



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



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ABSTRACT

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. This study assessed nontoxigenic type E *C. botulinum* and Group II *Clostridium* sp. growth in raw and HPP (550 MPa, 3 min, 10 °C) Thai coconut water (CCW; pH 5.2). No spore germination or growth occurred in HPP CCW inoculated with 10⁵ CFU/ml after 61 days regardless of oxygen concentration (< 0.5 – 11 mg/l) or storage temperature (4 and 20 °C). Spore concentration decreased by 3.0 ± 0.1 log CFU/ml in a worst-case scenario consisting of non-HPP filter-sterilized CCW (pH 7.0) under anoxic incubation at 30 °C during 61 days, suggesting spore germination followed by cellular death. Supplementing filter-sterilized CCW (pH 7.0) with selected germinants and free amino acids did not support spore development, but the addition of nutrient-rich laboratory media (TPGY broth) at low concentrations (6.25%) promoted growth, suggesting that a lack of nutrients prevents *C. botulinum* development in CCW. Further risk assessment will require evaluating other CCW 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 sensorial 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 ranged from 5.50 to 6.10) at 600 MPa/2 min/4 °C yielded an inactivation > 5 log 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

Abbreviations: DO₂, dissolved oxygen; LO₂, low dissolved oxygen concentration; UO₂, unaltered dissolved oxygen concentration; HO₂, high dissolved oxygen concentration; FS, filter sterilized; G, germinants; AA, amino acids; CDM, chemically defined medium

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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 (US-FDA, 2015a,b). According to this regulation, processing technologies must guarantee, at a minimum, a 5-log 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 (US-FDA, 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 7 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 °C) and pH 5.0 or above, whereas proteolytic *C. botulinum* (group I) can only grow under temperature abuse (> 10 °C) 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 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 neurotoxin formation 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. Nonetheless, to the best of authors' knowledge, no peer-reviewed studies have previously attempted to evaluate the behavior of *C. botulinum* on high-pressure processed beverages.

Although spores are not inactivated in HPP coconut water, their germination and growth potential depends on multiple interrelated intrinsic and extrinsic factors. Dissolved oxygen (DO₂) 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 formation as well, causing nutrient depletion or directly inhibiting growth by the modification of physicochemical properties of the media (e.g. 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 could decrease oxygen concentration, increase pH or make nutrients more accessible, which would potentially facilitate spore outgrowth (Hotchkiss et al., 2016; Kasai et al., 2016; Odlag & 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* sp. 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-toxicogenic mutants of *C. botulinum* type E NCTC 11,219 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-toxicogenic three-strain cocktail referred hereafter as *C. botulinum*. NCTC strains were genetically modified by deletion of the botulinum toxin gene (*bont/E*), whereas *Clostridium* sp. DSM 1985 is a natural nontoxicogenic strain genetically related to type E *C. botulinum* based on 16S rDNA sequence, and has been 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 by Peck, Fairbairn, & Lund (1992) with minor adjustments. Single colonies of each strain were grown in reinforced clostridial medium at 30 °C during 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 °C in a Whitley DG250 anaerobic workstation (flushed with 80% N₂, 10% CO₂ and 10% H₂) by centrifugation (3,400 × g, 4 °C, 15 min). The resulting pellet was washed four times with 0.85% sterile NaCl solution by centrifugation, concentrated 5-fold, and stored at 4 °C outside the anaerobic workstation. Purity of the spore stocks was determined as the difference between unheated and heated (65 °C, 10 min) aliquots, yielding > 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. Final concentration of spores obtained for strains NCTC 11219, NCTC 8266 and DSM 1985 was around 10⁸ CFU/ml. The three-strain cocktail was prepared by decimally diluting aliquots of each spore stock in peptone water to obtain a concentration of 10⁷ 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 °C) in sterile containers and shipped to Spain, and from there to Belgium. A certified laboratory (Agrolab Ibérica, Spain) analyzed the nutritional composition of

Table 1
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/3 min/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

coconut water and the report can be found in the [supplementary material](#). After thawing coconut water overnight at 4 °C, 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 10⁵ CFU/ml. In order to evaluate the effect of multiple intrinsic factors on *C. botulinum* growth, the dissolved oxygen concentration (DO₂), pH, background microbiota and nutrient profile of coconut water were modified for various experiments as summarized in [table 1](#) and described over the next lines.

