituents of the proposed cocktail for *E. coli* O157:H7 belonged to cluster A except for isolates CIP 105248 and CIP 105243. These strains were grouped in cluster B at pH 6.0, but were selected due to their resistance in acid solutions. At pH 4.6, both strains exhibited <2.5 log_{10} CFU/mL reductions, whereas the average reduction for all strains was 3.4 log_{10} CFU/mL. According to the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) it is recommended that isolates used in process validation exhibit tolerance to any stress that characterizes the target food, and fruit juices are typically characterized by their mild or high acidity (NACMCF, 2010). Isolate CIP 106326 (cluster A) is also a member of the proposed cocktail and has been included in an *E. coli* O157:H7 strain cocktail used in the validation of HPP blueberry, grape, apple and açaí juices (Gouvea et al., 2020; Kabir et al., 2019; Petrus, Churey, Humiston, Cheng, & Worobo, 2019; Petrus, Churey, & Worobo, 2019). Similarly, all proposed *L. monocytogenes* strains belonged to cluster A, except isolate FSL N3-008 (cluster B), which is included because it was associated with an outbreak related to a vegetable product (coleslaw) and also provides more genetic diversity (serovar 4b).

Table 19. Pathogens cocktails proposed for further validation of HPP juices.

Strain cocktail	Isolate	Serotype	Source
E. coli O157:H7	CIP[a] 105248	O157:H7	Human, enteritis
	ATCC[b] 43894	O157:H7	Human, stool, colitis outbreak
	CIP 105212	O157:H7	Human, stool
	CIP 105231	O157:H7	Human, stool
	CIP 105243	O157:H7	Human, diarrhea
	CIP 105245	O157:H7	Human, enteritis
	ATCC 51659	O157:H7	Human, clinical isolate
L. monocytogenes	FSL[c] J2-035	1/2b	Animal, goat
	FSL J1-049	3c	Human, sporadic listeriosis cases
	FSL J2-054	1/2a	Animal, sheep
	FSL J1-094	1/2c	Human, sporadic listeriosis cases
	FSL J1-031	4a	Human, sporadic listeriosis cases
	FSL J1-168	4a	Human, sporadic listeriosis cases
	FSL W1-110	4c	Unknown history
	FSL N3-008	4b	Coleslaw, Halifax outbreak
	FSL R2-503	1/2b	Human, Illinois outbreak
	ITA[d] 363	1/2b	Vegetable
S. enterica	FSL S5-540	Anatum	Human
	FSL S5-373	Braenderup	Human
	FSL R8-6671	Dessau	Peanut
	FSL S5-439	Dublin	Human
	FSL S5-487	Give	Human
	FSL S5-448	Heidelberg	Human
	FSL S5-480	Heidelberg	Human
	HUBU[e] 72732	Typhimurium	Human
	HUBU 71144	Typhimurium	Human
	HUBU 90196	Enteritidis	Human

[a]Pasteur Institute Collection.
[b]American Type Culture Collection.
[c]Food Safety Laboratory (Cornell University, ILSI strain).
[d]Instituto Tecnológico Agrario de Castilla y León (ITACyL).
[e]Hospital Universitario de Burgos.

Additionally, this specific isolate is closely related to strains FSL J1-107 and FSL J1-108, both isolated from the same coleslaw outbreak and used in *L. monocytogenes* cocktails for HPP juice validation (Petrus, Churey, Humiston, et al., 2019; Petrus, Churey, & Worobo, 2019). Finally, proposed strains of *S. enterica* were grouped in cluster A and were selected based on their pressure resistance and adaptation phenotypes and to cover a wide range of serotypes. Nonetheless, the pressure resistance exhibited by the pathogen in the model solutions studied in this work was considerably lower than that of *E. coli* O157:H7 and *L.*

monocytogenes. Similar results were observed in fruit juices by other authors, where mild processing conditions were able to achieve >5 $\log_{10}$ reductions of a *S. enterica* cocktail in grape (350 MPa for 3 min) or açaí (400 MPa for 3 min) juices (Gouvea et al., 2020; Petrus, Churey, & Worobo, 2019). Thus, the use of *S. enterica* in juice process validation requires more research.

4. Conclusions

Exposure of *E. coli* O157:H7, *L. monocytogenes* and *S. enterica* to moderate HPP conditions (500 MPa for 1 min at 10 °C) in model solutions at pH 4.5 and 6.0 revealed a high variability in their survival and recovery at 12 °C for 14 days. *E. coli* O157:H7 was the only species that survived the process at pH 4.5, which makes this pathogen of special relevance in high-acid foods such as fruit juices. On the other hand, *S. enterica* showed the lowest pressure resistance even in pH 6.0 model solutions. At this pH most strains of *S. enterica* adapted and managed to recover showing variable tendencies, but at a notably slower rate when compared to the other pathogens. *E. coli* O157:H7 and *L. monocytogenes* were the most pressure tolerant, and isolates displayed great variability towards HPP and recovery during subsequent storage at 12 °C.

E. coli O157:H7 survivors in pH 4.5 medium sustained 100 % sublethal injury and failed to adapt and recover, which suggests that combining mild HPP conditions (500 MPa for 1 min) with low pH (<4.5) and a citric acid concentration of 4.16 g/L may be an effective strategy in reducing pathogens. Future work would require the evaluation of pressure resistance in other systems including food products and identifying the acid concentration in which pathogens are still able to recover. This opens the possibility to optimize HPP cycle conditions and lower cost of production, since maintenance and operation costs of HPP equipment increase with pressure level. Furthermore, shorter pressure build-up and holding times increase the throughput of industrial HPP units.

The use of the strain cocktails proposed for each species would make process validation more robust, as required by some food safety agencies. Future research needs to challenge the proposed strain cocktails in real food systems under the same HPP parameters and storage conditions to validate the adequacy of this selection. In the particular case of fruit juices, this work and others here referenced suggest that *S. enterica* is more pressure sensitive and less prone to recover after HPP than *E. coli* O157:H7 and *L. monocytogenes* in acidic media, so the relevance of this pathogen in process validation should be questioned.

5. References

Alpas, H., Kalchayanand, N., Bozoglu, F., & Ray, B. (2000). Interactions of high hydrostatic pressure, pressurization temperature and pH on death and injury of pressure-resistant and pressure-sensitive strains of foodborne pathogens. *International Journal of Food Microbiology*, *60*, 33–42.

Alpas, H., Kalchayanand, N., Bozoglu, F., Sikes, A., Dunne, C. P., & Ray, A. B. (1999). Variation in Resistance to Hydrostatic Pressure among Strains of Food-Borne Pathogens. *Applied and Environmental Microbiology*, *65*(9), 4248–4251.

Arvizu-Medrano, S. M., & Escartín, E. F. (2005). Effect of acid shock with hydrochloric, citric, and lactic acids on the survival and growth of *Salmonella* Typhi and *Salmonella* Typhimurium in acidified media. *Journal of Food Protection*, *68*(10), 2047–2053. https://doi.org/10.4315/0362-028X-68.10.2047

Asioli, D., Aschemann-Witzel, J., Caputo, V., Vecchio, R., Annunziata, A., Næs, T., & Varela, P. (2017). Making sense of the “clean label” trends: A review of consumer food choice behavior and discussion of industry implications. *Food Research International*. Elsevier Ltd. https://doi.org/10.1016/j.foodres.2017.07.022

Barba, F. J., Koubaa, M., do Prado-Silva, L., Orlien, V., & Sant’Ana, A. de S. (2017). Mild processing applied to the inactivation of the main foodborne bacterial pathogens: A review. *Trends in Food Science and Technology*, *66*, 20–35. https://doi.org/10.1016/j.tifs.2017.05.011

Begot, C., Lebert, I., & Lebert, A. (1997). Variability of the response of 66 *Listeria monocytogenes* and *Listeria innocua* strains to different growth conditions. *Food Microbiology*, *14*(5), 403–412. https://doi.org/10.1006/fmic.1997.0097

Benito, A., Ventoura, G., Casadei, M., Robinson, T., & Mackey, B. (1999). Variation in resistance of natural isolates of *Escherichia coli* O157 to high hydrostatic pressure, mild heat, and other stresses. *Applied and Environmental Microbiology*, *65*(4), 1564–1569. https://doi.org/10.1128/aem.65.4.1564-1569.1999

Bruschi, C., Komora, N., Castro, S. M., Saraiva, J., Ferreira, V. B., & Teixeira, P. (2017). High hydrostatic pressure effects on *Listeria monocytogenes* and *L. innocua*: Evidence for variability in inactivation behaviour and in resistance to pediocin bacHA-6111-2. *Food Microbiology*, *64*, 226–231. https://doi.org/10.1016/j.fm.2017.01.011

Canadian Food Inspection Agency. (2017). *Food Microbiology-Targeted Surveys FINAL REPORT. Bacterial Pathogens, Viruses and Parasites in Unpasteurized Juices and High Pressure Processed Juices*.

Chen, H., Neetoo, H., Ye, M., & Joerger, R. D. (2009). Differences in pressure tolerance of *Listeria monocytogenes* strains are not correlated with other stress tolerances and are not based on

differences in *CtsR*. *Food Microbiology*, *26*(4), 404–408. https://doi.org/10.1016/j.fm.2009.01.007

Deliza, R., Rosenthal, A., Abadio, F. B. D., Silva, C. H. O., & Castillo, C. (2005). Application of high pressure technology in the fruit juice processing: Benefits perceived by consumers. *Journal of Food Engineering*, *67*(1–2), 241–246. https://doi.org/10.1016/j.jfoodeng.2004.05.068

Duru, I. C., Andreevskaya, M., Laine, P., Rode, T. M., Ylinen, A., Løvdal, T., … Auvinen, P. (2020). Genomic characterization of the most barotolerant *Listeria monocytogenes* RO15 strain compared to reference strains used to evaluate food high pressure processing. *BMC Genomics*, *21*(1), 1–14. https://doi.org/10.1186/s12864-020-06819-0

Erkmen, O., & Dogan, C. (2004). Effects of ultra high hydrostatic pressure on *Listeria monocytogenes* and natural flora in broth, milk and fruit juices. *International Journal of Food Science and Technology*, *39*(1), 91–97. https://doi.org/10.1046/j.0950-5423.2003.00754.x

Gänzle, M., & Liu, Y. (2015). Mechanisms of pressure-mediated cell death and injury in *Escherichia coli*: From fundamentals to food applications. *Frontiers in Microbiology*. Frontiers Media S.A. https://doi.org/10.3389/fmicb.2015.00599

Gayán, E., Rutten, N., Van Impe, J., Michiels, C. W., & Aertsen, A. (2019). Identification of novel genes involved in high hydrostatic pressure resistance of *Escherichia coli*. *Food Microbiology*, *78*(2019), 171–178. https://doi.org/10.1016/j.fm.2018.10.007

Gayán, E., Van den Bergh, B., Michiels, J., Michiels, C. W., & Aertsen, A. (2020). Synthetic reconstruction of extreme high hydrostatic pressure resistance in *Escherichia coli*. *Metabolic Engineering*, *62*, 287–297. https://doi.org/10.1016/j.ymben.2020.09.008

González-Angulo, M. (2015). *Validación de Listeria innocua como "surrogate" (sustituto) de Listeria monocytogenes en productos cárnicos tratados por altas presiones hidrostáticas*. Universidad de Burgos, Spain.

González-Angulo, M., Serment-Moreno, V., Queirós, R. P., & Tonello-Samson, C. (2021). Food and Beverage Commercial Applications of High Pressure Processing. In K. Muthukumarappan & K. Knoerzer (Eds.), *Innovative Food Processing Technologies* (pp. 39–73). Amsterdam: Elsevier. https://doi.org/10.1016/b978-0-12-815781-7.00009-3

González-Tejedor, G. A., Garre, A., Esnoz, A., Artés-Hernández, F., & Fernández, P. S. (2018). Effect of storage conditions in the response of *Listeria monocytogenes* in a fresh purple vegetable smoothie compared with an acidified TSB medium. *Food Microbiology*, *72*, 98–105. https://doi.org/10.1016/J.FM.2017.11.005

Gouvea, F. S., Padilla-Zakour, O. I., Worobo, R. W., Xavier, B. M., Walter, E. H. M., & Rosenthal, A. (2020). Effect of high-pressure processing on bacterial inactivation in açaí juices with varying pH and soluble solids content. *Innovative Food Science and Emerging Technologies*,

66, 102490. https://doi.org/10.1016/j.ifset.2020.102490

Jackson-Davis, A., Mendonca, A., Hale, S., Jackson, J., King, A., & Jackson, J. (2018). Microbiological Safety of Unpasteurized Fruit and Vegetable Juices Sold in Juice Bars and Small Retail Outlets. In S. Ricke, G. Atungulu, C. Rainwater, & S. Park (Eds.), *Food and Feed Safety Systems and Analysis* (pp. 213–225). London: Academic Press. https://doi.org/10.1016/B978-0-12-811835-1.00012-9

Jordan, S. L., Pascual, C., Bracey, E., & Mackey, B. M. (2001). Inactivation and injury of pressure-resistant strains of *Escherichia coli* O157 and *Listeria monocytogenes* in fruit juices. *Journal of Applied Microbiology*, *91*(3), 463–469. https://doi.org/10.1046/j.1365-2672.2001.01402.x

Kabir, M. N., Aras, S., Allison, A., Adhikari, J., Chowdhury, S., & Fouladkhah, A. (2019). Interactions of Carvacrol, Caprylic Acid, Habituation, and Mild Heat for Pressure-Based Inactivation of O157 and Non-O157 Serogroups of Shiga Toxin-Producing *Escherichia coli* in Acidic Environment. *Microorganisms*, *7*(5). https://doi.org/10.3390/microorganisms7050145

Koseki, S., & Yamamoto, K. (2006). Recovery of *Escherichia coli* ATCC 25922 in phosphate buffered saline after treatment with high hydrostatic pressure. *International Journal of Food Microbiology*, *110*(1), 108–111. https://doi.org/10.1016/j.ijfoodmicro.2006.01.039

Linton, M., Mcclements, J. M. J., & Patterson, M. F. (1999). *Survival of Escherichia coli O157:H7 during Storage in Pressure-Treated Orange Juice*. *Journal of Food Protection* (Vol. 62).

Liu, Y., Gill, A., McMullen, L., & Gänzle, M. G. (2015). Variation in heat and pressure resistance of verotoxigenic and nontoxigenic *Escherichia coli*. *Journal of Food Protection*, *78*(1), 111–120. https://doi.org/10.4315/0362-028X.JFP-14-267

Liu, Y., Ream, A., Joerger, R. D., Liu, J., & Wang, Y. (2011). Gene expression profiling of a pressure-tolerant *Listeria monocytogenes* Scott A *ctsR* deletion mutant. *Journal of Industrial Microbiology and Biotechnology*, *38*(9), 1523–1533. https://doi.org/10.1007/s10295-011-0940-9

McCollum, J. T., Cronquist, A. B., Silk, B. J., Jackson, K. A., O'Connor, K. A., Cosgrove, S., … Mahon, B. E. (2013). Multistate Outbreak of Listeriosis Associated with Cantaloupe. *New England Journal of Medicine*, *369*(10), 944–953. https://doi.org/10.1056/NEJMoa1215837

Miles, A. A., Misra, S. S., & Irwin, J. O. (1938). The estimation of the bactericidal power of the blood. *Journal of Hygiene*, *38*(6), 732–749. https://doi.org/10.1017/S002217240001158X

NACMCF. (2010). Parameters for determining inoculated pack/challenge study protocols. *Journal of Food Protection*, *73*(1), 140–202. https://doi.org/10.4315/0362-028X-73.1.140

Nakaura, Y., Morimatsu, K., Inaoka, T., & Yamamoto, K. (2019). *Listeria monocytogenes* cells injured by high hydrostatic pressure and their recovery in nutrient-rich or -free medium during cold storage. *High Pressure Research*, *39*(2), 324–333.

https://doi.org/10.1080/08957959.2019.1584895

Noma, S., Tomita, C., Shimoda, M., & Hayakawa, I. (2004). Response of *Escherichia coli* O157:H7 in apple and orange juices by hydrostatic pressure treatment with rapid decompression. *Food Microbiology*, *21*(4), 469–473. https://doi.org/10.1016/j.fm.2003.09.004

Patterson, M. F., McKay, A. M., Connolly, M., & Linton, M. (2012). The effect of high hydrostatic pressure on the microbiological quality and safety of carrot juice during refrigerated storage. *Food Microbiology*, *30*(1), 205–212. https://doi.org/10.1016/j.fm.2011.09.013

Petrus, R., Churey, J., Humiston, G., Cheng, R., & Worobo, R. (2019). The combined effect of high pressure processing and dimethyl dicarbonate to inactivate foodborne pathogens in apple juice. *Brazilian Journal of Microbiology*, *51*, 779–785. https://doi.org/10.1007/s42770-019-00145-8

Petrus, R., Churey, J., & Worobo, R. (2019). Searching for high pressure processing parameters for *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* reduction in Concord grape juice. *British Food Journal*, *122*(1), 170–180. https://doi.org/10.1108/BFJ-06-2019-0446

Podolak, R., Whitman, D., & Black, D. G. (2020). Factors Affecting Microbial Inactivation during High Pressure Processing in Juices and Beverages: A Review. *Journal of Food Protection*, *83*(9), 1561–1575. https://doi.org/10.4315/JFP-20-096

Reineke, K., Sevenich, R., Hertwig, C., Janßen, T., Fröhling, A., Knorr, D., … Schlüter, O. (2015). Comparative study on the high pressure inactivation behavior of the Shiga toxin-producing *Escherichia coli* O104:H4 and O157:H7 outbreak strains and a non-pathogenic surrogate. *Food Microbiology*, *46*, 184–194. https://doi.org/10.1016/j.fm.2014.07.017

Robey, M., Benito, A., Hutson, R. H., Pascual, C., Park, S. F., & Mackey, B. M. (2001). Variation in Resistance to High Hydrostatic Pressure and *rpoS* Heterogeneity in Natural Isolates of *Escherichia coli* O157:H7. *Applied and Environmental Microbiology*, *67*(10), 4901–4907. https://doi.org/10.1128/AEM.67.10.4901-4907.2001

Shahbaz, H. M., Yoo, S., Seo, B., Ghafoor, K., Kim, J. U., Lee, D. U., & Park, J. (2016). Combination of TiO_2-UV Photocatalysis and High Hydrostatic Pressure to Inactivate Bacterial Pathogens and Yeast in Commercial Apple Juice. *Food and Bioprocess Technology*, *9*(1), 182–190. https://doi.org/10.1007/s11947-015-1614-9

Sherry, A. E., Patterson, M. F., & Madden, R. H. (2004). Comparison of 40 *Salmonella enterica* serovars injured by thermal, high-pressure and irradiation stress. *Journal of Applied Microbiology*, *96*(4), 887–893. https://doi.org/10.1111/j.1365-2672.2004.02211.x

Syed, Q. A., Buffa, M., Guamis, B., & Saldo, J. (2013). Lethality and injuring the effect of compression and decompression rates of high hydrostatic pressure on *Escherichia coli*

O157:H7 in different matrices. *High Pressure Research*, *33*(1), 64–72. https://doi.org/10.1080/08957959.2013.767898

Tamber, S. (2018). Population-Wide Survey of *Salmonella enterica* Response to High-Pressure Processing reveals a Diversity of Responses and Tolerance Mechanisms. *Applied and Environmental Microbiology*, *84*(2), 1–12.

Teo, A. Y., Ravishankar, S., & Sizer, C. E. (2001). Effect of low-temperature, high-pressure treatment on the survival of *Escherichia coli* O157:H7 and *Salmonella* in unpasteurized fruit juices. *Journal of Food Protection*, *64*(8), 1122–1127.

U.S. Food and Drug Administration. (2001). Hazard analysis and critical control point (HACCP); procedures for the safe and sanitary processing and importing of juice; final rule, 21 CFR Part 120. *Federal Register*, *66*, 6137–6202.

