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The use of High-Pressure Processing (HPP) to improve the safety and quality of raw coconut (*Cocos nucifera* L) water

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ABSTRACT

This research investigated the use of high-pressure processing (HPP) for inactivating vegetative pathogens and spoilage microbiota in fresh unfiltered coconut water (*Cocos nucifera* L) from nuts obtained from Florida and frozen CW from Brazil with pH >5.0 and storage at 4 °C. Additionally, CW was evaluated to determine if it supported the growth and toxin production of *Clostridium botulinum* with or without the use of HPP when stored at refrigeration temperatures. Samples of fresh unfiltered CW were inoculated to 5.5 to 6.5 logs/mL with multiple strain cocktails of *E. coli* O157:H7, *Salmonella* spp. and *Listeria monocytogenes* and HPP at 593 MPa for 3 min at 4 °C. HPP and inoculated non-HPP controls were stored at 4 °C for 54 and 75 days for Florida CW and Brazil CW, respectively. Results of analyses showed HPP samples with <1 CFU/mL and no detection (negative/25 mL) with enrichment procedures for the 3 inoculated pathogens for all analyses. The non-HPP control samples did not show growth of the pathogens but a gradual decrease in levels to ca. 3-Logs/mL by day 54 in the fresh Florida CW and similarly in frozen Brazil CW by Day 75. Microbial spoilage of uninoculated samples was evaluated for normal spoilage microbiota through 120 days storage at 4 °C. Microbial counts remained at ca. 2-logs with no detectable signs of spoilage for HPP samples through 120 d. The non-HPP control samples spoiled within 2 weeks of storage at 4 °C with gas production, cloudiness, and off-odors. To evaluate if CW supports the growth and toxin production of *C. botulinum*, samples of unfiltered and filtered (0.2 μm) CW were inoculated with either proteolytic or non-proteolytic *C. botulinum* spores at 2 log CFU/mL that were processed at 593 MPa for 3 min and stored at 4 °C and 10 °C for 45 days. Inoculated positive and non-inoculated negative controls were prepared and stored as the HPP treated and non-HPP samples. No growth of *C. botulinum* or toxin production was detected in either the unfiltered or filtered CW regardless if products were HPP treated or not. All inoculated samples with *C. botulinum* spores were enriched at Day-45 in PYGS media to determine the viability of the inoculated spores at the end of shelf-life and screened for *C. botulinum* toxins. In all samples, *C. botulinum* toxin Types A, B and E were detected indicating spores were viable throughout the storage. Type F toxin was not detected possibly due to inherent conditions in the samples that may affected toxin screening.

1. Introduction

High pressure processing (HPP) is now a global mainstream food processing technology. The current commercial application is a non-thermal pasteurization method used to ensure food safety and provide extension of quality in food and beverages. Its use in the fruit and vegetable industries have grown exponentially with global sales forecast more than $10b US by 2028 and projected total increase to $64b US in all categories by 2028 (Visiongain, 2015). HPP is recognized by the US FDA as a method to satisfy the 5-Log reduction of vegetative pathogens such *E. coli* O157:H7, *Salmonella* and L. *monocytogenes* in fruit juices (United States Food and Drug Administration, 2004) and similarly in many other countries as a process to ensure the safety of raw, non-thermally treated fruit juice and other foods (Canadian Food Inspection Agency, 2018; Kurowska et al., 2016). Additionally, HPP is shown to be effective against the oocysts of *Cryptosporidium parvum*, one of the pertinent pathogens defined by the US FDA and Health Canada in fruit juice (Canadian Food Inspection Agency, 2018; Slifko et al., 2000) and other foods (Collins et al., 2005). Several HPP pathogen validation studies have shown similar inactivation of vegetative pathogens in both

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high acid (pH ≤ 4.6) and low acid (pH > 4.6) fruit juices, fruit juice-vegetable blends, plant nut "milks", and fresh coconut water (Lukas, 2013, Mor-Mur and Yuste, 2005; Morris, 2000; Pilavtepe-Celik et al., 2009, Pilavtepe-Celik, 2013; Raghubeer and Phan, unpublished results). However, in its current commercial application, HPP is ineffective against bacterial spores (Black et al., 2007).

The increasing demand by consumers for raw, non-thermally treated, healthy products has resulted in a large increase in the consumption of HPP foods and beverages including CW, particularly in North America and Europe with a Compound Annual Growth Rate (CAGR) of 25.03% during the period 2017–2021 in Europe (Reportlinker, 2018). Globally, the CW market is expected to exceed more than US$ 2.5 Billion by 2024 at a CAGR of 15% and growth in HPP equipment sales to over US$500 million (Reportlinker, 2018). The coconut tree is the most naturally widespread fruit plant on Earth (Lima et al., 2015) and is a major agricultural component in many countries in Asia. The top producing countries of coconut are Indonesia, The Philippines, India, Brazil, and Sri Lanka with the first 3 combining for 60% of the global market (Business Wire, 2019). The CW taken from fresh green coconuts is one of the fastest growing beverage categories in North America, Western Europe and Brazil with Indonesia, Philippines, Thailand, Vietnam, and India the top 5 exporters (CBI, 2018).

Several factors contribute to the growing demand for this beverage. Fresh, raw coconut water is low in calories and a natural source of electrolytes including sodium and potassium (Koslo, 2011; Prades et al., 2012, Yong et al., 2009; United States Department of Agriculture, 2018), fats, carbohydrates, proteins and other minerals (Effiong, 2003; Yartey et al., 1993). The top 5 commercial manufacturers of CW, Vita Coco, PepsiCo, Coco-cola, Green Coco Europe, Taste Nirvana (Market Watch, 2020), thermally processed their products due to the high pH and the concerns of the US FDA and other regulatory agencies on the potential risk of *C. botulinum*. Thermal treatment, however, results in off flavors (Narataruksa et al., 2010), significant loss of natural coconut flavor, vitamins and bioactive compounds which are largely unchanged by HPP and other non-thermal processes (Altemimi et al., 2014; Damar, 2006). Results presented here test the hypotheses that CW either contain a compound or compounds that inhibit the germination, growth and toxin production of strains of *C. botulinum* or, lack of essential components to facilitate growth and toxin production at refrigeration temperatures (≤10 °C). The former is probably more likely as several studies have shown the presence of antimicrobial factors in fresh CW (Fowoyo and Alamu, 2018; Mahayothee et al., 2015).

Many studies reported the presence of three antimicrobial peptides (AMP's) in CW that appear to be inhibitory to Gram-positive and Gram-negative human pathogens (Mandal et al., 2009; Nasimuddin et al., 2016). Others have reported antiviral and antibacterial activities due to the presence of lauric acid and other antimicrobial components (Cruz et al., 2014; Esquenazi et al., 2002; Meneguetti et al., 2017; Sarika and Rai, 2012; Sheri et al., 2006). Lauric acid in virgin coconut oil has been reported to inhibit the growth of *C. difficile* (Shilling et al., 2013) as well as inhibition of *C. difficile* by capric and caprylic acids also present in coconut oil. Wang and Ng (2005) isolated from CW a 10 kDa peptide with antifungal activity against *Fusarium oxysporum*, *Mycosphaerella arachidicola* and *Physalospora piricola*. Meneguetti et al. (2017) and Esquenazi et al. (2002) reported the presence of several phenolic compounds in coconut including Catechin and salicylic acid in the CW and gallic, caffeic, salicylic, and p-coumaric acids in the meat. These and other compounds have been reported to show antimicrobial effects against bacterial pathogens and other microorganisms (Fan et al., 2017; Lima et al., 2015; Effiong et al., 2010). In many areas of the world coconut water and other parts of the coconut fruit have been successfully used for the treatment of childhood diarrhea, gastroenteritis, cholera, and several other illnesses (Adams and Bratt, 1992; DebMandal and Mandal, 2011; De Souza Cândido et al., 2015; Parachin et al., 2012, Prades et al., 2012; Singh, 1986; Zakaria et al., 2006). The presence of these reported antimicrobial components, variety of acids, phenolic compounds together with no known foodborne illness outbreak, except the *Vibrio cholera* in coconut milk reported by the Centers for Disease Control (US CDC) in 1991, prompted this investigation for a better understanding of the use of HPP to inactivate pathogens in a product with pH > 4.6 and whether the product pose food safety hazard from *C. botulinum*. If HPP can satisfy the US FDA Juice HACCP (US FDA, 2004) for this low acid product and if the product does not permit the growth and toxin production of *C. botulinum* at refrigeration of ≤10 °C, then there will be a significant global market growth to meet the increasing demand for non-thermally treated CW.

2. Materials and methods

2.1. High Pressure (HP) equipment

Two different HP equipment were used in these studies. The first study on the inactivation of vegetative pathogens and shelf-life evaluation was done on a commercial AV-10 HPP unit (JBTC-Avure Technologies, Middletown OH, USA) located in Avure Technology's Corporate Food Laboratory (Erlanger OH, USA) with a come-up-time to 600 MPa pressure of 1 min 40 s using Flow's 30XQ pump with (4) intensifiers (Flow International Corp., Kent WA. USA). The study with *Clostridium botulinum* was done on a 24-L HPP unit (JBTC-Avure Technologies, Middletown OH, USA) located at The Institute for Food Safety and Health (IFSH), Bedford Park, IL USA with a come-up-time to 600 MPa of 1 min 24 s. using Flow's 7 × 690 MPa single intensifier pump (Flow International Corp., Kent WA. USA). All studies were done at 593 MPa, for 3 min with HPP vessel water temperature 4 ± 1 °C.