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 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 (US-FDA, 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 (DO₂) 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 × 30 mm) pierced through the bottle lid was used to inject sterile gas mixtures (Air Liquide, Germany) at 10 l/h flow rate during 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 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 (UO₂) contained a 7.0 ± 0.8 mg/l oxygen concentration. The injection of 100% N₂ yielded the lowest oxygen concentration used in this study (LO₂; < 0.5 ± 0.1 mg/l), whereas a 35% O₂ + 65% N₂ mixture yielded the highest (HO₂; 11.0 ± 0.5 mg/l). Non-invasive O₂ 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 predominant microbial species in HPP 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 DO₂ 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 a greater diversity. rDNA fragments were amplified with B27F forward primer (5'-AGAGTTTGA TCMTGGCTCAG-3') and U1492R reverse primer (5'-GGTTACCTTGTT ACGACTT-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 > 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 °C, 15 min) and subsequent microfiltration (0.22 µm pore size). 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 (LO₂; section 2.3.1), and adjusted to pH 7.0 with sterile 1 N NaOH (Ando & Lida, 1970; Strasdine, 1967).

2.4.1. Supplementation of microfiltered coconut water with germinant, 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 NaHCO₃, 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 NaHCO₃ 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 °C, anaerobic workstation). This concentration of cycloserine effectively inhibited the growth of spoilage microorganisms in agar plates but did not have an effect on the three-spore cocktail counts (supplementary material). 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 °C, 10 min), which only accounts for spores. Total aerobic counts were determined in plate count agar (PCA; 17.5 g/l [Oxoid, England]) by incubating plates at 30 °C 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 effect of HPP on spore inactivation and the extent of germination and/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/3 min/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 (DO₂) content (Fig. 1). 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 (supplementary material). Although growth of type E *C. botulinum* has been reported at temperature levels below 4 °C in laboratory media, 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 (Fig. 1A-1C).

During the 61 days of incubation, DO₂ concentration decreased by 1.86 ± 1.22 mg/l in UO₂ coconut water (Fig. 1B) and 4.13 ± 0.3 mg/l in HO₂ (Fig. 1C). 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

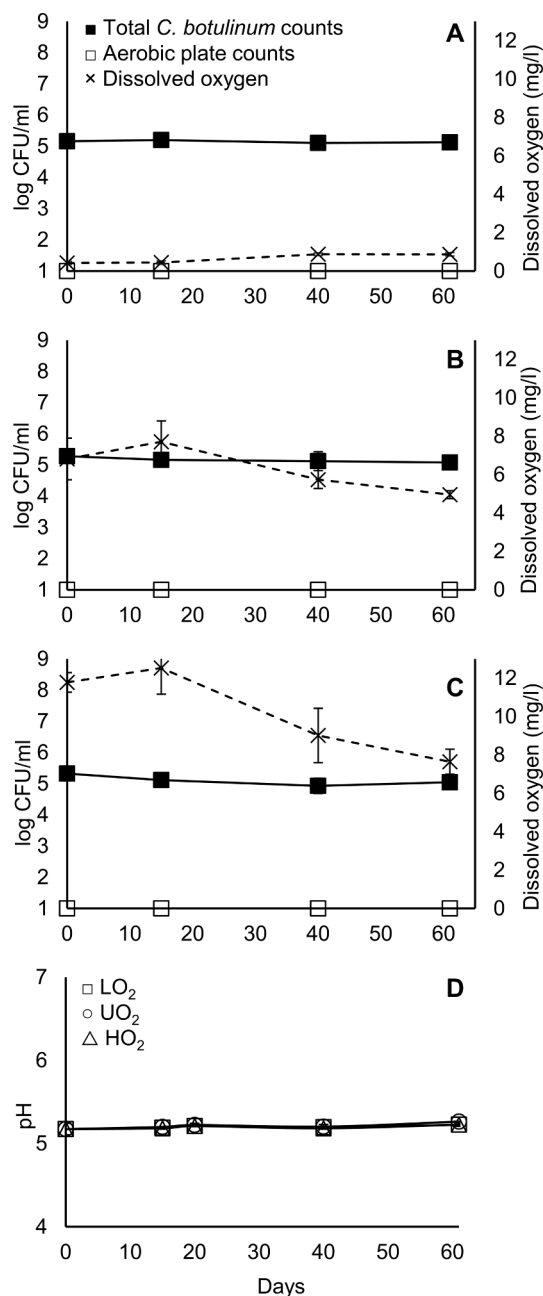


Fig. 1. *C. botulinum* total counts, aerobic plate counts and dissolved oxygen concentration in high-pressure processed coconut water (550 MPa, 3 min) stored at 4 °C with different initial dissolved oxygen concentration levels: (A) low (LO₂; $< 0.5 \pm 0.1$ mg/l); (B) unaltered (UO₂; 7.0 ± 0.8 mg/l); (C) high (HO₂; 11.0 ± 0.5 mg/l). pH evolution of coconut water (D) in LO₂, UO₂ and HO₂.

coconut water often indicates that pink discoloration can potentially occur (Happy Coco, 2019). In the case of LO₂ coconut water, dissolved oxygen remained between 0.1 and 0.45 mg/l after 40 days (Fig. 1A).