Usajewicz, I., & Nalepa, B. (2006). Survival of *Escherichia coli* O157:H7 in milk exposed to high temperatures and high pressure. *Food Technology and Biotechnology*, *44*(1), 33–39.

Van Boeijen, I. K. H., Chavaroche, A. A. E., Valderrama, W. B., Moezelaar, R., Zwietering, M. H., & Abee, T. (2010). Population Diversity of *Listeria monocytogenes* LO28: Phenotypic and Genotypic Characterization of Variants Resistant to High Hydrostatic Pressure. *Applied and Environmental Microbiology*, *76*(7), 2225–2233. https://doi.org/10.1128/AEM.02434-09

Van Boeijen, I. K. H., Moezelaar, R. O. Y., Abee, T., & Zwietering, M. H. (2008). Inactivation kinetics of three *Listeria monocytogenes* strains under high hydrostatic pressure. *Journal of Food Protection*, *71*(10), 2007–2013. https://doi.org/10.4315/0362-028X-71.10.2007

van der Veen, S., Moezelaar, R., Abee, T., & Wells-Bennik, M. H. J. (2008). The growth limits of a large number of *Listeria monocytogenes* strains at combinations of stresses show serotype- and niche-specific traits. *Journal of Applied Microbiology*, *105*(5), 1246–1258. https://doi.org/10.1111/j.1365-2672.2008.03873.x

Vanlint, D., Pype, B. J. Y., Rutten, N., Vanoirbeek, K. G. A., Michiels, C. W., & Aertsen, A. (2013). Loss of cAMP/CRP regulation confers extreme high hydrostatic pressure resistance in *Escherichia coli* O157: H7. *International Journal of Food Microbiology*, *166*(1), 65–71. https://doi.org/10.1016/j.ijfoodmicro.2013.06.020

Wells-Bennik, M., Karatzas, K. A., Moezelaar, R., & Abee, T. (2014). *Listeria monocytogenes* High Hydrostatic Pressure Resistance and Survival Strategies. In *High-Pressure Microbiology* (pp. 101–115). American Society of Microbiology. https://doi.org/10.1128/9781555815646.ch6

Wemekamp-Kamphuis, H. H., Wouters, J. A., De Leeuw, P. P. L. A., Hain, T., Chakraborty, T., & Abee, T. (2004). Identification of sigma factor σ^B-controlled genes and their impact on acid stress, high hydrostatic pressure, and freeze survival in *Listeria monocytogenes* EGD-e. *Applied and Environmental Microbiology*, *70*(6), 3457–3466.

https://doi.org/10.1128/AEM.70.6.3457-3466.2004

Whitney, B. M., Williams, R. C., Eifert, J., & Marcy, J. (2007). High-Pressure Resistance Variation of *Escherichia coli* O157:H7 Strains and *Salmonella* Serovars in Tryptic Soy Broth, Distilled Water, and Fruit Juice. *Journal of Food Protection*, *70*(9), 2078–2083. https://doi.org/10.4315/0362-028X-70.9.2078

Wilches-Pérez, D. (2015). *Variability of Listeria monocytogenes response to high hydrostatic pressure. A case study on meat products*. *PhD Thesis*. Universidad de Burgos, Spain.

Yuk, H. G., & Marshall, D. L. (2004). Adaptation of *Escherichia coli* O157:H7 to pH alters membrane lipid composition, verotoxin secretion, and resistance to simulated gastric fluid acid. *Applied and Environmental Microbiology*, *70*(6), 3500–3505. https://doi.org/10.1128/AEM.70.6.3500-3505.2004

Chapter 5

Assessing the potential of typical industrial high pressure processing (HPP) parameters for vegetative pathogen control and shelf-life extension of tender coconut water

CHAPTER 5

ASSESSING THE POTENTIAL OF TYPICAL INDUSTRIAL HIGH PRESSURE PROCESSING (HPP) PARAMETERS FOR VEGETATIVE PATHOGEN CONTROL AND SHELF-LIFE EXTENSION OF TENDER COCONUT WATER

High pressure processing (HPP) has been successfully implemented in the food industry to extend shelf-life and guarantee food safety in a wide range of juices and beverages. This study examined the adequacy of typical HPP industrial parameters (600 MPa for 3 min at 10 °C) to inactivate vegetative pathogens in different varieties of tender coconut (*Cocos nucifera* L.) water (6-8 months mature). Pressure resistant and acid adapted strain cocktails of *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella enterica* were inoculated in four varieties of tender coconut water (Thailand Green Dwarf, King Coconut, Pacific Tall and Pacific Tall × Yellow Dwarf hybrid). Reductions greater than 5 $\log_{10}$ CFU/mL were achieved in all varieties for all pathogens immediately after HPP (day 0). This reduction was sustained for 60 days of storage at 4 °C, except for *L. monocytogenes* in the Pacific Tall × Yellow Dwarf hybrid where a fraction survived HPP (1.2 $\log_{10}$ CFU/mL on day 0) and recovered during storage. Inoculated non-HPP samples revealed that the hybrid type of coconut water was the only one that supported growth of *L. monocytogenes*, whereas water from King Coconut and Pacific Tall coconut varieties exerted a lethal effect, which suggests the presence of natural antimicrobial compounds in some varieties. Physicochemical parameters of coconut waters did not change considerably immediately after HPP and during refrigerated shelf-life. Additionally, microbiological spoilage indicators remained stable through storage at 4 °C, ensuring quality and shelf-life extension for a period of at least 60 days. Future work requires the optimization of processing conditions to achieve a 5-$\log_{10}$ reduction in all types of coconut water. Identifying potential antimicrobial compounds in some varieties would serve to define processing conditions more accurately and consider their presence for risk assessment.

1. Introduction

Tender coconut water is a delicate tropical beverage extracted from green coconuts (harvested after 6 to 8 months of maturation in palm trees) gaining popularity in western society due to its unique flavor profile, natural hydrating properties, health-promoting effects, and because it represents a source of functional compounds (DebMandal & Mandal, 2011; Mantena, Jagadish, Badduri, Siripurapu, & Unnikrishnan, 2003). For commercialization and distribution purposes, tender coconut water has been traditionally heat sterilized and sold shelf-stable, but the fresh quality is reduced due to the intense conditions (Awua, Doe, & Agyare, 2011; Ma et al., 2019). Among alternate processing technologies developed to overcome these limitations, high pressure processing (HPP) has become a success in the market segment of premium juices. More than 24 % of globally installed HPP units are on juice processing facilities and annual production of HPP juices is estimated in 540 million L/year (González-Angulo, Serment-Moreno, Queirós, & Tonello-Samson, 2021). HPP is a suitable nonthermal processing technology to extend the shelf-life of multiple types of foods and beverages with a minimal impact on their nutritional and sensory properties. Fruit juices contain heat sensitive flavor and nutritional compounds that are not affected by pressure as they are by heat (Oey, Lille, Van Loey, & Hendrickx, 2008). HPP has been successfully used during the last three decades in the United States, Europe and Asia to preserve juices and coconut water stored under refrigeration with a shelf-life generally up to three months.

In the US, the Food and Drug Administration (FDA) approved the use of HPP as an alternative technology for the preservation of food and beverages. This opens the possibility to juice manufacturers to comply with the FDA's juice Hazard Analysis and Critical Control Point rule (21 CFR part 120.24) since HPP is able to deliver reductions larger than 5 $\log_{10}$ CFU/mL of vegetative pathogens (U.S. Food and Drug Administration, 2001). However, the spore-former *Clostridium botulinum* is currently considered a microorganism of concern in low-acid juices such as coconut water. At the conditions normally used in the food industry HPP does not inactivate bacterial spores (Black et al., 2007), so additional control measures should be implemented to prevent *C. botulinum* spores germination and growth in juices with pH greater than 4.6. Nonetheless, in the particular case of coconut water, preliminary research suggests that *C. botulinum* is not able to grow nor to produce the botulinum neurotoxin in the beverage after high pressure processing even under storage abuse conditions as previously discussed in Chapters 2 and 3 and elsewhere in the literature (Raghubeer, Dunne, Farkas, & Ting, 2000). Authors attribute this to the lack of essential nutrients in coconut water required for the growth of the pathogen, or to the presence of inhibitory compounds naturally present in the juice. Tender coconut is a well-known source of antimicrobial factors such as polyphenols

or peptides that if present at a sufficient concentration could exert a bactericidal effect against pathogens (Chakraborty & Mitra, 2008; Mandal et al., 2009). Unlike with thermal processing, HPP ensures that these functional compounds remain largely unchanged and active, as previously documented in other low-acid juices such as carrot juice (Patterson, McKay, Connolly, & Linton, 2012). Since coconut water appears to be an adverse medium for *C. botulinum* outgrowth, food safety interventions should focus in the control of vegetative pathogens. Fruit juices, and more specifically low-acid juices, are considered a substrate in which foodborne pathogens can grow. *Escherichia coli* O157:H7 and *Salmonella* spp. are typically associated with outbreaks related to the consumption of unpasteurized juices (Jackson-Davis et al., 2018). Although outbreaks linked to *Listeria monocytogenes* have not been reported in fruit juices, the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) suggested that the pathogen should be considered as a target microorganism because of its ubiquitous and psychrotrophic nature. Additionally, *L. monocytogenes* has been associated to foodborne outbreaks due to the consumption of mildly-acidic fruits, such as cantaloupe (McCollum et al., 2013).

HPP has shown the potential to inactivate pathogens in high-acid and low-acid beverages such as grape, apple, carrot or even coconut water (Patterson et al., 2012; Petrus, Churey, & Worobo, 2019, 2020; Raghubeer et al., 2020). This led some regulatory agencies (such as Health Canada) to conclude that HPP does not rise concerns related to food safety. Hence, process validation is no longer required to launch new products to the market (Canadian Food Inspection Agency, 2018). Although there are multiple studies dealing with the HPP inactivation of pathogens in fruit juices, some countries legally require HPP validation before commercialization. With specific regard to the United States, processors must validate on a case-by-case basis the processing conditions used to achieve a 5-$\log_{10}$ reduction of pertinent microorganisms as stated in the Juice HACCP rule (U.S. Food and Drug Administration, 2001). This is because microbial inactivation of pathogens by HPP is dependent on intrinsic properties of the product such as composition and physicochemical attributes (Rendueles et al., 2011). Additionally, the FDA recently communicated through an extensive review that the lack of consensus concerning the selection of representative strains for process validation remains an unresolved issue (Podolak, Whitman, & Black, 2020). Therefore, the present study was undertaken to evaluate the adequacy of standard processing conditions commonly used in the juice industry (600 MPa for 3 min at 10 °C) to inactivate previously identified pressure-resistant and acid habituated bacterial cocktails of *E. coli* O157:H7, *L. monocytogenes* and *S. enterica* in four varieties of tender coconut water, covering a wide range of geographical origins and physicochemical characteristics, aiming to assess the suitability of HPP to maintain safety of this tropical juice.

2. Materials and methods

2.1. Coconut water

Four varieties of raw and unprocessed tender coconut water (all extracted from 6- to 8-month-old coconuts) were shipped frozen to the University of Burgos (Table 20). Samples were kept at -18 °C until used. After thawing at 4 °C, 30 mL aliquots were aseptically dispensed in PET bottles (Sunbox, Spain) and placed in ice to keep them cool until further manipulation.

Table 20. Details of the coconut waters used in the study.

Code	Geographical origin	Cultivar
BE	Belize	Pacific Tall × Yellow Dwarf
SL	Sri Lanka	King Coconut (*aurantiaca*)
T1	Thailand	Thailand Green Dwarf (*Nam Hom*)
TP	The Philippines	Pacific Tall

2.2. Physicochemical characterization

Coconut waters' pH was measured with a Crison micropH 2001 pH meter (Crison Instruments, Spain). The total soluble solids (TSS) were determined using an Atago WM-7 refractometer and expressed as Brix degrees (°Brix). Total titratable acidity (TA) was determined by titration of 10 mL of coconut water to a pH endpoint of 8.2 using 0.1 M NaOH. TA was calculated as percentage of malic acid. All physicochemical analyses were performed in triplicate through the duration of the experiment.

2.3. Determination of phenolic content

The total content of phenolic compounds (TPC) of coconut waters was determined spectrophotometrically following the Folin-Ciocalteu method (Singleton & Rossi, 1965). The reaction mixture contained 0.5 mL of coconut water, 0.5 mL of Folin-Ciocalteu reagent (Merck, Germany) and 10 mL of a 75 g/L sodium carbonate (Thermo Fisher, US) solution. The final mixture was diluted to 25 mL with deionized water. The mixture was kept in the dark at ambient temperature for 1 h, and then the absorbance at 750 nm was measured on a U-1900 UV-visible spectrophotometer (Hitachi, Ltd., USA). Results were expressed as milligrams of gallic acid equivalent per liter of coconut water (mg GAE/L). TPC determinations were performed in triplicate through the duration of the experiment.

2.4. Bacterial strains, acid habituation and cocktail preparation

Strains used in this study were selected based on the characterization previously discussed on Chapter 4. This selection pressure resistant strains covered a wide range of genotypic and source variability (Table 19 in Chapter 4). Strains were cultured from frozen 20 % glycerol stocks (-80 °C) on tryptic soy agar plates with 0.6 % yeast extract (TSAYE) (Oxoid, UK) at 37 °C for 24 h. Isolated colonies were transferred to 10 mL of tryptic soy broth (Condalab, Spain) with 0.6 % yeast extract (TSBYE) and incubated at 37 °C for 24 h to obtain fully active cultures.

Acid habituation was conducted as described by Enache et al. (2011) with slight modifications. A loopful of actively growing cultures in TSBYE was transferred to 10 mL of TSBYE adjusted to pH 5.0 by the addition of a 10X filter-sterilized solution of malic acid. Final concentration of the organic acid in the broth medium was 0.24 % (w/v). Tubes were incubated at 37 °C for 24 h. After incubation, 2.5 mL of each acid-habituated strain were dispensed in 10 mL centrifuge tubes (Labbox, Spain) and washed by centrifugation at 12,000 rpm for 10 min at 4 °C in a 5810R Eppendorf centrifuge (Eppendorf, Germany). Pellets were resuspended in ice-cold Butterfield's buffer (pH 7.2) to avoid additional acid stress. Washing step was repeated twice and pellets were finally resuspended in 2.5 mL of each type of coconut water. Strain cocktails were created by mixing cultures suspended on the appropriate type of coconut water. Four cocktails containing the same strains suspended on each coconut water were finally obtained for *E. coli* O157:H7, *L. monocytogenes* and *S. enterica*, respectively.

2.5. Coconut water inoculation and HPP conditions

PET bottles containing 30 mL of each type of coconut water were individually and separately inoculated with 300 µL of the appropriate strain cocktail of *E. coli* O157:H7, *L. monocytogenes* or *S. enterica* targeting a final level of 7 $\log_{10}$ CFU/mL. Samples were processed at 600 MPa for 3 min in an industrial Hiperbaric 135 unit equipped with a 135 L horizontal vessel (Hiperbaric, Spain). Water at 10 °C was used as pressurizing fluid. Adiabatic heat increase due to compression was estimated in 3 °C/100 MPa. Pressurization rate was of 221 MPa/min, and pressure release after the 3 min holding period was <2 s. Triplicate samples were analyzed for non-pressure treated controls and HPP samples stored at 4 °C on days 20, 40 and 60, whereas five replicates were considered to evaluate the lethal effect of HPP immediately after processing (day 0). Separate non-inoculated samples for each type of coconut water were processed at the same conditions and incubated at 4 °C for shelf-life determination. Aerobic plate counts, lactic acid bacteria, *Enterobacteriaceae*, and molds &

yeasts counts were assessed in triplicate on non-treated samples and after HPP on days 0, 20, 40 and 60.

In parallel, duplicate 30-mL samples of each type of coconut water were separately inoculated with a 300-µL aliquot of decimally diluted bacterial cocktails of each pathogen to achieve an initial concentration between 5 and 6 $\log_{10}$ CFU/mL. Growth of *E. coli* O157:H7, *L. monocytogenes* and *S. enterica* was monitored for 20 days at 4 ° C. Coconut waters were high-pressure processed prior to inoculation (600 MPa for 3 min) to reduce the load of spoilage microorganisms and to consider any positive or negative effect of the treatment in the growth dynamics of the three pathogens studied.

2.6. Microbial enumeration

Serial dilutions of each sample were done using quarter strength Ringer's solution (Oxoid, UK). Undiluted and diluted aliquots of 100 µL were plated on selective agar plates for the enumeration of inoculated vegetative pathogens. Sorbitol MacConkey Agar with BCIG (Oxoid, UK) was used for *E. coli* O157:H7, Oxoid Chromogenic Listeria Agar (Oxoid, UK) for *L. monocytogenes* and Xylose-Lysine-Deoxycholate Agar (Biolife, Italy) for *S. enterica*, as described in the U.S. Food and Drug Administration's Bacteriological Analytical Manual (BAM) (Andrews, Wang, Jacobson, & Hammack, 2020; Feng, Weagant, & Jinneman, 2018; Hitchins, Jinneman, & Chen, 2017). Plates were incubated at 37 ° C for 24-48 h before enumeration. The level of microbial reduction was determined as the difference between average counts in non-treated and treated samples.

Aerobic plate counts, lactic acid bacteria, molds & yeasts and *Enterobacteriaceae* were determined by plating 1 mL of undiluted and diluted samples on Plate Count Agar (Oxoid, UK) (30 °C for 48 h); de Man, Rogosa and Sharpe agar (Oxoid, UK) (30 °C for 48 h); Sabouraud Dextrose Chloramphenicol agar (Oxoid, UK) (25 °C for 72 h); and Violet Red Bile Glucose agar (Oxoid, UK) (35 °C for 24 h), respectively.

2.7. Statistical analysis

Results are expressed as average of three or five replicates±standard deviation, unless otherwise stated. One-way analysis of variance (ANOVA) was used to identify significant differences over time within each type of coconut water in their physicochemical parameters, total phenolic content and spoilage microorganisms using Statgraphics Centurion XVIII (StatPoint Technologies, USA). Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Physicochemical characterization of tender coconut water

The inactivation of bacteria by high pressure processing depends on numerous factors, including intrinsic properties of the food (Rendueles et al., 2011). Parameters such as pH, total acidity, sugar content or polyphenols not only affect to pressure inactivation of microorganisms, but also may influence their recovery and growth during shelf-life. The four different varieties of coconut water studied covered ranges of pH (4.7 to 5.6), titratable acidity (0.034 to 0.094 % of malic acid) and total soluble solids (5.2 to 7.1 °Brix) that typically characterize the water extracted from green coconuts (6 to 8 months of development) (Jackson, Gordon, Wizzard, McCook, & Rolle, 2004; Tan, Cheng, Bhat, Rusul, & Easa, 2014) as shown in Table 21.

Table 21. pH, titratable acidity (TA), total soluble solids (TSS) and total phenolic content (TPC) before and after HPP (600 MPa for 3 min) for 60 days of cold storage (4 °C) in four different varieties of coconut water.