2.2. Preparation of fresh coconut water

Young coconuts (Florida Coconuts, Davie, FL, USA) were freshly picked and shipped to Avure's Corporate Laboratory and stored at 4 °C for 3 days before use. The coconuts were sprayed with a 70% w/v aqueous ethanol solution and allowed to dry for 3 to 5 min. Most of the fibrous mesocarp (husk) around the stem area was removed with a sterile stainless steel (SS) knife and the nut was punctured at around the center of the stem scar using a sterilized SS corer. Both the knives used for removing the husk and corers for puncturing the endocarp (shell) were sterilized by immersing in 95% denatured ethanol then flamed. The CW was drained into sterile containers (10 batches) and kept refrigerated for ca. 4 h at 4 °C. The pH measurements were obtained by using pH meter (Orion Dual Star, with Orion 9156DJWP probes, Thermo Scientific Waltham, MA USA) that was three-point calibrated daily (± 0.05 readability pH units) with standard buffer solutions (Traceable® Products, Webster, TX USA). Duplicate pH readings obtained for each of the 10 CW batches averaged 5.35 ± 0.18. A hand-held refractometer (Atago hand-held refractometer, Model ATC-1E, Brix 0–32%, Japan) was used to measure the Brix (°Bx) of the CW samples. The average °Bx was 5.6 ± 0.9 (n = 10) for the 10 batches of Florida CW.

Both unfiltered and filtered (0.2 μm; Millipore, Burlington, MA USA) CW were used for the study with *Clostridium botulinum*. These samples were placed in sterile plastic bottles, frozen at −20 °C and shipped in refrigerated coolers to The Institute for Food Safety and Health (IFSH), IL USA. For the shelf-life storage study at Avure Technologies, only unfiltered CW was used: the fresh unfiltered CW from Florida nuts, and the frozen, unfiltered CW received from Brazil (supplied by Pascal Salvati, Pure Brazilian, Dunedin, FL USA). The frozen CW from Brazil had average pH 5.23 ± 0.3 (n = 8) and °Bx 5.16 ± 0.6 (n = 8).

2.3. Inoculation of vegetative pathogens and high-pressure treatment

E. coli O157:H7 (7-strain cocktail), *Salmonella* (6-strain cocktail), and *Listeria monocytogenes* (5-strain cocktail; Table 1) were used in the

Table 1
Pathogen strains used for inoculum.

Lab. ID	Description	Source	Provider
E. coli O157:H7 (8 strains)			
FDA 1	Seattle 13A24; SLT 1, 2	Meat Loaf	FDA, Seattle WA
FDA 2	Seattle 13A46; SLT 1, 2	Hamburger	FDA, Seattle WA
FDA 3	Seattle 13A29; SLT 1, 2	Dry Salami	FDA, Seattle WA
FDA 4	Sea 6318; SLT 1, 2	Jack-In-The-Box Hamburger	FDA, Seattle WA
FDA 5	Sea 6458; SLT 1, 2	Jack-In-The-Box Hamburger	FDA, Seattle WA
FDA 6	Sea 13B88; SLT 1, 2	Apple juice strain Unpasteurized Juice	FDA, Seattle WA
NFFEc 1	ATCC 43889, SLT 2	Stool, HUS Patient	ATCC purchase
NFFEc 2	ATCC 43895, SLT 1, 2	Raw Hamburger	ATCC purchase
Listeria monocytogenes (7 strains)			
AFLLm5	ATCC BAA-751	Animal	ATCC Purchase
NFFLm 1	Scott A	Chocolate Milk	FDA, Seattle WA
NFFLm2	R1950	Pasteurized Salted Egg Yolk	Nalley's Lab Isolate
AFLLm1	ATCC 7644	Human	ATCC Purchase
AFLLm2	ATCC 11914	Animal Brain Tissue	ATCC Purchase
AFLLm3	ATCC 11915	Human	ATCC Purchase
AFLLm4	V-7	(UW Student Research)	FDA, Seattle WA
Salmonella (9 strains)			
Sea 2518	Salmonella group C	Specific food source information not available. Isolated by FDA from food during Salmonella outbreak investigations	FDA, Seattle WA
Sea 2521	Salmonella group G2		FDA, Seattle WA
Sea 2522	Salmonella group C		FDA, Seattle WA
Sea 2523	Salmonella group C1		FDA, Seattle WA
Sea 2525	Salmonella Poly A (+)		FDA, Seattle WA
NFPA1	*S. senftenberg* ATCC 43845: *S. enterica* subsp. *enterica* serovar senftenberg	Egg powder	NFL, Dublin, CA
AFLsal1	*S. enteritidis* ATCC 13076: *Salmonella enterica* subsp. *enterica* serovar Enteritidis	Animal	ATCC Purchase
AFLsal2	*S. choleraesuis (typhimurium) ATCC 13311*	Mutton	ATCC purchase
AFLsal3	*Salmonella* spp. Species NA	Turkey	Silliker Laboratory for AOAC International methods evaluation
C. botulinum (4 proteolytic and 7 non-proteolytic strains): Inoculation level 2-logs			
NA	Proteolytic Type A (62A, 69A)	NA	IFSH Culture Collection
NA	Proteolytic Type B 213B, 7273B	NA	IFSH Culture Collection
NA	Non-proteolytic Type B (2B, 17B, Kap9-B)	NA	IFSH Culture Collection
NA	Non-proteolytic Type E (Beluga E, Birmingham-E, Alaska-E)	NA	IFSH Culture Collection
NA	Non-proteolytic type F (610F)	NA	IFSH Culture Collection

Inoculation levels of vegetative culture cocktails (listed above) are shown in Tables 3 and 4.
ATCC = American Typed Culture Collection; FDA = Food & Drug Administration, Bothell (Seattle), WA; NFPA = National Food Processors Association, Dublin, CA; **NFF** = Nalley's Fine Foods (Birdseye Foods) Microbiological Laboratory. NA = Not Available. NFL: National Food Laboratory (Eurofins). AFLsal: Avure Food Laboratory *Salmonella* strain. AOAC: Association of Official Agricultural Chemists. IFSH: Institute for Food Safety and Health.

inoculation of fresh, unfiltered CW from Florida at 5 to 6-log CFU/mL (Table 3). All inoculations were conducted in duplicate with duplicate analyses and HPP at 593 Mpa for 3 min with CW temperature 4 ± 2 °C and HPP process water temperature at 4 ± 1 °C. A second study with the same strains used for the fresh Florida CW was done in the frozen CW from Brazil with a slightly higher inoculation level (>6 log CFU/mL; Table 4) using the same processing conditions described above for the Florida CW. Prior to the inoculation of CW samples, each strain of pathogens was added to filter sterilized (0.2 μm; Millipore, Burlington, MA USA) CW and refrigerated at 4 ± 1 °C for 48 ± 2 h as a pre-conditioning step. The strains were recovered by spread plating the inoculated CW sample on respective selective agar plates and stored on nutrient agar (VWR Scientific Radnor, PA USA) slants. For the inoculation study, each of the preconditioned bacterial strain was grown independently to late stationary phase at 35 ± 2 °C for ca. 20 h in Brain Heart Infusion (BHI) media (VWR Scientific Radnor, PA USA) and pooled by organism type prior to inoculation. Inoculation levels were estimated spectrophotometrically at 600 nm (Lambda Bio + Spectrophotometer Waltham, MA USA) and at time of product inoculation (Day-0) by plating of the pooled inoculum on respective selective media (United States Food and Drug Administration 1998/2007 (2015). Bacteriological Analytical Manual (BAM) Online). Duplicate samples were prepared for both inoculated, non-HPP controls and the inoculated, HPP treated samples. Duplicate samples were obtained by processing inoculated products in two different cycles (2 runs) in the HPP vessel under the same processing conditions (593 MPa for 3 min 4 ± 1 °C water temperature). One sample from each run makes up the duplicate samples for each of the analysis times shown in Tables 3 and 4 for both vegetative pathogen inoculation studies. Microbiological analyses were conducted in duplicate on both inoculated, non-HPP controls and HPP treated samples on each date of analysis.

2.4. Enumeration of inoculated vegetative pathogens and spoilage microorganisms

Only unfiltered samples were used for the vegetative pathogen inoculation and spoilage microbiota shelf-life studies. Serial dilutions of inoculated samples, both HPP and non-HPP controls were prepared in 0.1% sterile peptone water as described in the US Food and Drug Administration's (US FDA) Bacteriological Analytical Manual (BAM) Online2015 Chapters 3, 4, 5, 10, 17, and 18. Increased sensitivity to <1 CFU per mL was achieved by spread plating 1 mL of the undiluted sample (ca. 0.333 mL each) onto 3 agar media as well as the 1:10 dilutions to eliminate any inhibitory effect that may be present in the undiluted CW. For subsequent higher serial dilutions, 0.1 mL was spread plated on each agar plate. Confirmation of suspected colonies was done as described in US FDA (BAM) Online 2015. For the recovery of pressure stressed cells, 25 mL of each HPP sample were enriched in

respective modified selective broth medium with positive or negative results reported per 25 mL. Confirmation of growth in enrichment was done on respective selective agar plates.