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 (Fig. 2). Spore concentration determined by plate counting of heated

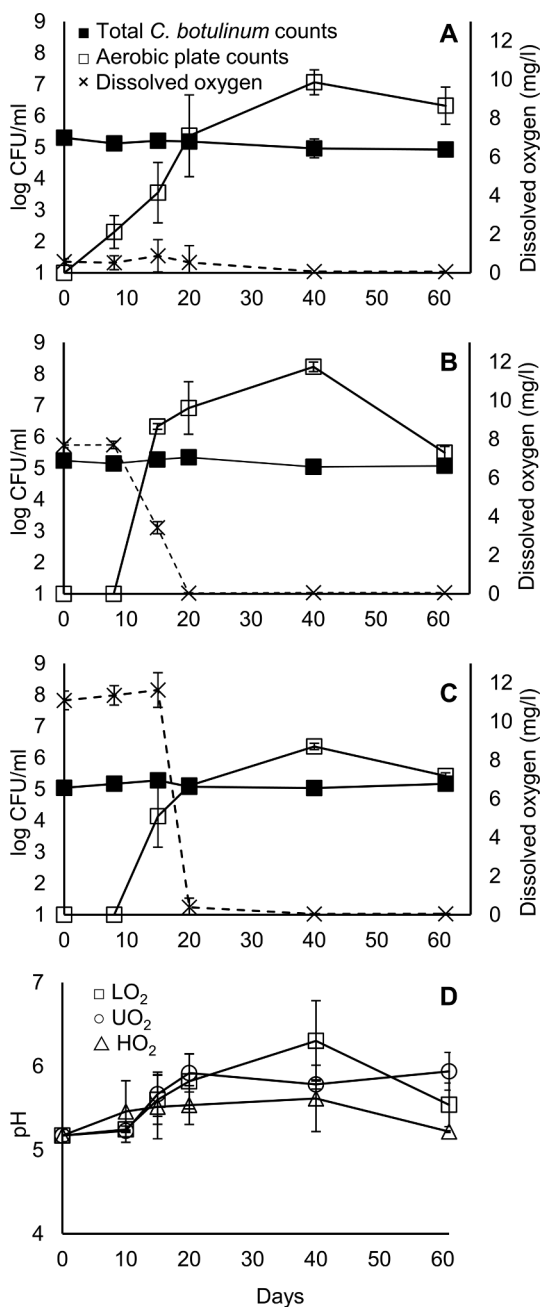


Fig. 2. *C. botulinum* total counts, aerobic plate counts and dissolved oxygen concentration in high-pressure processed coconut water (550 MPa, 3 min) stored at 10 °C with initial different dissolved oxygen concentration levels: (A) low (LO₂; $< 0.5 \pm 0.1 \text{ mg/l}$); (B) unaltered (UO₂; $7.0 \pm 0.8 \text{ mg/l}$); (C) high (HO₂; $11.0 \pm 0.5 \text{ mg/l}$). pH evolution of coconut water (D) in LO₂, UO₂ and HO₂.

aliquots (65 °C, 10 min) after 61 days was constant, suggesting that spores remained dormant despite temperature abuse, independently of oxygen concentration (supplementary material). 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 ± 0.1 to $8.2 \pm 0.2 \text{ log CFU/ml}$ depending on initial oxygen concentration (Fig. 2A-2C). Growth of spoilage microorganisms recovered after HPP caused severe degradation in the aspect of coconut water, which became turbid and viscous.

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 DO₂ and incubation temperature: *Tatumella tyseos* (99% identity, 100% query cover) and *Xanthomonas* sp. (100% identity and query cover). Both are facultative anaerobes associated to plant degradation and are cause of plant diseases (Bull et al., 2014; Marín-Cevada et al., 2010). Their presence might be a consequence of cross contamination during coconut water extraction. *T. tyseos* 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 (Fig. 2D). 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.