Coconut water	Day	pH	TA (% malic acid)	TSS (°Brix)	TPC (mg GAE/L)
T1	No HPP	5.6±0.0^{a}	0.034±0.000^{b}	7.13±0.23^{a}	22.4±2.4^{b}
	0	5.5±0.0^{a}	0.042±0.002^{a}	6.90±0.00^{b}	30.9±2.8^{a}
	20	5.5±0.1^{b}	0.043±0.001^{a}	6.77±0.06^{bc}	29.2±2.3^{a}
	40	5.5±0.0^{ab}	0.040±0.003^{a}	6.67±0.06^{c}	25.2±0.5^{b}
	60	5.6±0.0^{a}	0.039±0.006^{a}	6.90±0.00^{b}	21.7±1.6^{b}
SL	No HPP	4.8±0.0^{b}	0.094±0.000^{a}	5.20±0.10^{a}	149.9±3.4^{b}
	0	4.8±0.0^{a}	0.084±0.003^{b}	5.10±0.00^{a}	187.4±14.7^{a}
	20	4.7±0.0^{c}	0.100±0.003^{a}	4.70±0.10^{c}	155.3±6.4^{b}
	40	4.7±0.0^{c}	0.102±0.003^{a}	4.80±0.00^{bc}	129.0±4.6^{c}
	60	4.8±0.0^{c}	0.101±0.010^{a}	4.90±0.00^{b}	125.6±3.0^{c}
TP	No HPP	5.3±0.0^{b}	0.047±0.000^{b}	5.80±0.10^{a}	37.6±5.2^{c}
	0	5.4±0.0^{a}	0.064±0.003^{a}	5.70±0.00^{b}	49.0±3.1^{a}
	20	5.3±0.0^{bc}	0.060±0.004^{a}	5.67±0.06^{bc}	46.5±1.2^{ab}
	40	5.3±0.0^{c}	0.060±0.002^{a}	5.60±0.00^{c}	41.8±1.2^{bc}
	60	5.4±0.0^{a}	0.061±0.006^{a}	5.70±0.00^{b}	41.2±0.9^{c}
BE	No HPP	5.3±0.0^{ab}	0.067±0.000^{b}	6.67±0.06^{a}	26.1±5.6^{a}
	0	5.3±0.0^{a}	0.055±0.002^{c}	6.57±0.06^{b}	20.5±5.0^{ab}
	20	5.2±0.0^{c}	0.072±0.000^{b}	6.47±0.06^{c}	17.7±0.7^{b}
	40	5.1±0.0^{d}	0.094±0.002^{a}	6.30±0.00^{d}	16.9±0.8^{b}
	60	5.3±0.0^{b}	0.093±0.009^{a}	6.43±0.06^{c}	19.4±1.2^{b}

Different letters within columns indicate significant differences ($p < 0.05$) for each type of coconut water.

Total phenolic content of T1, TP and BE coconut waters was within the reported by Santos et al., (2013), but below the documented in other works (Mahayothee et al., 2016; Tan et al., 2014). Coconut water SL exhibited the highest phenolic content (149.9 mg GAE/L). Although significant differences were observed in pH following HPP (600 MPa for 3 min) and through subsequent storage ($p < 0.05$), fluctuations in all cases did not exceed ±0.12 units during the 60-day incubation period at 4 °C. Titratable acidity progressively increased after HPP in all varieties. Same tendency has been observed in coconut water (Ma et al., 2019) and in other fruit juices and purées following HPP (Picouet, Sárraga, Cofán, Belletti, & Guàrdia, 2015; Yuan et al., 2018). This phenomenon is associated to enzymes present in the beverage that remain active after HPP (Yuan et al., 2018) or to the formation of organic acids due to microbial growth (Ma et al., 2019). The latter correlates well with the observed slight but significant decrease in total soluble solids ($p < 0.05$), since microorganisms surviving HPP (Table 23) could use sugars as substrate for growth. Total phenolic content increased in all cases immediately after HPP (day 0) except in BE coconut water, which remained unchanged ($p > 0.05$). Previous work revealed that HPP (500 MPa for 5 min) did not affect total phenolic content of mature coconut water (Ma et al., 2019). However, it is widely reported that HPP can improve the concentration of phenolic compounds in fruit and vegetable products. Authors attribute this to changes in the structure of vegetable tissue, which enhances the bioavailability of intracellular chemical compounds (Serment-Moreno, Jacobo-Velázquez, Torres, & Welti-Chanes, 2018). HPP improved total phenolic content by 1-27 % in a papaya beverage, blackberry and strawberry purées, and blueberry and pomegranate juices (Barba, Esteve, & Frigola, 2013; Chen et al., 2015; Patras, Brunton, Da Pieve, & Butler, 2009; Varela-Santos et al., 2012). Coconut waters used in this study were not filtered and contained varying amounts of endosperm tissue, which in the particular case of coconut is well known to represent a source of phenolic compounds (Mahayothee et al., 2016). Concentration of total phenols steadily decreased during incubation period at 4 °C. This may be consequence of enzymatic activity. Polyphenol oxidase (PPO) is a pressure resistant enzyme present in coconut water that catalyzes the oxidation of polyphenols. This enzymatic oxidation is responsible of the characteristic pink discoloration of fresh coconut water (Prades, Dornier, Diop, & Pain, 2012), a phenomenon that was observed in the present study through the incubation period of the samples.

Physicochemical characterization of food products is important to define processing parameters and to assess the performance of preservation methods. Additionally, evolution of the beverage during refrigerated shelf-life also depends on these parameters since pathogens or spoilage microorganisms may adapt and recover, or progressively die after processing. Their stability over shelf-life also indicate quality from sensory and nutritional perspectives.

3.2. HPP safety validation study

Subjecting the acid-habituated multiple-strain cocktails of *E. coli* O157:H7, *L. monocytogenes* and *S. enterica* to HPP (600 MPa for 3 min) in the four types of coconut water achieved more than a 5-$\log_{10}$ reduction in all cases immediately after processing (day 0) (Table 22). *S. enterica* was inactivated below the detection limit (<1 $\log_{10}$ CFU/mL) in the four varieties of coconut water and showed no recovery through the 60-day incubation period at 4 °C. Similarly, *E. coli* O157:H7 displayed counts below the detection limit (<1 $\log_{10}$ CFU/mL) in all coconut water types except in BE coconut water (1.1 $\log_{10}$ CFU/mL on days 0 and 20). Nonetheless, the observed inactivation exceeded 5-$\log_{10}$ units over incubation time and it is likely that survivors failed to adapt and progressively died, since *E. coli* O157:H7 was not detected on days 40 and 60. Conversely, *L. monocytogenes* was the most pressure resistant pathogen as survivors were observed immediately after HPP (day 0) in T1 and BE coconut waters. Further enumeration in coconut water T1 yielded counts below the detection limit (<1 $\log_{10}$ CFU/mL), but *L. monocytogenes* progressively grew in BE coconut water during incubation at 4 °C. As a consequence, inactivation decreased from 5.9 $\log_{10}$ CFU/mL on day 0 to 3.2 $\log_{10}$ CFU/mL on day 60. It is noteworthy to mention that TP and T1 coconut waters had a higher pH (5.6 and 5.55, respectively) than coconut water BE (pH 5.3) (Table 21), so other intrinsic factors aside from acidity may account for the enhanced pressure tolerance of *L. monocytogenes*.

Previous work suggests that HPP (593 MPa for 3 min) can effectively achieve more than a 6-$\log_{10}$ reduction of the three pathogens in coconut waters from Florida (pH 5.4) and Brazil (pH 5.2) over a period of 54 and 75 days, respectively, at 4 °C (Raghubeer et al., 2020). The enhanced resistance observed in the present study can be attributed to the higher inoculation level (between 7.1 and 7.6 $\log_{10}$ CFU/mL, compared to 5.5 and 6.5 $\log_{10}$ CFU/ mL), differences in the pressure tolerance of isolates, different procedure for the habituation of strains to acidic conditions or differences in the intrinsic characteristics of coconut waters. As similarly reported for coconut water, HPP has also shown the potential to control pathogens in other low-acid juices. Processing carrot juice (pH 6.0 to 6.2) achieved a reduction of *E. coli* O157:H7 and *S. enterica* greater than 6.4 $\log_{10}$ CFU/mL when processed at 615 MPa for 2 min (Teo, Ravishankar, & Sizer, 2001) and a reduction of *L. monocytogenes* greater than 6.0 $\log_{10}$ CFU/mL at 500 MPa for 1 min (Patterson et al., 2012). Subjecting non-acidified cantaloupe purée (pH 6.9) to 500 MPa for 5 min inactivated *L. monocytogenes* and *S. enterica* (>6.0 and >6.8 $\log_{10}$ CFU/mL reductions, respectively) for 10 days at 4 °C (Mukhopadhyay et al., 2016). Similarly, a reduction of *L. monocytogenes* greater than 6.0 $\log_{10}$ CFU/mL was obtained in peach juice (pH 5.2) after processing at 600 MPa for 2.5 min (Erkmen & Dogan, 2004).

Table 22. *E. coli* O157:H7, *L. monocytogenes* and *S. enterica* counts (log_{10} CFU/mL) before and after HPP (600 MPa for 3 min) and logarithmic reductions (log_{10} CFU/mL) for 60 days of cold storage (4 °C) in four different varieties of coconut water.

Coconut water	Day	*E. coli* O157:H7 (log_{10} CFU/mL)		*L. monocytogenes* (log_{10} CFU/mL)		*S. enterica* (log_{10} CFU/mL)	
		Counts	Reduction	Counts	Reduction	Counts	Reduction
T1	No HPP	7.6±0.3		7.3±0.5		7.5±0.0	
	0	<1.0	>6.6	1.2±0.4	6.1	<1.0	>6.5
	20	<1.0	>6.6	<1.0	>6.3	<1.0	>6.5
	40	<1.0	>6.6	<1.0	>6.3	<1.0	>6.5
	60	<1.0	>6.6	<1.0	>6.3	<1.0	>6.5
SL	No HPP	7.5±0.5		7.1±0.2		7.6±0.1	
	0	<1.0	>6.5	<1.0	>6.1	<1.0	>6.6
	20	<1.0	>6.5	<1.0	>6.1	<1.0	>6.6
	40	<1.0	>6.5	<1.0	>6.1	<1.0	>6.6
	60	<1.0	>6.5	<1.0	>6.1	<1.0	>6.6
TP	No HPP	7.6±0.1		7.0±0.1		7.5±0.2	
	0	<1.0	>6.6	<1.0	>6.0	<1.0	>6.5
	20	<1.0	>6.6	<1.0	>6.0	<1.0	>6.5
	40	<1.0	>6.6	1.2±0.4	5.8	<1.0	>6.5
	60	<1.0	>6.6	<1.0	>6.0	<1.0	>6.5
BE	No HPP	7.1±0.4		7.1±0.5		7.7±0.2	
	0	1.1±0.3	6.0	1.2±0.5	5.9	<1.0	>6.7
	20	1.1±0.2	6.0	1.8±0.4	5.3	<1.0	>6.7
	40	<1.0	>6.1	2.9±0.8	4.2	<1.0	>6.7
	60	<1.0	>6.1	3.9±0.4	3.2	<1.0	>6.7

3.3. Survival of pathogens in coconut water

The growth evolution of *E. coli* O157:H7, *L. monocytogenes* and *S. enterica* at 4 °C in the four coconut waters studied is shown in Figure 21. A steady decrease was observed in *E. coli* O157:H7 and *S. enterica* counts through the 20 days of storage regardless of the variety of coconut water (between 0.5 and 3.6 and $\log_{10}$ CFU/mL), but both pathogens remained viable after 20 days of incubation. Previous work showed a similar behavior for *S. enterica* inoculated in tender coconut water (pH 5.3) stored at 5 °C (Beristaín-Bauza et al., 2018). Nonetheless, higher temperatures promoted growth of *S. enterica* and *E. coli* in the beverage (Awua, Doe, & Agyare, 2012; Beristaín-Bauza et al., 2018). However, *L. monocytogenes* behaved differently on each type of coconut water. Counts of the pathogen were stable through storage in coconut water T1, but a sharp decrease was observed in SL and TP varieties with reductions of 3.7 and 3.6 $\log_{10}$ CFU/mL, respectively.

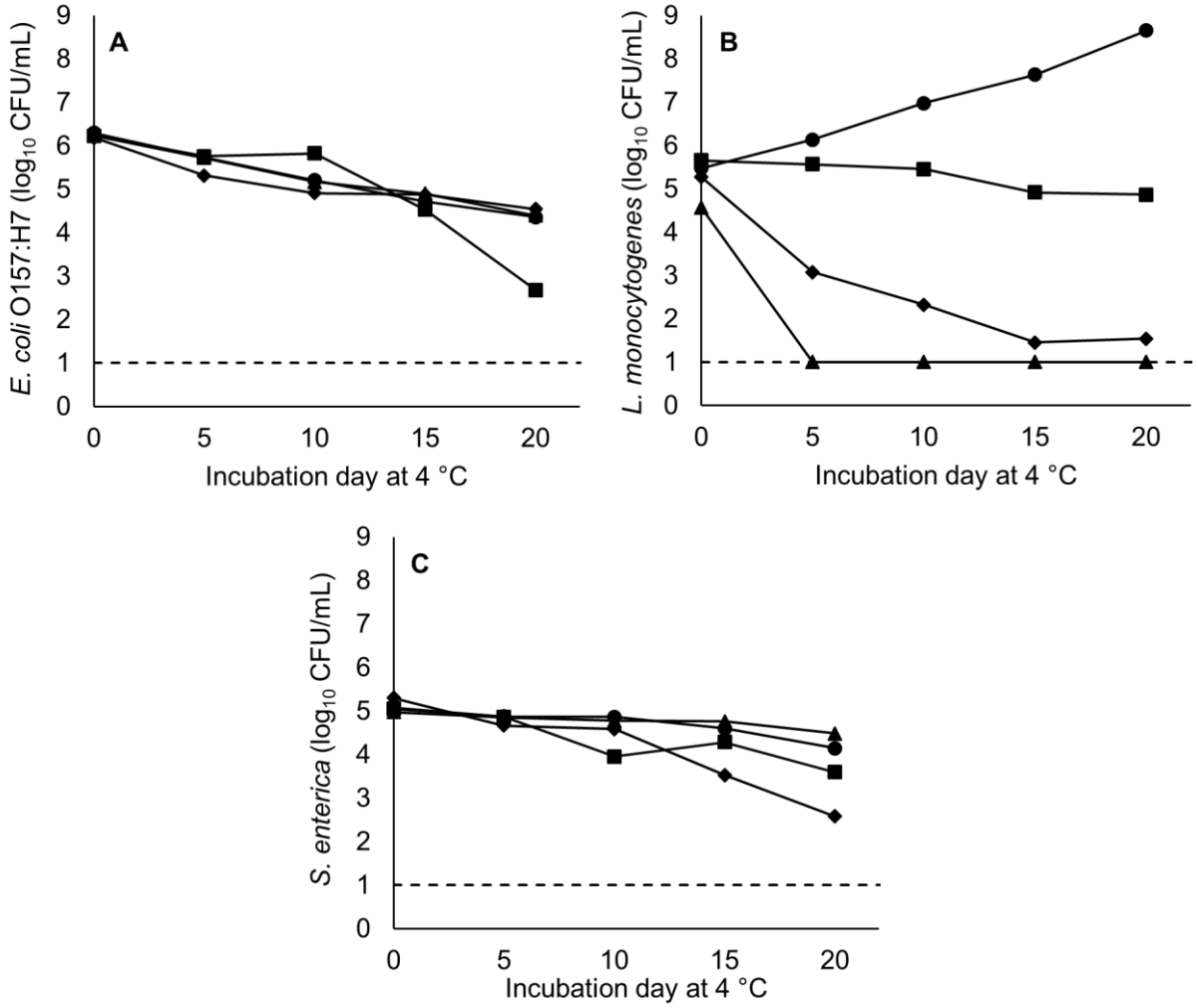

Figure 21. Growth evaluation in non-treated samples at 4 °C of *E. coli* O157:H7 (A), *L. monocytogenes* (B) and *S. enterica* (C) in four different varieties of coconut water (■ T1, ♦ SL, ▲ TP, and ● BE). Results are expressed as the average of two replicates (n = 2). Dashed line represents the detection limit.

In the particular case of TP coconut water, *L. monocytogenes* counts remained below the detection limit (<1 $\log_{10}$ CFU/mL) from day 5 of storage. This is likely due to an antimicrobial effect rather than low pH, because SL coconut water had a lower pH than TP coconut water (pH 4.8 and 5.3, respectively). Conversely, BE coconut water supported growth of *L. monocytogenes* with counts reaching 8.7 $\log_{10}$ CFU/mL after 20 days of storage at 4 °C. This goes in agreement with the observed recovery of the pathogen after HPP in the BE coconut water, but not in the other cultivars (Table 22). It has been previously reported that coconut water obtained from coconuts of the same Green Dwarf variety (pH 4.9) can support growth of *L. monocytogenes* under refrigeration (4 °C) or abuse conditions (10 and 35 °C) (Walter, Kabuki, Esper, Sant'Ana, & Kuaye, 2009). However, other work reported that inoculated *E. coli* O157:H7, *L. monocytogenes* and *S. enterica* showed a sharp decline during storage at 4 °C in two varieties of coconut water from Florida (pH 5.6) and Brazil (pH 5.2). Authors attributed this to the presence of naturally-occurring antimicrobial factors (Raghubeer et al., 2020). Coconut water is source of defence peptides with bactericidal properties. For instance, peptide *Cn*-AMP1 was isolated from tender coconut water and showed minimum inhibitory concentrations for *E. coli*, *B. subtilis*, *P. aeruginosa* and *S. aureus* between 75-85 µg/mL (Mandal et al., 2009), although it is not known if coconut waters used in the present study contained this particular peptide at sufficient concentration.

3.4. Shelf-life of HPP coconut water

Aerobic plate counts, lactic acid bacteria, molds, yeasts and *Enterobacteriaceae* levels were periodically assessed in the four types of coconut water after HPP for 60 days at 4 °C. Initial microbial load varied among coconut waters, but lactic acid bacteria and aerobic plate counts were reduced after HPP (600 MPa for 3 min) and remained below 3.8 $\log_{10}$ CFU/mL in T1 coconut water and below 1.7 $\log_{10}$ CFU/mL in SL, TP and BE coconut waters (Table 23). Molds, yeasts and *Enterobacteriaceae* were completely inactivated in the four varieties and remained undetectable (<1 CFU/mL) during storage period. Unprocessed T1 coconut water had the highest lactic acid bacteria load and also showed the lowest inactivation after HPP. It is generally recognized that Gram-positive bacteria, and more specifically some lactic acid bacteria species are among the most pressure-tolerant microorganisms (Hoover & Farkas, 1989). Nonetheless, counts were stable during refrigerated storage and ensured microbiological quality of coconut waters. Previous work discussed in Chapter 2 of the present thesis showed similar results for a different variety of tender coconut water processed at 550 MPa for 3 min, where total aerobes remained below the detection limit during storage at 4 °C for 61 days (Figure 9). Nonetheless, total aerobes rapidly increased in samples stored at 10 and 20 °C after HPP (Figure 10Figure 11, respectively).

Table 23. Total plate counts, lactic acid bacteria, molds & yeasts and *Enterobacteriaceae* counts before and after HPP (600 MPa for 3 min) for 60 days of cold storage (4 °C) of four different varieties of coconut water.

Coconut waters	Day	log_{10} CFU/mL			
		Aerobic plate counts	Lactic acid bacteria	Molds and yeasts	*Enterobacteriaceae*
T1	No HPP	5.0±0.0[a]	5.0±0.1[a]	2.1±0.1[a]	1.4±0.3[a]
	0	3.1±0.3[b]	1.8±0.0[b]	ND	ND
	20	3.8±1.4[ab]	3.6±1.9[ab]	ND	ND
	40	2.5±0.8[c]	1.9±1.1[ab]	ND	ND
	60	2.1±0.4[c]	2.4±1.8[b]	ND	ND
SL	No HPP	1.5±0.1[a]	1.2±0.1[a]	1.7±0.1[a]	ND
	0	1.0±0.1[b]	0.4±0.3[b]	ND	ND
	20	0.9±0.2[b]	0.2±0.3[b]	ND	ND
	40	0.5±0.2[c]	0.1±0.2[b]	ND	ND
	60	0.8±0.2[b]	ND*	ND	ND
TP	No HPP	2.0±0.1[b]	0.2±0.2[a]	0.1±0.2[a]	ND
	0	1.7±0.1[cd]	ND	ND	ND
	20	2.3±0.0[a]	ND	ND	ND
	40	1.8±0.2[c]	ND	ND	ND
	60	1.6±0.0[c]	ND	ND	ND
BE	No HPP	2.7±0.6[a]	2.9±0.1[a]	2.7±0.0[a]	2.2±0.1[a]
	0	0.2±0.3[c]	ND	ND	ND
	20	1.3±0.1[b]	ND	ND	ND
	40	0.4±0.4[c]	ND	ND	ND
	60	1.3±0.1[b]	ND	ND	ND

ND: not detected (<1 CFU/mL).