Shelf-life samples were analyzed for the aerobic plate count levels (APC), yeast, molds, lactic acid bacteria, and total coliform bacteria. Serial dilutions of controls and HPP samples were prepared in 0.1% sterile peptone water, pH 7.1 ± 0.2 (US FDA BAM Online, 2015) and plated either on 3 M Petri Films as(3 M Corp. St. Paul MN, USA) as or spread plated on De Man, Rogosa and Sharpe (MRS) agar (VWR Scientific Radnor, PA USA). Enumeration of APC, yeast, mold, and total coliform were done using 3 M Petri Films according to the directions of the supplier. Lactic acid bacteria (LAB) levels were determined by spread plating on MRS agar plates and anaerobic incubation at 30 °C as described in the Compendium of methods for the microbiological examination of foods (American Public Health Association, 2015).

2.5. Clostridium botulinum strains and preparation of spore crop

Four proteolytic *C. botulinum* strains and seven non-proteolytic strains (Table 1) were selected from IFSH Culture Collection for use in the challenge study, based on published literature (Doyle, 1998; United States Department of Agriculture, 1992). Spore crops were prepared using the biphasic method (Anellis et al., 1972) and prior to cocktail preparation, all spore crops were cultured on peptone yeast extract glucose starch agar (PYGS) to determine final spore counts and confirmation of toxin production using DIG-ELISA (Sharma et al., 2006). Proteolytic *C. botulinum* cultures were incubated anaerobically at 37 ± 2 °C for 72 h and non-proteolytic *C. botulinum* cultures were incubated anaerobically at 28 ± 2 °C for 72 h.

Spore mixtures to be used as inoculums contained approximately equal numbers of spores of each strain of *C. botulinum* in the cocktail. Proteolytic *C. botulinum* spore cocktails consisted of strains 62A, 69A, 213B, and 7273B were suspended in 50 mL sterile DI water to a final concentration of 4.91 log CFU/mL. A 50 mL cocktail consisting of non-proteolytic *C. botulinum* spore strains 2B, 17B, Kap9-B, Beluga-E, Birmingham-E, Alaska-E, and 610F was similarly produced and suspended in sterile DI water to a final concentration of 5.30 log CFU/mL.

2.6. Inoculation of fresh, filtered coconut water and high-pressure treatment

Coconut water samples were inoculated with *C. botulinum* (proteolytic and non-proteolytic) cocktails in a ratio of 1:100 to achieve a final inoculated concentration of approximately 2 log CFU/mL. Inoculated samples (100 mL) were portioned into pouches according to IFSH SOP High Pressure SOP MC-SAFE-24L-SOP001-V01 with the use of a disinfectant e.g. peracetic acid in between the primary and secondary containment. Samples were then high pressure processed for 3 min at 593 MPa with an initial product temperature of 4 ± 2 °C and processing water temperature of 4 ± 1 °C.

The experimental matrix (Table 2a and b) included two formulations (fresh and filtered CW) inoculated with either proteolytic or non-proteolytic *C. botulinum* spores at 2 log CFU/mL that were high pressure treated at 593 MPa for 3 min and then stored at two storage temperatures (4 and 10 °C) over a shelf life of 45 days. Control samples for both CW products were tested in parallel with high pressure treated CW samples; the positive control samples were inoculated with 2 logs (CFU/mL) of *C. botulinum* spores while the negative controls were not inoculated. All inoculations were conducted in triplicate with duplicate analyses of each inoculated sample.

2.7. Analysis of normal microbiota in CW samples stored at IFSH

Samples were stored at 4 or 10 °C for up to 45 days. Triplicate samples of each sample type were pulled for analysis at each time point [(Days 0, 9 pre-HPP), 1, 30 and 45 days)] and storage temperature, and plate counts conducted in duplicate for each sample (25 mL) to enumerate *C. botulinum* levels, total count and total anaerobic counts by plating onto appropriate media. The remaining portion of each sample was frozen for subsequent analysis of *C. botulinum* toxin production by DIG ELISA.

Table 2
a. Study design for *C. botulinum* inoculated into filtered CW.
b. Study design for *C. botulinum* inoculated into and raw unfiltered CW.

a.

	CW sample	Inoculation	Treatment	Storage temperature (°C)	Analyses
1a	Filter sterilized	Non-proteolytic and proteolytic spore cocktails	None control	4	Total count Spore count ELISA[a]
1b	Filter sterilized	Non-proteolytic and proteolytic spore cocktails	HPP	4	Total count Spore count ELISA
1c	Filter sterilized	Non-proteolytic and proteolytic spore cocktails	None control	10	Total count Spore count ELISA
1d	Filter sterilized	Non-proteolytic and proteolytic spore cocktails	HPP	10	Total count Spore count ELISA
1e	Filter sterilized	None	None control	4	Total count Spore count ELISA
1f	Filter sterilized	None	HPP	4	Total count Spore count ELISA
1g	Filter sterilized	None	None control	10	Total count Spore count ELISA
1h	Filter sterilized	None	HPP	10	Total count Spore count ELISA

b.

	CW sample	Inoculation	Treatment	Storage temperature (°C)	Analyses
2a	Raw untreated	Non-proteolytic and proteolytic spore cocktails	None control	4	Total count Spore count ELISA[a]
2b	Raw untreated	Non-proteolytic and proteolytic spore cocktails	HPP	4	Total count Spore count ELISA
2c	Raw untreated	Non-proteolytic and proteolytic spore cocktails	None control	10	Total count Spore count ELISA
2d	Raw untreated	Non-proteolytic and proteolytic spore cocktails	HPP	10	Total count Spore count ELISA
2e	Raw untreated	None	None control	4	Total count Spore count ELISA
2f	Raw untreated	None	HPP	4	Total count Spore count ELISA
2g	Raw untreated	None	None control	10	Total count Spore count ELISA
2h	Raw untreated	None	HPP	10	Total count Spore count ELISA

[a] DIG-ELISA (Sharma et al., 2006).

2.8. Statistical analysis

Data were analyzed using Excel 2016 Data Analysis Tool Pack. When levels of the inoculated pathogens decreased to undetectable

levels (Negative/25 mL) with HPP treatment a value of zero was used for determination of arithmetic mean.

3. Results and discussions

3.1. Results of vegetative pathogens study

Both enumeration by spread plating and enrichment procedures were used to determine the log reduction and elimination (absence or presence/25 mL) of the inoculated strains of *Salmonella*, *Listeria monocytogenes* and *E. coli* O157:H7 (EHEC) as shown in Table 1 in both fresh, unfiltered CW (Florida) and raw, unfiltered, frozen CW from Brazil. The CW product from Brazil had slightly lower pH 5.23 ± 0.3 (n = 8) and °Bx 5.16 ± 0.6 (n = 8) levels compared to the CW from Florida with mean pH 5.35 ± 0.18 (n = 10) and mean 5.6 ± 0.9°Bx (n = 10).

The first microbiological analyses of CW samples were done ca. 18 h following HPP treatment of 593 MPa for 3 min with water temperature at 4 ± 1 °C and storage at 4 °C. The levels in the HPP samples showed a decrease from the initial inoculated levels (determined at time of HPP treatment) of ca. 6-Log CFU/mL of all 3 pathogens to <1 CFU/mL and no detection (absence/25 mL) with enrichment procedures (Tables 3 and 4; Fig. 1a, b). These results were also obtained for all analyses during the 54 days of storage at 4 °C for the Florida CW. Similarly, the enumeration and enrichment results obtained in the frozen CW from Brazil showed <1 CFU/mL and no detection (negative/25 mL) in all analyses during the 75 days of storage at 4 °C. There was no significant difference ($P > .05$) in the inactivation of the inoculated pathogens by HPP in the raw CW from Florida and the raw, frozen CW from Brazil.

For the non-HPP controls in both Florida and Brazilian frozen CW, a slight decrease in inoculated pathogen levels were observed at the Day-1 (18 h) analyses followed by a sharper decline in levels through Day-28 (Fig. 1A and B). This decrease in levels of the inoculums in the controls was greater ($p < .05$) in the unfiltered CW from Brazil than in the Florida CW. This is likely due to the higher initial levels of lactic acid bacteria (LAB) in the CW from Brazil compared to that in the Florida CW with 17,000 CFU/mL and 10 CFU/mL respectively (Fig. 2) and not the effect of slightly lower pH and Brix levels. All the inoculated non-HPP controls for both CW products had positive readings/25 mL with enrichment procedures throughout the storage periods of 54 and 75 days. However, no increase in the levels (CFU/mL) were observed in the products stored at 4 °C (Figure, 1A and 1B). This indicated that the CW did not support the growth of the vegetative pathogens at both 4 and 10 °C. Similar findings were reported on vegetative pathogens in pathogen inoculated studies in CW and other juice products (Health Canada, 2006; Considine et al., 2008; Lukas, 2013; Raghubeer and Phan, unpublished results).

3.2. Shelf-life of raw unfiltered CW (Florida and Brazil)

The aerobic plate count (APC), yeast, mold, total coliform bacteria count and the levels of lactic acid bacteria (LAB) were monitored on unfiltered, raw CW from both Florida nuts and frozen samples from Brazil. During the 120 days of storage at 4 °C, 10 analyses were conducted on non-HPP controls and HPP samples of Florida CW and 9 analyses on Brazil CW. The lowest dilution plated for CW samples from Florida was the undiluted CW (results expressed as <1 CFU/mL) followed by serial dilutions in 0.1% peptone water. The lowest dilution plated for the unfiltered CW from Brazil was the 1:10 dilution in 0.1% peptone water with results reported as <10 CFU/ mL. The reason is to negate any inhibitory effect undiluted CW may have on surviving organism which is commonly seen when plating undiluted acidic products such as fruit juice products. For HPP samples the APC and LAB levels were approximately at 1 to 2 logs throughout the 120 days of storage for both products (Fig. 2). Yeast, mold and total coliform bacteria were not detected (<10 CFU/mL) in the CW from Florida (Fig. 2) as well as in the product from Brazil (<10 CFU/mL). These findings are usually seen in acidic juice products such as apple, citrus, acai berry, cranberry, and pomegranate (Raghubeer and Phan, unpublished results) where the high acidity is too inhibitory for the recovery of pressure injured surviving microorganisms that are native to the food. It is not uncommon for HPP juice products with pH > 5.0 to show 3 to 4 logs of APC and LAB by 120 days of storage at 4 °C. The low levels (<2 logs) in both CW samples (pH > 5.0) may suggest that compounds in CW described in several reports (Pilavtepe-Celik et al., 2009; Pilavtepe-Celik, 2013; Lukas, 2013; Mor-Mur and Yuste, 2005; Morris, 2000) may be exhibiting similar inhibitory effects on pressure injured spoilage microorganisms as the high acidity effects reported in fruit juices (Canadian Food Inspection Agency, 2018; Palou et al., 2002; Patterson, 2005; Raghubeer and Phan, unpublished results).