DO₂ content of UO₂ and HO₂ samples showed a sharp decrease between days 15 and 20 that can be attributed to the microbial growth observed (Fig. 2B-2C). By the end of incubation period, DO₂ 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 (García-Ochoa, Gomez, Santos, & Merchuk, 2010). Oxygen demand might be so high that DO₂ 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 (García-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 O₂ concentrations. Growth and toxin production was observed in broccoli florets after 9 days of incubation at 12 °C (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 °C during 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 LO₂, UO₂ and HO₂ (Fig. 3). However, a decrease of $1.7 \pm 0.3 \text{ log CFU/ml}$ occurred in LO₂ coconut water after 61 days (Fig. 3A). Similarly, spore counts slightly decreased between 0.8 and 1.1 log CFU/ml in UO₂ and HO₂ coconut water after 20 days of incubation, respectively (Fig. 3B and 3C). This was observed right after DO₂ 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 LO₂ samples where initial DO₂ concentration was already low. Additionally, pH increased

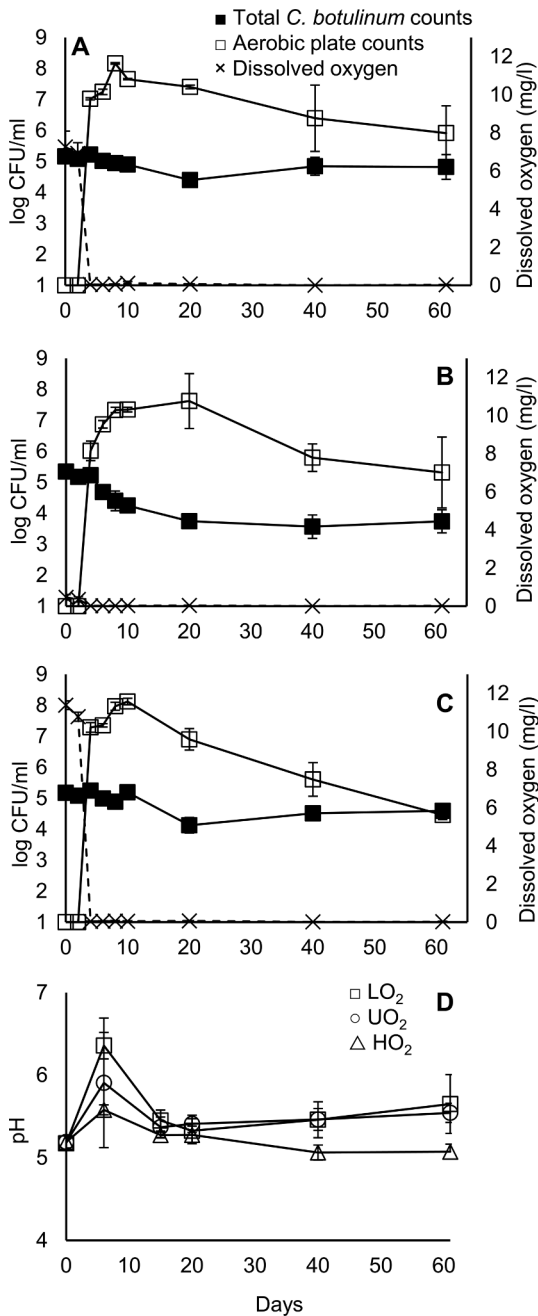


Fig. 3. *C. botulinum* total counts, aerobic plate counts and dissolved oxygen concentration in high-pressure processed coconut water (550 MPa, 3 min) stored at 20 °C with initial different dissolved oxygen concentration levels: (A) low (LO₂; <math>< 0.5 \pm 0.1 \text{ mg/l}</math>); (B) unaltered (UO₂; $7.0 \pm 0.8 \text{ mg/l}$); (C) high (HO₂; $11.0 \pm 0.5 \text{ mg/l}$). pH evolution of coconut water (D) in LO₂, UO₂ and HO₂.

between 0.4 and 1.8 units (Fig. 3D). 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 of the samples (supplementary material).