Other authors showed as well the potential of HPP to control spoilage microorganisms in coconut water. Ma et al., (2019) reported that total aerobic bacteria, molds and yeasts counts remained below 2.0 $\log_{10}$ CFU/mL after HPP (500 MPa for 5 min) for 30 days at 4 °C. Similarly, subjecting two types of coconut water to 593 MPa for 3 min maintained aerobic bacteria, yeasts, total coliforms and lactic acid bacteria counts below 2.0 $\log_{10}$ CFU/mL in both types over a period of 120 days at 4 ° C (Raghubeer et al., 2020). HPP can also extend the shelf-life of other low-acid juices. For instance, processing carrot juice (pH 6.3) at 600 MPa for 5 min achieved 3-4 $\log_{10}$ CFU/mL reductions in aerobic bacteria, molds, yeasts and lactic acid bacteria. Counts of these indicators remained below 2.0 $\log_{10}$ CFU/mL for 12 days of storage at 4 °C (Stinco et al., 2019). Shelf-life of sugarcane (pH 5.0) and melon (pH 6.2) juices was extended up to 28 days under refrigeration after processing at 500 MPa for 5 min and 400 MPa for 10 min, respectively, with aerobic bacteria counts below 2.0 $\log_{10}$ CFU/mL and molds and yeasts counts below the detection limit (<1 CFU/mL) (Huang, Chang, & Wang, 2015; Pei, Hou, Wang, & Chen, 2018). Similarly, molds and yeasts were completely inactivated (<1 CFU/mL) and aerobic bacteria counts remained for 20 days below 1 $\log_{10}$ CFU/mL in cucumber juice (pH 5.7) after processing at 500 MPa for 5 min (Liu, Zhang, Zhao, Wang, & Liao, 2016).

4. Conclusions

Typical HPP parameters used by the juice industry (600 MPa for 3 min) can cause population reductions of pertinent vegetative pathogens greater than the 5-$\log_{10}$ required by the FDA's HACCP rule (21 CFR part 120) for a period of 60 days in tender coconut waters T1, SL and TP. Despite the same HPP conditions yielded a 5-$\log_{10}$ reduction of pathogens in BE coconut water, this inactivation was not sustained for *L. monocytogenes* during the 60-day storage period at 4 °C. Growth curves on each coconut water revealed that other intrinsic factors of the beverage aside from acidity may account for this behavior. Coconut water BE was the only type that supported growth of *L. monocytogenes*, whereas TP and SL coconut waters exerted a lethal effect on the pathogen during storage at 4 °C.

From a quality perspective it was observed that processing tender coconut water at 600 MPa for 3 min had a limited effect on physicochemical characteristics of the beverage during refrigerated storage (pH, total soluble solids, and titratable acidity), although typical pink discoloration due to polyphenol oxidation was observed. Additionally, total aerobic counts, lactic acid bacteria, *Enterobacteriaceae,* molds & yeasts were effectively controlled by HPP. Despite the fact that total aerobes and lactic acid bacteria were not completely inactivated in all coconut water varieties, counts remained stable during the 60-day storage period at 4 °C, which extended the commercial shelf-life of the beverage.

Future work requires the optimization of processing parameters since typical conditions used in the juice industry for high-acid beverages (600 MPa for 3 min) failed to maintain the required 5-$\log_{10}$ reduction in all coconut water varieties up to 60 days at 4 °C. To this end, BE coconut water would be used to validate extended holding times to control *L. monocytogenes*, which managed to adapt and grow in this particular type of coconut water. New processing conditions would be used to assess the inactivation of *E. coli* O157:H7, *L. monocytogenes* and *S. enterica* under normal (4 °C) and moderate temperature abuse (8 °C) conditions, as required by 21 CFR 120.24. The influence of inoculum size (6 to 7 $\log_{10}$ CFU/mL) would be evaluated as well to define the importance of this parameter in achieving the 5-$\log_{10}$ reduction of pertinent microorganisms during shelf-life. Finally, optimized parameters identified would be validated in multiple varieties of tender coconut water.

5. References

Andrews, W. H., Wang, H., Jacobson, A., & Hammack, T. S. (2020). *Salmonella*. Retrieved May 30, 2020, from https://www.fda.gov/food/Laboratory-methods-food/bam-chapter-5-salmonella

Awua, A. K., Doe, E. D., & Agyare, R. (2011). Exploring the influence of sterilisation and storage on some physicochemical properties of coconut (*Cocos nucifera* L.) water. *BMC Research Notes*, *4*. https://doi.org/10.1186/1756-0500-4-451

Awua, A. K., Doe, E. D., & Agyare, R. (2012). Potential Bacterial Health Risk Posed to Consumers of Fresh Coconut (*Cocos nucifera* L.) Water. *Food and Nutrition Sciences*, *3*, 1136–1143. https://doi.org/10.4236/fns.2012.38149

Barba, F. J., Esteve, M. J., & Frigola, A. (2013). Physicochemical and nutritional characteristics of blueberry juice after high pressure processing. *Food Research International*, *50*(2), 545–549. https://doi.org/10.1016/j.foodres.2011.02.038

Beristaín-Bauza, S., Martínez-Niño, A., Ramírez-González, A. P., Ávila-Sosa, R., Ruíz-Espinosa, H., Ruiz-López, I. I., & Ochoa-Velasco, C. E. (2018). Inhibition of *Salmonella Typhimurium* growth in coconut (*Cocos nucifera* L.) water by hurdle technology. *Food Control*, *92*, 312–318. https://doi.org/10.1016/j.foodcont.2018.05.010

Black, E. P., Setlow, P., Hocking, A. D., Stewart, C. M., Kelly, A. L., & Hoover, D. G. (2007). Response of Spores to High-Pressure Processing. *Comprehensive Reviews in Food Science and Food Safety*, *6*(4), 103–119. https://doi.org/10.1111/j.1541-4337.2007.00021.x

Canadian Food Inspection Agency. (2018). Novel Food Information: High Pressure Processing (HPP)-Treated Fruit and Vegetable-Based Juices. Retrieved October 5, 2020, from https://www.canada.ca/en/health-canada/services/food-nutrition/genetically-modified-foods-other-novel-foods/approved-products/novel-food-information-high-pressure-processing-treated-fruit-vegetable-based-juices.html

Chakraborty, M., & Mitra, A. (2008). The antioxidant and antimicrobial properties of the methanolic extract from *Cocos nucifera* mesocarp. *Food Chemistry*, *107*(3), 994–999. https://doi.org/10.1016/j.foodchem.2007.08.083

Chen, D., Pang, X., Zhao, J., Gao, L., Liao, X., Wu, J., & Li, Q. (2015). Comparing the effects of high hydrostatic pressure and high temperature short time on papaya beverage. *Innovative Food Science & Emerging Technologies*, *32*, 16–28. https://doi.org/10.1016/J.IFSET.2015.09.018

DebMandal, M., & Mandal, S. (2011). Coconut (*Cocos nucifera* L.: *Arecaceae*): In health promotion and disease prevention. *Asian Pacific Journal of Tropical Medicine*, *4*(3), 241–247. https://doi.org/10.1016/S1995-7645(11)60078-3

Enache, E., Mathusa, E. C., Elliott, P. H., Glenn Black, D., Chen, Y., Scott, V. N., … Schaffner, D. W. (2011). Thermal Resistance Parameters for Shiga Toxin-Producing *Escherichia coli* in Apple Juice. *Journal of Food Protection*, *74*(8), 1231–1237. https://doi.org/10.4315/0362-028X.JFP-10-488

Erkmen, O., & Dogan, C. (2004). Effects of ultra high hydrostatic pressure on *Listeria monocytogenes* and natural flora in broth, milk and fruit juices. *International Journal of Food Science and Technology*, *39*(1), 91–97. https://doi.org/10.1046/j.0950-5423.2003.00754.x

Feng, P., Weagant, S. D., & Jinneman, K. (2018). Diarrheagenic *Escherichia coli*. Retrieved May 30, 2020, from https://www.fda.gov/food/Laboratory-methods-food/bam-chapter-4a-diarrheagenic-escherichia-coli

González-Angulo, M., Serment-Moreno, V., Queirós, R. P., & Tonello-Samson, C. (2021). Food and Beverage Commercial Applications of High Pressure Processing. In K. Muthukumarappan & K. Knoerzer (Eds.), *Innovative Food Processing Technologies* (pp. 39–73). Amsterdam: Elsevier. https://doi.org/10.1016/b978-0-12-815781-7.00009-3

Hitchins, A. D., Jinneman, K., & Chen, Y. (2017). Detection of *Listeria monocytogenes* in Foods and Environmental Samples, and Enumeration of Listeria monocytogenes in Foods. Retrieved May 30, 2020, from https://www.fda.gov/food/Laboratory-methods-food/bam-chapter-10-detection-listeria-monocytogenes-foods-and-environmental-samples-and-enumeration

Hoover, D., Farkas, D., Farkas, D., & Farkas, D. (1989). Biological effects of high hydrostatic pressure on food microorganisms. *Food Technology*, *43*(3), 99–107.

Huang, H. W., Chang, Y. H., & Wang, C. Y. (2015). High Pressure Pasteurization of Sugarcane Juice: Evaluation of Microbiological Shelf-life and Quality Evolution During Refrigerated Storage. *Food and Bioprocess Technology*, *8*(12), 2483–2494. https://doi.org/10.1007/s11947-015-1600-2

Jackson-Davis, A., Mendonca, A., Hale, S., Jackson, J., King, A., & Jackson, J. (2018). Microbiological Safety of Unpasteurized Fruit and Vegetable Juices Sold in Juice Bars and Small Retail Outlets. In S. Ricke, G. Atungulu, C. Rainwater, & S. Park (Eds.), *Food and Feed Safety Systems and Analysis* (pp. 213–225). London: Academic Press. https://doi.org/10.1016/B978-0-12-811835-1.00012-9

Jackson, J. C., Gordon, A., Wizzard, G., McCook, K., & Rolle, R. (2004). Changes in chemical composition of coconut (*Cocos nucifera*) water during maturation of the fruit. *Journal of the Science of Food and Agriculture*, *84*(9), 1049–1052. https://doi.org/10.1002/jsfa.1783

Liu, F., Zhang, X., Zhao, L., Wang, Y., & Liao, X. (2016). Potential of high-pressure processing and high-temperature/short-time thermal processing on microbial, physicochemical and sensory assurance of clear cucumber juice. *Innovative Food Science and Emerging Technologies*, *34*, 51–58. https://doi.org/10.1016/j.ifset.2015.12.030

Ma, Y., Xu, L., Wang, S., Xu, Z., Liao, X., & Cheng, Y. (2019). Comparison of the quality attributes of coconut waters by high-pressure processing and high-temperature short time during the refrigerated storage. *Food Science & Nutrition*, *7*(4), 1512–1519. https://doi.org/10.1002/fsn3.997

Mahayothee, B., Koomyart, I., Khuwijitjaru, P., Siriwongwilaichat, P., Nagle, M., & Müller, J. (2016). Phenolic Compounds, Antioxidant Activity, and Medium Chain Fatty Acids Profiles of Coconut

Water and Meat at Different Maturity Stages. *International Journal of Food Properties*, *19*(9), 2041–2051. https://doi.org/10.1080/10942912.2015.1099042

Mandal, S. M., Dey, S., Mandal, M., Sarkar, S., Maria-Neto, S., & Franco, O. L. (2009). Identification and structural insights of three novel antimicrobial peptides isolated from green coconut water. *Peptides*, *30*(4), 633–637. https://doi.org/10.1016/j.peptides.2008.12.001

Mantena, S. K., Jagadish, Badduri, S. R., Siripurapu, K. B., & Unnikrishnan, M. K. (2003). In vitro evaluation of antioxidant properties of *Cocos nucifera* Linn. water. *Molecular Nutrition & Food Research*, *47*(2), 126–131. https://doi.org/10.1002/food.200390023

McCollum, J. T., Cronquist, A. B., Silk, B. J., Jackson, K. A., O'Connor, K. A., Cosgrove, S., … Mahon, B. E. (2013). Multistate Outbreak of Listeriosis Associated with Cantaloupe. *New England Journal of Medicine*, *369*(10), 944–953. https://doi.org/10.1056/NEJMoa1215837

Mukhopadhyay, S., Sokorai, K., Ukuku, D., Fan, X., Juneja, V., Sites, J., & Cassidy, J. (2016). Inactivation of *Salmonella enterica* and *Listeria monocytogenes* in cantaloupe purée by high hydrostatic pressure with/without added ascorbic acid. *International Journal of Food Microbiology*, *235*, 77–84. https://doi.org/10.1016/j.ijfoodmicro.2016.07.007

Oey, I., Lille, M., Van Loey, A., & Hendrickx, M. (2008). Effect of high-pressure processing on colour, texture and flavour of fruit- and vegetable-based food products: a review. *Trends in Food Science and Technology*. https://doi.org/10.1016/j.tifs.2008.04.001

Patras, A., Brunton, N. P., Da Pieve, S., & Butler, F. (2009). Impact of high pressure processing on total antioxidant activity, phenolic, ascorbic acid, anthocyanin content and colour of strawberry and blackberry purées. *Innovative Food Science and Emerging Technologies*, *10*(3), 308–313. https://doi.org/10.1016/j.ifset.2008.12.004

Patterson, M. F., McKay, A. M., Connolly, M., & Linton, M. (2012). The effect of high hydrostatic pressure on the microbiological quality and safety of carrot juice during refrigerated storage. *Food Microbiology*, *30*(1), 205–212. https://doi.org/10.1016/j.fm.2011.09.013

Pei, L., Hou, S., Wang, L., & Chen, J. (2018). Effects of high hydrostatic pressure, dense phase carbon dioxide, and thermal processing on the quality of Hami melon juice. *Journal of Food Process Engineering*, *41*(6), e12828. https://doi.org/10.1111/jfpe.12828

Petrus, R., Churey, J., & Worobo, R. (2019). Searching for high pressure processing parameters for *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* reduction in Concord grape juice. *British Food Journal*, *122*(1), 170–180. https://doi.org/10.1108/BFJ-06-2019-0446

Petrus, R., Churey, J., & Worobo, R. (2020). High-pressure processing of apple juice: the most effective parameters to inactivate pathogens of reference. *British Food Journal*, *122*(12), 3969–3979. https://doi.org/10.1108/BFJ-03-2020-0178

Picouet, P. A., Sárraga, C., Cofán, S., Belletti, N., & Dolors Guàrdia, M. (2015). Effects of thermal and high-pressure treatments on the carotene content, microbiological safety and sensory properties of acidified and of non-acidified carrot juice. *LWT - Food Science and Technology*,

62(1), 920–926. https://doi.org/10.1016/J.LWT.2014.07.027

Podolak, R., Whitman, D., & Black, D. G. (2020). Factors Affecting Microbial Inactivation during High Pressure Processing in Juices and Beverages: A Review. *Journal of Food Protection*, *83*(9), 1561–1575. https://doi.org/10.4315/JFP-20-096

Prades, A., Dornier, M., Diop, N., & Pain, J.-P. (2012). Coconut water preservation and processing: a review. *Fruits*, *67*(3), 157–171. https://doi.org/10.1051/fruits/2012009

Raghubeer, E. V., Phan, B. N., Onuoha, E., Diggins, S., Aguilar, V., Swanson, S., & Lee, A. (2020). The use of High-Pressure Processing (HPP) to improve the safety and quality of raw coconut (*Cocos nucifera* L) water. *International Journal of Food Microbiology*, *331*, 108697. https://doi.org/10.1016/j.ijfoodmicro.2020.108697

Raghubeer, E. V., Dunne, C. P., Farkas, D. F., & Ting, E. Y. (2000). Evaluation of batch and semicontinuous application of high hydrostatic pressure on foodborne pathogens in salsa. *Journal of food protection*, *63*(12), 1713-1718. https://doi.org/10.4315/0362-028X-63.12.1713

Rendueles, E., Omer, M. K., Alvseike, O., Alonso-Calleja, C., Capita, R., & Prieto, M. (2011). Microbiological food safety assessment of high hydrostatic pressure processing: A review. *LWT - Food Science and Technology*. Academic Press. https://doi.org/10.1016/j.lwt.2010.11.001

Santos, J. LA, Bispo, V. S., Filho, A. B., Pinto, I. F., Dantas, L. S., Vasconcelos, D. F., … Matos, H. R. (2013). Evaluation of Chemical Constituents and Antioxidant Activity of Coconut Water (*Cocus nucifera* L.) and Caffeic Acid in Cell Culture. *Annals of the Brazilian Academy of Sciences*, *85*(4), 1235–1246. https://doi.org/10.1590/0001-37652013105312

Serment-Moreno, V., Jacobo-Velázquez, D. A., Torres, J. A., & Welti-Chanes, J. (2018). Microstructural and Physiological Changes in Plant Cell Induced by Pressure: Their Role on the Availability and Pressure-Temperature Stability of Phytochemicals. *Food Engineering Reviews*, *9*(4), 314–334. https://doi.org/10.1007/s12393-017-9158-6

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *American Journal of Enology and Viticulture*, *16*(3), 144–158.

Stinco, C. M., Szczepańska, J., Marszałek, K., Pinto, C. A., Inácio, R. S., Mapelli-Brahm, P., … Meléndez-Martínez, A. J. (2019). Effect of high-pressure processing on carotenoids profile, colour, microbial and enzymatic stability of cloudy carrot juice. *Food Chemistry*, *299*, 125112. https://doi.org/10.1016/j.foodchem.2019.125112

Tan, T. C., Cheng, L. H., Bhat, R., Rusul, G., & Easa, A. M. (2014). Composition, physicochemical properties and thermal inactivation kinetics of polyphenol oxidase and peroxidase from coconut (*Cocos nucifera*) water obtained from immature, mature and overly-mature coconut. *Food Chemistry*, *142*, 121–128. https://doi.org/10.1016/j.foodchem.2013.07.040

Teo, A. Y., Ravishankar, S., & Sizer, C. E. (2001). Effect of low-temperature, high-pressure

treatment on the survival of *Escherichia coli* O157:H7 and *Salmonella* in unpasteurized fruit juices. *Journal of Food Protection*, *64*(8), 1122–1127.

U.S. Food and Drug Administration. (2001). Hazard analysis and critical control point (HACCP); procedures for the safe and sanitary processing and importing of juice; final rule, 21 CFR Part 120. *Federal Register*, *66*, 6137–6202.

Varela-Santos, E., Ochoa-Martinez, A., Tabilo-Munizaga, G., Reyes, J. E., Pérez-Won, M., Briones-Labarca, V., & Morales-Castro, J. (2012). Effect of high hydrostatic pressure (HHP) processing on physicochemical properties, bioactive compounds and shelf-life of pomegranate juice. *Innovative Food Science & Emerging Technologies*, *13*, 13–22. https://doi.org/10.1016/J.IFSET.2011.10.009

Walter, E. H. M., Kabuki, D. Y., Esper, L. M. R., Sant'Ana, A. S., & Kuaye, A. Y. (2009). Modelling the growth of *Listeria monocytogenes* in fresh green coconut (*Cocos nucifera* L.) water. *Food Microbiology*, *26*(6), 653–657. https://doi.org/10.1016/j.fm.2009.04.003

Yuan, B., Danao, M. G. C., Stratton, J. E., Weier, S. A., Weller, C. L., & Lu, M. (2018). High pressure processing (HPP) of aronia berry purée: Effects on physicochemical properties, microbial counts, bioactive compounds, and antioxidant capacities. *Innovative Food Science and Emerging Technologies*, *47*, 249–255. https://doi.org/10.1016/j.ifset.2018.03.009

General discussion

GENERAL DISCUSSION AND CONCLUSION

Foodborne outbreaks related to fresh juice consumption remain an unresolved issue in food safety (Jackson-Davis et al., 2018). Juices can be contaminated by the use of raw materials that came into contact with pathogens during irrigation or collection, by human handling or by direct contact with contaminated surfaces during processing operations. In this regard, the main pathogens that require attention are *E. coli* O157:H7, *S. enterica* and *L. monocytogenes* (Nguyen-The, 2012). Although coconut water has not been directly associated with outbreaks of foodborne illnesses, food regulatory standards require the implementation of intervention methods to inactivate pathogens and ensure food safety. Additionally, the intrinsic characteristics of coconut water require consideration of additional hazards, such as spore formers. Due to the low acidity of the beverage (pH >4.6) it is assumed *C. botulinum* can germinate, grow and produce the botulinum neurotoxin under favorable conditions (*i.e.* temperature abuse, anoxic environment and absence of competitive microorganisms). This justifies the higher prevalence of the pathogen in heat-treated products, where these favorable conditions are easier to find (Nguyen-The, 2012). Traditional thermal interventions are typically applied to inactivate pathogens and spoilage microorganisms in coconut water; however, quality is also affected (Awua, Doe, & Agyare, 2011). High pressure processing (HPP) is a nonthermal preservation technology that preserves fresh quality attributes of food, including coconut water (Ma et al., 2019). Nevertheless, in its current commercial application (up to 600 MPa between 4 and 25 °C), HPP is not effective against bacterial spores. In addition, vegetative pathogens exhibit a great inter- and intraspecies variability towards high pressure. Therefore, this thesis aimed to validate the safety of high-pressure processed tender coconut water. To this end, the potential for proteolytic *C. botulinum* and nonproteolytic *C. botulinum* to grow in the beverage was evaluated to establish if the pathogen is of concern. Additionally, wide populations of *E. coli* O157:H7, *L. monocytogenes* and *S. enterica* were characterized to identify representative pressure-resistant strains that were later used to validate the ability of HPP to control them in tender coconut water.