However, in the control (non-HPP) samples for the shelf-life studies, the levels of the non-pressure stressed spoilage microorganisms increased rapidly within the first 2 weeks of storage (Fig. 2). The inhibition of bacterial growth was observed only with the inoculated pathogens (Fig. 1) in the non-HPP controls and on the surviving spoilage organisms (normal flora) in HPP shelf-life samples. The lack of growth of low-level surviving spoilage microorganisms following HPP has been observed across several product categories regardless of pH, °Bx, water activity levels with LAB being the predominant surviving bacteria (Pilavtepe-Celik, 2013; Raghubeer and Phan, unpublished results).

3.3. Growth and toxin production of C. botulinum

Samples of unfiltered and filtered (0.2 μm) raw CW were inoculated with either cocktails of proteolytic or non-proteolytic *C. botulinum*

Table 3
Effects of HPP on *inoculated vegetative pathogens compared to non-HPP controls in fresh CW from Florida. Average CFU/mL (n = 2).

Days after HPP	Sample	Spread plate enumeration (avg. CFU/mL): n = 2			Enrichment for presence/absence per 25 mL (n = 2)		
		E. coli O157:H7	*Salmonella*	*L. monocytogenes*	*E. coli* O157:H7	*Salmonella*	*L. monocytogenes*
1	Control[a]	1,500,000	143,000	141,000	Positive	Positive	Positive
	HPP[b]	<1[c]	<1	<1	Negative	Negative	Negative
14	Control	200,000	2000	9000	Positive	Positive	Positive
	HPP	<1	<1	<1	Negative	Negative	Negative
21	Control	40,000	1000	4000	Positive	Positive	Positive
	HPP	<1	<1	<1	Negative	Negative	Negative
54	Control	1500	1000	1000	Positive	Positive	Positive
	HPP	<1	<1	<1	Negative	Negative	Negative

*$E. coli$ O157:H7 = 2.4 × 10^6 CFU/ML; *Salmonella* = 8.9 × 10^5 CFU/mL; *L. monocytogenes* = 4.4 × 10^5 CFU/mL.
[a] Inoculated, non-HPP controls.
[b] HPP 593 MPa (86,000 psi) for 3 min at pressure with water temperature 4 ± 1C.
[c] Spread plate of 1 mL of undiluted product on 3 separate plates to increase sensitivity of enumeration.

Table 4
Effects of HPP on *inoculated vegetative pathogens compared to non-HPP controls in unfiltered raw, frozen CW from Brazil. Average CFU/mL (n = 2).

Days after HPP	Sample	Spread plate enumeration (mean CFU/mL): n = 2			Enrichment for presence/absence per 25 mL (n = 2)		
		E. coli O157:H7	*Salmonella*	*L. monocytogenes*	*E. coli* O157:H7	*Salmonella*	*L. monocytogenes*
1	Control[a]	1,690,000	2,150,000	1,000,000	Positive	Positive	Positive
	HPP[b]	<1[c]	<1	<1	Negative	Negative	Negative
7	Control	692,000	4470	8100	Positive	Positive	Positive
	HPP	<1	<1	<1	Negative	Negative	Negative
14	Control	33,000	1380	3350	Positive	Positive	Positive
	HPP	<1	<1	<1	Negative	Negative	Negative
28	Control	560	570	380	Positive	Positive	Positive
	HPP	<1	<1	<1	Negative	Negative	Negative
45	Control	320	210	110	Positive	Positive	Positive
	HPP	<1[c]	<1	<1	Negative	Negative	Negative
60	Control	200	150	50	Positive	Positive	Positive
	HPP	<1	<1	<1	Negative	Negative	Negative
75	Control	140	50	30	Positive	Positive	Positive
	HPP	<1	<1	<1	Negative	Negative	Negative

*$E. coli$ O157:H7 = 3.9×10^6 CFU/mL; *Salmonella* = 4.1×10^6 CFU/mL; *L. monocytogenes* = 1.09×10^6 CFU/mL.
[a] Inoculated, non-HPP controls.
[b] HPP at 593 MPa (86,000 psi) for 3 min at pressure with water temperature 4 °C.
[c] Spread plate of 1 mL of undiluted product on 3 separate plates to increase sensitivity of enumeration.

spores (approximately 2 logs CFU/mL) and were treated with HPP or left untreated prior to storage at 4 and 10 °C for 45 days.

The levels of proteolytic *C. botulinum* spores inoculated into filtered CW remained stable over the 45 days storage (Fig. 3). Although the total levels of anaerobic bacteria in unfiltered samples stored at 10 °C increased at Day 45, this increase could be attributed to indigenous anaerobic bacteria present in the sample at the start of shelf life storage, possibly LAB. Similar results on the growth of LAB have been consistently observed in our laboratory when samples of HPP and non-HPP were stored at 10 °C (data not shown). Inoculated samples that were not HPP treated and stored at 10 °C were approximately 2 log CFU/mL higher that samples that were HPP treated and were likely due to the increase of LAB. Inoculated samples that were HPP treated (593 MPa, 3 min) and stored at 4 °C had no significant increase in total anaerobic counts and remained stable over the 45-day storage. All samples were screened for the presence of *C. botulinum* toxins (Types A,

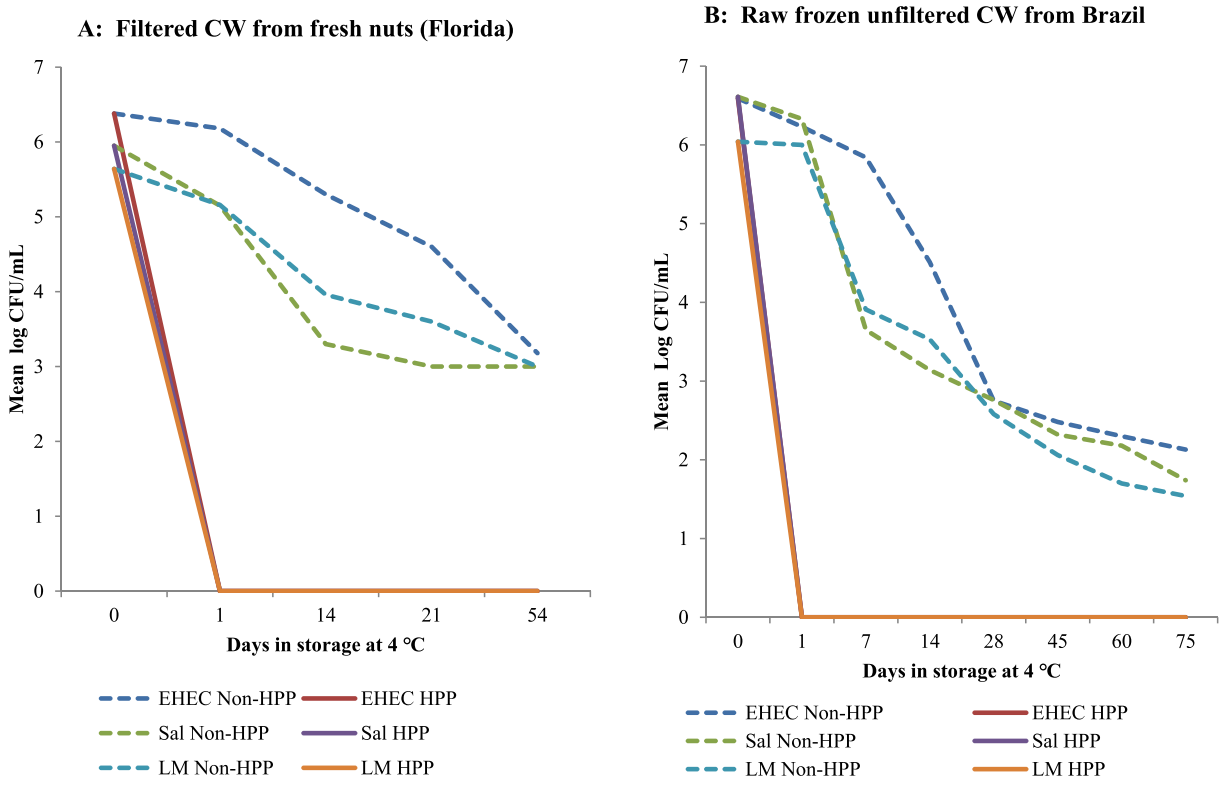

Fig. 1. (A, B). Effects of HPP (593 MPa for 3 min; Solid Lines) on inoculated strains of *E. coli* 0157:H7 (EHEC), *Salmonella* (Sal), and *Listeria monocytogenes*. Compared to Inoculated non-HPP samples (Dashed Lines). Average results of duplicate samples. Inoculation study (A, left chart) was done on CW from raw nuts purchased from Florida with analyses on samples through 54 days of storage. Inoculation levels for A (pooled culture plated at time of inoculation): *E. coli* O157:H7 = 2.4×10^6 CFU/ML; *Salmonella* = 8.9×10^5 CFU/mL; *L. monocytogenes* = 4.4×10^5 CFU/mL. Replicated inoculation study (B, right chart) was done on raw, frozen CW received from Brazil. Analysis was done over a period of 75 days of storage at 4 °C with Inoculation levels for B: *E. coli* O157:H7 = 3.9×10^6 CFU/mL; *Salmonella* = 4.1×10^6 CFU/mL; *L. monocytogenes* = 1.09×10^6 CFU/mL.