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

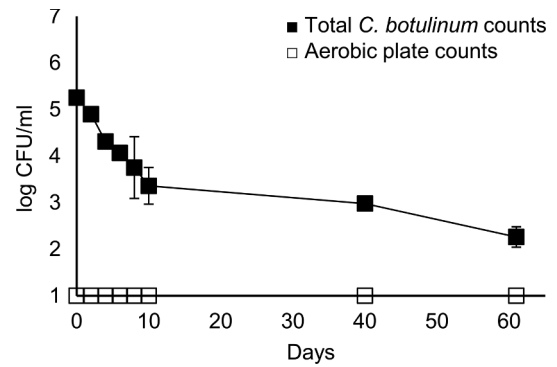


Fig. 4. *C. botulinum* total counts and aerobic plate counts in FS coconut water (pH 7.0) incubated at 30 °C under strict anaerobic conditions.

concentration of DO₂ adjusted to 0.013 atm O₂ (~0.58 mg/l O₂) (Lund, Knox, & Sims, 1984), whereas in the present study the bacterium failed to grow even at lower concentrations (<math>< 0.50 \text{ mg/l O}_2</math>). 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 DO₂ 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 spore germination and growth, or a combination of some of these factors.

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

C. botulinum did not grow in HPP tender coconut water with different initial oxygen concentrations, therefore 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, *C. botulinum* counts decreased by $1.9 \pm 0.4 \text{ log CFU/ml}$ (Fig. 4). Furthermore, *C. botulinum* counts steadily continued to decrease during the incubation period, reaching a final concentration of $2.3 \pm 0.2 \text{ log CFU/ml}$ after 61 days with no vegetative cells detected (Fig. 4). This reduction was slightly greater than that observed in LO₂ coconut water incubated at 20 °C ($1.7 \pm 0.3 \text{ log CFU/ml}$) after the 20-day incubation period (Fig. 3A). Optimum growth conditions for *C. botulinum* in coconut water (pH 7.0, strictly anoxic atmosphere, 30 °C and absence of background microbiota) did apparently favor spore germination followed by subsequent germinated spores’ death since no vegetative cells were identified. It is

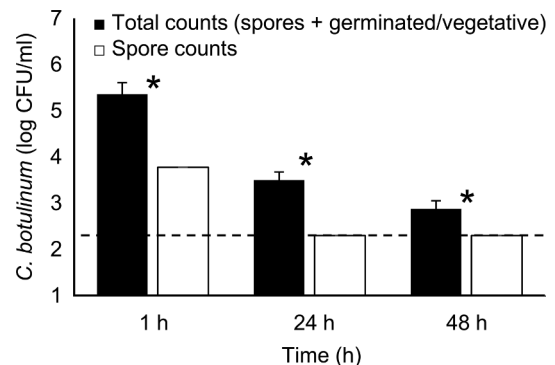


Fig. 5. *C. botulinum* total counts and spore counts in FS + G coconut water after incubation during 48 h at 30 °C under strict anaerobic conditions. *Significant difference ($p < 0.05$) (dashed line: detection limit).

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* sp. germination and growth when present at sufficient concentration (Bowles & Miller, 1994; Juneja, Bari, Inatsu, Kawamoto, & Friedman, 2007; Pacheco et al., 2017). Both of these approaches were furtherly studied in the present work.

3.3. Supplementation with germinants

Heating FS coconut water aliquots supplemented with germinants (FS + G) at 65 °C for 10 min reduced the concentration of *C. botulinum* by 1.6 ± 0.2 log CFU/ml after 1 h of incubation (Fig. 5). This difference with respect to the initial spore count (5.36 log CFU/ml) was significant ($p < 0.05$; Fig. 5), which indicates that a fraction of spores germinated and lost their heat resistance. *C. botulinum* total counts further decreased to reach 3.5 ± 0.2 and 2.9 ± 0.2 log CFU/ml, after 24 and 48 h, respectively. Heating at 65 °C for 10 min reduced counts below the detection limit (2.3 log CFU/ml), which suggests that > 1.9 log CFU/ml and > 0.6 log 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 CFU/ml the concentration of spores without the addition of germinants (Fig. 4), whereas it only took 48 h to achieve a similar spore concentration reduction under the same conditions in germinant-supplemented coconut water (Fig. 5). 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 CFU/ml in black and oolong teas incubated at 30 °C under strict anaerobic conditions during 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 °C 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).

3.4. Supplementation with free amino acids

Amino acids are a key element for non-proteolytic *C. botulinum* growth and toxin formation (Perkins & Tsuji, 1962; Strasdine & Melville, 1968; Whitmer & Johnson, 1988). Based on compositional analysis performed on the coconut water used in this study (supplementary material), 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 CFU/ml of spores (Fig. 6). Total *C. botulinum* counts decreased in 48 h from 5.2 ± 0.1 to 4.1 ± 0.2 log CFU/ml, as previously observed during the first 48 h in FS coconut water (Fig. 4). Heating aliquots at 65 °C 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 UFC/ml, $p < 0.05$) but similar to that observed in FS + G coconut water (0.88 log UFC/ml, $p > 0.05$) (Fig. 5). 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 2) provided to coconut water all the above-mentioned amino acids at a concentration higher than the established 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 vitamins) (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 3).