1. Risk of *C. botulinum* growth in tender HPP coconut water

Despite the lack of epidemiology and scientific evidences, no other parameters apart from acidity are considered by food safety agencies to control the risk of *C. botulinum* growth and neurotoxin production in high-pressure processed coconut water (pH >4.6). Chapter 2 of this thesis determined that nontoxigenic spores of non-proteolytic *C. botulinum* type E and *Clostridium* spp. failed to grow in tender coconut water (pH 5.2) from Thailand Green Dwarf

coconut variety (T2) stored at 4 and 10 °C for 61 days, although they remained viable after HPP (550 MPa for 3 min at 10 °C). This is consistent with the findings published by Raghubeer et al. (2020), who observed that spores of proteolytic *C. botulinum* and nonproteolytic *C. botulinum* remained viable but failed to produce the botulinum neurotoxin after HPP (593 MPa for 3 min at 10 °C) in two varieties of tender coconut water from Brazil (pH 5.2) and Florida (pH 5.4) stored at 4 and 10 °C for 45 days. Moreover, work performed in this thesis evidenced that growth from spores was not observed after HPP even when samples of coconut water were stored under severe temperature abuse (20 °C) and absence of dissolved oxygen (<0.5 mg/L O_2). Spore concentration remained constant or slightly decreased during the 61-day incubation period, regardless of storage temperature or dissolved oxygen concentration. Additionally, it was also observed that other pressure-resistant bacterial species (*i.e. Tatumella ptyseos* and *Xanthomonas* spp.) managed to survive and progressively recovered and grew after HPP in coconut water samples incubated at 10 and 20 °C. This caused severe spoilage and depleted dissolved oxygen levels in all samples below 0.5 mg/L O_2. Despite of anaerobic conditions created by growth of spoilage microorganisms and favorable storage temperature (10-20 °C), *C. botulinum* spores failed to grow in tender coconut water (pH >4.6).

Aiming to elucidate the intrinsic parameters that condition *C. botulinum* spore germination and growth in tender coconut water, the next steps involved the modification of some of the characteristics of the beverage that are known to affect spore behavior, and potentially stimulating germination and growth. Increasing pH to optimum values (pH 7), removing dissolved oxygen (<0.5 mg/L O_2), sterilizing by filtration before spore inoculation to avoid competition with spoilage microbiota, and incubating samples of coconut water from Thailand Green Dwarf coconuts at 30 °C (optimum growth temperature) resulted in a marked decrease of nonproteolytic type E *C. botulinum* and *Clostridium* spp. spore concentration during storage at 30 °C for 61 days. This suggests that this fraction of spores germinated but did not resume vegetative growth under extremely favorable conditions. To ascertain whether failure to grow was attributable to naturally present antimicrobial compounds in coconut water or to the lack of specific nutrients required by *C. botulinum*, the beverage was supplemented with external sources of nutrients. Supplementation with 2 % casein hydrolysate + 0.1 g/L tryptophan did not support spore growth. Conversely, the addition of tryptone-peptone-glucose-yeast extract (TPGY) broth was sufficient to promote spore growth based on total count determination. The low concentration of TPGY broth required to stimulate germination and growth in tender coconut water (between 6.25-12.5 %) presumptively discarded the presence of antimicrobial compounds, at least at sufficient concentration.

The previously discussed study was conducted using nontoxigenic strains and determining growth based on total plate counts. To assess more accurately the safety of HPP tender coconut water, next experiments (Chapter 3) used separate nine-strain cocktails of toxigenic proteolytic *C. botulinum* and nonproteolytic *C. botulinum* carefully selected based on their outbreak history. Additionally, growth and botulinum neurotoxin production were determined by multiplex PCR and ELISA, respectively. Growth potential of proteolytic *C. botulinum* and nonproteolytic *C. botulinum* spore cocktails was evaluated in multiple varieties of tender coconut water. Intrinsic characteristics of coconut waters were modified aiming to determine their influence on the growth of the spores. Redox potential was reduced by continuous bubbling with a 10 % H_2/90 % N_2 gas mixture. This method was preferred over N_2 bubbling (used in Chapter 2) because in addition to oxygen removal, the presence of H_2 creates more reduced environments that facilitate spore germination and growth. Additionally, background microbiota was inactivated by gamma irradiation (25 kGy), which is considered a noninvasive cold sterilization intervention. This technique was preferred over microfiltration (used in Chapter 2) because it does not remove the pulp (*i.e.* solid endosperm) from coconut water, which may represent a source of nutritional or antimicrobial compounds (Mahayothee et al., 2016). Finally, pH of coconut waters was adjusted to values above natural pH (from 5.5 to 6.8), and incubation temperature for inoculated samples was of 30 °C, which is close to the optimum value for proteolytic *C. botulinu*m and nonproteolytic *C. botulinum*.

Despite optimum incubation temperature, redox potential and absence of competing microorganisms, none of the anoxic natural (pH 4.6 to 5.3) and pH-adjusted (pH 5.5 to 5.8) coconut water samples supported growth of psychrotrophic, nonproteolytic *C. botulinum* determined as gas production and botulinum neurotoxin gene (*bont*) detection. Same result was obtained for natural and pH-adjusted coconut water samples inoculated with proteolytic *C. botulinum*, except for one replicate of Thailand Green Dwarf coconut water (T2) adjusted to pH 5.6. Conversely, all positive controls consisting of 1:1 mixtures of each type of coconut water with peptone-glucose-yeast extract-starch (PYGS) broth were positive for growth of proteolytic *C. botulinum* and nonproteolytic *C. botulinum*. A similar observation was reported by Raghubeer et al. (2020) in two types of filter-sterilized coconut water (pH 5.4 and 5.2) inoculated with proteolytic *C. botulinum* and nonproteolytic *C. botulinum* spores. Samples were subjected to HPP (593 MPa for 3 min at 4 °C) and stored for 45 days under slight temperature abuse (10 °C). Botulinum neurotoxin formation (BoNT) was not detected at the end of the 45-day period, but subsequent enrichment with PYGS broth showed the presence of toxins. It is noteworthy to mention that dissolved oxygen concentration and redox potential of coconut waters used in the abovementioned study were not modified and remained the same as those of commercial HPP coconut water, which probably do not allow spore growth.

Aiming to understand the importance of pH on *C. botulinum* growth, Thailand Green Dwarf coconut water (T2) was chosen for further study as it was the only one that supported growth of proteolytic *C. botulinum* when adjusted to pH 5.6. Interestingly, samples of natural coconut water adjusted to pH 6.4 and 6.0 supported growth of proteolytic *C. botulinum* types A and B, whereas only samples adjusted to pH 6.4 supported growth of nonproteolytic *C. botulinum* type B. These observations support the hypothesis that coconut water does not contain inhibitory compounds that prevent spore germination and/or cell multiplication, and that pH is a critical factor when it is higher than that of natural coconut water (pH 4.7-5.3). To confirm this premise and to establish the intrinsic factors in coconut water that in combination with pH may limit growth of *C. botulinum*, Thailand Green Dwarf coconut water (T2) was adjusted to the highest pH value at which growth was not observed in previous experiments (*i.e.* pH 5.5 for proteolytic *C. botulinum* and pH 6.0 for nonproteolytic *C. botulinum*) and supplemented with (i) PYGS at decreasing concentrations, and (ii) different groups of nutrients. Growth evidenced as turbidity took place when low concentrations of PYGS broth (6.25 %) were used to supplement coconut water. These findings agree with previous work conducted with non-toxigenic nonproteolytic *C. botulinum* type E and *Clostridium* spp. spores (Chapter 2). Similarly, supplementing coconut water (pH 5.5 and 6.0) with free amino acids, vitamins and minerals supported growth of proteolytic *C. botulinum* type B and nonproteolytic *C. botulinum* type F in samples incubated at 30 °C based on *bont* gene detection. Failure of strains of other toxin types to grow could be attributed to suboptimal pH or different nutrient requirements between phenotypically and genotypically diverse *C. botulinum* groups or toxin types. This hampers the identification of specific nutrients that are limiting in coconut water which may determine *C. botulinum* growth in the liquid endosperm of coconuts.

Hence, in order to evaluate the potential of *C. botulinum* to produce the toxin, a range of five representative varieties of gamma-irradiated and anoxic coconut water were adjusted to different pH values (from their natural pH to pH 6.7), and inoculated with proteolytic *C. botulinum* and nonproteolytic *C. botulinum* spore cocktails. After 50 days of anoxic incubation at 30 °C, growth and/or toxin formation were not detected in any of the coconut waters tested at their natural pH (pH 4.7-5.3), except for proteolytic *C. botulinum* in the previously studied coconut water from the Thailand Green Dwarf variety (T2), where a small quantity of BoNT/B was produced (79 pg/mL or 10 MLD_{50}/mL). Similarly, growth and/or toxin formation were not detected in any of the coconut waters adjusted to pH 5.5, except for proteolytic *C. botulinum* in the two Thailand Green Dwarf varieties studied (T1 and T2). This aligns with previous results, where gas formation and *bont* genes were detected at this pH. Finally, BoNT production by proteolytic *C. botulinum* was detected in all coconut water samples adjusted to the highest pH (6.2-6.7), except in coconut water extracted from the King Coconut variety (SL).

On the other hand, nonproteolytic *C. botulinum* failed to grow in all coconut waters at their natural pH (4.7-5.3) and coconut waters adjusted to pH 5.5. Growth was only evidenced as gas production at the highest pH (6.2-6.7) in all coconut waters, except in the King Coconut variety (SL) and in one from the Thailand Green Dwarf (T1) varieties. Botulinum neurotoxin and *bont* genes were not detected in any of the samples inoculated with nonproteolytic *C. botulinum* spores. These results demonstrated that adequate risk assessment of HPP tender coconut water should consider that growth and toxin production by proteolytic *C. botulinum* and nonproteolytic *C. botulinum* under realistic storage conditions (presence of dissolved oxygen, occurrence of pressure-resistant competitive microbiota, pH 4.7-5.3) is highly unlikely even under severe temperature abuse because the beverage is a poor substrate for growth.

2. Assessing strain variability for process validation of HPP coconut water

Following the failure of *C. botulinum* to grow in tender coconut water under commercial conditions, and from a prevalence perspective (Nguyen-The, 2012), it is considered that enterohemorrhagic *E. coli*, *L. monocytogenes* and *Salmonella* spp. are the three major pathogens of concern that require attention from juice and coconut water producers. Despite commercial success of HPP in the juice industry, and the fact that some regulatory agencies concluded that HPP does not raise concerns related to food safety (such as in the European Union or Canada), other countries legally require the validation of processing conditions to consistently achieve a 5-$\log_{10}$ reduction of pertinent pathogens throughout shelf-life (U.S. Food and Drug Administration, 2001). In order to fulfill US regulations and to optimize processing conditions (pressure and holding time), the use of well characterized strains that show resistance to the processing technology to be validated is encouraged in validation studies. However, publicly available pressure-resistant strains recommended for robust validations of juices have not been identified yet for HPP. Moreover, it is well established that different species of pathogens and different strains within the same species exhibit considerable variation in pressure resistance (Alpas et al., 1999). Varying physicochemical characteristics of fruit and vegetable juices, and conflicting results in published articles do not provide enough evidence to conclude that HPP can control the potential hazards in all juices, and more specifically in low acid juices such as coconut water, where available scientific literature is scarce. Hence, the identification of representative strains would serve to design more robust validation studies and address the inter- and intraspecies variability issue (Podolak, Whitman, & Black, 2020).

In order to select appropriate strains to conduct validation studies, Chapter 4 investigated the response of multiple strains of *E. coli* O157:H7 (34 isolates), *L.*

monocytogenes (44 isolates) and *S. enterica* (45 isolates) to moderate HPP conditions (500 MPa for 1 min at 10 °C) in model solutions consisting of TSBYE adjusted to pH 4.5 and 6.0 with 4.16 and 1.47 g/L of citric acid, respectively. This matrix was selected to resemble physicochemical characteristics of high-acid (pH 4.5) and low-acid (pH 6.0) juices, and to assess adaptation and recovery patterns of individual strains following HPP. Pressure resistance widely varied between bacterial species and between strains of the same species. The greatest pressure resistance at pH 6.0 was exhibited by *E. coli* O157:H7 and *L.* monocytogenes. Intraspecies variability for both species was evidenced by counts spreading between 2.0-6.5 $\log_{10}$ CFU/mL in both cases following HPP. Similar behavior has been described for multiple strains of *E. coli* O157:H7 and *L. monocytogenes* when subjected to HPP in PBS or TSBYE, respectively (Liu, Gill, McMullen, & Gänzle, 2015; Bruschi, 2017). On the other hand, *S. enterica* showed the lowest pressure resistance with 82 % of the strains evaluated displaying counts below the detection limit after HPP (<2.0 $\log_{10}$ CFU/mL). In addition to previously published works, Chapter 4 evaluated adaptation and recovery behaviors following HPP. Despite 100 % of sublethal injury among *L. monocytogenes* survivors after HPP at pH 6.0, all strains adapted to the medium and showed median counts of 7.9 $\log_{10}$ CFU/mL after 7 days of storage at 12 °C, which were the highest among the three species studied. Both, *E. coli* O157:H7 and *S. enterica* followed a similar recovery pattern reaching median counts of 3.6 and 5.2 $\log_{10}$ CFU/mL after 7 days of storage, respectively, but *S. enterica* showed the greatest count dispersion. After 14 days of storage of pH 6.0 model solutions, most strains of the three species studied most likely reached stationary growth with median counts clearly above 8 $\log_{10}$ CFU/mL. Interestingly, 76 % of *E. coli* O157:H7 isolates survived HPP at pH 4.5, whereas none of *S. enterica* and *L. monocytogenes* did at this pH value (<2 $\log_{10}$ CFU/mL). Nonetheless, 100 % of *E. coli* O157:H7 that survived at pH 4.5 sustained sublethal injury and failed to recover during storage at 12 °C.

Principal component (PC) analysis served to identify the variables that allow the classification of strains of each species in different groups based on their behavior. Whereas two components explained more than 85 % of the variability for *L. monocytogenes* and *S. enterica*, a third component was required to explain the same variability for *E. coli* O157:H7 due to the pressure resistance of the pathogen at pH 4.5. The highest factor loadings for *L. monocytogenes* and *E. coli* O157:H7 were attributed to immediate pressure resistance (day 0) at pH 6.0 and 4.5, and to their ability to adapt and recover after 7 days of incubation at 12 °C. The high pressure sensitivity of *S. enterica* accounts for the higher factor loadings observed on days 7 and 14, which suggests that this species is mainly described by adaptability and recovery phenotypes. The relevant variables identified by PC analysis were used to classify individual strains using cluster analysis. Cluster A incorporated strains with the highest median

counts immediately after HPP. Additionally, isolates in cluster A generally showed the greatest ability to adapt and recover following HPP, as evidenced by growth rates between days 1-7. Cluster B gathered strains with intermediate pressure resistance but able to adapt and recover after HPP. Conversely, strains grouped in cluster C showed the lowest growth rates between days 1-7, which suggests that they required more time to adapt and recover after HPP. Therefore, isolates grouped in cluster A were considered more suited to conduct validation studies as they would represent a worst-case scenario from a food safety perspective. This, together with an individualized analysis of genotypic characteristics and isolation source of the strains served to propose a bacterial cocktail to use in HPP juice validation studies (Table 24).

Table 24. Summary of the characteristics of strains proposed for HPP validation.

Species	Strain	Cluster	Characteristics
E. coli O157:H7	CIP 105248	B	Extreme pressure resistance at pH 4.5.
	ATCC 43894	A	Extreme pressure resistance at pH 4.5 and 6.0. Great adaptation and recovery after HPP.
	CIP 105212	A	Extreme pressure resistance at pH 4.5. Great adaptation and recovery after HPP at pH 6.0.
	CIP 105231	A	Extreme pressure resistance at pH 4.5. Great adaptation and recovery after HPP at pH 6.0.
	CIP 105243	B	High pressure resistance at pH 4.5 and 6.0.
	CIP 105245	A	High pressure resistance at pH 4.5 and 6.0. Great adaptation and recovery after HPP.
	ATCC 51659	A	High pressure resistance at pH 6.0. Great adaptation and recovery after HPP at pH 6.0.
L. monocytogenes	FSL J2-035	A	Extreme pressure resistance at pH 6.0. Serotype 1/2b.
	FSL J1-049	A	Extreme pressure resistance at pH 6.0. Serotype 3c.
	FSL J2-054	A	Extreme pressure resistance at pH 6.0. Serotype 1/2a.
	FSL J1-094	A	Extreme pressure resistance at pH 6.0. Serotype 1/2c.
	FSL J1-031	A	Extreme pressure resistance at pH 6.0. Serotype 4a.
	FSL J1-168	A	Extreme pressure resistance at pH 6.0. Serotype 4a.
	FSL W1-110	A	Extreme pressure resistance at pH 6.0. Serotype 4c.
	FSL N3-008	B	Great adaptation and recovery after HPP at pH 6.0. Serotype 4.
	FSL R2-503	A	Extreme pressure resistance at pH 6.0. Serotype 1/2b.
	ITA 363	A	Extreme pressure resistance at pH 6.0. Serotype 1/2b.

Table 24 (cont.) Summary of the characteristics of strains proposed for HPP validation.

Species	Strain	Cluster	Characteristics
S. enterica	FSL S5-540	A	Counts above detection limit after HPP at pH 6.0. Great adaptation and recovery after HPP. Serovar Agona.
	FSL S5-373	A	Counts above detection limit after HPP at pH 6.0. Great adaptation and recovery after HPP. Serovar Braenderup.
	FSL R8-6671	A	Great adaptation and recovery after HPP. Serovar Dessau.
	FSL S5-439	A	Counts above detection limit after HPP at pH 6.0. Great adaptation and recovery after HPP. Serovar Dublin.
	FSL S5-487	A	Great adaptation and recovery after HPP. Serovar Give.
	FSL S5-448	A	Great adaptation and recovery after HPP. Serovar Heidelberg.
	FSL S5-480	A	Great adaptation and recovery after HPP. Serovar Heidelberg.
	HUBU 72732	A	Counts above detection limit after HPP at pH 6.0. Great adaptation and recovery after HPP. Serovar Typhimurium.
	HUBU 71144	A	Counts above detection limit after HPP at pH 6.0. Great adaptation and recovery after HPP. Serovar Typhimurium.
	HUBU 90196	A	Great adaptation and recovery after HPP. Serovar Enteritidis.