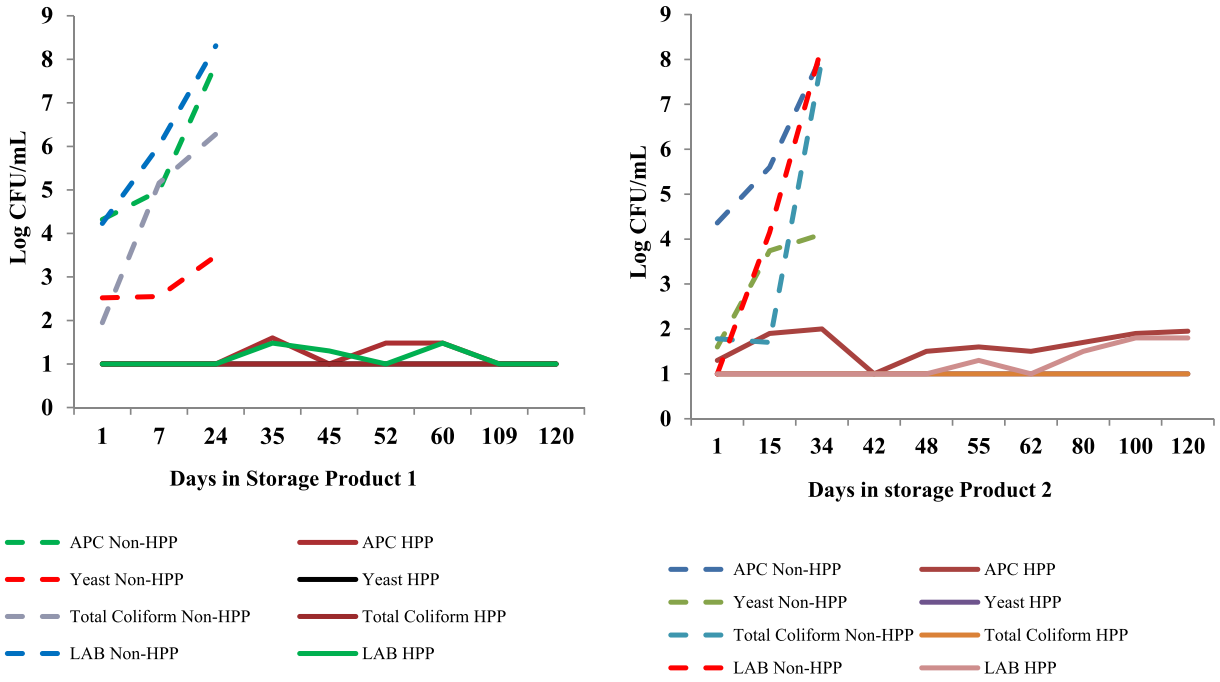

Fig. 2. Product 1. Microbial shelf-life of unfiltered raw, frozen CW from Brazil. Non-HPP treated controls are shown by in dashed lines. Lowest dilution plated was 1:10 (<10 CFU/mL). Non-HPP controls were not tested (NT) after the Day-14 analysis due to spoilage of all control samples. Product 2: Microbial shelf-life of unfiltered raw, fresh CW from nuts purchased from Florida. Non-HPP treated controls are shown by dashed lines. Lowest dilution plated was undiluted sample (<1 CFU/mL). Non-HPP controls were not tested (NT) after the Day-14 analysis due to complete spoilage. All samples stored at 4 °C.

B, E and F) by DIG-ELISA and no positive detection of *C. botulinum* toxin was observed which indicates the increased in total anaerobic bacteria levels observed on Day 45 were not *C. botulinum* and most likely from the growth of LAB which may have been introduced in the filtered CW from errors in the filtration and/or during transfer from filter units into sterile containers. Similarly, the levels of total anaerobic bacteria in uninoculated filtered CW not HPP treated and stored at 4 °C increased by 1.2 log CFU/mL over 45 days while samples that were HPP treated had no significant increase ($P > 0.05$) in total anaerobic bacteria levels. However, when samples, whether HPP treated or not HPP treated, were stored at 10 °C, significant increase ($P < 0.05$) in total anaerobic bacteria levels were observed at Days 30 and 45 and total anaerobic bacteria levels from samples that were HPP treated were approximately 1 log CFU/mL lower than the untreated samples (Fig. 4). All samples were screened for the presence of *C. botulinum* toxins (Types A, B, E and F) by DIG-ELISA and no positive detection of *C. botulinum* toxin was observed indicating the increased levels of total anaerobic bacteria observed on Days 30 and 45 were not due to the presence *C. botulinum*

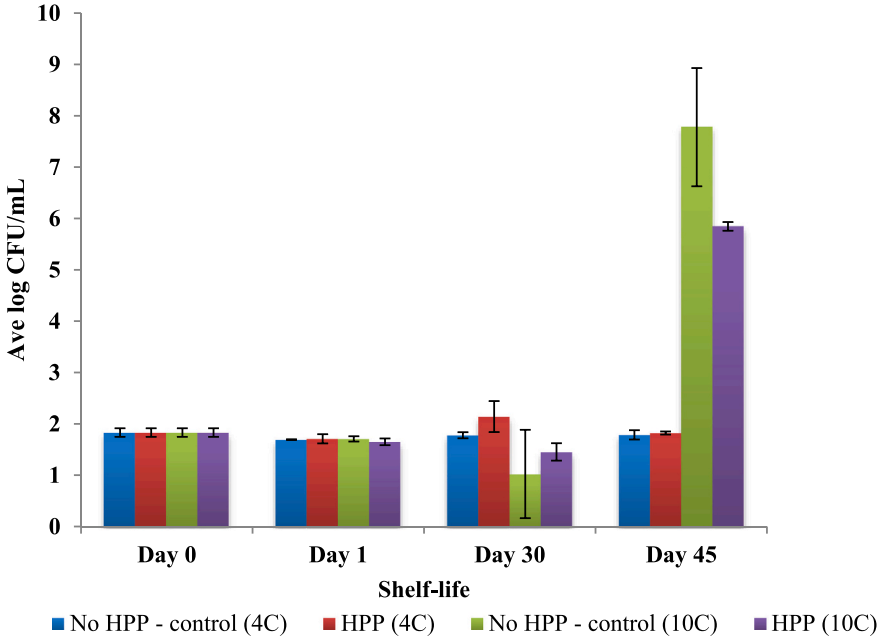

Fig. 3. Total levels of anaerobic bacteria in unfiltered CW inoculated with 2 log CFU/mL proteolytic *C. botulinum* spores (HPP and no HPP treatment) and stored at 4 and 10 °C for 45 days.

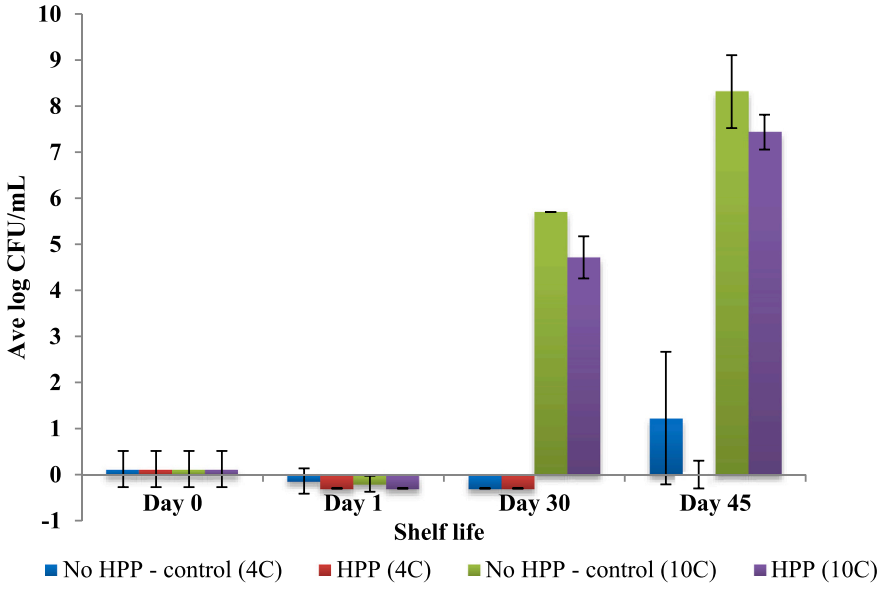

Fig. 4. Total levels of anaerobic bacteria in filtered CW (HPP and no HPP treatment) and stored at 4 and 10 °C for 45 days.

but possibly from anaerobic bacteria, likely LAB present in the samples at the start of the storage.