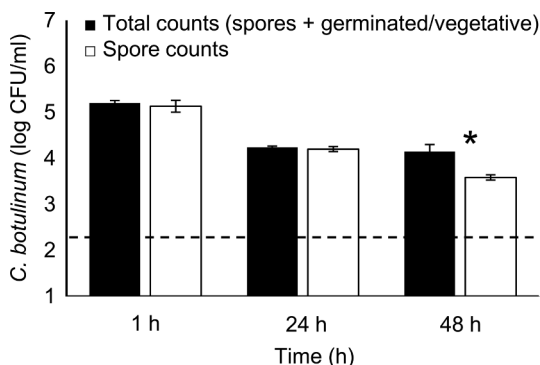


Fig. 6. *C. botulinum* total counts and spore counts in FS + AA coconut water after incubation during 48 h at 30 °C under strict anoxic conditions. *Significant difference ($p < 0.05$) (dashed line: detection limit).

Table 2

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

Table 3
Vitamin content in chemically defined media (CDM) and coconut water.

Vitamin	Concentration reported in CDM for <i>C. botulinum</i> growth	Concentration reported in coconut water
Biotin	0.01 mg/l (Strasidine & 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 (Strasidine & Melville, 1968; Whitmer & Johnson, 1988) 1 mg/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 (Strasidine & Melville, 1968; Ward & Carroll, 1966; Whitmer & Johnson, 1988)	0.32 mg/l (Yong et al., 2009)
Nicotinamide	1 mg/l (Strasidine & Melville, 1968; Ward & Carroll, 1966; Whitmer & Johnson, 1988)	0.64 mg/l (Gopikrishna et al., 2008) 0.8 mg/l (Yong et al., 2009)
Folate	1 mg/l (Strasidine & 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 (Strasidine & Melville, 1968) 50 mg/l (Whitmer & Johnson, 1988)	NA ^a

^a Not available

3.5. Supplementation with TPGY broth

To understand 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. Serial dilutions of the beverage (FS + TPGY) with the laboratory broth revealed that between 6.25 and 12.5% TPGY was sufficient to stimulate outgrowth from 5 log CFU/ml of spores to a cell concentration of 6.5 ± 1.7 log 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% (Fig. 7). 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.

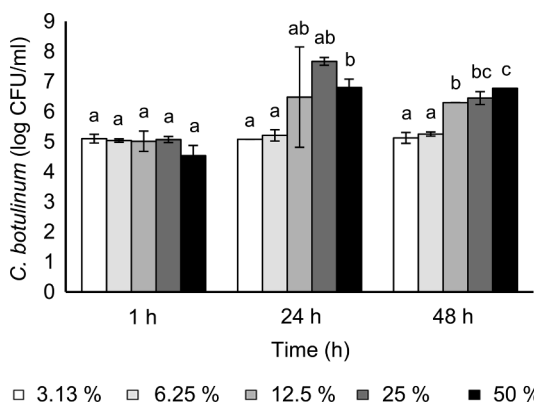


Fig. 7. *C. botulinum* total counts in coconut water supplemented with TPGY broth at different concentrations after incubation during 48 h at 30 °C under strictly anoxic conditions. Different letters indicate significant differences ($p < 0.05$).

4. Conclusions

HPP Thai raw coconut water (pH 5.2) used in this study did not support outgrowth of *C. botulinum* spores after 61 days of storage at refrigeration and moderate temperature abuse (4 – 10 °C), and only germination 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* to grow.

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 (groups I and II) and botulinum neurotoxin determination rather than plate counts would assess the risk more precisely.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

CRediT authorship contribution statement

Mario González-Angulo: Investigation, Methodology, Writing - original draft. **Charlien Clauwers:** Investigation, Validation, Formal analysis. **Rania Harastani:** Investigation, Validation. **Carole Tonello:** Conceptualization, Writing - review & editing. **Isabel Jaime:** Writing - review & editing. **Jordi Rovira:** Writing - review & editing. **Chris W. Michiels:** Project administration, Supervision, Methodology, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodres.2020.109278>.

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