Work described in Chapter 5 evaluated the potential of typical HPP parameters used by the food industry (600 MPa for 3 min at 10 °C) to control previously selected vegetative pathogens (Chapter 4) in tender coconut water. Physicochemical characterization of the four different varieties of coconut water studied [Thailand Green Dwarf (T1), King Coconut (SL), Pacific Tall (TP) and Pacific Tall × Yellow Dwarf (BE)] showed that pH, total soluble solids and titratable acidity were not affected by HPP and remained stable through the 60-day storage period at 4 °C. Total phenolic content significantly increased after HPP in all cases, except in BE coconut water, which remained constant. A steady decrease of phenolic content was observed during shelf-life. The determination of physicochemical characteristics is important because the inactivation of bacteria depends on numerous factors, including intrinsic properties of juices and beverages. In addition, these factors may affect recovery of injured microorganisms (Nasiłowska, Sokołowska, & Fonberg-Broczek, 2018; Rendueles et al., 2011). Subjecting to HPP (600 MPa for 3 min) the four varieties of coconut water inoculated with acid-adapted strain cocktails of *E. coli* O157:H7, *L. monocytogenes* and *S. enterica* (Table 24) achieved more than a 5-$\log_{10}$ reduction in all cases. Microbial counts were below the detection

limit in all coconut waters (<1 log_{10} CFU/mL) for 60 days of storage at 4 °C (>6-log_{10} reduction), except for *E. coli* O157:H7 in BE coconut water, and for *L. monocytogenes* in T1 and BE coconut waters. However, plate counts of *E. coli* O157:H7 on day 40 in BE coconut water, and of *L. monocytogenes* on day 20 in T1 coconut water were below the detection limit, which suggests that survivors failed to adapt and progressively died. Nevertheless, *L. monocytogenes* managed to adapt and recovered in BE coconut water. As a consequence, inactivation of the pathogen was below the required 5-log_{10} reduction by day 40 of storage at 4 °C. This is not in agreement with previous findings by Raghubeer et al. (2020), where a full pathogen inactivation (>5 log_{10} CFU/mL) of the three species was reported for 54 and 75 days of refrigerated storage at 4 °C in two different types of coconut water [from Brazil (Brazilian Green Dwarf) and from Florida (unknown variety)] processed at 593 MPa for 3 min. This can be explained by differences in the strains or coconut waters used. In the current thesis, TP and T1 coconut waters had a higher pH (5.3 and 5.6, respectively) than BE coconut water (pH 5.3). This suggests that other intrinsic factors of the beverage may account for the increased pressure tolerance of *L. monocytogenes* in the BE variety. In order to test this hypothesis, the cocktails of the three pathogenic species were inoculated in the four varieties of coconut water and not subjected to HPP, and growth evolution was studied during storage at 4 °C. Counts of *E. coli* O157:H7 and *S. enterica* remained stable or decreased between 0.5 and 3.6 log_{10} CFU/mL during the 20-day storage period, regardless of the variety. However, *L. monocytogenes* behaved differently on each type of coconut water. Counts were stable during storage in T1 coconut water. Conversely, a sharp decrease of 3.7 and 3.6 log_{10} CFU/mL was observed in SL and TP coconut waters, respectively. In the particular case of TP coconut water, *L. monocytogenes* counts remained below the detection limit (<1 log_{10} CFU/mL) from day 5 of storage, which suggests an antimicrobial effect. On the other hand, BE coconut water supported growth of *L. monocytogenes*. Counts of the pathogen reached 8.2 log_{10} CFU/mL after 20 days of storage at 4 °C. This finding is in agreement with the observed recovery of *L. monocytogenes* after HPP in BE coconut water, but not in the other varieties. Conflicting results have been published regarding growth potential of *L. monocytogenes* in coconut water. Some studies showed that sterile coconut water from the Brazilian Green Dwarf variety (pH 4.88) can support growth of the pathogen at 4, 10 and 35 °C (Walter, Kabuki, Esper, Sant'Ana, & Kuaye, 2009). Alternatively, other researchers reported a sharp decrease in two varieties of coconut water from Brazil (Brazilian Green Dwarf) (pH 5.2) and Florida (unknown variety) (pH 5.5) during storage at 4 °C (Raghubeer et al., 2020). Different results obtained even within the same variety of coconut water can be attributed to the use of different *L. monocytogenes* strain cocktails, differences in the composition of the beverage, or a possible competitive effect of the microbiota that was not evidenced in the sterile coconut water samples.

From a quality perspective, microbial spoilage indicators were effectively controlled by HPP (600 MPa for 3 min) in the four types of coconut water. Aerobic plate counts and lactic acid bacteria remained stable for 60 days of storage at 4 °C. *Enterobacteriaceae* and molds & yeasts were completely inactivated and remained below the detection limit (<1 CFU/mL) during storage. Recent work showed the potential of similar HPP conditions (593 MPa for 3 min) to extend microbiological shelf-life of coconut water up to 120 days at 4 °C. Authors reported that aerobic bacteria, yeasts, coliforms and lactic acid bacteria counts were below 2 $\log_{10}$ CFU/mL during shelf-life (Raghubeer et al., 2020). Results from the present work demonstrated that typical HPP parameters used by the food industry (600 MPa for 3 min at 10 °C) can achieve more than 5-$\log_{10}$ reductions of vegetative pathogens in coconut water, and extend refrigerated shelf-life of the beverage. However, extending pressure holding time is recommended when recovery and growth is observed for any of the pertinent pathogens during shelf-life.

3. References

Alpas, H., Kalchayanand, N., Bozoglu, F., Sikes, A., Dunne, C. P., & Ray, A. B. (1999). Variation in Resistance to Hydrostatic Pressure among Strains of Food-Borne Pathogens. *Applied and Environmental Microbiology*, *65*(9), 4248–4251.

Awua, A. K., Doe, E. D., & Agyare, R. (2011). Exploring the influence of sterilisation and storage on some physicochemical properties of coconut (*Cocos nucifera* L.) water. *BMC Research Notes*, *4*. https://doi.org/10.1186/1756-0500-4-451

Bruschi, C., Komora, N., Castro, S. M., Saraiva, J., Ferreira, V. B., & Teixeira, P. (2017). High hydrostatic pressure effects on *Listeria monocytogenes* and *L. innocua*: Evidence for variability in inactivation behaviour and in resistance to pediocin bacHA-6111-2. *Food microbiology*, *64*, 226-231.

Jackson-Davis, A., Mendonca, A., Hale, S., Jackson, J., King, A., & Jackson, J. (2018). Microbiological Safety of Unpasteurized Fruit and Vegetable Juices Sold in Juice Bars and Small Retail Outlets. In S. Ricke, G. Atungulu, C. Rainwater, & S. Park (Eds.), *Food and Feed Safety Systems and Analysis* (pp. 213–225). London: Academic Press. https://doi.org/10.1016/B978-0-12-811835-1.00012-9

Liu, Y., Gill, A., McMullen, L., & Gänzle, M. G. (2015). Variation in heat and pressure resistance of verotoxigenic and nontoxigenic *Escherichia coli*. *Journal of Food Protection*, *78*(1), 111–120. https://doi.org/10.4315/0362-028X.JFP-14-267

Ma, Y., Xu, L., Wang, S., Xu, Z., Liao, X., & Cheng, Y. (2019). Comparison of the quality attributes of coconut waters by high-pressure processing and high-temperature short time during the refrigerated storage. *Food Science & Nutrition*, *7*(4), 1512–1519. https://doi.org/10.1002/fsn3.997

Mahayothee, B., Koomyart, I., Khuwijitjaru, P., Siriwongwilaichat, P., Nagle, M., & Müller, J. (2016). Phenolic Compounds, Antioxidant Activity, and Medium Chain Fatty Acids Profiles of Coconut Water and Meat at Different Maturity Stages. *International Journal of Food Properties*, *19*(9), 2041–2051. https://doi.org/10.1080/10942912.2015.1099042

Nasiłowska, J., Sokołowska, B., & Fonberg-Broczek, M. (2018). Long-Term Storage of Vegetable Juices Treated by High Hydrostatic Pressure: Assurance of the Microbial Safety. *BioMed Research International*, *2018*. https://doi.org/10.1155/2018/7389381

Nguyen-The, C. (2012). Biological hazards in processed fruits and vegetables–Risk factors and impact of processing techniques. *LWT-Food Science and Technology*, *49*(2), 172-177.

Podolak, R., Whitman, D., & Black, D. G. (2020). Factors Affecting Microbial Inactivation during High Pressure Processing in Juices and Beverages: A Review. *Journal of Food Protection*, *83*(9), 1561–1575. https://doi.org/10.4315/JFP-20-096

Raghubeer, E. V., Phan, B. N., Onuoha, E., Diggins, S., Aguilar, V., Swanson, S., & Lee, A. (2020). The use of High-Pressure Processing (HPP) to improve the safety and quality of raw coconut (*Cocos nucifera* L) water. *International Journal of Food Microbiology*, *331*, 108697. https://doi.org/10.1016/j.ijfoodmicro.2020.108697

Rendueles, E., Omer, M. K., Alvseike, O., Alonso-Calleja, C., Capita, R., & Prieto, M. Microbiological food safety assessment of high hydrostatic pressure processing: A review. *LWT - Food Science and Technology*. Academic Press. https://doi.org/10.1016/j.lwt.2010.11.001

U.S. Food and Drug Administration. (2001). Hazard analysis and critical control point (HACCP); procedures for the safe and sanitary processing and importing of juice; final rule, 21 CFR Part 120. *Federal Register*, *66*, 6137–6202.

Walter, E. H. M., Kabuki, D. Y., Esper, L. M. R., Sant'Ana, A. S., & Kuaye, A. Y. (2009). Modelling the growth of *Listeria monocytogenes* in fresh green coconut (*Cocos nucifera* L.) water. *Food Microbiology*, *26*(6), 653–657. https://doi.org/10.1016/j.fm.2009.04.003

Conclusions and future work

CONCLUSIONS AND FUTURE WORK

1. Although there have been no outbreaks associated with coconut water consumption, pertinent microorganisms that require control are *E. coli* O157:H7, *L. monocytogenes* and *S. enterica*. Mild processing conditions (500 MPa for 1 min at 10 °C) used for the identification of pressure resistant strains of the three pathogens showed high variability at strain and species level in citric acid model solutions simulating high-acid (pH 4.5) and low-acid (pH 6.0) juices. Strains of *E. coli* O157:H7 and *L. monocytogenes* showed the highest pressure resistance at pH 6.0, whereas *S. enterica* the lowest. Additionally, *L. monocytogenes* and *S. enterica* were completely inactivated at pH 4.5, but not *E. coli* O157:H7. Pressure resistance and acid tolerance of *E. coli* O157:H7, and growth potential of *L. monocytogenes* under refrigeration make these two species the most relevant in process validation of HPP juices.

2. Representative strain cocktails of *E. coli* O157:H7, *L. monocytogenes* and *S. enterica* subjected to typical HPP conditions used in the juice industry (600 MPa for 3 min at 10 °C) were reduced by more than a 5-$\log_{10}$ in various types of tender coconut water with different physicochemical characteristics. However, *L. monocytogenes* was the only species able to recover and grow in one variety of tender coconut water stored at 4 °C. Recovery of *L. monocytogenes* during refrigerated storage does not depend on acidity, but on other intrinsic compositional properties of coconut water.

3. Tender coconut water is a poor substrate for growth and neurotoxin production by proteolytic *C. botulinum* and nonproteolytic *C. botulinum* due to a combination of low pH and nutrient limitation. Under highly favorable conditions (anoxic sterile coconut water incubated at 30 °C for 50 days), growth can be achieved by rising the pH and/or nutrient supplementation. Therefore, growth and neurotoxin production under commercial conditions is highly unlikely even in conditions of temperature abuse. These findings discard *C. botulinum* as the pertinent microorganism in pure tender high-pressure processed coconut water.

4. Tender coconut water from King Coconut, Pacific Tall and one of the two Thailand Green Dwarf varieties tested did not support growth of proteolytic *C. botulinum* and nonproteolytic *C. botulinum* at their natural pH under highly favorable conditions. Slight growth was observed in the remaining Thailand Green Dwarf variety, although studied conditions did not resemble realistic commercial scenarios. Additionally,

processing the same tender coconut waters at 600 MPa for 3 min achieved a 5-$\log_{10}$ reduction of bacterial cocktails of vegetative pathogens for a period of 60 days during storage at 4 °C. Therefore, this work validated 600 MPa for 3 min as a suitable intervention to ensure safety of tender coconut water extracted from tender King Coconut, Pacific Tall and Thailand Green Dwarf coconut varieties.

Following studies about vegetative pathogen inactivation should target the validation of adequate HPP parameters in tender coconut water stored under temperature abuse conditions. Since preliminary tests (data not shown) suggest that the 5-$\log_{10}$ reduction is not sustained for *E. coli* O157:H7, *L. monocytogenes* and *S. enterica* during storage of coconut water at 12 °C, extended holding time at 600 MPa should be evaluated in order to establish safe processing conditions. Future research with *C. botulinum* could investigate the intrinsic characteristics of tender coconut water that in combination with acidity play a role in restricting growth and toxin production by the pathogen, with a special focus in the differences between the varieties that do support growth of *C. botulinum* and those that do not. However, even if the differences between the compositions of coconut water are found, it would be challenging to implement them as controls in HACCP plans.

Appendix

APPENDIX TO CHAPTER 2

Table A1. Nutritional composition of raw Thai coconut water used in the study.

Parameters	Specific compounds	Concentration
Water		92.60 g/100 ml
Total fat		<0.50 g/100 ml
Total protein		<1 g/100 ml
Free aminoacids	Alanine	<0.01 g/100 ml
	Arginine	<0.01 g/100 ml
	Asparagine	<0.01 g/100 ml
	Cysteine	<0.02 g/100 ml
	Glutamic acid	<0.01 g/100 ml
	Glycine	<0.01 g/100 ml
	Histidine	<0.01 g/100 ml
	Isoleucine	<0.01 g/100 ml
	Leucine	<0.01 g/100 ml
	Methionine	<0.01 g/100 ml
	Phenylalanine	<0.01 g/100 ml
	Proline	<0.01 g/100 ml
	Serine	<0.01 g/100 ml
	Threonine	<0.01 g/100 ml
	Tyrosine	<0.01 g/100 ml
	Valine	<0.02 g/100 ml
Sugars	Fructose	2.61 g/100 ml
	Glucose	2.60 g/100 ml
	Sucrose	0.794 g/100 ml
Vitamins	Folic acid	<5.00 µg/100 ml
	L-ascorbic acid	<1 mg/100 ml
	Riboflavin	<0.05 mg/100 ml
Ash	Calcium	18.8 mg/100 ml
	Magnesium	<10 mg/100 ml
	Potassium	236 mg/100 ml

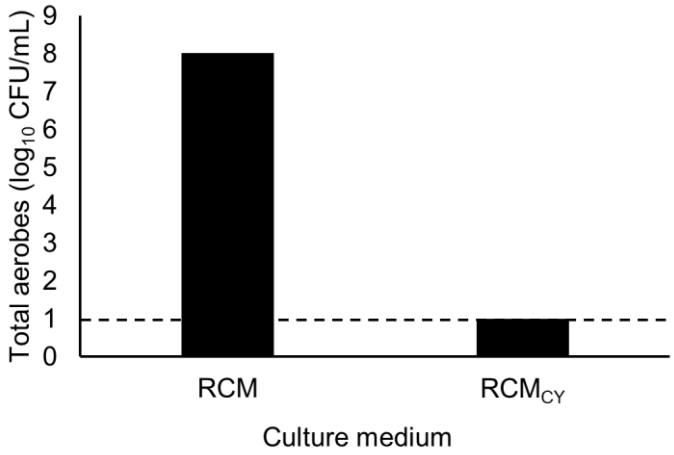

Figure A1. Growth of background microbiota present in HPP coconut water (550 MPa for 3 min) after 20 days of incubation at 10 °C assessed in reinforced clostridial medium (RCM) and selective RCM_{CY} (Reinforced Clostridial Medium supplemented with 100 µg/mL of cycloserine) after 48 h at 30 °C in an anaerobic workstation (dashed line: detection limit).

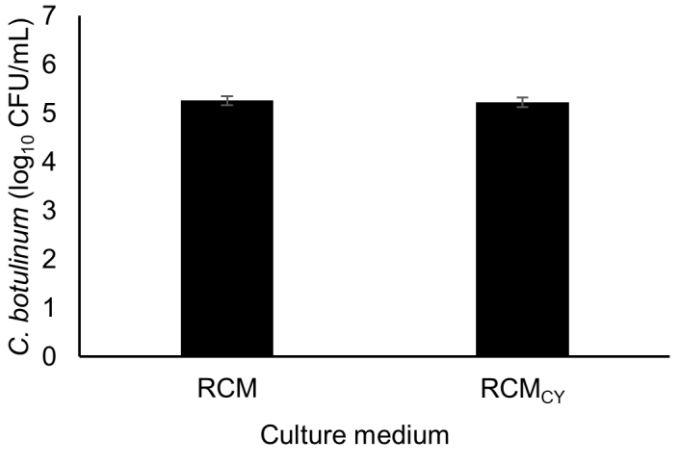

Figure A2. Three-spore cocktail suspension counts assessed in reinforced clostridial medium (RCM) and selective RCM_{CY} (Reinforced Clostridial Medium supplemented with 100 µg/mL of cycloserine) after 24 h of incubation at 30 °C in an anaerobic workstation.

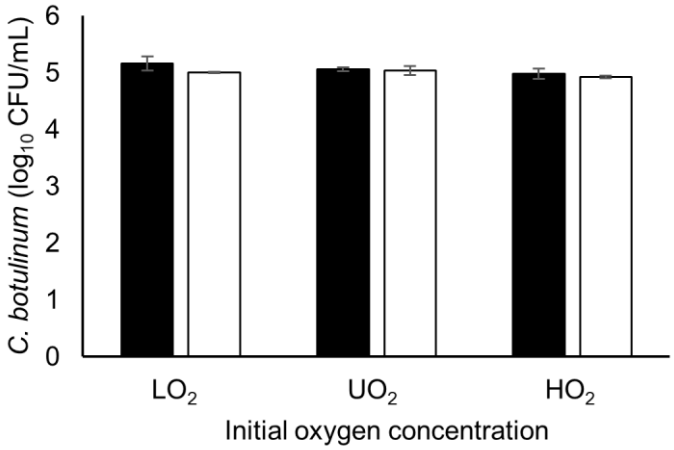

Figure A3. *C. botulinum* counts in coconut water with initial high oxygen concentration (HO_2, 11.0±0.5 mg/L O_2), low oxygen concentration (LO_2, <0.5±0.1 mg/L O_2) and unaltered (UO_2, 7.0±0.8 mg/L O_2) after 61 days of incubation at 4 °C.

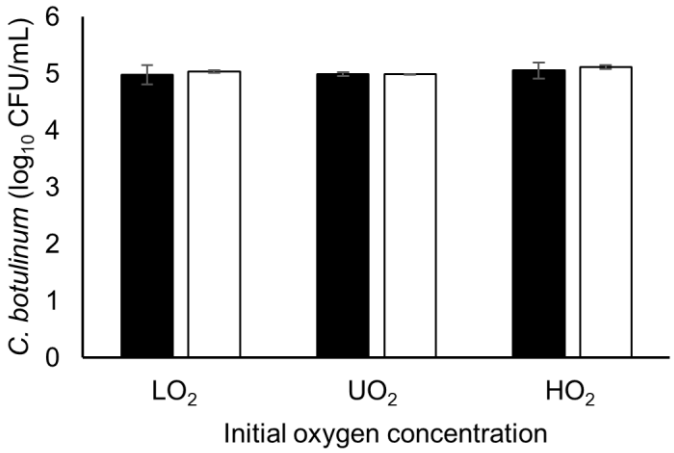

Figure A4. *C. botulinum* counts in coconut water with initial high oxygen concentration (HO_2, 11.0±0.5 mg/L O_2), low oxygen concentration (LO_2, <0.5±0.1 mg/L O_2) and unaltered (UO_2, 7.0±0.8 mg/L O_2) after 61 days of incubation at 10 °C.