The levels of non-proteolytic *C. botulinum* spores inoculated into filtered CW remained stable over the 45-day storage at 4 °C (Fig. 5). However, the total levels of anaerobic bacteria in samples, regardless of HPP treatment or without HPP treatment, stored at 10 °C increased significantly at Day 45 with increases of between 4 and 5.5 log CFU/mL with samples HPP treated having 1.5 log CFU/mL lower total anaerobic levels compared to non-HPP treated samples. Inoculated samples that were not HPP treated and stored at 10 °C had higher total anaerobic bacteria levels than the similarly HPP treated samples by approximately 3 log CFU/mL (Fig. 3). All samples were screened for the presence of *C. botulinum* toxins (Types A, B, E and F) by DIG-ELISA and no positive detection of *C. botulinum* toxin was observed which indicated the increased in total anaerobic bacteria levels observed on Day 45 were not *C. botulinum*.

The levels of non-proteolytic *C. botulinum* spores inoculated into raw unfiltered CW and HPP treated also remained stable over the 45-day storage (Fig. 6) at 4 °C while untreated samples increased in total anaerobic counts from Day 1 and Day 30 from samples stored at 10 °C and 4 °C respectively. The total anaerobic bacteria in HPP treated samples stored at 10 °C increased to 7.5 log CFU/mL on Day 30 of storage. The results indicate storage at 4 °C in combination with HPP treatment was effective in preventing the growth of anaerobic bacteria in the samples. All samples were similarly screened for the presence of *C. botulinum* toxins (Types A, B, E and F) by DIG-ELISA and no positive detection of *C. botulinum* toxin was observed which confirming the increased in total anaerobic bacteria levels observed on Day 45 were not *C. botulinum* but most likely due to growth of LAB.

As controls, filtered CW were either HPP treated or no HPP applied and stored at 4 °C and 10 °C for 45 days (Fig. 7). Samples stored at 4 °C had no significant increase in total anaerobic and aerobic bacteria counts except for Day 45 from samples without HPP treatment where 1.2 log CFU/mL and 1.8 log CFU/mL increase were observed in

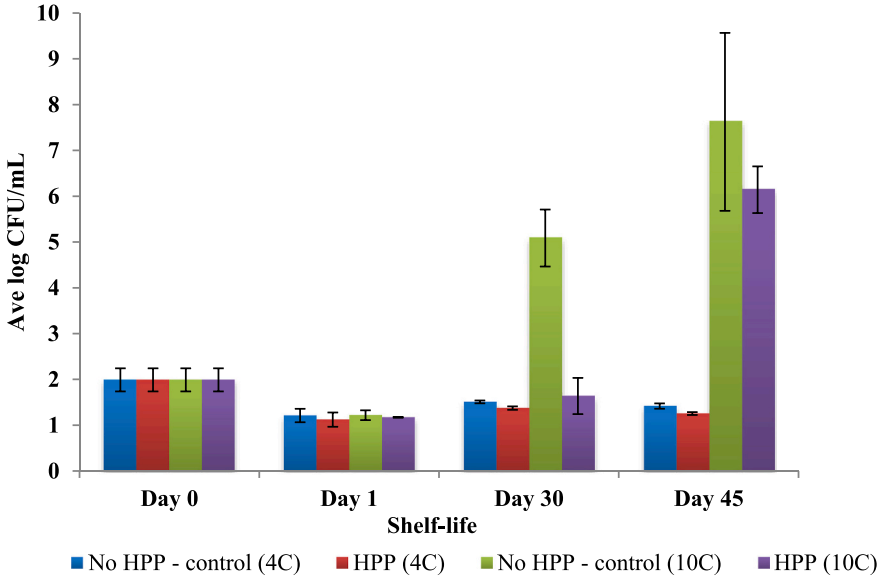

Fig. 5. Total levels of anaerobic bacteria in filtered CW inoculated with 2 log CFU/mL non-proteolytic *C. botulinum* spores (HPP and no HPP treatment) and stored at 4 and 10 °C for 45 days.

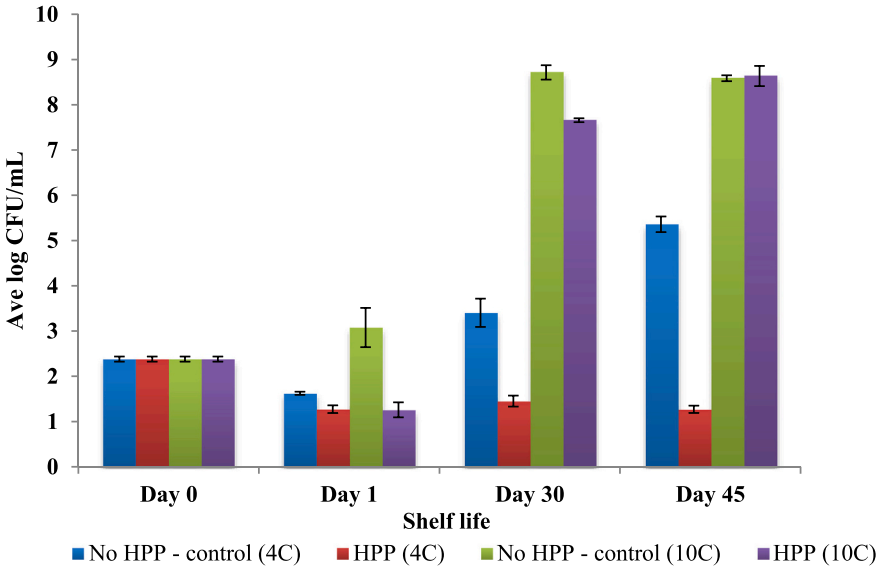

Fig. 6. Total levels of anaerobic bacteria in raw CW inoculated with 2 log CFU/mL non-proteolytic *C. botulinum* spores (HPP and no HPP treatment) and stored at 4 and 10 °C for 45 days.

anaerobic and aerobic bacteria counts respectively. The presence of microorganisms in the filtered CW may be due to errors in filtration and/or low-level contamination during transfer from filtration units to sterile plastic bottles.

Raw unfiltered CW were similarly prepared and processed as described above with either HPP treated or no HPP applied and stored at 4 °C and 10 °C for 45 days (Fig. 8). Samples that were HPP treated and stored at 4 °C had no significant increase in total anaerobic and aerobic bacteria counts to Day 45. HPP treated samples significantly reduced background microbiota to almost undetectable levels and remained low when stored at 4 °C. However, when HPP treated samples were stored at 10 °C, any surviving microorganism after HPP treatment showed increased levels at Day 30 and Day 45, the anaerobic bacteria levels were 6.7 and 8.9 log CFU/mL respectively while aerobic bacteria levels at Day 30 and Day 45 were at 3.0 and 8.7 log CFU/mL. These were more likely the growth of lactic acid bacteria, particularly homofermentative strains which tend to grow better at temperatures above 8 °C. Samples without HPP treatment and stored at 4 and 10 °C increased significantly over shelf-life storage from Day 1 suggesting the presence of psychrotrophic bacteria in sample.

All inoculated samples with proteolytic and non-proteolytic *C. botulinum* spores were enriched at Day 45 in PYGS media to determine the viability of the inoculated spores at the end of shelf-life storage and screened for the presence of *C. botulinum* toxin. In all samples, *C. botulinum* toxin Types A, B and E were detected indicating spores that were introduced into CW were viable but did not grow throughout shelf-life storage. Type F toxin was not detected and could be due to inherent conditions in samples such as pH that may affect toxin screening.

4. Conclusions

HPP at 593 MPa for 3 min is effective in eliminating inoculated strains of *E. coli* O157:H7, *Salmonella* and L. *monocytogenes*. Non-HPP control samples showed that the inoculated vegetative pathogens did not grow but slowly decrease in levels during refrigerated storage suggesting the presence of antimicrobial compounds against bacterial pathogens that were reported in several studies.

Coconut water samples treated with HPP at 593 MPa for 3 min gave microbiologically stable (<2-logs) product during storage at 4 °C for

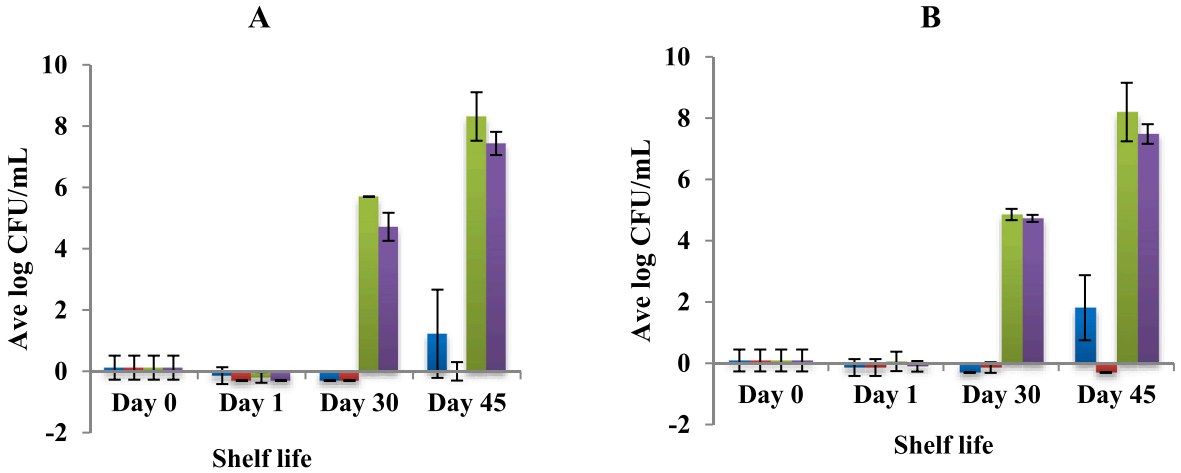

Fig. 7. Total anaerobic bacteria count (A) and total aerobic bacteria (B) in filtered uninoculated CW (HPP and no HPP treatment) and stored at 4 and 10 °C for 45 days. ■ represents No HPP control stored at 4 °C, ■ represents HPP treated stored at 4 °C, ■ represents No HPP control stored at 10 °C, and, ■ represents HPP treated stored at 10 °C.