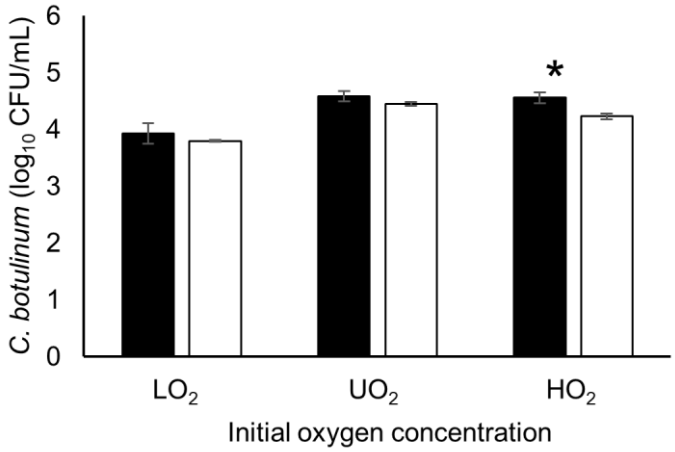

Figure A5. *C. botulinum* counts in coconut water with initial high oxygen concentration (HO_2, 11.0±0.5 mg/L O_2), low oxygen concentration (LO_2, <0.5±0.1 mg/L O_2) and unaltered (UO_2, 7.0±0.8 mg/L O_2) after 61 days of incubation at 20 °C (* $p < 0.05$)

APPENDIX TO CHAPTER 3

Table A2. Individual nutrients used to supplement T2 coconut water and final concentration in the mixture.

Nutrient group	Compound	Final concentration
Amino acids	Alanine	4.45 g/L
	Arginine	3.00 g/L
	Phenylalanine	1.00 g/L
	Glutamate	0.50 g/L
	Methionine	0.10 g/L
	Tryptophan	0.10 g/L
	Valine	0.10 g/L
	Serine	0.10 g/L
	Glycine	0.10 g/L
	Histidine	0.10 g/L
	Isoleucine	0.10 g/L
	Leucine	0.10 g/L
	Tyrosine	0.05 g/L
Minerals	Sodium L-lactate	11.20 g/L
	$NaH_2PO_4 \times H_2O$	4.70 g/L
	K_2HPO_4	11.14 g/L
	K_2SO_4	3.48 g/L
	$(NH_4)_2SO_4$	2.64 g/L
	$NaHCO_3$	8.40 g/L
	$FeSO_4 \times 7\ H_2O$	0.28 mg/L
	$ZnCl_2$	0.14 mg/L
	$CaCl_2 \times 2\ H_2O$	1.47 mg/L
	$MgS_2O \times 7\ H_2O$	74.00 mg/L
	$MnCl_2$	0.20 mg/L
Vitamins	*p*-aminobenzoic acid	0.40 mg/L
	Pyridoxamine	1.00 mg/L
	Biotin	0.20 mg/L
	Nicotinic acid	1.00 mg/L
	Thiamine	0.40 mg/L
	Folic acid	0.25 mg/L
	Choline	50.00 mg/L
	Nicotinamide	1.00 mg/L
	Calcium pantothenate	50.00 mg/L

Table A3. Combinations of nutrient groups used to separately supplement samples of T2 coconut water.

Experiment	Combination of nutrient groups
Supplement 1	Amino acids
Supplement 2	Minerals
Supplement 3	Vitamins
Supplement 4	Amino acids + Minerals
Supplement 5	Amino acids + Vitamins
Supplement 6	Minerals + Vitamins
Supplement 7	Amino acids + Minerals + Vitamins

Table A4. Proteolytic *C. botulinum* growth visualized as turbidity and/or gas production in various types of natural coconut water, coconut water adjusted to a higher pH and mixtures of coconut water with PYGS broth incubated anaerobically at 30 °C.

Coconut water	Condition	pH	Number of replicates (out of three) positive for growth on day:																						
			27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
Be	Natural	5.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Adjusted	5.8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	+PYGS	6.1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Br	Natural	4.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Adjusted	5.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	+PYGS	5.7	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
SL	Natural	4.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Adjusted	5.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	+PYGS	5.7	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
T1	Natural	5.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Adjusted	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	+PYGS	6.1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
T2	Natural	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Adjusted	5.6	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	+PYGS	6.1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
T3	Natural	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Adjusted	5.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	+PYGS	6.1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3

Table A4 (cont.) Proteolytic *C. botulinum* growth visualized as turbidity and/or gas production in various types of natural coconut water, coconut water adjusted to a higher pH and mixtures of coconut water with PYGS broth incubated anaerobically at 30 °C.

Coconut water	Condition	pH	Number of replicates (out of three) positive for growth on day:																						
			27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
T4	Natural	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Adjusted	5.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	+PYGS	6.1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
TP	Natural	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Adjusted	5.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	+PYGS	6.1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3

*Growth observations could only commence on day 27 due to laboratory shut down for maintenance purposes

Table A5. Nonproteolytic *C. botulinum* growth visualized as turbidity and/or gas production in various types of natural coconut water, coconut water adjusted to a higher pH and mixtures of coconut water with PYGS broth incubated anaerobically at 30 °C.

Coconut water	Condition	pH	Number of replicates (out of three) positive for growth on day:																						
			27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
Be	Natural	5.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Adjusted	5.8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	+PYGS	6.1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Br	Natural	4.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Adjusted	5.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	+PYGS	5.7	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
SL	Natural	4.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Adjusted	5.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	+PYGS	5.7	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
T1	Natural	5.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Adjusted	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	+PYGS	6.1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
T2	Natural	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Adjusted	5.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	+PYGS	6.1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
T3	Natural	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Adjusted	5.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	+PYGS	6.1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3

Table A5 (cont.) Nonproteolytic *C. botulinum* growth visualized as turbidity and/or gas production in various types of natural coconut water, coconut water adjusted to a higher pH and mixtures of coconut water with PYGS broth incubated anaerobically at 30 °C.

Coconut water	Condition	pH	Number of replicates (out of three) positive for growth on day:																						
			27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
T4	Natural	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Adjusted	5.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	+PYGS	6.1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
TP	Natural	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Adjusted	5.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	+PYGS	6.1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3

*Growth observations could only commence on day 27 due to laboratory shut down for maintenance purposes.

Table A6. Proteolytic *C. botulinum* growth visualized as turbidity and/or gas production in T2 coconut water, mixtures of T2 coconut water with PYGS broth (T2+PYGS) and PYGS broth adjusted to different pH values incubated anaerobically at 30 °C.

Sample type	pH	Number of replicates (out of five) positive for growth on day:																										
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
T2	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	2	2
	6.4	0	0	0	0	0	0	0	4	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
T2+PYGS	5.2	0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	5.5	0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	6.3	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	6.7	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
PYGS	5.3	0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	5.5	0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	6.2	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	6.9	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Table A6 (cont.) Proteolytic *C. botulinum* growth visualized as turbidity and/or gas production in T2 coconut water, mixtures of T2 coconut water with PYGS broth (T2+PYGS) and PYGS broth adjusted to different pH values incubated anaerobically at 30 °C.

Sample type	pH	Number of replicates (out of five) positive for growth on day:																						
		28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
T2	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6.0	2	2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	6.4	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
T2+PYGS	5.2	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	5.5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	6.3	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	6.7	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
PYGS	5.3	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	5.5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	6.2	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	6.9	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Table A7. Nonproteolytic *C. botulinum* growth visualized as turbidity and/or gas production in T2 coconut water, mixtures of T2 coconut water with PYGS broth (T2+PYGS) and PYGS broth adjusted to different pH values incubated anaerobically at 30 °C.

Sample type	pH	Number of replicates (out of five) positive for growth on day:																										
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
T2	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6.4	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	2	2	2	2	2	2
T2+PYGS	5.2	4	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	5.5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	6.3	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	6.7	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
PYGS	5.3	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	5.5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	6.2	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	6.9	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Table A7 (cont.) Nonproteolytic *C. botulinum* growth visualized as turbidity and/or gas production in T2 coconut water, mixtures of T2 coconut water with PYGS broth (T2+PYGS) and PYGS broth adjusted to different pH values incubated anaerobically at 30 °C.

Sample type	pH	Number of replicates (out of five) positive for growth on day:																						
		28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
T2	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6.4	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
T2+PYGS	5.2	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	5.5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	6.3	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	6.7	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
PYGS	5.3	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	5.5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	6.2	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	6.9	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Table A8. Proteolytic *C. botulinum* growth visualized as turbidity and/or gas production in various types of natural and pH-adjusted coconut water incubated anaerobically at 30 °C.

Coconut water	pH	Number of replicates (out of five) positive for growth on day:																										
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Be	5.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6.7	0	0	0	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
SL	4.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T1	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T2	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	6.2	0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
TP	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1

Table A8 (cont.) Proteolytic *C. botulinum* growth visualized as turbidity and/or gas production in various types of natural and pH-adjusted coconut water incubated anaerobically at 30 °C.

Coconut water	pH	Number of replicates (out of five) positive for growth on day:																						
		28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
Be	5.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6.7	2	2	2	2	3	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
SL	4.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T1	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6.5	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2
T2	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5.5	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	6.2	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
TP	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6.3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Table A9. Nonproteolytic *C. botulinum* growth visualized as turbidity and/or gas production in various types of natural and pH-adjusted coconut water incubated anaerobically at 30 °C.

Coconut water	pH	Number of replicates (out of five) positive for growth on day:																										
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Be	5.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6.7	0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
SL	4.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T1	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T2	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6.2	0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
TP	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6.3	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Table A9 (cont.) Nonproteolytic *C. botulinum* growth visualized as turbidity and/or gas production in various types of natural and pH-adjusted coconut water incubated anaerobically at 30 °C.

Coconut water	pH	Number of replicates (out of five) positive for growth on day:																						
		28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
Be	5.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6.7	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
SL	4.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T1	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T2	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6.2	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
TP	5.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6.3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

APPENDIX TO CHAPTER 4

Table A10. Details of the thirty-four *E. coli* strains used in the study.

Strain	Serotype	Virulence genes			Source
		stx$_1$	*stx*$_2$	*eae*	
ATCC 43888	O157:H7	-	+	+	Human, stool
ATCC 43889	O157:H7	+	-	+	Human, stool, hemolytic uremic syndrome
ATCC 43890	O157:H7	+	+	+	Human, stool
ATCC 43894	O157:H7	+	+	+	Child, stool, hemorrhagic colitis outbreak
ATCC 43895	O157:H7	NA	NA	+	Hamburger, hemorrhagic colitis outbreak
ATCC 51657	O157:H7	+	+	NA	Human
ATCC 51658	O157:H7	+	+	NA	Human
ATCC 51659	O157:H7	+	+	NA	Human
ATCC 700728	O157:H7	-	-	+	Human, diarrhea
CIP 105212	O157:H7	+	+	NA	Human, stool
CIP 105213	O157:H7	+	+	NA	Human, stool
CIP 105230	O157:H7	NA	NA	NA	Child, stool
CIP 105231	O157:H7	NA	NA	NA	Child, stool
CIP 105243	O157:H7	NA	NA	NA	Child, diarrhea
CIP 105245	O157:H7	NA	NA	NA	Human, enteritis
CIP 105246	O157:H7	NA	NA	NA	Human, enteritis
CIP 105248	O157:H7	NA	NA	NA	Human, enteritis
CIP 105249	O157:H7	NA	NA	NA	Human, enteritis
CIP 107190	O157:H7	NA	NA	NA	Calf
CIP 107872	O157:H7	NA*	NA	NA	Duck
LREC 19999	O157:H7	+	-	+	Bovine, stool
LREC 20006	O157:H7	+	+	+	Bovine, stool
LREC 20008	O157:H7	+	+	+	Bovine, stool
LREC 20016	O157:H7	-	+	+	Ovine, stool
LREC 20021	O157:H7	+	+	+	Bovine, stool
LREC 20028	O157:H7	-	+	+	Bovine, stool
LREC 20030	O157:H7	-	+	+	Bovine, stool
LREC 20041	O157:H7	+	+	+	Bovine, stool
LREC 20045	O157:H7	+	-	+	Bovine
LREC 20046	O157:H7	-	+	+	Ovine, stool
LREC 20062	O157:H7	-	+	+	Sheep milk
LREC 20071	O157:H7	-	+	+	Sheep milk
LREC 20085	O157:H7	-	+	+	Goat milk
LREC 20098	O157:H7	-	+	+	Goat, stool

*NA: information not available

Table A11. Details of the forty-four *L. monocytogenes* strains used in the study.

Strain	Lineage	Serotype	Virulence genes			Source
			inlA	*inlC*	*inlJ*	
FSL C1-056	II	1/2a	+	+	+	Human, sporadic case
FSL C1-115	II	3a	+	+	+	Human, sporadic case
FSL C1-122	I	4b	+	+	+	Human, sporadic case
FSL J1-031	III	4a	+	+	+	Human, sporadic case
FSL J1-049	I	3c	+	+	+	Human, sporadic case
FSL J1-094	II	1/2c	+	+	+	Human, sporadic case
FSL J1-101	II	1/2a	+	+	+	Human, hot dog, sporadic case
FSL J1-116	I	4b	+	+	+	Human, epidemic (UK, 1988)
FSL J1-119	I	4b	+	+	+	Human, epidemic (US, 1985)
FSL J1-123	I	4b	+	+	+	Human, epidemic (Switzerland)
FSL J1-126	I	4b	+	+	+	Human, sporadic case
FSL J1-158	IV	4b	+	+	-	Goat
FSL J1-168	III	4a	+	+	+	Human, sporadic case
FSL J1-169	I	3b	+	+	+	Human, sporadic case
FSL J1-177	I	1/2b	+	+	+	Human, sporadic case
FSL J1-225	I	4b	+	+	+	Human, epidemic (Scott A)
FSL J2-020	II	1/2a	+	+	+	Cow
FSL J2-031	II	1/2a	+	+	+	Bovine
FSL J2-035	I	1/2b	-	-	+	Goat
FSL J2-054	II	1/2a	+	+	+	Sheep
FSL J2-063	II	1/2a	+	+	+	Sheep
FSL J2-064	I	1/2b	+	+	+	Cow
FSL M1-004	II	NA*	+	+	+	Human, sporadic case
FSL N1-225	I	4b	+	+	+	Human, epidemic (US, 1998)
FSL N1-227	I	4b	+	+	+	RTE meat, epidemic (US, 1998)
FSL N3-008	I	4b	+	+	+	Coleslaw, epidemic (US, 1981)
FSL N3-013	I	4b	+	+	+	Pâté, epidemic (UK, 1988-1990)
FSL N3-022	I	4b	+	+	+	Cheese, epidemic (Switzerland)
FSL N3-031	II	1/2a	+	+	+	Hot dog, sporadic case
FSL R2-499	II	1/2a	+	+	+	Human, sliced turkey, epidemic
FSL R2-500	I	4b	+	+	+	Cheese, epidemic (US, 2000)
FSL R2-501	I	4b	+	+	+	Human, epidemic (US, 2000)
FSL R2-502	I	1/2b	-	+	+	Chocolate, epidemic (US, 1994)
FSL R2-503	I	1/2b	-	+	+	Human, epidemic (US, 1994)
FSL R2-763	I	4b	-	+	+	Human, epidemic (US, 2002)
FSL R2-764	I	4b	-	+	+	Sliced deli meat
FSL R2-765	I	4b	-	+	+	Environmental
FSL W1-110	III	4c	+	+	+	NA
FSL W1-111	IV	4c	+	+	-	NA
FSL W1-112	IV	4a	+	+	-	NA
ITA 358	NA	NA	+	+	+	Strawberries
ITA 359	NA	NA	+	+	+	Mushrooms
ITA 360	NA	NA	+	+	+	Lettuce
ITA 363	NA	1/2b	+	+	+	Vegetable (undefined)

Table A12. Details of the forty-five *S. enterica* strains used in the study.

Strain	Serotype	Source
FSL A4-633	Mississippi	Human
FSL R6-540	Typhi	Human
FSL R8-5221	Tennessee	Peanut
FSL R8-5223	Hartford	Peanut
FSL R8-5224	G (1):b,-	Peanut
FSL R8-6670	Meleagridis	Peanut
FSL R8-6671	Dessau	Peanut
FSL R8-7229	Dessau	Peanut
FSL S5-373	Braenderup	Human
FSL S5-406	Javiana	Human
FSL S5-408	Stanley	Human
FSL S5-415	Enteritidis	Human
FSL S5-438	Weltevreden	Human
FSL S5-439	Dublin	Human
FSL S5-447	Paratyphi B var. Java	Human
FSL S5-448	Heidelberg	Human
FSL S5-451	Mbandaka	Human
FSL S5-458	Schwarzengrund	Human
FSL S5-480	Heidelberg	Human
FSL S5-483	Enteritidis	Human
FSL S5-487	Give	Human
FSL S5-490	Worthington	Human
FSL S5-504	Muenchen	Human
FSL S5-517	Agona	Human
FSL S5-523	Thompson	Human
FSL S5-536	Typhimurium	Human
FSL S5-540	Anatum	Human
FSL S5-543	Hadar	Human
FSL S5-580	4,5,12:I:-	Bovine
FSL S5-639	Newport	Human
FSL S5-642	Oranienburg	Human
FSL S5-648	Blockley	Human
FSL S5-649	Saintpaul	Human
FSL S5-658	Senftenberg	Human
FSL S5-961	Virchow	Human
FSL W1-029	Typhimurium U302	Human
FSL W1-030	Typhimurium DT104	Human
HUBU 34301	Typhimurium	Human, child
HUBU 37190	Typhimurium	Human
HUBU 71110	Enteritidis	Human, child
HUBU 71144	Typhimurium	Human, child
HUBU 72732	Typhimurium	Human, child
HUBU 790	Typhimurium	Human
HUBU 90108	Typhimurium	Human
HUBU 90196	Enteritidis	Human, child

Table A13. *E. coli* O157:H7 counts in model solutions of TSBYE at pH 6.0 and 4.5 after HPP (500 MPa for 1 min) and during subsequent storage at 12 °C.