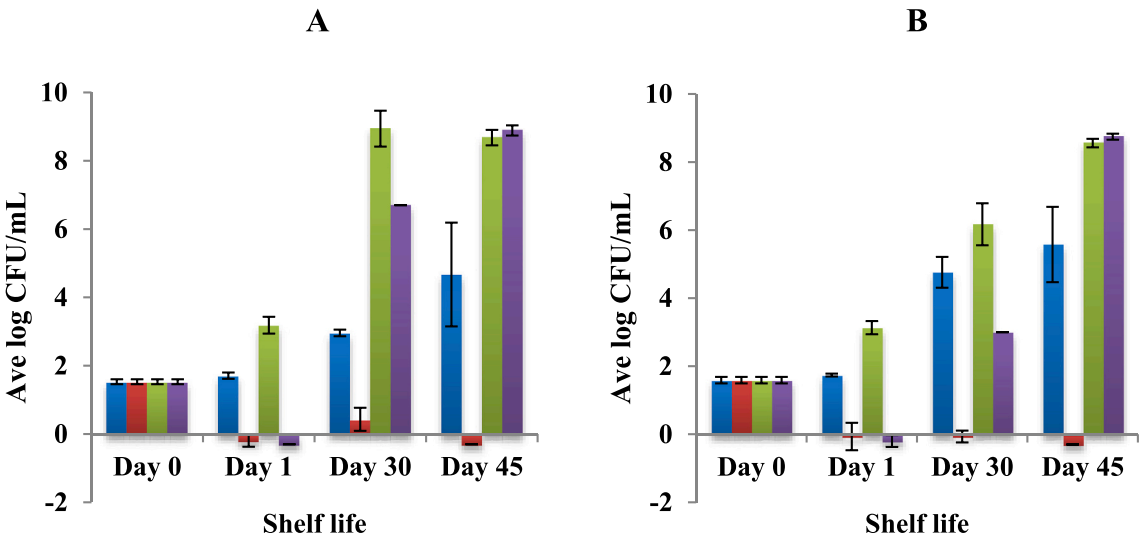

Fig. 8. Total anaerobic bacteria count (A) and total aerobic bacteria (B) in raw uninoculated CW (HPP and no HPP treatment) and stored at 4 and 10 °C for 45 days. ■ represents No HPP control stored at 4 °C, ■ represents HPP treated stored at 4 °C, ■ represents No HPP control stored at 10 °C, and, ■ represents HPP treated stored at 10 °C.

120 days with no off odors and similar taste to fresh CW. In contrast, non-HPP treated CW showed elevated microbial counts, gas production, cloudiness, and off-odor within two weeks of storage at 4 °C.

Spores of proteolytic and non-proteolytic strains of *C. botulinum* inoculated in raw unfiltered and filtered CW did not grow or produce toxins when stored for 45 days at 4 and 10 °C, regardless if the product was HPP or not. The inoculated spores remained viable during the 45 days in the preparations of coconut water as they were recovered from the products and evaluated for viability and toxin production in PYGS medium. It appears that antimicrobial compounds such as lauric acids, antimicrobial peptides and other substances reportedly present in fresh coconut water are inhibitory to the growth and toxin production of both non-proteolytic and proteolytic strains of *C. botulinum* when stored at ≤10 °C.

The use of HPP can significantly reduce background microbiota and in combination with appropriate storage temperature, e.g. 4 °C prevent the growth of spoilage microbiota in both filtered and unfiltered CW. However, when storage temperature increases e.g. 10 °C, microbiota present in samples regardless of filtration or HPP treatment proliferated to high numbers. Several studies conducted in our laboratory confirmed the rapid spoilage of CW and other juice products mainly from the growth of LAB.

Filtered CW inoculated with proteolytic or non-proteolytic *C. botulinum* spores increased in anaerobic bacteria levels when stored at 10 °C regardless of HPP treatment which was probably due to errors in filtration, transfer from filter units to sterile PET bottles or low-level contamination during spore inoculation. The combined use of filtration, HPP and 4 °C storage prevented the outgrowth of both anaerobic and aerobic bacteria and no *C. botulinum* growth and subsequent toxin formation was detected. It is well reported that HPP under the conditions used in these studies does no inactivate *C. botulinum* spores (Black et al., 2007) and the inhibition of growth observed during shelf life storage could be due to the presence of inhibitory compounds naturally present in CW. The enrichment of *C. botulinum* inoculated samples in PYGS after 45 days showed the presence of toxins indicating that the inoculated spores were still viable in CW but unable to grow and produce toxin in CW. Should the inhibitory compound(s) in CW be used as a preventive control for *C. botulinum* in CW, these compounds may need to be identified and correlated to determine levels that would be inhibitory to *C. botulinum*, not only in CW but may also have applicability for the control of *C. botulinum* growth and toxin production in low acid juices and nut milks. However, it is more likely that a combination of inhibitory compounds as reported in several studies referenced above are present in CW that collectively prevented the growth and toxin production of both non-proteolytic and proteolytic of strains *C. botulinum* when kept at refrigeration temperature (≤10 °C).

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References

Adams, W., Bratt, D.E., 1992. Young coconut water for home rehydration in children with mild gastroenteritis. Trop. Geogr. Med. 44 (1–2), 149–153.

Altemimi, A., Choudhary, R., Watson, D.G., Lightfoot, D.A., 2014. Effects of ultrasonic treatments on the polyphenol and antioxidant content of spinach extracts. Ultrason. Sonochem. https://doi.org/10.1016/j.ultsonch.2014.10.023.

American Public Health Association, 2015. The Compendium of Methods for the Microbiological Examination of Foods. APHA, Washington DC, pp. 20036.

Anellis .D., A, Berkowitz, D., Kemper, D., Rowley, D.B., 1972. Production of types A and B spores of *Clostridium botulinum* by the biphasic method: effect on spore population, radiation resistance, and toxigenicity. Appl. Microbiol. 23 (4), 734–739.

Black, E.P., Setlow, P., Hocking, A.D., Stewart, C., Kelly, A.L., Hoover, D.G., 2007. Response of spores to high-pressure processing. Compr. Rev. Food Sci. Food Saf. 6, 103–121.

Business wire, 2019. World Coconuts Market Analysis, Forecast, Size, Trends and Insights Report. https://www.businesswire.com/news/home/20190919005726/en/World-Coconuts-Market-Analysis-Forecast-Size-Trends.

Canadian Food Inspection Agency, 2018. Food Microbiology – Targeted Surveys FINAL REPORT: Bacterial Pathogens, Viruses and Parasites in Unpasteurized Juices and High Pressure Processed Juices April 1, 2016 – August 31, 2017. 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.

CBI, Ministry of Foreign Affairs, 2018. Exporting Coconut Water to Europe. https://www.cbi.eu/market-information/processed-fruit-vegetables-edible-nuts/coconut-water/europe/.

Collins, M.V., Flick, G.J., Smith, S.A., Fayer, R., Croonrnberghs, R., O'Keefe, S., Lindsay, D.S., 2005. The effect of high-pressure processing on infectivity of *Cryptosporidium parvum* oocysts recovered from experimentally exposed eastern oysters (*Crassostrea virginica*). J. Eukaryot. Microbiol. 52 (6), 500–504. ©2005 by the International Society of Protistologists. https://doi.org/10.1111/j.550-7408.2005.00059.x.

Considine, K.M., Kelly, A.L., Fitzgerald, G.F., Hill, Sleator, R.D., 2008. High-pressure processing – effects on food safety food quality. FEMS Microbiol Lett 281, 1–9.

Cruz, J., Guzmán .F., C., Fernández-Lafuente, R., Torres, R., 2014. Antimicrobial peptides: promising compounds against pathogenic microorganisms. Curr. Med. Chem. 21