Strain	pH 6.0 (log_{10} CFU/mL)					pH 4.5 (log_{10} CFU/mL)				
	Control	Day 0	Day 1	Day 7	Day 14	Control	Day 0	Day 1	Day 7	Day 14
ATCC 43888	7.2 ± 0.1	3.1 ± 0.3	2.0 ± 0.0	2.7 ± 0.9	8.1 ± 0.1	7.2 ± 0.1	5.9 ± 0.3	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
ATCC 43889	7.3 ± 0.0	3.2 ± 0.2	2.0 ± 0.0	2.3 ± 0.4	9.1 ± 0.1	7.2 ± 0.2	4.4 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
ATCC 43890	7.2 ± 0.1	2.6 ± 0.3	7.6 ± 0.8	6.1 ± 2.7	8.6 ± 0.7	7.2 ± 0.0	2.4 ± 0.3	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
ATCC 43894	7.2 ± 0.0	6.4 ± 0.2	4.0 ± 0.2	6.8 ± 0.3	8.7 ± 0.1	7.1 ± 0.1	6.3 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
ATCC 43895	7.2 ± 0.2	5.8 ± 0.4	5.6 ± 0.3	5.6 ± 0.3	9.1 ± 0.1	7.2 ± 0.1	2.4 ± 0.5	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
ATCC 51657	7.1 ± 0.1	2.6 ± 0.5	2.0 ± 0.0	5.9 ± 3.4	8.5 ± 0.3	7.2 ± 0.1	2.8 ± 1.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
ATCC 51658	7.2 ± 0.1	3.1 ± 0.1	2.1 ± 0.2	5.1 ± 1.1	8.2 ± 0.2	7.1 ± 0.1	2.5 ± 0.5	2.0 ± 0.0	2.0 ± 0.0	2.2 ± 0.4
ATCC 51659	7.2 ± 0.1	6.4 ± 0.1	3.3 ± 0.3	6.2 ± 0.3	8.3 ± 0.0	7.1 ± 0.3	4.2 ± 0.4	2.0 ± 0.0	2.0 ± 0.0	3.0 ± 1.1
ATCC 700728	7.2 ± 0.1	2.9 ± 0.3	2.0 ± 0.0	2.0 ± 0.0	7.9 ± 0.5	7.2 ± 0.1	4.2 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
CIP 105212	7.1 ± 0.2	4.6 ± 0.1	3.4 ± 0.2	7.1 ± 0.3	8.1 ± 0.2	7.1 ± 0.1	6.2 ± 0.1	2.0 ± 0.0	3.0 ± 0.0	2.2 ± 0.3
CIP 105213	7.1 ± 0.1	2.8 ± 0.3	3.4 ± 0.3	6.3 ± 0.8	8.1 ± 0.1	7.0 ± 0.0	6.2 ± 0.3	2.4 ± 0.2	2.0 ± 0.0	2.0 ± 0.0
CIP 105230	6.8 ± 0.2	2.4 ± 0.6	2.6 ± 0.1	7.5 ± 0.4	8.2 ± 0.1	7.2 ± 0.1	6.0 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
CIP 105231	7.1 ± 0.1	5.5 ± 0.3	4.1 ± 0.2	7.2 ± 0.6	7.8 ± 0.3	7.2 ± 0.1	6.5 ± 0.1	2.2 ± 0.3	2.0 ± 0.0	2.0 ± 0.0
CIP 105243	7.1 ± 0.1	4.0 ± 0.7	2.0 ± 0.0	2.0 ± 0.0	6.6 ± 1.4	7.1 ± 0.1	4.5 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
CIP 105245	7.1 ± 0.1	4.6 ± 0.1	3.8 ± 0.1	4.4 ± 0.1	8.7 ± 0.4	7.0 ± 0.1	4.6 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
CIP 105246	7.0 ± 0.1	3.6 ± 0.2	2.4 ± 0.3	4.0 ± 1.3	8.9 ± 0.2	7.1 ± 0.1	4.6 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
CIP 105248	7.2 ± 0.1	4.2 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	9.1 ± 0.1	7.1 ± 0.2	4.6 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
CIP 105249	6.9 ± 0.1	4.5 ± 0.1	3.4 ± 0.0	3.1 ± 0.1	8.8 ± 0.2	7.1 ± 0.1	2.9 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
CIP 107190	7.3 ± 0.1	3.6 ± 0.2	2.1 ± 0.2	4.1 ± 0.1	8.3 ± 0.0	7.2 ± 0.1	5.8 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.5 ± 0.9
CIP 107872	6.9 ± 0.1	6.2 ± 0.0	3.4 ± 0.0	8.3 ± 0.3	7.9 ± 0.8	7.1 ± 0.1	3.9 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
LREC 19999	6.9 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	6.8 ± 4.2	7.0 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
LREC 20006	6.9 ± 0.1	3.6 ± 0.5	3.1 ± 0.3	6.3 ± 0.7	8.4 ± 0.2	6.8 ± 0.2	4.4 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
LREC 20008	6.9 ± 0.1	2.9 ± 0.1	2.2 ± 0.3	2.0 ± 0.0	2.0 ± 0.0	6.8 ± 0.1	2.6 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
LREC 20010	6.6 ± 0.1	3.5 ± 0.2	2.7 ± 0.1	2.5 ± 0.5	7.7 ± 0.4	6.8 ± 0.1	2.1 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
LREC 20016	7.2 ± 0.1	2.9 ± 0.4	2.0 ± 0.0	5.8 ± 1.4	8.4 ± 0.1	7.2 ± 0.1	2.3 ± 0.3	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
LREC 20021	6.9 ± 0.1	6.2 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.8 ± 1.1	7.0 ± 0.0	4.0 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
LREC 20028	6.9 ± 0.1	2.5 ± 0.5	2.0 ± 0.0	2.0 ± 0.0	4.1 ± 0.1	6.9 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
LREC 20030	7.1 ± 0.2	3.4 ± 0.5	3.4 ± 0.3	3.9 ± 0.5	8.5 ± 0.1	7.2 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
LREC 20041	6.9 ± 0.1	2.1 ± 0.2	2.0 ± 0.0	4.0 ± 1.7	8.3 ± 0.3	6.9 ± 0.2	3.4 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
LREC 20045	6.9 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	6.7 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
LREC 20046	7.1 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	3.7 ± 0.2	7.2 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0

Table A13 (cont.) *E. coli* O157:H7 counts in model solutions of TSBYE at pH 6.0 and 4.5 after HPP (500 MPa for 1 min) and during subsequent storage at 12 °C.

Strain	pH 6.0 (log_{10} CFU/mL)					pH 4.5 (log_{10} CFU/mL)				
	Control	Day 0	Day 1	Day 7	Day 14	Control	Day 0	Day 1	Day 7	Day 14
LREC 20062	7.1 ± 0.1	2.2 ± 0.3	3.6 ± 0.6	3.6 ± 0.6	8.7 ± 0.4	7.2 ± 0.0	2.0 ± 0.0	2.6 ± 0.7	2.0 ± 0.0	2.0 ± 0.0
LREC 20071	7.2 ± 0.1	2.2 ± 0.4	2.0 ± 0.0	2.0 ± 0.0	8.3 ± 0.0	7.1 ± 0.2	2.0 ± 0.0	2.1 ± 0.2	2.0 ± 0.0	2.0 ± 0.0
LREC 20085	7.1 ± 0.1	2.9 ± 0.3	2.0 ± 0.0	2.6 ± 1.1	6.2 ± 2.8	7.0 ± 0.1	2.2 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
LREC 20098	7.3 ± 0.1	4.5 ± 0.1	2.7 ± 0.5	3.0 ± 0.2	7.9 ± 0.1	7.2 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0

Table A14. *L. monocytogenes* counts in model solutions of TSBYE at pH 6.0 and 4.5 after HPP (500 MPa for 1 min) and during subsequent storage at 12 °C.

Strain	pH 6.0 (log_{10} CFU/mL)					pH 4.5 (log_{10} CFU/mL)				
	Control	Day 0	Day 1	Day 7	Day 14	Control	Day 0	Day 1	Day 7	Day 14
FSL C1-056	6.9 ± 0.2	3.2 ± 0.1	4.1 ± 0.2	6.8 ± 0.6	8.5 ± 0.0	7.6 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL C1-115	7.1 ± 0.1	4.4 ± 0.5	5.6 ± 0.5	9.2 ± 0.1	9.1 ± 0.1	6.7 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL C1-122	7.9 ± 0.1	2.0 ± 0.0	2.7 ± 0.2	4.6 ± 1.5	8.9 ± 0.4	7.4 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL F6-154	7.4 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	6.5 ± 1.4	8.5 ± 0.1	5.3 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL J1-031	7.2 ± 0.1	5.6 ± 0.0	5.6 ± 0.0	9.8 ± 0.1	8.8 ± 0.2	7.9 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL J1-049	7.5 ± 0.1	5.4 ± 0.1	5.6 ± 0.3	9.5 ± 0.2	9.0 ± 0.2	7.5 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL J1-094	7.4 ± 0.1	5.9 ± 0.1	6.0 ± 0.1	6.8 ± 0.2	9.1 ± 0.1	7.3 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL J1-101	7.5 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	6.2 ± 2.5	8.8 ± 0.2	7.5 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL J1-110	6.1 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.7 ± 0.9	8.5 ± 0.2	7.4 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL J1-116	7.4 ± 0.5	2.5 ± 0.2	3.8 ± 0.3	9.4 ± 0.0	8.8 ± 0.1	7.4 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL J1-119	7.2 ± 0.1	2.3 ± 0.4	2.9 ± 0.1	4.4 ± 0.5	9.0 ± 0.2	7.1 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL J1-123	7.4 ± 0.1	2.0 ± 0.0	3.1 ± 0.2	7.5 ± 0.2	8.1 ± 0.2	7.2 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL J1-126	7.7 ± 0.1	2.2 ± 0.3	2.9 ± 0.3	8.6 ± 0.3	9.2 ± 0.1	7.4 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL J1-158	7.4 ± 0.1	4.7 ± 0.0	4.5 ± 0.2	8.6 ± 0.6	9.2 ± 0.1	6.9 ± 0.3	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL J1-168	7.6 ± 0.1	5.1 ± 0.2	5.4 ± 0.2	9.0 ± 0.5	8.9 ± 0.3	7.3 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL J1-169	7.5 ± 0.3	2.7 ± 0.1	2.8 ± 0.7	3.7 ± 2.9	8.8 ± 0.1	7.5 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL J1-177	7.4 ± 0.1	4.4 ± 0.0	4.5 ± 0.1	4.8 ± 1.5	8.6 ± 0.1	7.3 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL J1-225	7.5 ± 0.0	2.6 ± 0.5	2.6 ± 1.0	6.6 ± 0.2	8.7 ± 0.2	7.5 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL J2-020	7.4 ± 0.1	2.5 ± 0.5	2.8 ± 0.8	6.4 ± 0.4	8.9 ± 0.1	7.0 ± 0.4	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL J2-031	7.0 ± 0.1	2.3 ± 0.2	3.1 ± 0.2	7.5 ± 0.3	8.8 ± 0.3	6.9 ± 0.4	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL J2-035	7.4 ± 0.0	5.4 ± 0.2	5.8 ± 0.1	3.8 ± 3.1	8.8 ± 0.2	7.4 ± 0.3	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL J2-054	7.2 ± 0.1	5.2 ± 0.1	4.9 ± 0.5	9.2 ± 0.7	9.2 ± 0.0	7.4 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL J2-063	7.2 ± 0.2	4.8 ± 0.1	4.2 ± 0.0	7.5 ± 0.0	8.4 ± 0.2	7.4 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL J2-064	7.4 ± 0.1	6.3 ± 0.1	2.1 ± 0.2	8.2 ± 0.8	9.0 ± 0.1	6.9 ± 0.3	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL M1-004	7.5 ± 0.1	4.1 ± 0.1	4.5 ± 0.2	5.8 ± 0.2	9.0 ± 0.2	6.4 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL N1-225	6.7 ± 0.2	3.0 ± 0.1	3.2 ± 0.1	6.4 ± 0.3	8.7 ± 0.2	7.3 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL N1-227	7.3 ± 0.0	2.0 ± 0.0	2.7 ± 0.4	8.2 ± 0.1	8.2 ± 0.2	7.6 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL N3-008	6.8 ± 0.0	2.2 ± 0.4	2.0 ± 0.0	7.3 ± 0.2	8.5 ± 0.2	7.5 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL N3-013	7.4 ± 0.1	2.5 ± 0.2	4.1 ± 0.4	9.0 ± 0.2	8.5 ± 0.1	7.4 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL N3-022	7.6 ± 0.1	2.0 ± 0.0	3.9 ± 0.2	9.1 ± 0.1	8.9 ± 0.2	7.3 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL N3-031	7.3 ± 0.1	2.0 ± 0.0	2.2 ± 0.3	9.2 ± 0.0	8.2 ± 0.0	7.6 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0

Table A14 (cont.) *L. monocytogenes* counts in model solutions of TSBYE at pH 6.0 and 4.5 after HPP (500 MPa for 1 min) and during subsequent storage at 12 °C.

Strain	pH 6.0 (log_{10} CFU/mL)					pH 4.5 (log_{10} CFU/mL)				
	Control	Day 0	Day 1	Day 7	Day 14	Control	Day 0	Day 1	Day 7	Day 14
FSL R2-499	7.4 ± 0.1	2.0 ± 0.0	2.9 ± 0.5	9.3 ± 0.0	9.2 ± 0.1	7.4 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL R2-500	7.3 ± 0.2	2.0 ± 0.0	3.1 ± 0.2	8.9 ± 0.4	9.2 ± 0.1	7.2 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL R2-501	7.4 ± 0.0	2.0 ± 0.0	3.5 ± 0.0	8.8 ± 0.4	9.0 ± 0.2	7.4 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL R2-502	7.4 ± 0.0	6.3 ± 0.2	6.3 ± 0.2	8.8 ± 0.2	9.0 ± 0.1	7.3 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL R2-503	7.4 ± 0.0	6.3 ± 0.1	6.2 ± 0.3	8.9 ± 0.2	9.0 ± 0.1	7.3 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL R2-763	7.4 ± 0.0	2.0 ± 0.0	4.2 ± 0.2	7.6 ± 0.5	8.2 ± 0.2	7.3 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL R2-764	7.4 ± 0.0	2.0 ± 0.0	4.2 ± 0.1	7.8 ± 0.7	8.1 ± 0.1	7.4 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL R2-765	7.4 ± 0.0	6.2 ± 0.2	2.8 ± 0.3	7.5 ± 1.4	8.8 ± 0.2	7.4 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL W1-110	7.2 ± 0.1	6.2 ± 0.1	6.2 ± 0.2	8.4 ± 0.0	9.0 ± 0.2	7.3 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL W1-111	7.1 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	7.5 ± 0.7	9.0 ± 0.2	7.5 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL W1-112	7.4 ± 0.0	4.5 ± 0.1	2.0 ± 0.0	4.0 ± 0.1	8.0 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
ITA 358	7.6 ± 0.1	2.0 ± 0.0	3.4 ± 0.1	8.5 ± 1.2	8.9 ± 0.1	7.5 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
ITA 359	7.4 ± 0.1	2.0 ± 0.0	2.9 ± 0.4	8.4 ± 0.4	8.9 ± 0.1	7.4 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
ITA 360	7.5 ± 0.1	2.0 ± 0.0	2.5 ± 0.4	8.7 ± 0.0	8.9 ± 0.2	7.6 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
ITA 363	7.4 ± 0.1	6.4 ± 0.0	4.2 ± 0.5	8.1 ± 0.9	8.8 ± 0.1	7.3 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0

Table A15. *S. enterica* counts in model solutions of TSBYE at pH 6.0 and 4.5 after HPP (500 MPa for 1 min) and during subsequent storage at 12 °C.

Strain	pH 6.0 (log_{10} CFU/mL)					pH 4.5 (log_{10} CFU/mL)				
	Control	Day 0	Day 1	Day 7	Day 14	Control	Day 0	Day 1	Day 7	Day 14
FSL A4-633	6.6 ± 0.0	2.3 ± 0.2	2.1 ± 0.2	2.0 ± 0.0	8.5 ± 0.5	6.6 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL R6-540	6.6 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	4.2 ± 1.2	7.5 ± 0.2	6.5 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL R8-5221	7.0 ± 0.1	2.0 ± 0.0	2.1 ± 0.2	5.6 ± 0.0	9.7 ± 0.0	7.0 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL R8-5223	6.5 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	4.3 ± 0.5	7.5 ± 1.3	6.6 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL R8-5224	6.9 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	6.2 ± 2.5	9.0 ± 0.3	6.9 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL R8-6670	7.0 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	4.2 ± 0.6	8.5 ± 0.0	7.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL R8-6671	6.6 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	8.4 ± 0.1	8.2 ± 0.1	6.6 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL R8-7229	6.6 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.8 ± 0.8	8.5 ± 0.1	6.6 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-373	6.6 ± 0.1	2.5 ± 0.5	2.0 ± 0.0	7.2 ± 1.5	7.0 ± 0.2	6.6 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-406	6.6 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	5.5 ± 0.0	9.6 ± 0.0	6.2 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-408	6.6 ± 0.0	2.0 ± 0.0	2.4 ± 0.3	4.8 ± 0.0	9.6 ± 0.0	6.6 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-415	6.8 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	5.0 ± 0.6	9.3 ± 0.3	6.9 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-438	6.9 ± 0.4	2.0 ± 0.0	2.0 ± 0.0	4.0 ± 1.1	8.8 ± 1.1	6.5 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-439	6.8 ± 0.1	3.1 ± 0.0	2.0 ± 0.0	5.6 ± 0.5	7.7 ± 0.0	6.9 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-447	7.1 ± 0.1	2.0 ± 0.0	2.6 ± 0.2	4.5 ± 1.0	9.6 ± 0.0	7.1 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-448	6.5 ± 0.0	2.0 ± 0.0	2.1 ± 0.2	6.7 ± 0.3	8.6 ± 0.1	6.4 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-451	6.7 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	5.9 ± 0.5	8.4 ± 0.9	6.7 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-458	6.4 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	3.1 ± 0.0	8.9 ± 0.5	6.4 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-480	7.0 ± 0.1	2.0 ± 0.0	2.5 ± 0.2	5.8 ± 0.0	8.5 ± 0.2	6.9 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-483	6.7 ± 0.3	2.0 ± 0.0	2.0 ± 0.0	5.8 ± 0.1	7.6 ± 0.0	6.4 ± 0.4	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-487	7.0 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	7.7 ± 0.1	9.2 ± 0.0	7.0 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-490	6.8 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	5.0 ± 0.0	9.7 ± 0.0	6.9 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-504	6.9 ± 0.1	2.0 ± 0.0	2.1 ± 0.2	3.8 ± 1.3	9.7 ± 0.0	7.0 ± 0.3	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-517	6.4 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	5.4 ± 0.0	8.6 ± 0.2	6.4 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-523	6.9 ± 0.0	2.1 ± 0.2	2.0 ± 0.0	5.6 ± 0.0	9.7 ± 0.0	6.8 ± 0.3	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-536	6.6 ± 0.3	2.0 ± 0.0	2.0 ± 0.0	5.1 ± 0.4	6.6 ± 0.0	7.0 ± 0.3	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-540	6.3 ± 0.2	2.1 ± 0.2	2.0 ± 0.0	6.8 ± 1.7	8.4 ± 0.7	6.3 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-543	6.4 ± 0.5	2.0 ± 0.0	2.2 ± 0.3	2.5 ± 0.5	6.6 ± 0.8	6.8 ± 0.3	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-580	6.5 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	5.6 ± 0.7	9.4 ± 0.0	6.5 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-639	6.6 ± 0.0	2.8 ± 0.2	2.8 ± 0.1	5.6 ± 0.8	8.5 ± 0.1	6.6 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-642	6.4 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	3.9 ± 1.0	9.6 ± 0.0	6.5 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-648	6.2 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	3.5 ± 0.5	8.7 ± 0.4	6.4 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0

Table A15 (cont.) *S. enterica* counts in model solutions of TSBYE at pH 6.0 and 4.5 after HPP (500 MPa for 1 min) and during subsequent storage at 12 °C.

Strain	pH 6.0 (log_{10} CFU/mL)					pH 4.5 (log_{10} CFU/mL)				
	Control	Day 0	Day 1	Day 7	Day 14	Control	Day 0	Day 1	Day 7	Day 14
FSL S5-649	6.8 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	4.0 ± 2.1	9.4 ± 0.0	6.7 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-658	6.7 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.7 ± 0.0	7.8 ± 0.5	6.4 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL S5-961	6.5 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	6.4 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL W1-029	6.6 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	4.0 ± 0.0	8.5 ± 0.0	6.8 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
FSL W1-030	6.5 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	4.8 ± 0.4	9.6 ± 0.1	6.6 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
HUBU 34301	6.8 ± 0.6	2.0 ± 0.0	2.0 ± 0.0	2.6 ± 0.5	7.1 ± 2.8	6.9 ± 0.4	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
HUBU 37190	6.4 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	5.3 ± 0.6	9.9 ± 0.0	6.4 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
HUBU 71110	6.5 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	5.7 ± 0.6	9.1 ± 0.1	6.6 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
HUBU 71144	6.5 ± 0.0	2.1 ± 0.2	2.0 ± 0.0	6.0 ± 0.0	8.6 ± 0.5	6.5 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
HUBU 72732	7.6 ± 0.1	2.3 ± 0.2	2.4 ± 0.4	6.0 ± 0.0	9.2 ± 0.3	7.2 ± 0.4	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
HUBU 790	6.5 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	4.8 ± 1.6	9.4 ± 0.3	6.4 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
HUBU 90108	6.4 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	5.5 ± 0.0	9.0 ± 0.2	6.4 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
HUBU 90196	6.5 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	6.6 ± 0.0	9.2 ± 0.4	7.1 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0