(20), 2299–2321. Available at. https://www.ncbi.nlm.nih.gov/pubmed/24533812.
Damar, S., 2006. Processing of Coconut Water with High Pressure Carbon Dioxide. Ph.D. Dissertation. University of Florida, Gainesville. FL Available at. http://ufdcimages.uflib.ufl.edu/UF/E0/01/55/41/00001/damar_s.pdf.
De Souza Cândido, E., e Silva Cardoso, M.H., Sousa, D.A., Viana, J.C., de Oliveira-Júnior, N.G., Miranda, V., Franco, O.L., 2015. Review: the use of versatile plant antimicrobial peptides in agribusiness and human health. Peptides 55, 65–78. https://doi.org/10.1016/j.peptides.2014.02.003. Epub 2014 Feb 16.
DebMandal, M., Mandal, S., 2011. Coconut (*Cocos nucifera* L.: Arecaceae): in health promotion and disease prevention. Asian Pac J Trop Med 3, 241–247. Available at. https://www.ncbi.nlm.nih.gov/pubmed/21771462.
Doyle, M.P., 1998. Evaluating the potential risk from extended-shelf-life refrigerated foods by *Clostridium botulinum* inoculation studies. Food Tech 4, 154–156.
Effiong, G.S., 2003. Characterization and chemical composition of coconut water and coconut milk. J. Pure and Appl. Sc. 6 (1), 26–32.
Effiong, G.S., Ebong, P.E., Eyong, E.U., Uwah, A.J., Ekong, U.E., 2010. Amelioration of chloramphenicol induced toxicity in rats by coconut water. J. Appl. Sci. Res. 6 (4), 331–335.
Esquenazi, D., Wigg, M.D., Miranda, M.M., Rodríguez, H.M., Tostes, J.B., Rozental, S., da Silva, J., Alviano, C.S., 2002. Antimicrobial and antiviral activities of polyphenolics from Cocos nucifera Linn. (Palmae) husk fiber extract. Res. Microbiol. 153 (10), 647–652. Available at. https://www.ncbi.nlm.nih.gov/pubmed/12558183.
Fan, X., Wagner, K., Sokorai, K.J.B., Ngo, H., 2017. Inactivation of Gram-positive bacteria by novel phenolic branched-chain fatty acids. J. Food Prot. 80 (1), 6–14.
Fowoyo, P., Alamu, J., 2018. Nutritional composition and antimicrobial activity of coconut water against selected gastrointestinal pathogens. International Journal of Microbiology and Application 5 (1), 1–8. http://www.openscienceonline.com/journal/ijma.
Health Canada, 2006. Guidelines for the safety assessment of novel foods. Food directorate health products and Food Branch Health Canada. https://www.canada.ca/en/health-canada/services/food-nutrition/legislation-guidelines/guidance-documents.htmL.
Koslo, J., 2011. Coconut Water: Is it Really nature's Sports Drink. Kaplan University School of health Available at. http://healthandwellness.kaplan.edu/articles/nutrition/coconut.
Kurowska, A., Szaikowska, S., Van der Meulen, B.M.J., 2016. EU regulatory approach to high-pressure processing. In: High Pressure Processing of Food-Principles, Technology and Application, https://doi.org/10.1007/978-1-4939-3234-4_30.
Lima, E.B.C., Sousa, C.N., Meneses, L.N., Ximenes, N.C., Santos, M.A., Lima, N.B., Patrocínio, M.C., Macedo, D., Vasconcelos, S.M., 2015. *Cocos nucifera* (L.) (Arecaceae): a phytochemical and pharmacological review. Braz. J. Med. Biol. Res. 48 (11), 953–964. https://doi.org/10.1590/1414-431X20154773.
Lukas, A.R., 2013. Use of High-Pressure Processing to Reduce Foodborne Pathogens in Coconut Water. Ms. Thesis. Virginia Polytechnic Institute and State University Dept. Fd. Sci. and Technol. https://vtechworks.lib.vt.edu/bitstream/handle/10919/24760/Lukas_AR.
Mahayothee, B., Koomyart, I., Khuwijitjaru, P., Siriwongwilaichat, M., Nagle, M., Müller, J., 2015. Phenolic compounds, antioxidant activity, and medium chain fatty acids profiles of coconut water and meat at different maturity stages. Intern. J. Food properties 19 (9), 2041–2051.
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, 633–637.
Market Watch, 2020. Global coconut water market (2020): global industry size, share, top countries data, future challenges, revenue, demand, industry growth and top players analysis to 2024. https://www.marketwatch.com/press-release/global-coconut-water-market-2020-global-industry-size-sharetop-countries-data-future-challenges-revenue-demand-industry-growth-and-top-players-analysis-to-2024-2020-03-04.
Meneguetti, B.T., dos Santos Machado, L., Oshiro, G.N., Nogueira, M.L., Carvalho, C.M.E., Franco, O.L., 2017. Antimicrobial peptides from fruits and their potential use as biotechnological tools—a review and outlook. Front. Microbiol. https://doi.org/10.3389/fmicb.2016.02136. 10 January 2017.
Mor-Mur, M., Yuste, J., 2005. Microbiological aspects of high-pressure processing. In: Sun, D.W. (Ed.), Emerging Technologies for Food Processing. Elsevier Academic Press, London, UK, pp. 47–65.
Morris, C.E., 2000. US developments in non-thermal juice processing. Food Engineering and Ingredients 25 (6), 26–30.
Narataruksa, P., Pichitvittayakarn, W., Heggs, P.J., Tia, S., 2010. Fouling behavior of coconut milk at pasteurization temperatures. Applied Thermal Eng 30 (11 − 12), 1387–1395.
Nasimuddin, S., Rajan, P.D., Sumathi, G., 2016. A study of invitro antimicrobial activity of coconut water and coconut oil on gram positive and gram-negative bacteria. World Journal of Pharmaceutical Research 5 (8), 696–700.
Palou, E, Lopez-Malo, A, Welti-Chanes, J, 2002. Innovative fruit preservation using high pressure. Engineering and food for the twenty first century. Welti-Chanes, J, Barbosa-Canovas, G.V., Aguilera, J.M. (Eds.), Food preservation technology CRC, Boca Raton, pp. 715–726.
Parachin, N.S., Mulder, K.C., Viana, A.A., Dias, S.C., Franco, O.L., 2012. Expression systems for heterologous production of antimicrobial peptides. Peptides 38 (2), 446–456.
Patterson, M.F., 2005. A review microbiology of pressure-treated foods. J. Appl. Microbiol. 98, 1400–1409.
Pilavtepe-Celik, M., 2013. High hydrostatic pressure (HHP) inactivation of foodborne pathogens in low-acid juices. Int. J. Food Sci. Technol. 48, 673–677.
Pilavtepe-Celik, M., Buzrul, S., Alpas, H., Bozoglu, F., 2009. Development of a new mathematical model for inactivation of Escherichia coli O157:H7 and Staphylococcus aureus by high hydrostatic pressure in carrot juice and peptone water. J. Food Eng. 90, 388–394.
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.
Raghubeer, E., Phan, B.N., 2020. HPP pathogen validation studies in fruit and fruit juice-based vegetable juices. In: Manuscript in preparation, unpublished results.
Reportlinker, 2018. High Pressure Processing (HPP) Equipment and Foods Market 2018–2028. https://www.reportlinker.com/p05574234/High-Pressure-Processing-HPP-Equipments-and-Foods-Market.html.
Sarika, I.M.A., Rai, A., 2012. Biotic stress resistance in agriculture through antimicrobial peptides. Peptides 36 (2), 322–330.
Sharma, S.K., Ferreira, J.L., Eblen, B.S., Whiting, R.C., 2006. Detection of Type A, B, E and F *Clostridium botulinum* neurotoxins in foods by using an amplified enzyme-linked immunosorbent assay with digoxigenin-labeled antibodies. Appl. Environ. Microbiol. 72, 1231–1238.
Sheri, L., Mary, G., Preuss, H.G., 2006. A review of monolaurin and lauric acid: natural viricidal and bactericidal agents. Alternative and Complementary Therapies 12 (6), 310–318.
Shilling, M., Matt, L., Rubin, E., Visitacion, M.P., Haller, N.A., Grey, S.F., Woolverton, C.J., 2013. Antimicrobial effects of virgin coconut oil and its medium-chain fatty acids on *Clostridium difficile*. J. Med. Food 6 (12), 1079–1085.
Singh, Y.N., 1986. Traditional medicine in Fiji: some herbal folk cures used by Fiji Indians. J. Ethnopharmacol. 15, 57–88.
Slifko, T.R., Raghubeer, E.V., Rose, J.B., 2000. Effect of high hydrostatic pressure on *Cryptosporidium parvum* infectivity. J. Food Prot. 63 (9), 1262–1267.
United States Department of Agriculture, 1992. Vacuum or Modified Atmosphere Packaging for Refrigerated Raw Fishery Products. National Advisory Committee on the Microbiological Criteria for Foods Adopted March 20, 1992. https://www.fsis.usda.gov/wps/portal/fsis/topics/regulations/advisory-committees/nacmcf-reports/nacmcf-map-fishery-products.
United States Department of Agriculture, 2018. USDA Branded Food Products Database. Coconut Water. https://ndb.nal.usda.gov/ndb/foods/show/45349011.
United States Food and Drug Administration, 2004. Juice HACCP hazards and controls guidance. In: US Food and Drug Administration Center for Food Safety and Applied Nutrition, . http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/Juice/ucm072557.htm.
United States Food and Drug Administration 1998/2007, 2015. Bacteriological Analytical Manual Online. AOAC International, Gaithersburg, MD 20877 USA. http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm063346.htm.
Visiongain, 2015. The Food High Pressure Processing (HPP) Technologies Market Forecast. https://www.visiongain.com/Report/1406/-2015-2025.
Wang, H.X., Ng, T.B., 2005. An antifungal peptide from the coconut. Peptides 26 (12), 2392–2396.
Yartey, J., Harisson, E.K., Brakohiapa, L.A., Nkrumah, F.K., 1993. Carbohydrate and electrolyte content of some home-available fluids used for oral rehydration in Ghana. J. Trop. Pediatr. 39, 234–237.
Yong, J.W., Ge, L., Y., F., S., N., 2009. The chemical composition and biological properties of coconut (Cocos nucifera L.) water. Molecules 14 (12), 5144–5164.
Zakaria, Z.A., Reezal, I., Mat Jais, A.M., Somchit, M.N., Sulaiman, M.R., Marmin, A.H.I., Sidek, H., Husin, S.H., Rahim, M.H.A., Abdul-Rahman, L., 2006. The anti-inflammatory, anti-pyretic and wound healing activities of Cocos nucifera (MATAG types) fresh juice and kernel extract in experimental animals. J. Pharmacol. Toxicol. 1, 516–526.